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January 2012

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On the Cover

Dioskurides of Samos,
***Musici Ambulanti*,**
Mosaic (c. 2200 years ago)
(41 cm × 43 cm)
Museo Archeologico Nazionale, Piazza Museo Nazionale, Napoli, Italy

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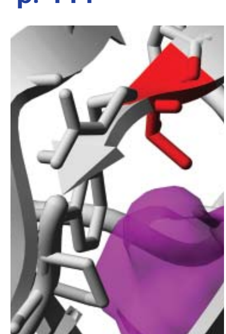

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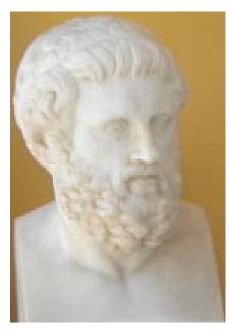

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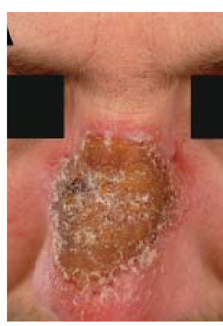

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Intestinal Toxemia Botulism in 3 Adults, Ontario, Canada, 2006–2008

Yolanda D. Sheppard, Dean Middleton, Yvonne Whitf eld, Felix Tyndel, Shariq Haider, Jamie Spiegelman, Richard H. Swartz, Mark P. Nelder, Stacey L. Baker, Lisa Landry, Ross MacEachern, Sherri Deamond, Lorrie Ross, Garth Peters, Michelle Baird, David Rose, Greg Sanders, and John W. Austin

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Learning Objectives

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

- Evaluate the epidemiology and microbiology of adult intestinal toxemia botulism.
- Analyze risk factors for adult intestinal toxemia botulism.
- Assess the clinical presentation of adult intestinal toxemia botulism.
- Evaluate the prognosis of adult intestinal toxemia botulism.

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Five cases of intestinal toxemia botulism in adults were identified within an 18-month period in or near Toronto, Ontario, Canada. We describe findings for 3 of the 5 case-patients. Clinical samples contained *Clostridium botulinum* spores and botulinum neurotoxins (types A and B) for extended periods (range 41–61 days), indicative of intestinal toxemia botulism. Patients' clinical signs improved with supportive care and administration of botulinum antitoxin. Peanut butter from the residence of 1 case-patient yielded *C. botulinum* type A, which corresponded with type A spores found in the patient's feces. The food and clinical isolates from this case-patient could not be distinguished by pulsed-field gel electrophoresis. Two of the case-patients had Crohn disease and had undergone previous bowel surgery, which may have contributed to infection with *C. botulinum*. These cases reinforce the view that an underlying gastrointestinal condition is a risk factor for adult intestinal toxemia botulism.

Botulism is a neuroparalytic condition, typified by cranial nerve palsies that may be followed by descending symmetrical flaccid paralysis, which can lead to respiratory arrest and, in some instances, death (*1*). Intestinal toxemia botulism is an infectious form of botulism in which illness results from ingesting spores, which is followed by spore germination and intraluminal production of botulinum neurotoxins over an extended period (*2*). Intestinal toxemia botulism is rarely reported in adults; <20 cases have been described in the literature since the first reports in 1980 and 1981 (*3*,*4*). The rarity of this condition contributes to its obscurity, an obscurity that may negatively affect those infected through a lack of rapid diagnosis and treatment. Countries with published case reports of intestinal toxemia botulism in adults are the United States (*3–10*), Italy (*11*,*12*), Iceland (*9*), and Japan (*13*). The etiologic agents involved in intestinal toxemia botulism in adults are *Clostridium botulinum* types A, B, and F (*6–9*,*13*,*14*) and, more rarely, *C. baratii* type F (*10*), and *C. butryicum* type E (*11*,*12*).

Although considered a rare condition, intestinal toxemia botulism was reported in 5 adults in or near Toronto, Ontario, Canada, during November 2006–May 2008. We report the clinical, laboratory, and epidemiologic findings for 3 of the 5 case-patients. Details pertaining to the clinical course of illness of 1 of the patients were previously published in a case report (*15*). In this case series, we document 3 cases of adult intestinal toxemia botulism, which occurred in a small geographic area in a short period, and provide further evidence for the potential role of host factors, such as Crohn disease, previous bowel surgery, and short bowel syndrome, which may predispose a person to intestinal toxemia botulism.

We reviewed case investigation files from the Ontario Ministry of Health and Long-Term Care and the local public health units in or near Toronto. Physicians involved in the cases provided clinical summaries. Consent to publish information pertaining to the diagnosis of intestinal toxemia botulism was obtained from the 3 case-patients. Two additional cases were reported in November 2006 and May 2008; however, consent was not obtained from those patients.

Serum, stool, gastric aspirate, and enema samples were assayed for botulinum toxin by using the mouse bioassay (*16*). Stool and enema specimens were cultured for *C. botulinum* as described (*16*). Botulinum neurotoxin was identified by neutralization with specific antitoxin types A, B, E, and F obtained from the Centers for Disease Control and Prevention (Atlanta, GA, USA). *C. botulinum* was identified and differentiated from other botulinum toxin–producing clostridia on the basis of Gram stain reaction, morphologic features, motility, lipase and lecithinase reactions on egg yolk agar, sugar fermentations, esculin hydrolyis, gelatin liquefaction, and production of botulinum neurotoxin. Pulsed-field gel electrophoresis (PFGE) was done as described (*17*). Cases of adult intestinal toxemia botulism were confirmed by detection of *C. botulinum* in stool specimens over prolonged periods in conjunction with clinical observations consistent with botulism. One of these cases has been reported separately as a case study (*15*).

Case Reports

Case-Patient 1

A 63-year-old woman was admitted to a hospital on November 22, 2006, after a 2-day history of abdominal pain, blurred vision, diarrhea, dysarthria, dysphagia, horizontal binocular diplopia, imbalance, and weakness in the arms and hands. No recent infections, wounds, or antimicrobial drug use were reported. After admission to the hospital, she experienced respiratory arrest, for which intubation and ventilation were required. The patient's medical history included Crohn disease, bowel surgery, short bowel syndrome, hypertension, breast cancer, nephrolithiasis, and small fiber polyneuropathy (motor function was normal on nerve conduction studies 6 years earlier). A pathology report from 20 years earlier noted acute and chronic inflammation of the patient's large and small bowels. No acute wounds or punctures were apparent on examination.

The neurologic examination showed a normal level of consciousness and mental state; reduced pupillary light responses; bilateral, symmetrical ophthalmoplegia; bilateral ptosis; bifacial lower motor neuron weakness; moderate proximal and distal symmetrical limb weakness; absent tendon reflexes; and flexor plantar responses. Muscle tone was decreased; neither atrophy nor fasciculation occurred, and sensation was intact. Results of routine blood and urine tests showed only a mild elevation of the serum amylase

level. Botulism was strongly suspected. However, other conditions that affect the central nervous system, including the brainstem and cranial nerves, were considered. Results of tests for syphilis, Lyme disease, West Nile virus infection, acetylcholine receptor antibodies, and IgG against GQ1b were negative.

Results of cerebrospinal fluid studies, including cytology and viral PCR studies, were also normal. A magnetic resonance imaging scan of the brain had unremarkable findings. Electrodiagnostic studies showed mildly reduced compound motor action potential amplitudes and normal sensory responses. Posttetanic facilitation was marked, with a 700% increase in compound motor action potential amplitudes. Electromyography showed that lower limb muscles were electrically silent. Enema fluid, stool, and gastric aspirate specimens were positive for type A botulinum neurotoxin and viable *C. botulinum* type A spores, with organisms persisting in stool for 61 days. Botulinum antitoxin was administered in the evening of November 23, 2006.

The environmental investigation found that the patient ate a limited diet of tea, bagels, peanut butter, occasional honey, and weekly Chinese take-out food. An opened jar of peanut butter from the patient's residence yielded positive test results for *C. botulinum* type A spores, containing ≈14 *C. botulinum* spores/kg.

Both heated and nonheated samples from the enema fluid were positive for *C. botulinum* type A, indicating the presence of spores. Analysis of clinical and peanut butter isolates by PFGE indicated identical PFGE patterns (Figure).

The patient was still mechanically ventilated as of December 15, 2006, and required regular sedation. Once sedation wore off, the patient experienced random, spastic-like, nonpurposeful movements of all extremities. Her condition improved over the next 6 months, and she could walk by the time she was discharged.

Case-Patient 2

Bulbar symptoms developed in a 50-year-old woman with ophthalmoparesis on February 7, 2007, and her symptoms worsened until she was admitted to the hospital on February 10, 2007, with complete ophthalmoplegia, dysphagia, and quadriparesis. The patient's medical history included a diagnosis of Crohn disease in 1979 and 4 previous bowel resections with ileocolonic anastomosis, complicated by enterocutaneous fistulas (in 1986, 1995, 2001, and 2002). She had no wounds, and no chest or abdominal pains were reported before the onset of symptoms. The patient experienced oropharyngeal discomfort and difficulty speaking but was able to respond to questions with a yes or no. A repeat computed tomography scan was carried out, and results were normal. Lumbar puncture was conducted for full studies, including tests for West Nile virus. Over the next 24 hours, she had a decreased level of consciousness, a depressed Glasgow coma scale score, tachycardia, and difficulty breathing, with eventual respiratory failure requiring ventilation support.

The neurologic examination showed minimal orbicularis oculi contractions and quadriparesis with normal reflexes. The differential diagnoses being considered for this case-patient included the following: viral encephalitis, meningitis, brain stem lesion, myasthenia gravis, and infectious agents, specifically, *C. botulinum* and enteric organisms linked to Guillain-Barré syndrome, such as *Campylobacter*, *Salmonella*, and *Shigella* spp. The initial assessment by the consulting neurology team was that of Miller Fischer variant of Guillain-Barré syndrome and Eaton-Lambert syndrome or acute motor axonal neuropathy. Electromyography studies showed myopathic-like motor unit action potential. Results of a Tensilon test

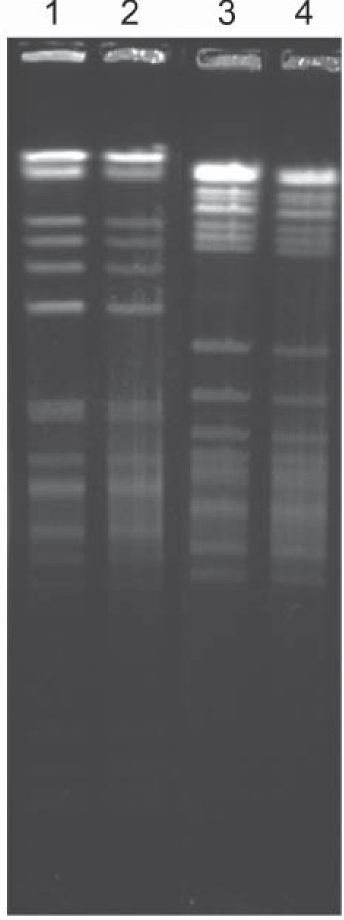

Figure. Results of pulsed-field gel electrophoresis of samples from case-patient 1, who had intestinal toxemia botulism, Ontario, Canada, 2006–2008. Lanes 1, 3: *Clostridium botulinum* type A isolated from enema sample; lanes 2, 4: *C. botulinum* type A isolated from peanut butter obtained from residence of patient. Samples in lanes 1 and 2 were digested with *Sma*I; samples in lanes 3 and 4 were digested with *Xho*I.

were negative. Additional tests, including a test for antibody against acetylcholine, and repeat electromyography studies with high frequency stimulation were negative (no incremental response and a negative acetylcholine antibody test results), which indicated that myasthenia gravis and Eaton-Lambert syndrome were unlikely. A presumptive diagnosis of botulism from intestinal toxemia, based in part on the patient's history of Crohn disease and previous bowel surgeries, was made on February 13, 2007. The patient received botulinum antitoxin on February 23, 2007. She remained dependent on a ventilator for 5 months and had a percutaneous endoscopic gastrostomy tube for feeding, leading to line sepsis on 2 occasions, ventilator-assisted pneumonia, and a tracheostomy.

The patient's food history included consumption of toast with peanut butter, coffee, chicken soup, and home-prepared beef tacos with grated cheese, lettuce, tomatoes, and salsa. Before the onset of symptoms, the patient consumed peanut butter daily. An opened jar of peanut butter from the home yielded positive test results for *C. botulinum* type A.

Of 7 serum samples, the first sample was positive for botulinum neurotoxin; however, the quantity was insufficient for serotype determination. Subsequent serum sample test results were negative for botulinum neurotoxin. Type A botulinum toxin was detected in the first stool specimen, which was collected on February 21, 2007. All subsequent stool specimen test results were negative for botulinum toxin. Test results for 5 of 8 stool specimens were positive for either *C. botulinum* type A or type B spores over 56 days. Three subsequent stool samples collected in May 2007 yielded negative test results for *C. botulinum*.

The patient was transferred to a rehabilitation hospital on June 17, 2007, where she required nightly ventilation support and received therapy for swallowing and speech as well as physiotherapy and occupational therapy. In September 2007, ventilatory support was gradually withdrawn; the patient learned to eat on her own and was discharged, but she continued to receive outpatient therapy for an additional 3 months. During a follow-up visit in June 2009, the patient was walking independently, and a respiratory evaluation showed normal ventilatory capacity of forced expiratory volume of 2.58 L, 99% of predicted normal inspiratory muscle strength, and only minimal expiratory muscle weakness.

Case-Patient 3

A 45-year-old man with no known history of gastrointestinal problems and otherwise healthy was admitted to the hospital on January 8, 2008, with a distended abdomen and blurry vision (*15*). The patient's abdomen continued to distend, and bowel sounds were diminished despite nasogastric suctioning. No recent infections or antimicrobial drug therapy were noted. The distended abdomen signaled a possible bowel obstruction, and scans confirmed the presence of an obstruction with unknown cause. A neurologic consultation was necessary after 5 days to assess the patient's worsening neurologic symptoms. Guillain-Barré syndrome and myasthenia gravis were initially considered, but results of a nerve conduction study were classic for botulism. Botulism was suspected on January 14, 2008, on the basis of neurophysiologic findings, coupled with the clinical observations suggestive of botulism.

Clinical samples were subsequently sent for analysis, and through consultation with the physician, neurologist, and public health practitioners, it was determined that the patient would still benefit from antitoxin, even though it had been more than 24 hours since the onset of symptoms. Antitoxin was administered the evening of January 14, 2008. The patient remained hospitalized until February 27, 2008, when clinical symptoms improved. *C. botulinum* type B was detected in the patient's stool at weeks 2 and 8 after the onset of symptoms. Botulinum neurotoxin was not detected in the stool extracts. Resulting of testing of both heated samples were positive for *C. botulinum* type B, indicating the presence of spores in the sample. Two subsequent fecal samples at weeks 11 and 15 yielded negative test results for *C. botulinum*. The persistence of *C. botulinum* type B over a period of 41 days supported the diagnosis of intestinal toxemia botulism. The patient had a history of eating primarily commercial canned food, although no specific types were reported, and none of the patient's contacts were reported to be ill.

Conclusions

Diagnosis of intestinal toxemia botulism was determined through repeated detection of botulinum neurotoxins, viable *C. botulinum,* or both in clinical samples over extended periods. Colonization with *C. botulinum* persisted in case-patients 1, 2, and 3 for 61, 56, and 41 days, respectively. In addition to the persistence of organisms in stool samples, the diagnoses were supported by the presence of clinical features compatible with botulism (Table). In previously published reports, *C. botulinum* was present in stool specimens for periods ranging from 2 to 119 days (*9*). Two additional cases of intestinal toxemia botulism in adults were identified in close proximity and time to the 3 cases reported here; however, consent was not obtained to publish the findings pertaining to these cases.

Intestinal toxemia botulism in adults is described in detail in earlier reports (*3*), and Arnon summarized 10 previous cases (*2*). Since the summary of Arnon, 3 cases have been reported in Italy (*11*), and a case occurred in Japan in 1999 (*13*). Previously cited risk factors for intestinal toxemia botulism in adults include structural and

Table. Clinical summary of 3 intestinal toxemia botulism cases in adults, Ontario, Canada, 2006–2008

Case-patient no.	Age, y/sex	Dates admitted to hospital and discharged	Clinical findings	Gastrointestinal history	*Clostridium botulinum* serotype	No. days stool sample positive for *C. botulinum**	Supportive care; antitoxin; administration date
1	63/F	2006 Nov 22	Abdominal pain, diarrhea, dysphagia, diplopia, weakness	Crohn disease, short bowel syndrome, remote bowel surgery	A	61	Intubation and ventilation; bivalent type A and B, monovalent type E; 2006 Nov 23
2	50/F	2007 Feb 8; 2007 Sep	Dysphagia, double vision, weakness, difficulty breathing	Crohn disease, bowel resections	A and B	56	Intubation and ventilation; bivalent type A and B, monovalent type E; 2007 Feb 23
3	45/M	2008 Jan 8; 2008 Feb 27	Abdominal distension and obstruction, aphonia, diplopia, dry mouth, dysphagia, dysphonia, ptosis	Unremarkable	B	41	Bivalent type A and B, monovalent type E; 2008 Jan 14

*Total number of days that *C. botulinum* spores or neurotoxins were detected in clinical samples.

functional gastrointestinal abnormalities, including Crohn disease (*8*), surgical alterations of the intestine (*6*,*9*), Meckel diverticulum (*12*,*18*), and modified intestinal flora caused by prolonged antimicrobial drug therapy (*6–10*,*13*). Two of the patients described in this report had Crohn disease and previous bowel surgery. In addition, case-patient 1 also had short bowel syndrome. Unlike the first 2 case-patients, case-patient 3 was previously healthy and had no history of gastrointestinal problems. Three other published reports of intestinal toxemia botulism in adults occurred in patients with no preexisting medical conditions or antimicrobial drug use reported. These cases occurred in Kentucky, USA (*9*); Iceland (*9*); and Japan (*13*). The initial symptoms of acute abdominal distension and small bowel obstruction experienced by case-patient 3 are uncommon and suggest that the differential diagnosis for small bowel obstruction in an otherwise healthy patient should include botulism (*15*).

Several factors could lead to underdiagnosis of intestinal toxemia botulism. Symptoms of botulism are similar to other conditions affecting the central nervous system, and botulism may be misdiagnosed as Guillain-Barré syndrome, myasthenia gravis, Eaton-Lambert syndrome, stroke syndromes, or tick paralysis (*1*,*19*). Similar to botulism in infants, cases of intestinal toxemia botulism in adults occur sporadically and may be missed unless the physician has seen a case of botulism previously. Two cases of foodborne botulism, both attributed to carrot juice, had been identified in the same region only 2 months before the first case of intestinal toxemia botulism described here (*20*), perhaps contributing to a period of higher awareness of botulism cases.

Given that intestinal toxemia botulism in adults is rare, the identification of multiple cases in a small geographic area in a short period is unusual. Two cases of intestinal toxemia botulism in adults, caused by *C. butyricum* which produced type E neurotoxin, were reported in Italy (*11*,*12*). The cases in Italy occurred in 2 communities ≈30 km apart and were separated by 12 months (*12*).

Two of the case-patients described in our report had Crohn disease and prior bowel surgery. Such conditions are consistent with previously published cases, providing further evidence that host susceptibility may play a substantial role in this disease. Although intestinal toxemia botulism is a rare disease worldwide, it is a serious disease that causes substantial illness and high health care costs. Public health practitioners and physicians investigating suspected botulism cases should thoroughly consider the patient's medical history, particularly for underlying gastrointestinal conditions that may make them susceptible to intestinal toxemia botulism.

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Ms Sheppard was an MPH student at the University of Guelph, Guelph, Ontario, Canada, at the time this research was conducted. Her research interests include the epidemiology of enteric and infectious diseases.

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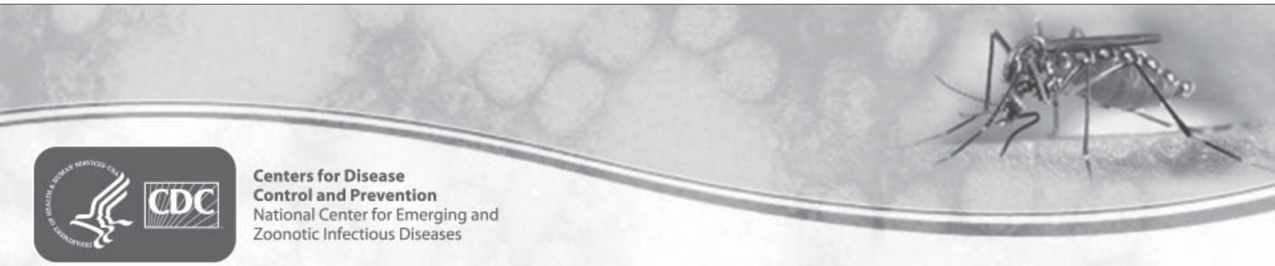

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Serious Invasive Saffold Virus Infections in Children, 2009

Alex Christian Yde Nielsen, Blenda Böttiger, Jytte Banner, Thomas Hoffmann, and Lars Peter Nielsen

The first human virus in the genus *Cardiovirus* was described in 2007 and named Saffold virus (SAFV). Cardioviruses can cause severe infections of the myocardium and central nervous system in animals, but SAFV has not yet been convincingly associated with disease in humans. To study a possible association between SAFV and infections in the human central nervous system, we designed a real-time PCR for SAFV and tested cerebrospinal fluid (CSF) samples from children ≤4 years of age. SAFV was detected in 2 children: in the CSF and a fecal sample from 1 child with monosymptomatic ataxia caused by cerebellitis; and in the CSF, blood, and myocardium of another child who died suddenly with no history of illness. Virus from each child was sequenced and shown to be SAFV type 2. These findings demonstrate that SAFV can cause serious invasive infection in children.

Molecular biology has revolutionized the diagnostics of infectious diseases through the introduction of more sensitive and specific diagnostic tests. Despite these advances, the etiologic agents of many apparent infections are still unidentified. For example, the etiologic agent is unknown for many cases of apparent pneumonia (*1*); in a study conducted in California, USA, despite extensive testing and evaluation, an underlying cause of encephalitis was unidentified for 207 (62%) of 334 patients (*2*).

During the past few years, intensive searches for new viruses, using conventional virologic methods and metagenomics, have resulted in the discovery of several new viruses. During the past decade, the family *Picornaviridae* has grown as the number of recognized genera has increased from 6 to 12 (*3,4*); the numbers of species, types, and subtypes have increased even more. However, only viruses from 3 genera (*Enterovirus*, *Hepatovirus*, and *Parechovirus*) have been firmly established as being capable of causing clinically significant disease in humans. Viruses from other genera (*Cardiovirus*, *Cosavirus*, and *Kobuvirus*) have so far been detected only in noninvasively collected human sample material such as fecal and respiratory samples, and their clinical significance remains to be fully elucidated. (Invasively collected sample material is that from tissues considered sterile, i.e., devoid of microorganisms.)

The phylogenetic relationships of human picornaviruses are shown in Figure 1. Most picornaviruses that are pathogenic to humans are ubiquitous viruses capable of causing a variety of diseases, from monosymptomatic febrile infection to severe infection in the central nervous system and myocardium. However, most infections with these viruses are asymptomatic (*5*).

Saffold virus (SAFV) was discovered by Jones et al. in 2007 by sequence-independent genomic amplification of virus isolated from a fecal sample (*6*). The sample had been obtained in 1981 from an 8-month-old child with fever of unknown origin. The genetic sequence of the virus indicated that the virus belonged to the species *Theilovirus* of the genus *Cardiovirus*, which contains 3 other members: Theiler's murine encephalomyelitis virus (TMEV), Vilyuisk human encephalomyelitis virus (VHEV), and Thera virus. In mice, TMEV is capable of causing infection in the central nervous system, and some variants of this virus cause a persistent infection and even multiple sclerosis–like disease (*7*). VHEV was isolated in the 1950s from cerebrospinal fluid (CSF) from a patient with Vilyuisk encephalomyelitis, a progressive neurologic

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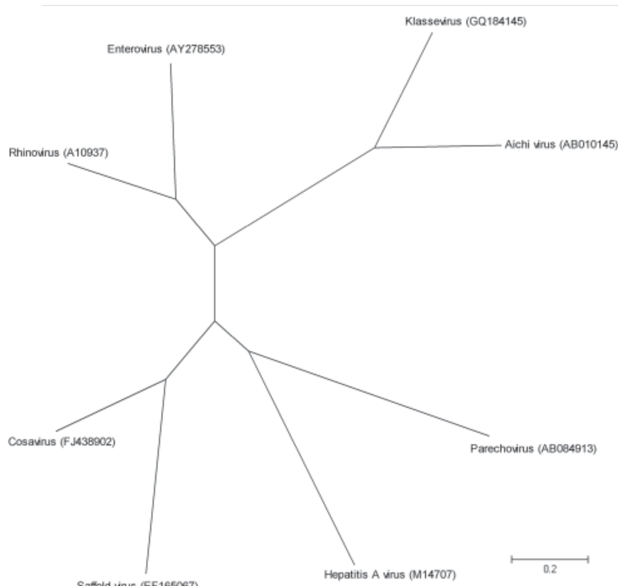

Figure 1. Phylogenetic tree based on full-genome sequences of all known human picornavirus species, represented with 1 virus strain each. The tree was constructed by the neighbor-joining method by using MEGA4 software (www.megasoftware.net). Scale bar indicates nucleotide substitutions per site.

disorder that occurs in indigenous populations of an isolated part of eastern Siberia (*8*). However, the correlation between VHEV and Vilyuisk encephalomyelitis is still uncertain because VHEV has been isolated by multiple passages in mice and thus may represent a highly divergent strain of TMEV. Thera virus (previously named Theiler-like rat virus) has been isolated from rats, but the clinical significance of infections with this virus is unknown (*9*,*10*). The genus *Cardiovirus* also contains a second species called encephalomyocarditis virus. Only 1 serotype is known, and it is capable of causing encephalitis and myocarditis in various animals (*11*,*12*).

Since the discovery of SAFV, several articles have provided insight into its epidemiologic and, to a minor degree, clinical significance. Saffold viruses are distributed worldwide (*6*,*13–19*), and 2 serologic studies have demonstrated that infection occurs early in life (*14*,*20*). However, finding an association with human disease has thus far been elusive. Most studies (*13*,*15*,*17*,*20–22*) have tried to associate SAFV with gastroenteritis, but no convincing results have been produced. A few studies (*16*,*18*,*21*,*23*) analyzed the clinical significance of SAFV virus in the respiratory system, but no substantial association between the virus and respiratory symptoms or disease has been made. Only 1 study (*21*) reports having tested invasively collected sample material (CSF samples), but no findings were positive.

To investigate the possible invasive potential of SAFV in humans, we developed a diagnostic PCR and tested CSF samples from a group of children. SAFV was detected in 2 of these children.

Materials and Methods

We tested previously submitted CSF samples for SAFV, reviewed the patients' medical records, and sequenced the viruses isolated. The study was approved by the local ethics committee, De Videnskabsetiske Komiteer for Region Hovedstaden, Denmark, protocol no. H-2–2010–019.

CSF Specimens

We tested 332 consecutively submitted CSF samples from 319 patients $\leq$4 years of age from Denmark that had been submitted to Statens Serum Institut, Copenhagen, from January 2006 through December 2009 for viral diagnostic testing. We tested for the following viruses: herpes simplex virus types 1 and 2 (228 samples), varicella zoster virus (228 samples), human enterovirus (261 samples), and human parechovirus (88 samples since November 2008). For all samples, initial test results were negative.

Fecal Samples

We selected fecal samples from 479 children $\leq$5 years of age with gastroenteritis that had been submitted for viral diagnostic testing from September 2009 through February 2010 and tested them for SAFV. Nucleic acid extracted from these samples was combined into 48 pools, with 9 or 10 samples per pool. Samples from pools with positive results were identified, and new extractions from these pools were tested individually. However, enough sample material for new extractions was available for only about half of the samples.

Nucleic Acid Purif cation

Nucleic acids were extracted from 200 µL of CSF or blood (from SAFV-positive patients) by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) and semiautomatic extraction on the QIAcube instrument (QIAGEN). Nucleic acid was extracted from 200-µL fecal suspension (10% in phosphate-buffered saline) by using the Total Nucleic Acid Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) on the MagnaPure LC instrument (Roche Diagnostics GmbH).

Nucleic Acid Amplif cation and Detection

Five microliters of extracted material was used per reverse transcription PCR (total volume 25 µL) by using the OneStep RT-PCR Kit (QIAGEN). The reaction mixtures contained 1 µmol/L of each primer and 0.2 µmol/L of probe.

Design of the primers and probe was based on an alignment of all available SAFV sequences in GenBank (www.ncbi.nlm.nih.gov/genbank) in July 2010 by using ClustalW (www.clustal.org) and Primer3 (http://frodo.wi.mit.edu/primer3) software. The primers and probe are selective for a highly conserved part of the 5′ untranslated region (Table). The Strategene Mx3005P real-time thermocycler instrument (Agilent Technologies A/S, Horsholm, Denmark) was used for amplification and detection with the following settings: 50°C for 20 min, 95°C for 15 min, followed by 45 cycles of 95°C for 15 s and 55°C for 1 min.

Genotyping by PCR and Sequencing

Genotyping was conducted by nested PCR and sequencing of parts of the viral protein (VP) 1 and VP2 regions of the capsid gene by using primers listed in the Table. The inner VP1 and VP2 primers amplified DNA fragments of ≈599 and 577 bp, respectively. PCR products were purified by using the High Pure PCR Purification Kit (Roche Diagnostics GmbH) before sequencing, which was performed by using the inner PCR primers on an ABI automated sequencer and BigDye version 1.1 chemistry (both from Applied Biosystems, Darmstadt, Germany). Sequences were aligned, and phylogenetic analysis with known reference sequences was performed by using MEGA4 software (www.megasoftware.net). The sequences have been submitted to GenBank under accession nos. JF693612–23.

Results

SAFV was detected in CSF from 2 of the 319 children. Additional sample material from these 2 children was subsequently obtained and tested. From child 1, blood and CSF collected at the same time and a fecal sample collected 2 weeks later were tested; only test results for the fecal sample were positive for SAFV. From child 2, a postmortem blood sample and a myocardial biopsy sample were tested; test results for each sample were positive for SAFV.

Child 1

Child 1 was a 16-month-old, previously healthy boy who became ill in May 2009. The boy had a fever 6 days before hospital admission, followed 1 day later by sudden onset of monosymptomatic ataxia, with no fever. The ataxia fluctuated from causing an insecure gait to walking into things and falling. The patient also had intermittent difficulty controlling his arms when trying to eat. No history of recent travel was reported. The boy was in otherwise good health; he had no abnormal psychological symptoms and retained a normal degree of consciousness throughout the acute phase of the disease. Differential diagnoses at hospital admission were intracranial tumor or viral cerebellitis. The boy's 4-year-old sister remained healthy.

Laboratory test results are listed in the online Appendix Table (wwwnc.cdc.gov/EID/article/18/1/11-0725-TA1.htm). At hospital admission, CSF values (leukocyte count, protein level, and glucose level) were within reference ranges, and no microorganisms were detected. A magnetic resonance imaging scan of the brain showed a small venous anomaly in the left frontal lobe but no tumor, hemorrhage, or inflammation. A fecal sample collected 2 weeks later yielded positive test results for parechovirus type 3 and negative results for enterovirus and adenovirus. Parechovirus was not found in the CSF or blood.

During the next 2 months, the ataxia remitted completely without sequelae. The diagnosis at this time was viral encephalitis, possibly caused by parechovirus type 3.

Child 2

Child 2 was a 27-month-old, previously healthy girl who was found dead in her bed in August 2009; she had no

Table. Primers and probe used for diagnostic PCR and primers used to sequence Saffold virus, 2009*

Assay, primer	Type	Sequence, 5′ → 3′
Diagnostic RT-PCR		
Saffold F	Forward primer	CTA WCA TGC CTC CCC GAT T
Saffold R	Reverse primer	GYT TAG ACC GGG GGA ACC
Saffold Probe	Probe	TTT CTG CCC TGC TGG GCG G
VP2 typing		
SAFV VP2 OF	Outer forward primer	GAR ATG ACY AAY CTB TCW GAY AGA GT
SAFV VP2 OR	Outer reverse primer	CCR TTR AAN ACS GGY TTN AC
SAFV VP2 IF	Inner forward primer	CGG CYA YAA ACA CKC AAT C
SAFV VP2 IR	Inner reverse primer	TTD GCR TGY TGN GTC CA
VPI typing, type 2 specific		
SAFV-2 VP1 OF1	Outer forward primer 1	GCA GAA AAA GGA AAG GTT GC
SAFV-2 VP1 OF2	Outer forward primer 2	GCA GAA AAA GGC AAA GTT GC
SAFV-2 VP1 OR	Outer reverse primer	TCT TGG RCA AAA CAC TCT CA
SAFV-2 VP1 IF	Inner forward primer	CYA TAG CTC TTC CTG AAA AYC A
SAFV-2 VP1 IR	Inner reverse primer	TGR ACC GAA AAY CTG TCT GC

*RT-PCR, real-time reverse transcription PCR; SAFV, Saffold virus: VP, viral protein.

known history of disease or symptoms. During necropsy, signs of cerebral herniation were detected. A small vascular malformation surrounded by edema was found in the brain. No signs of encephalitis or hemolytic uremic syndrome were found.

The following sample materials were collected: CSF, blood, feces, myocardium, pericardial aspirate, lung tissue, and respiratory secretions (online Appendix Table). In the CSF, mononuclear pleocytosis was noted. Results of routine bacteriologic culture found coagulase-negative staphylococci in the CSF, a few nonhemolytic streptococci in the lung tissue, and a few nonhemolytic streptococci and a few *Staphylococcus aureus* organisms in the pericardial aspirate. Verotoxin-producing *Escherichia coli* was cultured from the fecal sample. The conclusion of the autopsy and laboratory findings was that cerebral herniation was the immediate cause of death.

Virus from each of the 2 children was characterized by sequencing part of the VP1 region of the capsid gene, and the sequences were compared with those of other SAFVs detected in fecal samples from the patients with gastroenteritis (Figure 2). Of the 48 fecal sample pools, 10 were positive for SAFV by PCR. From these pools, 6 individual samples were available for further testing; the VP2 capsid region was successfully sequenced for 4 of these 6 samples, and they were all SAFV type 2. Later, an SAFV type 2–specific VP1 PCR was designed, which provided VP1 sequences from all 6 fecal samples and samples from the 2 children reported here: the fecal sample from child 1 and the blood sample from child 2. The phylogenetic analysis (Figure 2) showed that all viruses were SAFV type 2 and that the sequences arranged in 2 clusters with 8% nt differences and 5% aa differences between the clusters.

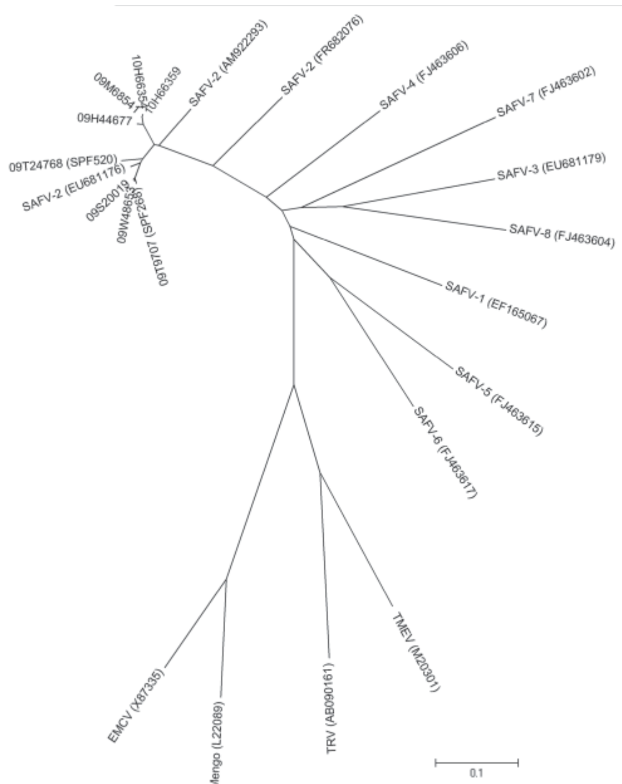

Figure 2. Phylogenetic tree showing partial viral protein 1 capsid sequences (588 nt) from the Saffold virus strains identified from 2 children from Denmark, 2009, and representative strains from other cardioviruses. The tree was constructed by the neighbor-joining method by using MEGA4 software (www.megasoftware.net). Scale bar indicates nucleotide substitutions per site. SAFV, Saffold virus; TMEV, Theiler's murine encephalomyelitis virus; TRV, Thera virus; EMCV, encephalomyocarditis virus.

Discussion

Several novel viruses have recently been discovered by using microarrays or mass-sequencing methods (*6*,*21*). Almost all of these viruses have been found in noninvasively collected patient materials, making the correlation to clinical disease difficult to establish. For the picornavirus group, this issue is further complicated by knowledge that this virus family can cause a wide variety of diseases with a high proportion of nonspecific symptoms or asymptomatic infections (*5*).

We report 2 cases of invasive infection with SAFV type 2 in children. For each child, SAFV was detected in at least 1 compartment other than the central nervous system. This finding strengthens the evidence of an acute infection as the cause of clinical disease. In child 1, SAFV virus was found in the CSF and a fecal sample. In child 2, findings were even more convincing because SAFV was found in 3 invasively collected samples: CSF, myocardium, and blood. No other credible cause of infection was found for either of the 2 children. In child 1, the only other positive finding was parechovirus type 3 in a fecal sample taken 2 weeks after onset of disease. Parechovirus was not found in the CSF and therefore seems unlikely as the cause of the acute symptoms. From child 2, several types of bacteria were identified, but these seem unlikely to be the cause of death because culture of postmortem samples often grows commensal organisms. The bacteria from this child were not found consistently in the tissue samples, thus making systemic bacterial infection less likely. Verotoxin-producing *E. coli* also seems an unlikely cause of death because no diarrhea or signs of hemolytic uremic syndrome were present before the child's death or at autopsy.

At autopsy of child 2, a small vascular malformation surrounded by edema was found. This edema could be the cause of the herniation. However, the edema might also have been caused by cerebral infection or septicemia. This possibility is supported by the finding of mononuclear

cells in the spinal fluid and the direct virus identification in the CSF and blood. The exact cause of death and whether there is a connection between the infection and the changes surrounding the malformation are unclear. However, our investigations show that before her death, the child's blood contained SAFV.

Child 1 had monosymptomatic ataxia preceded by 1 day of fever. The symptoms receded over the next few months, and the patient recovered fully. Child 2 died without any preceding symptoms or any known predisposing factors; this clinical picture is sometimes found for patients with enteroviral infections (*24*).

The finding of SAFV in invasively collected samples from the 2 children described here fits well with the knowledge about the picornavirus group. SAFV probably behaves similarly to the viruses in the enterovirus group, producing mainly asymptomatic infections but also producing nonspecific symptoms in other patients and severe disease in a few patients. Enteroviruses are known to cause many more or less organ-specific diseases such central nervous system infection (meningitis, encephalitis, and myelitis), myocarditis, enanthema, exanthema, and septicemia. These 2 cases fit well within the expected range of diseases attributed to picornaviruses, but further studies are needed to determine the exact correlation of SAFV to disease in humans.

In a previous study, Chiu et al. looked for SAFV in 400 CSF samples but found none (*21*). Lack of detection could be explained by different assay sensitivities or by the selection of samples tested. In their study, patient selection was based on neurologic disease and patient ages were not reported.

Our study has also shown that Saffold virus type 2 circulated in Denmark in 2009, the same year that the children reported here became ill. Other studies have shown type 2 to be a common type of SAFV and to be circulating worldwide (*13*,*15*,*16*,*18*,*20*,*21*,*23*). Because SAFVs are single-stranded RNA viruses, a nucleotide variation of 8% among the restricted number of samples in this study is expected.

In conclusion, we have established SAFV virus as a cause of invasive infection and a highly probable cause of severe disease in children. More studies are needed to further illuminate the role of SAFV as a human pathogen.

Dr A.C.Y. Nielsen is a medical doctor pursuing a PhD at the Department of Virology at Statens Serum Institut in Denmark, where he is researching the occurrence and clinical relevance of new human picornaviruses. Other research interests include microbiology (especially virology) and infectious diseases.

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Modeling Insights into *Haemophilus influenzae* Type b Disease, Transmission, and Vaccine Programs

Michael L. Jackson,[1] Charles E. Rose, Amanda Cohn, Fatima Coronado, Thomas A. Clark, Jay D. Wenger, Lisa Bulkow, Michael G. Bruce, Nancy E. Messonnier, and Thomas W. Hennessy

In response to the 2007–2009 *Haemophilus influenzae* type b (Hib) vaccine shortage in the United States, we developed a flexible model of Hib transmission and disease for optimizing Hib vaccine programs in diverse populations and situations. The model classifies population members by age, colonization/disease status, and antibody levels, with movement across categories defined by differential equations. We implemented the model for the United States as a whole, England and Wales, and the Alaska Native population. This model accurately simulated Hib incidence in all 3 populations, including the increased incidence in England/Wales beginning in 1999 and the change in Hib incidence in Alaska Natives after switching Hib vaccines in 1996. The model suggests that a vaccine shortage requiring deferral of the booster dose could last 3 years in the United States before loss of herd immunity would result in increasing rates of invasive Hib disease in children <5 years of age.

Routine use of *Haemophilus influenzae* type b (Hib) conjugate vaccines has dramatically reduced the incidence of Hib disease in children <5 years of age in numerous populations (*1–4*). Vaccination programs have also led to herd immunity through reduced Hib transmission, as shown by declines in the prevalence of oropharyngeal Hib colonization among vaccinated children and unvaccinated children and adults (*2,4–6*). However, even successful vaccination programs have not eliminated Hib colonization (*7,8*). Thus, the continued success of Hib control programs depends on maintaining age-appropriate Hib vaccine coverage. Such coverage can, however, be threatened by changes in vaccine supply, as indicated by the 2007–2009 Hib vaccine shortage in the United States (*9,10*).

To manage that shortage, the Centers for Disease Control and Prevention and partner organizations recommended that providers defer giving the 12–15-month booster dose to all children except those at high risk for invasive Hib disease (*9*). This recommendation was based on expert opinion about the predicted effects of a shortage initially expected to last <9 months (*9*). When it became clear that the shortage would last longer, we sought to develop a model of Hib transmission and disease to predict the effects of continued booster dose deferral and to guide vaccine policy. Such a model could also be useful for optimizing the introduction of Hib vaccines into new populations. Furthermore, it could provide insights into the dynamics of Hib transmission and colonization, which would inform the uncertainty over the types of Hib vaccines that are most appropriate for populations at high risk for invasive Hib disease, such as Alaska Natives (*11*). We present the model and show its application to various populations and vaccination scenarios.

Methods

Model Structure, Parameters, and Starting Conditions

We developed an age-structured mathematical model to describe Hib transmission, colonization, and disease (Figure 1). The model assumes that populations can be

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divided into mutually exclusive states on the basis of age, Hib antibody levels (high, low, and none), and Hib infection status (susceptible, colonized, and diseased), with an additional state (immune) for infants passively immunized with bacterial polysaccharide immunoglobulin. This model can be expressed as a set of partial differential equations (online Technical Appendix 1, wwwnc.cdc.gov/EID/pdfs/11-0336-Techapp1.pdf), with rate parameters governing the movement of the population between model states. As an example, the age-specific force of infection ($\lambda(a)$) is the rate at which susceptible persons of age a become colonized. We set values for the rate parameters by using published and unpublished data on birth and death rates, Hib colonization and incidence, and Hib vaccine uptake and effectiveness (online Technical Appendix 2, wwwnc.cdc.gov/EID/pdfs/11-0336-Techapp2.pdf).

We tested the model in 3 populations: persons in the United States as a whole; England and Wales; and Alaska Natives (defined as the indigenous residents of Alaska). These populations reflect major diversity in Hib epidemiology and vaccine policy (*1,3,4*). In the United States, Hib conjugate vaccines were first recommended in 1988 as a single dose for children 18 months of age, and in 1991, they were recommended as a primary series starting at 2 months of age, with a booster dose at 12–15 months. In England and Wales, Hib conjugate vaccines were introduced in 1992 as a primary series starting at 2 months of age and a 1-time catch-up campaign for children ≤4 years of age.

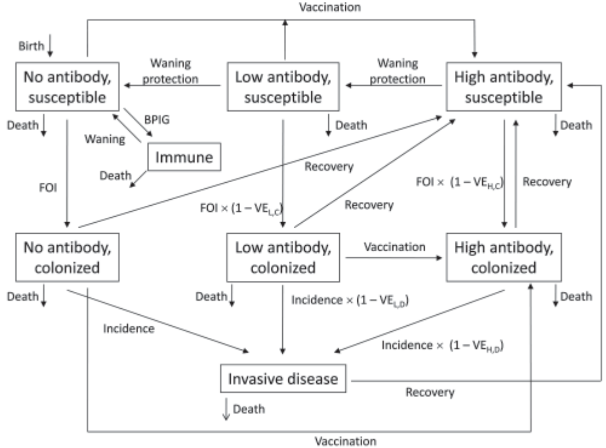


Figure 1. Structure of *Haemophilus influenzae* type b (Hib) simulation model. Persons are born into the no-antibody, disease-susceptible state and can die in any of the model states. Hib-susceptible persons become colonized based on the force of infection (FOI), which is reduced by protection from low ($VE_{L,C}$) or high ($VE_{H,C}$) antibody levels. Persons colonized with Hib develop invasive disease, which is reduced by protection from low ($VE_{L,D}$) or high ($VE_{H,D}$) antibody levels. Colonized and diseased persons recover to the high-antibody, disease-susceptible state. As immunity wanes, susceptible persons move from high to low antibody and from low to no antibody. Some persons are vaccinated and move from no or low antibody states to the high antibody state. For the Alaska Native population, use of bacterial polysaccharide immunoglobulin (BPIG) starting at birth temporarily moves persons to an immune state; as BPIG wanes, immune persons return to the susceptible state. See Technical Appendix 1 (wwwnc.cdc.gov/EID/pdfs/11-0336-Techapp1.pdf) for a formal description of the model structure.

We began the simulations for the US and Alaska Native populations in 1980 and for the England and Wales population in 1985. We used census data to determine the size and age structure of each population. In the starting year, we divided the populations among the model states so that Hib transmission was in or nearly in equilibrium. Modeling and subsequent analyses were all implemented by using SAS version 9.2 (SAS Institute Inc., Cary NC, USA).

Evaluating Model Fit

We verified model fit by using pseudo-R^2 to compare the age-specific point prevalence of Hib colonization from the model with observed prevalence data in the time period before vaccine introduction (*4,12–14*). In a similar manner, we compared the annual incidence rate of invasive Hib among children <5 years of age from the simulated populations with observed incidence data (*1,3,4,15–23*).

Effects of Vaccine Shortage in the United States

Before the 2007–2009 vaccine shortage, Merck & Co., Inc. (Whitehouse Station, NJ, USA), and Sanofi Pasteur (Bridgewater, NJ, USA) were licensed to produce Hib vaccines for the United States. The shortage was triggered when Merck recalled certain lots of their Hib vaccine and suspended vaccine production. In Merck's Hib vaccine, the Hib polyribosylribitol phosphate (PRP) polysaccharide is conjugated to *Neisseria meningitidis* outer membrane protein (OMP). PRP-OMP conjugate vaccines induce a strong immune response with a first dose at 2 months of age and are given as a 2-dose primary series (*24*). In Sanofi Pasteur's vaccine, PRP is conjugated to tetanus toxoid (T). For the primary series, PRP-T vaccines achieve antibody titers comparable to those achieved by PRP-OMP vaccines, but PRP-T vaccines require a 3-dose primary series (*24*). *Haemophilus* b conjugate (HbOC) vaccine, a third Hib vaccine formerly used in the United States, couples PRP oligosaccharides to CRM_{197} (cross-reacting material 197, a nontoxic mutant of diphtheria toxin). HbOC vaccines have immunogenic properties similar to those for PRP-T and require a 3-dose primary series (*24*).

For the US population, we modeled the effect of an extended vaccine shortage to explore what might have happened if the shortage had lasted >18 months (*10*). We first ran the model assuming that vaccine coverage from 2008 onward remained the same as that in 2007 (a complete series scenario). In this scenario, 50% of vaccinated children were assumed to receive PRP-OMP vaccine and 50% PRP-T vaccine, as determined by Merck and Sanofi Pasteur's preshortage Hib vaccine market shares. We then

ran the model assuming that the booster dose was deferred for all children starting in 2008 (a no-booster scenario) and that all vaccinated children received PRP-T. Last, we ran the model assuming that the booster dose was deferred starting in 2008 and that primary series coverage decreased by 10 percentage points, as suggested by some coverage surveys during the shortage (*25*) (a no-booster minus scenario). Again, all children were assumed to receive PRP-T. We compared annual incidence of invasive Hib in children <5 years of age under these 3 scenarios.

For the Alaska Native population we modeled the effect of switching Hib vaccines starting in 2010. During June 1991–1995 and from July 1997 onward, Alaska Native populations received PRP-OMP; during January 1996–June 1997, they received HbOC vaccines. Hib incidence in Alaska Native children rose in 1996–1997 when HbOC was used, prompting a switch back to PRP-OMP (*4*). During the 2007–2009 shortage, PRP-OMP vaccines from the Strategic National Stockpile were used for Alaska Natives (*9*). If Merck had not returned its vaccine to the market as expected, Alaska Natives would eventually have had to switch to PRP-T vaccines. To predict the effects of this switch, we compared predicted incidence in children <5 years of age from 2 models: 1 model assumed that PRP-OMP continued to be used from 2010 onward, and the other model assumed that PRP-T was used starting in 2010. We also modeled the effect of 1-time PRP-T booster campaigns, which occurred in conjunction with the switch to PRP-T, for all children 1–4 or 5–10 years of age.

Alternative Approaches to Vaccine Introduction

This model can also be used to explore strategies for introducing Hib conjugate vaccines to new populations. To illustrate this strategy, we modeled hypothetical vaccination programs in 2 populations with the age distribution and transmission patterns of the United States or of the Alaska Native population.

We compared predicted Hib incidence in children <5 years of age in 4 vaccination scenarios in the hypothetical populations: 1) a primary series starting at 2 months of age and a booster dose at 12–15 months of age; 2) only a primary series starting at 2 months of age; 3) only a single dose at 12–15 months of age; and 4) a primary series at 2 months of age and a 1-time catch-up campaign for children <5 years of age. We assumed the strategies used PRP-T for all vaccine doses, with 90% vaccine coverage achieved within 3 years of vaccine implementation.

Sensitivity Analyses

All model parameters taken from the literature are estimates based on samples of the population, and these estimates have some degree of uncertainty. We conducted detailed sensitivity analyses to determine whether our model conclusions would differ had we used different parameter values (online Technical Appendix 3, wwwnc.cdc.gov/EID/pdfs/11-0336-Techapp.pdf). We ran the model 10,000 times, each time randomly varying 3 parameters, and we looked for individual parameters and combinations of parameters that caused major differences between observed and modeled incidence in children <5 years of age. To test the effect of the rate of recovery from colonization, we also refit the model and ran several vaccination scenarios under extreme values for this parameter.

Results

Model Fit

The model accurately reproduced the observed prevalence of carriage by age group before vaccine introduction for the United States as a whole (pseudo-R^2 0.74) and for Alaska Natives (pseudo-R^2 0.98), the 2 populations for which carriage data were available. The model also accurately reproduced the observed annual incidence of invasive Hib in children <5 years of age in the United States (pseudo-R^2 0.97), in England and Wales (pseudo-R^2 0.91), and among Alaska Natives (pseudo-R^2 0.90) (Figure 2). Of note, the model captured the rise in Hib incidence in the United Kingdom beginning in 1999 and

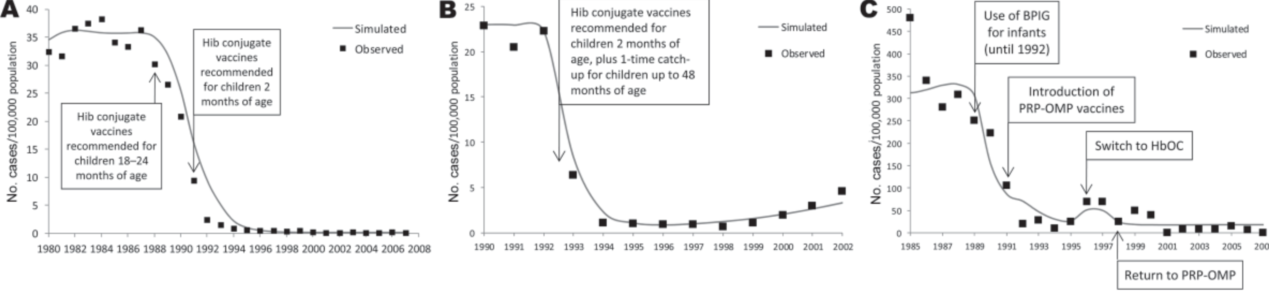


Figure 2. Observed and simulated incidence of invasive *Haemophilus influenzae* type b in children <5 years of age in 3 populations: (A) United States; (B) England and Wales; (C) Alaska Natives. PRP-OMP, Hib polyribosylribitol phosphate polysaccharide conjugated to *Neisseria meningitidis* outer membrane protein; HbOC, *Haemophilus* b conjugate vaccine; PRP-T, polyribosylribitol phosphate polysaccharide conjugated to tetanus toxoid.

the rise in invasive disease among Alaska Natives that was associated with the switch to HbOC vaccines in 1996/1997.

Force of Infection

For the United States and England and Wales, the best-fit force of infection suggests that Hib transmission before introduction of vaccine was driven by children 2–4 years of age (Table). Persons of all ages in both populations are primarily colonized through contact with children in this age group. For example, in the United States population, the annual force of infection on children <2 years of age was 36.3 infections/1,000 children, of which 24.3 (66.9%) were caused by children in the 2- to 4-year-old age group.

Furthermore, the model suggests that the dynamics of Hib transmission are different in Alaska Native populations than in the other 2 modeled populations. In Alaska Native populations, most Hib transmission before introduction of vaccine came through contact with children 5–9 rather than 2–4 years of age (Table). A stronger element of assortative mixing was also present, in that children <2 years of age acquired infection from other children <2 years of age, and persons ≥10 years of age acquired infection from other persons ≥10 years of age.

Model Predictions of Possible Effects of Hib Vaccine Shortage

If the Hib vaccine shortage and deferral of the 12–15 month booster dose in the United States extended indefinitely, the model predicts relatively little change in the incidence of invasive Hib in children <5 of age for the first 3 years under either shortage scenario (Figure 3, panel A). Beginning in 2011, the model predicts that Hib incidence would increase more substantially in the no-booster shortage scenario (from 0.14 cases/100,000 children in 2007 to 0.72/100,000 in 2012 and 5.7/100,000 by 2020), with slightly greater increases in the no-booster minus shortage scenario.

If Alaska Native populations would have had to switch from PRP-OMP to PRP-T vaccine, the model predicts that the incidence of Hib in children <5 years of age would more than double (from 17.9 cases/100,000 children in 2009 to 46.2/100,000 in 2011) (Figure 3, panel B). Given that children 5–9 years of age appear to drive transmission in Alaska Native populations, we modeled the effect of adding a 1-time vaccination campaign for children 5–9 years of age in 2010 to the switch from PRP-OMP to PRP-T vaccine. This model predicts that such a vaccination campaign would keep the incidence of Hib below that for the PRP-T vaccine scenario for 8 years (Figure 3, panel B). The effect of a 1-time booster campaign for children 1–4 years of age was similar (Figure 3, panel B).

Alternative Approaches to Vaccine Introduction

In a hypothetical population with the age distribution and transmission patterns of the United States, the most effective strategy for vaccine introduction would have been to introduce the vaccine as a primary series plus a booster at 12–15 months of age (Figure 4, panel A). Using PRP-T vaccines, we found that this strategy resulted in a rapid decline in incidence and the lowest equilibrium incidence, i.e., 0.22 cases/100,000 children <5 years of age. A strategy of offering only 1 dose of vaccine at 12–15 months of age, without a primary series, was predicted to have nearly as great an effect on Hib incidence, with an equilibrium

Table. Estimated annual prevaccination force of infection from Hib infectious persons to persons with susceptible, no-antibody status and estimated annual prevalence of Hib colonization in 3 modeled populations, stratified by age group*

Susceptible population, age group, y	Hib infections caused by infectious persons/1,000 susceptible persons, by age group, y				
	0–1	2–4	5–9	≥10	Total no. cases
United States					
0–1	0.2	24.3	11.6	0.2	36.3
2–4	0.1	77.4	1.4	0.3	79.2
5–9	10.1	136.2	15.5	2.0	163.8
≥10	7.0	78.5	9.2	0.8	95.5
Prevalence	1.1%	2.9%	5.3%	3.2%	NA
England and Wales					
0–1	0.6	15.4	10.7	1.7	28.4
2–4	3.1	62.8	11.2	1.9	79.0
5–9	10.1	133.6	16.8	1.9	162.5
≥10	6.6	78.4	8.4	2.4	95.8
Prevalence	1.0%	3.0%	4.9%	3.0%	NA
Alaska Natives					
0–1	109.5	5.8	52.3	1.2	168.8
2–4	28.4	15.8	49.2	5.4	98.8
5–9	28.9	144.3	357.8	5.9	536.9
≥10	28.4	21.7	80.3	64.7	195.1
Prevalence	5.2%	3.9%	9.9%	4.5%	NA

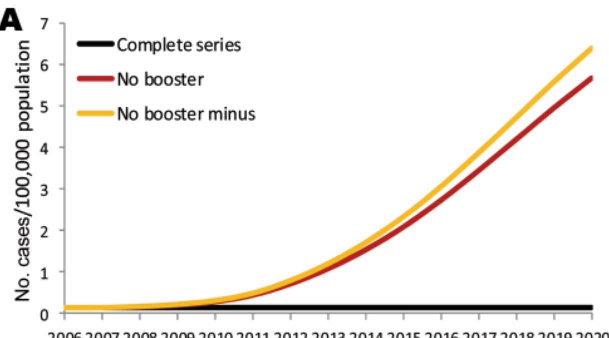
*Values are no. infections except as indicated. Data are based on Hib simulation model. Hib, *Haemophilus influenzae* type b; NA, not applicable.

incidence of 0.47 cases/100,000 children <5 years of age. Strategies using a primary series only or a primary series with a 1-time catch-up campaign were much less effective, resulting in an equilibrium incidence of 11.0 cases/100,000 children <5 years of age.

In a hypothetical population with the age distribution and transmission patterns of Alaska Natives, the most effective strategy with PRP-T vaccines again would have been vaccinating with a primary series and a booster dose at 12–15 months, which yielded an equilibrium incidence of 50.4 cases/100,000 children <5 years of age (Figure 4, panel B). Both the primary series alone and the primary series with a 1-time catch-up yielded equilibrium incidence rates of 136.2 cases/100,000 children <5 years of age. As with the US population, the strategy of a single dose at 12–15 months of age was superior to a primary series alone, with or without a 1-time catch-up. However, the equilibrium incidence of 101.7 cases/100,000 children <5 years of age was substantially higher than that for the primary plus booster strategy.

Sensitivity Analyses

We found that the model was robust to variations in all parameters except the mean rate of recovery from colonization (online Technical Appendix 3). To see whether conclusions about Hib epidemiology from our model would differ on the basis of the value of this recovery rate, we tried fitting the model assuming a fast and slow recovery rate. The results showed that our conclusions about Hib dynamics and the effect of vaccination programs would be unchanged even under extremely different values for the mean rate of recovery from carriage.



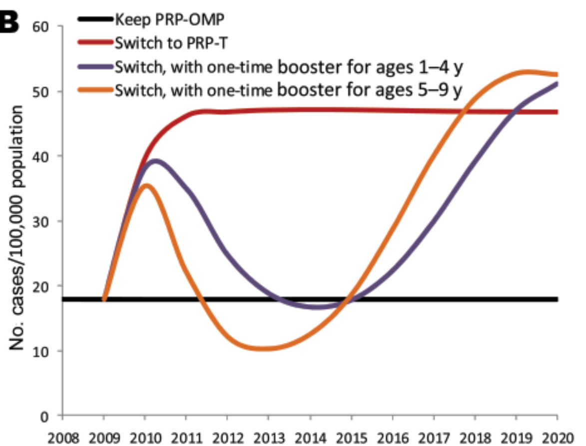


Figure 3. Predicted effects of extended *Haemophilus influenzae* type b (Hib) vaccine shortage on the incidence of invasive Hib disease in the United States (A) and of switching from PRP-OMP to PRP-T vaccine in the Alaska Native population (B). See text for complete description of shortage scenarios. PRP-OMP, Hib polyribosylribitol phosphate polysaccharide conjugated to *Neisseria meningitidis* outer membrane protein; PRP-T, polyribosylribitol phosphate polysaccharide conjugated to tetanus toxoid.

Discussion

We have developed a flexible model of Hib transmission and disease that can be applied to multiple contexts. This model can account for many essential features of Hib epidemiology, including the rapid decline in Hib incidence in the United States after vaccine introduction; the rise in Hib incidence in the United Kingdom 7 years after the catch-up campaign; and the increase in Hib incidence among Alaska Native populations when vaccine was switched from PRP-OMP to HbOC in 1996.

Our model suggests several essential insights into the epidemiology of Hib and into the design of Hib vaccination programs. First, our model suggests that in the United States and England and Wales, Hib transmission is driven by children 2–4 years of age. This is in contrast to prior Hib simulation models, which have suggested that transmission to persons of a given age group primarily occurs from others of the same age group (assortative mixing) (*26–28*). One model suggests that adults also play a major role across age groups (*27*). However, those transmission patterns do not explain the rapid decline in Hib incidence in the United States in 1988–1990, when Hib conjugate vaccine was only being offered to children 18–24 months of age. During that time, incidence declined even among children <1 year of age, an effect that is only possible if children ≥18 months of age are major drivers of Hib transmission.

Second, our model suggests that Hib transmission dynamics differ across populations. Unlike the findings from the best-fit model for the United States and England and Wales, the best-fit model for the Alaska Native population suggests that transmission is mainly driven by children 5–9 years of age, with some element of assortative mixing. These differences have major consequences for the design of Hib vaccination programs. For example, our model suggests that in the United States and England and Wales, giving 1 dose at 12–15 months of age would be nearly as effective, at a population level, as a full primary series plus a booster at 12–15 months of age.

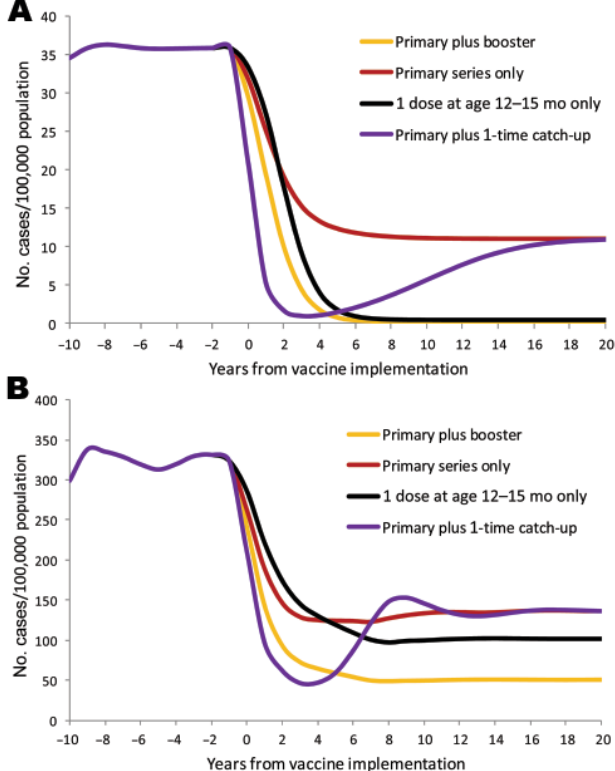


Figure 4. Predicted effects of different vaccination programs on the incidence of invasive *Haemophilus influenzae* type b disease, applied to a population with age structure and transmission dynamics like the United States population (A) and like the Alaska Native population (B).

In contrast, offering only a single dose at 12–15 months would be considerably less effective for the Alaska Native population. Furthermore, PRP-T vaccine was predicted to be much less effective than PRP-OMP vaccine for Alaska Native populations because in Alaska Natives, the force of infection is high even for young infants and PRP-OMP stimulates protective antibodies after the first dose at 2 months of age. A high force of infection in Alaska Native infants is consistent with the observed jump in Hib incidence in Alaska Natives in 1996–1997 with the switch to HbOC vaccine, which does not induce protective antibodies until the third dose at 6 months of age (*11*). Planning an optimal vaccination program should include some assessment of the Hib transmission dynamics in the target population. Our model can be used to estimate those dynamics from the age-specific prevalence of colonization and age-specific incidence of invasive Hib.

The World Health Organization recommends that all routine infant vaccination programs include conjugate Hib vaccines in infancy, with or without a booster later in life (*29*). Most countries with Hib vaccination programs are in line with these guidelines (*30*). Our study suggests 2 potential practical applications for the design of Hib vaccination programs. First, there may be populations for which a policy of a single dose at 12–15 months of age would reduce invasive Hib nearly as much as would a 3-dose primary series plus a booster. Furthermore, in some populations a single dose at 12–15 months may reduce Hib disease more than a 3-dose primary series without a booster. Additional exploration of the potential utility of a single dose of Hib conjugate vaccine at 12–15 months of age as a complete routine immunization schedule is needed. Second, countries planning to add Hib vaccines to their routine immunization programs could apply this model to local or regional data on Hib disease and colonization to characterize the potential effect of vaccination regimens under consideration.

Our study has a few limitations worth highlighting. First, as with all models, ours necessarily simplifies the underlying reality. We combined all persons ≥10 years of age into a single group because Hib colonization and incidence data were insufficient to reliably model more age groups within this broad category. The estimated transmission dynamics for this age group thus represent an average of adolescents and adults and may mask heterogeneity between these groups. Second, a model is only as good as the source data; if the estimates of model parameters from the literature are inaccurate, our model may be inaccurate. We conducted extensive sensitivity analyses to explore this (online Technical Appendix 3), and found that the model is robust to variation in most parameters. The exception is the rate of recovery from Hib colonization. This finding makes sense because duration of infectiousness is a major determinant of disease transmission. However, we are reassured that this sensitivity does not affect our conclusions because using widely varied values for this rate does not change the basic model conclusions. Third, we assume that immunity following natural infection is the same as immunity following vaccination, while, in reality, natural infection may induce longer-lasting protection. Our model could reproduce Hib incidence in England and Wales more accurately than a model that used different parameters for vaccine-induced versus natural immunity (*28*). This suggests the difference in protection between vaccination and natural infection may not be epidemiologically essential; however, exploring this is a topic for future research.

A strength of our study is that our model is complex enough to successfully model Hib in a variety of populations yet simple enough that the transmission parameters can be estimated from relatively limited carriage and incidence data. A second strength is that, like Leino et al. (*27*), we used an iterative process to refine initial estimates of the transmission parameters. This process enables greater

flexibility than that available when constraining the matrix to certain combinations of parameters (*26*) or choosing initial values by hand without further refinement (*28*).

Our Hib simulation model can be a useful tool for public health planners in countries that are considering implementing Hib vaccination programs and for countries that must respond to Hib vaccine shortages. The model suggests the importance of young children in the transmission of Hib, the need for a dose at 12–15 months of age to maintain herd immunity against Hib disease, and the importance of evaluating Hib transmission dynamics for optimizing vaccine programs.

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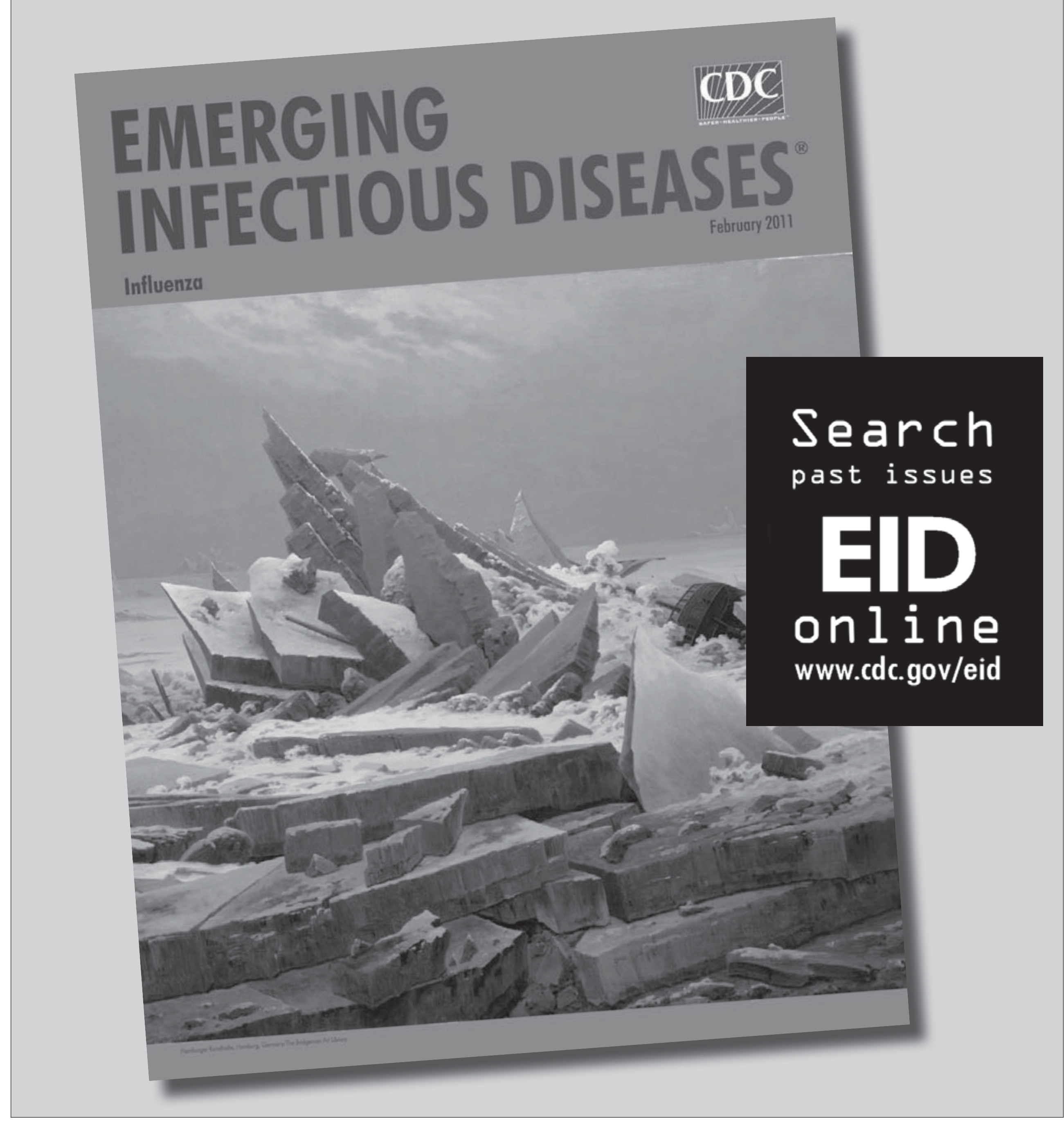

Assessing Prion Infectivity of Human Urine in Sporadic Creutzfeldt-Jakob Disease

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Prion diseases are neurodegenerative conditions associated with a misfolded and infectious protein, scrapie prion protein (PrP^{Sc}). PrP^{Sc} propagates prion diseases within and between species and thus poses risks to public health. Prion infectivity or PrP^{Sc} presence has been demonstrated in urine of experimentally infected animals, but there are no recent studies of urine from patients with Creutzfeldt-Jakob disease (CJD). We performed bioassays in transgenic mice expressing human PrP to assess prion infectivity in urine from patients affected by a common subtype of sporadic CJD, sCJDMM1. We tested raw urine and 100-fold concentrated and dialyzed urine and assessed the sensitivity of the bioassay along with the effect of concentration and dialysis on prion infectivity. Intracerebral inoculation of transgenic mice with urine from 3 sCJDMM1 patients failed to demonstrate prion disease transmission, indicating that prion infectivity in urine from sCJDMM1 patients is either not present or is <0.38 infectious units/mL.

Prion diseases, a group of neurodegenerative disorders affecting humans and animals, have received considerable attention largely because of their intriguing pathogenetic mechanism and the threat they pose to public health because of their insidious infectivity. Despite their heterogeneity, all classic prion diseases are characterized by the presence of an abnormal isoform of the normal cellular prion protein (PrP^{C}), which predominantly accumulates in the central nervous system (*1*). The abnormal isoform, identified as scrapie PrP or PrP^{Sc}, is thought to form from a posttranslational change in conformation of PrP^{C}. Prion diseases can also be transmitted by an infectious mechanism because exogenous PrP^{Sc} can impose its conformation on to the host's PrP^{C} through a PrP^{Sc}-templated conversion process (*1*).

The most common form of human prion disease, Creutzfeldt-Jakob disease (CJD), can be sporadic, inherited, or acquired by infection; the sporadic form alone accounts for the great majority of all the cases of CJD (*2*). Following protease digestion, the unglycosylated form of PrP^{Sc} exhibits the electrophoretic mobilities of either 21 kDa or 19 kDa (*3*). These 2 PrP^{Sc} isoforms, named PrP^{Sc} types 1 and 2, respectively, along with the methionine/valine polymorphism at codon 129 of the PrP gene, led to the current classification of sporadic CJD (sCJD) in 5 phenotypically distinct subtypes (*2*,*3*). The sCJDMM1 subtype (affecting persons homozygous for methionine at codon 129 and carrying the PrP^{Sc} type 1) accounts for ≈70% of all cases of sCJD and unquestionably is the most prevalent type of human prion disease (*2*,*3*).

PrP^{Sc} is generally considered the major, if not the sole, component of the infectious agent in prion diseases (*1*,*4*,*5*). The presence of PrP^{Sc}, prion infectivity, or both has been found in several tissues and organs outside the central nervous system in prion-affected humans and animals (*6*–*10*). These findings have caused mounting concerns regarding the risk for human transmission of the disease from a variety of sources, including the consumption of prion contaminated meat and other animal products, the use of contaminated surgical instruments and medicinal

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products, and the exposure to waste from infected humans and animals. In this context, body fluids such as saliva, milk, and urine have received particular attention as they may support horizontal transmission and environmental contamination, which in turn may contribute to the propagation of ovine scrapie and chronic wasting disease (CWD) of cervids. Indeed, saliva has been reported to be a source of prion in scrapie-infected sheep and CWD-infected deer (*11*–*14*), whereas milk has been found to contain prions in scrapie-infected sheep (*15*,*16*).

The detection of PrP^{Sc} by immunoblotting has been previously reported in urine of prion-affected hamsters and humans (*17*). However, this observation has not been confirmed in 3 subsequent studies, which instead have suggested that the original immunoblot finding resulted from nonspecific cross-reaction either with contaminating bacterial proteins (*18*) or urinary IgG fragments (*19*,*20*). Recently, prion infectivity has been detected in urine from experimentally prion-infected animals, including hamsters (*21*,*22*), deer (*13*), and mice, in association with lymphocytic nephritis (*23*). However, infectivity in urine from naturally prion-affected animals has never been reported.

It is difficult to extrapolate the animal data on urine infectivity to human urine, especially for sCJD, because this form of prion disease is believed to start spontaneously in the brain rather than being caused by exogenous infection. Nevertheless, the possibility that PrP^{Sc} is indeed present in urine of sCJD patients exists because small quantities of PrP^{Sc} have been identified in peripheral organs of sCJD patients (*6*,*7*,*10*). Furthermore, we have recently shown that normal urine contains discrete amounts of a C-terminal fragment of PrP, matching the so-called C1 fragment but not full-length PrP (*24*). Although C1 may not be a good substrate for PrP^{Sc} replication, and its conversion to PrP^{Sc} has never been reported, the possibility of conversion may not be ruled out (*25*,*26*).

The presence of prions in urine of CJD patients would obviously pose serious risks relating to the medicinal use of urine-extracted proteins, hormones, and urokinase as well as collection and disposal of patient urine. Indeed, it has recently been reported that fragments of PrP, consistent with the urine PrP present in normal urine described above, co-purify with urine-derived gonadotropins (*24*,*27*). This finding has prompted the claim that PrP^{Sc} may also co-purify with urinary gonadotropins (*27*).

With the exception of early failed attempts to transmit prion disease to rodents and nonhuman primates with urine from CJD patients (*28*,*29*), no investigation on prion infectivity of human urine has been reported. We searched for prion infectivity in urine obtained from patients with sCJDMM1, the most common form of sCJD, by bioassay that used transgenic (Tg) mice expressing human PrP (*30*).

Materials and Methods

Patients

Samples from 4 patients (patients 1–4) affected by typical sCJDMM1 that was histologically and immunochemically confirmed (with no clinical signs of inflammatory kidney disease) were provided by the National Prion Disease Pathology Surveillance Center. The patients were 54, 68, 60, and 69 years of age with disease durations of ≈2, 2, 3, and 2 months, respectively (*2*). Approvals from the Institutional Review Board and the Institutional Animal Care and Use Committee were obtained.

Urine

Urine samples from sCJDMM1 patients 1–3 collected 2 weeks, 1 month, and 1 week before death, and from 3 healthy controls, were concentrated 100-fold by ultrafiltration with Centricon Plus-70 (Millipore, Billerica, MA, USA) (10-kDa cutoff) by using a Beckman (Miami, FL, USA) centrifuge at 3,000 × *g*. Concentrated urine was dialyzed against phosphate-buffered saline (PBS) (4 L each time) at 4°C by using the Pierce Slide-A-Lyzer cassette (Thermo Fisher Scientific, Inc., Rockford, IL, USA) (10-kDa cutoff) with 2 additional changes over 2 days.

Brain Homogenates and Microsomal Fractions

Brain homogenates (BH) from inoculated mice (10%, wt/vol) were prepared at 4°C in PBS cleared by centrifugation at 1, 000 × *g* for 5 min. Microsomal fractions (MFs) were prepared from the frontal cortex of the sCJDMM1 patients as described (*31*). BH (10%, wt/vol) in PBS were centrifuged at 700 × *g* for 10 min at 4°C. The supernatant was collected and the pellet, resuspended in PBS at 30% (wt/vol), was centrifuged as above. This supernatant, pooled with the previous one, was recentrifuged at 10,000 × *g* for 7 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C. The final pellet, representing the MF, was resuspended in PBS at 10% (wt/vol). Transgenic mice expressed full-length human PrP-129M at wild type level in mouse PrP null background, Tg(HuPrP-129M)$Prnp^{0/0}$ (Tg40) (*30*).

Effect of Concentrated and Dialyzed Urine on Prion Infectivity

MF of sCJDMM1 patient 4 was spiked in 100× concentrated and dialyzed normal urine. The sample was 10-fold serially diluted and intracerebrally injected into Tg40 mice.

Determination of Infectivity Loss during Concentration and Dialysis

MF (100 μL) of sCJDMM1 patient 3 was spiked into normal urine (100 mL) either before or after 100×

concentration and dialysis. Subsequently, 30 µL of the differently processed urine samples, both containing an equivalent amount of 3 µL of MF, were intracerebrally injected into Tg40 mice. The infectivity titers of the 2 differently processed MF spiked urine samples were compared on the basis of their mean incubation times in Tg40 mice.

Inoculation

Thirty microliters of MF from sCJDMM1 patients 1–4 suspended in PBS or in 100× concentrated and dialyzed normal urine were intracerebrally injected into Tg40 mice (Tables 1–4). Infectivity of urine from 3 sCJDMM1 patients and 3 healthy subjects was assayed as above by inoculation of 30 µL of 100× concentrated and dialyzed urine; raw urine from sCJDMM1 patient 1 was also similarly bioassayed in 33 Tg40 mice. Mice were euthanized within 3 days of becoming symptomatic.

Infectivity Titration

Infectivity in MF from sCJDMM1 patients 1 and 4, the latter spiked and diluted in 100× concentrated and dialyzed normal urine, was estimated by endpoint dilution by using probit regression analysis (STATA version 8.2 software; StataCorp LP, College Station, TX, USA). Incubation periods (Figure 1, x-axis) of each mouse injected with known infectivity titers (Figure 1, y-axis) of sCJDMM1 patient 1, were plotted and the experimental points fitted either by linear or segmental linear regression curves (GraphPad Prism version 5.0b for Macintosh; GraphPad Software, San Diego, CA, USA), and the 2 models were compared by the extra sum-of-squares F test for determining the best model fitting the experimental points. The more complicated model (segmental linear regression) did not significantly ($p = 0.4033$) fit the experimental points better than the simpler linear regression model. The linear regression model was therefore selected for estimating the infectivity titers in brain preparations of sCJDMM1 patients 2 and 3, and PBS spiked with MF preparation from sCJDMM1 patient 4. Mean incubation periods of animals with positive test results were interpolated to the dose-incubation-period curve of patient 1 to estimate infectivity titers.

The amount of prion infectivity that is below the threshold of detectability of our bioassay but that might be present in the urine of the sCJDMM1 patients was estimated in infectious unit (IU) per milliliter with 95% confidence interval (CI) (rather than in 50% infectious dose [ID_{50}]) by assuming a Poisson distribution in the response variable (*32*). The total volume of native urine assayed in sCJDMM1 patients 1–3 combined was 84 mL when the 100-fold concentration was taken into account (0.03 mL × 10 mice each in patients 1 and 2 and 0.03 mL × 8 mice in patient 3 × 100-fold concentration). However, considering the 20-fold loss of prion infectivity caused by the use of 100-fold concentrated and dialyzed urine as carrier (see Results), the injected urine equivalents might be 20× lower, i.e., 4.2 (1.5 mL in patients 1 and 2 and 1.2 mL in patient 3). Urine infectivity in patient 1 was also estimated by assuming a Poisson distribution based on the volume of raw urine bioassayed from this patient which was 0.99 mL (30 µL × 33 mice).

Histopathologic and Prion Protein Immunohistochemical Analyses

Half brains fixed in formalin and immersed in 98% formic acid for 30 min were sliced into 4 coronal sections and processed for histologic and PrP immunohistochemical analyses (*30*). Aliquots of BH or sodium phosphotungstate (NaPTA)–precipitated samples, with or without treatment with proteinase K (PK) (specific activity 44 units/mg; Sigma Aldrich, St. Louis, MO, USA) at a concentration of 2 U/mL (45 µg/mL), were loaded onto 15% Tris-glycine sodium dodecylsulfate–polyacrylamide gels, subjected to electrophoresis, and immunoblotted with monoclonal antibody 3F4 (to PrP residues 109–112) (*9*,*33*).

Results

Prion Infectivity Titer of Brain Tissue from sCJDMM1

All mice inoculated with brain MF from the 4 sCJDMM1 patients showed brain histologic lesions, PrP^{Sc}

Table 1. Infectivity titers in microsomal fraction of 4 patients with sCJDMM1 infection*

sCJDMM1 inoculum	Brain† dilution	Incubation time, d , mean ± SEM	Distribution	Prion titer, ID_{50}/g tissue†
Patient 1	10^{-1}	256 ± 5	7/7	3.0×10^{6}
	10^{-2}	297 ± 18	4/4	
	10^{-3}	314 ± 15	5/5	
	10^{-4}	339 ± 16	5/5	
	10^{-5}	420, 437	2/5	
	10^{-6}	>681	0/5	
Patient 2	10^{-4}	382 ± 12	5/10	2.2×10^{6}
Patient 3	10^{-4}	348 ± 12	10/10	1.2×10^{7}
Patient 4	10^{-2}	267 ± 14	5/5	7.2×10^{6}

*sCJD, sporadic Creutzfeldt-Jakob disease; ID_{50}, 50% infectious dose.
†Brain tissue equivalent.

Table 2. Prion infectivity in microsomal fraction prepared from sCJDMM1 patient 4 and suspended in PBS or 100× concentrated and dialyzed urine*

Carrier	Brain† dilution	Incubation time, d, mean ± SEM	Distribution	Prion titer, ID_{50}/g tissue†
PBS‡	10^{-2}	267 ± 14	5/5	7.2×10^6
100× concentrated and dialyzed normal human urine	10^{-1}	262 ± 4	5/5	3.3×10^5
	10^{-2}	281 ± 7	5/5	
	10^{-3}	377 ± 35	6/6	
	10^{-4}	311, 335	2/4	
	10^{-5}	>793	0/6	
	10^{-6}	>702	0/6	

*sCJD, sporadic Creutzfeldt-Jakob disease; PBS, phosphate-buffered saline; ID_{50}, 50% infectious dose.
†Brain tissue equivalent.
‡Titer was the same as calculated in Table 1.

deposition patterns, and immunoblot profile of PK-resistant PrP^{Sc} observed following sCJDMM1 transmission (Figure 2). The prion infectivity titer in the MF obtained from sCJDMM1 patient 1 was 3.0×10^6 ID_{50} per gram of tissue equivalent as determined by endpoint dilution bioassay in Tg40 mice (Table 1; Figure 1). We then used incubation times of each mouse inoculated with different brain tissue equivalent dilutions to build up the dose-incubation period curve of Figure 1 (slope ± SE –0.022 ± 0.002; Y-intercept ± SE 10.69 ± 0.73; R^2 0.75; p<0.0001). Titers from brain MF of sCJDMM1 patients 2–4 were estimated by interpolating the mean incubation times of the inoculated Tg40 mice to the dose-incubation period curve of Figure 1. The results showed an infectivity titer between 2 and 12×10^6 ID_{50} per gram of brain tissue equivalent (Table 1). These data indicated that the frontal cortex of all 4 sCJDMM1 patients contained high and comparable prion infectivity titers.

Urine Concentration and Dialysis

To maximize the sensitivity of bioassay, urine samples were concentrated 100× by ultrafiltration and dialyzed against PBS before bioassay in the humanized Tg40 mice. The potential toxic effect of intracerebral injection of concentrated and dialyzed urine was assessed by injecting 3 Tg40 mice with 100× concentrated and dialyzed urine collected from a healthy subject. The inoculated mice showed no clinical signs and were euthanized at 7, 14, and 21 days postinoculation (dpi). The histologic examination revealed no lesions (data not shown). These data show that 100× concentrated and dialyzed urine is not toxic following intracerebral injection into Tg40 mice.

Effect of Concentrated and Dialyzed Urine on Prion Infectivity

The effect on prion infectivity of the concentrated and dialyzed urine when used as carrier was assessed by comparing infectivity titers of MF from cerebral cortex of sCJDMM1 patient 4 diluted either in PBS or in 100× concentrated and dialyzed urine. The bioassay analyses showed that by using concentrated and dialyzed urine as carrier, the infectivity titer of MF was reduced ≈20-fold (1.3 log reduction) (Table 2), indicating that 100× concentrated and dialyzed urine can support prion infectivity although less efficiently than PBS.

Effect of Concentration and Dialysis Procedures on Prion Infectivity

The possible loss of prion infectivity during concentration and dialysis procedures was assessed by bioassay. We compared the infectivity titers of brain MF from sCJDMM1 patient 3 spiked in normal urine before the concentration and dialysis procedure with the infectivity of the same amount of MF spiked in normal urine already concentrated and dialyzed (Table 3). Similar mean incubation periods of 265 days and 268 days, respectively, and prion titers of 8.2×10^6 ID_{50}/g and 6.9×10^6 ID_{50}/g, respectively, were found in the 2 differently processed samples, showing that the concentration and dialysis procedure per se had no detectable effect on infectivity.

Bioassay of Concentrated and Dialyzed Urine and Raw Urine from sCJDMM1 Patients

To detect the prion infectivity in urine from sCJDMM1 patients, urine samples collected from 3

Table 3. Determination of infectivity loss of MF from sCJDMM1 patient 3 spiked in normal urine, during concentration and dialysis procedures*

Inoculum	Brain† dilution	Incubation time, d, mean ± SEM	Distribution	Prion titer, ID_{50}/g tissue†
MF in normal urine, then dialyzed and 100× concentrated	10^{-2}	265 ± 12‡	8/9	8.2×10^6
MF in 100× concentrated and dialyzed normal human urine	10^{-2}	268 ± 10‡	8/8	6.9×10^6

*MF, microsomal fraction; sCJD, sporadic Creutzfeldt-Jakob disease; ID_{50}, 50% infectious dose.
†Brain tissue equivalent.
‡p = 0.42 by *t*-test.

Table 4. Native prion infectivity of concentrated and dialyzed as well as raw urine from 3 sCJDMM1 patients by bioassay in Tg40 mice*

Inoculum	Incubation time, d	Distribution
100× concentrated and dialyzed urine (sCJDMM1 patient 1)	>736	0/10
100× concentrated and dialyzed (sCJDMM1 patient 2)	>788	0/10
100× concentrated and dialyzed (sCJDMM1 patient 3)	>788	0/8
100× concentrated and dialyzed (normal control 1)	>719	0/5
100× concentrated and dialyzed (normal control 2)	>756	0/5
100× concentrated and dialyzed (normal control 3)	>752	0/5
Raw urine (sCJDMM1 patient 1)	>857	0/33

*sCJD, sporadic Creutzfeldt-Jakob disease.

end-stage sCJDMM1 patients were concentrated 100× by ultrafiltration followed by dialysis against PBS. The concentrated and dialyzed urine from the 3 donors was intracerebrally inoculated into 8–10 Tg40 mice. Over the expected normal lifespan (up to 788 dpi), no mice showed any evidence of clinical disease (Table 4). No PK-resistant PrP^{Sc} was detected by Western blot, even after enrichment with NaPTA precipitation (Figure 3, panel A). Results of histopathologic and PrP immunohistochemical examinations also were negative (Figure 3, panel B).

On the basis of these negative results, we estimate that prion infectivity in urine of sCJDMM1 patients to be 0 IU/total volume of inoculated urine, which by Poisson distribution is less than 0.37 IU for patients 1 and 2 and 0–0.46 IU for patient 3 (upper limit of 95% CI). However, if we take into account the 100× concentration of urine and the finding that 100× concentrated and dialysized urine may reduce prion infectivity by ≈20-fold (Table 2), the estimates of the highest prion titers possible (i.e., the upper 95% CI) of urine in sCJDMM1 are 0.24 IU/mL for patients 1 and 2 and 0.38 IU/mL for patient 3 (see Materials and Methods).

To directly assess the prion infectivity in urine without concentration and dialysis, we inoculated 33 Tg40 mice with raw urine from sCJDMM1 patient 1. No inoculated mice showed clinical signs of prion disease over their normal lifespan (up to 857 dpi) (Table 4). Similarly, histopathologic and PrP immunohistochemical examination were negative, and no PK-resistant PrP^{Sc} was detected by Western blot in the brain even after PrP^{Sc} enrichment with NaPTA (data not shown). Thus, the estimated infectivity titer in raw urine of sCJDMM1 by Poisson distribution is 0–0.11 IU/mL (95% CI), not dissimilar to the value (0–0.24) obtained with the same urine after concentration and dialysis.

Discussion

The present study demonstrates that the urine from patients affected by advanced sCJDMM1, the most common sCJD subtype that alone accounts for ≈60% of all human prion diseases, contains either no prion infectivity or an infectivity titer that is below the detection limit of our bioassays. The bioassays were done in Tg mice expressing human PrP-129M (Tg40) following inoculation with urine obtained from patients with sCJDMM1 and a variety of positive and negative controls. In limit dilution experiments, Tg40 mice inoculated with MF preparations obtained from the brains of 3 urine donors with sCJDMM1 had prion disease develop at up to 10^5 or 10^4 dilutions of the brain tissue equivalent depending on whether the MF preparations were inoculated directly or after spiking into concentrated and dialyzed normal human urine.

To enhance the sensitivity of our system, urine samples were concentrated and dialyzed before inoculation. Similar procedures have been used in all the previous studies on prion infectivity of urine (*13*,*22*,*23*), except for the study by Gregori et al. (*21*). Our procedure is similar to that used by Seeger et al., who reported the detection of prion infectivity in urine from scrapie inoculated mice affected by nephritis (*23*). Although we demonstrated in the spiking experiment with MF from sCJDMM1 that the 100× concentration and dialysis procedure did not cause infectivity loss (Table 3), the infectivity of the prion-spiked preparation decreased

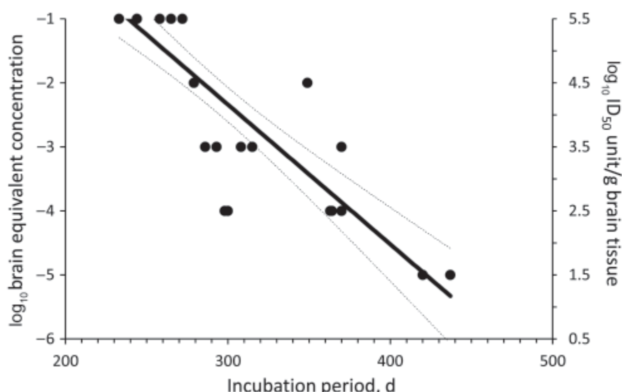


Figure 1. Dose-incubation period curve of brain microsomal fraction from sporadic Creutzfeldt-Jakob disease MM1 (patient 1) intracerebrally injected into Tg40 mice. Each solid circle represents the incubation time for single animal (x-axis) at different brain tissue equivalent dilution. Animals with the same incubation times have overlapping solid circles. Right y-axis is the amount of infectivity present in the inoculum at each brain tissue equivalent dilution (left y-axis). The experimental points were fitted by linear regression curve and 50% infectious dose (ID_{50}) calculated by using probit-nonlinear regression analysis. Dashed lines indicate 95% confidence interval.

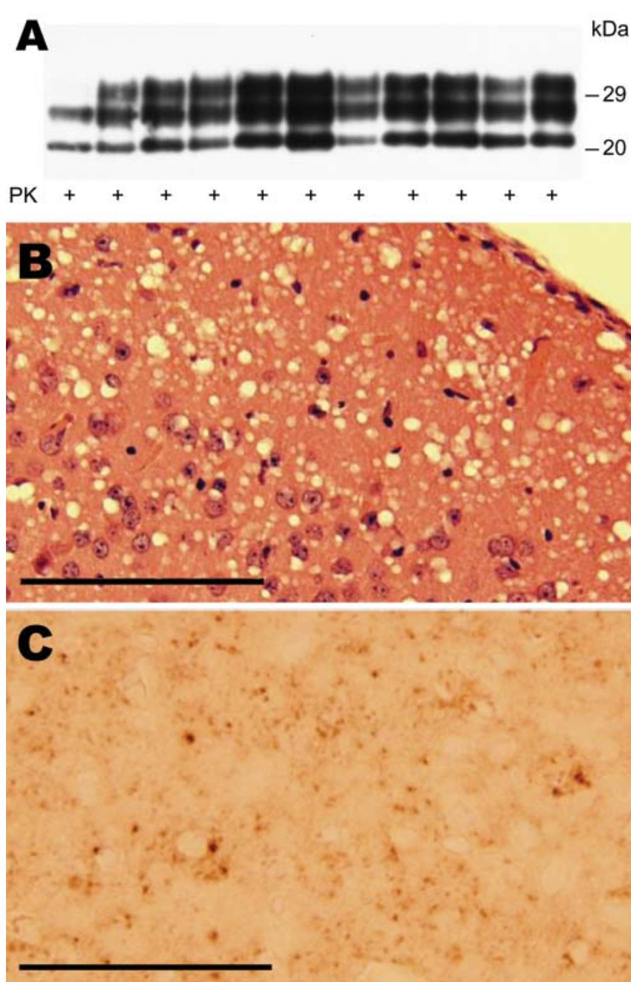
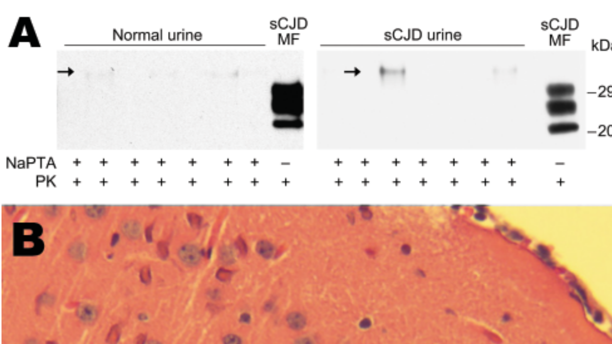


Figure 2. Immunochemical and histopathologic study of humanized transgenic (Tg) mice inoculated with sporadic Creutzfeldt-Jakob disease MM1 (sCJDMM1) microsomal fraction (MF). A) Immunoblot of proteinase K (PK)–resistant scrapie prion protein (PrP^{Sc}) from brains of 10 Tg40 mice inoculated with MF from a patient with sCJDMM1. The inoculum sCJDMM1 MF is shown as control in the first lane. Histologic (B) and immunohistochemical (C) studies show widespread spongiform degeneration and punctate PrP^{Sc} immunostaining of the cerebral cortex from the inoculated mice. Monclonal antibody 3F4 was used for all immunostaining. Scale bar in B = 100 μm. Scale bar in C = 50 μm.

20-fold when 100× concentrated and dialyzed urine was used as carrier compared with PBS. On the basis of these findings, we estimated that if prion infectivity is present at all in sCJDMM1 urine, it is at most 0.38 IU/mL if the 20-fold infectivity loss is factored in. Because the nature of the potential PrP^{Sc} in urine from CJD patients is not known, this urine PrP^{Sc} species might show even higher loss of infectivity in the concentrated and dialyzed urine carrier than the brain PrP^{Sc} preparations used in the spiking experiments. To address this concern, we inoculated 33 Tg40 mice with raw urine from one of the 3 donors with sCJDMM1. No recipient mice showed evidence of prion disease suggesting an infectivity ranging from 0.0 and 0.11 IU/per mL (upper limit of 95% CI) as estimated by the Poisson distribution.

Although asymptomatic disease in recipient mice associated with NaPTA- undetectable minute amounts of PrP^{Sc} cannot be excluded, our inability to detect prion infectivity in human urine of patients with sCJDMM1 differs from several recent experimental studies on urine of prion-affected animals. Low prion infectivity has been demonstrated in urine from scrapie-infected hamsters (*21*,*22*), CWD-infected deer (*13*), and in scrapie-infected mice affected by lymphocytic nephritis. In the last study, however, no urine infectivity was found in non-nephritic mice (*23*). Three additional studies have demonstrated the presence of PrP^{Sc} in urine from scrapie-infected hamsters and CWD-infected deer using protein misfolding cyclic amplification (PMCA) (*13*,*34*,*35*). However, this highly sensitive procedure can detect prion concentrations below the level of detectability of bioassays.

The most likely explanation for the discrepancy between our negative results on human urine and the positive findings by bioassay in urine from animals resides in the different locale and mode of formation of the prion agents. In all the published animal experiments, including bioassays and PMCA, the prion disease was induced by intracerebral or oral administration of exogenous prions, whereas we examined urine infectivity in a naturally occurring sporadic human prion disease. In exogenously acquired prion diseases, PrP^{Sc} is much more likely to be widely present in nonneural peripheral organs, including blood, kidney, and bladder, which is likely the result of early exposure of peripheral organs to the inoculated prions. In sCJD, which is believed to occur spontaneously in the brain (rather than being acquired by infection from exogenous prions), only minute amounts of PrP^{Sc} have been detected in a few nonneural peripheral organs and tissues such as skeletal muscle and spleen (*6*–*8*,*10*). In contrast, in variant CJD (vCJD), the form of CJD acquired by consumption of bovine spongiform encephalopathy–infected beef, the spread of PrP^{Sc} to peripheral organs is much wider and typically involves lymph nodes, tonsil, spleen, portions of the intestinal tract, and the skeletal muscle (*7*,*10*,*36*,*37*), as well as kidney and other organs (*9*). These considerations indicate that vCJD (not sCJD) in principle is more similar to the exogenously acquired animal prion diseases that have been used to study prion infectivity in urine. Therefore, vCJD urine that is more likely to contain prion infectivity should be tested by PMCA or bioassay. However, the reported infectivity of animal urine might also result, at least in some instances, from contamination with feces.



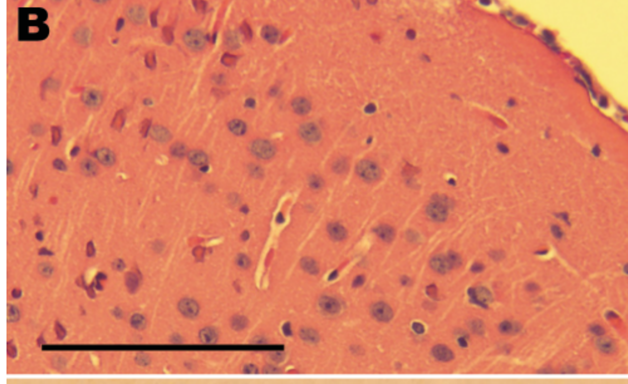


Figure 3. Immunochemical and histopathologic study of humanized transgenic (Tg) mice inoculated with urine from patients with sporadic Creutzfeldt-Jakob disease MM1 (sCJDMM1). A) Immunoblot of brain homogenates (BH) from Tg40 mice inoculated with 100-fold concentrated and dialyzed urine from a sCJDMM1 patient show no proteinase K (PK)-resistant scrapie prion protein (PrP^{Sc}). Before immunoblotting, BH samples were treated with sodium phosphotungstate (NaPTA) to concentrate the PrP^{Sc} possibly present. BH from a Tg40 mouse inoculated with sCJDMM1 MF is shown as a positive control. A nonspecific band ≈32 kDa was detected in normal and sCJDMM1 urine (arrow). All samples were treated with PK. Histologic (B) and immunohistochemical (C) examinations show neither lesions nor abnormal PrP deposits in the cerebral cortex from urine-inoculated mice. Monoclonal antibody 3F4 was used for all immunostaining. Scale bar in B = 100 μm. Scale bar in C = 50 μm.

Recent data have proved that feces from hamsters infected with scrapie by the oral route and to a lesser extent through intracerebral and intraperitoneal inoculation, contain a discrete amount of PrP^{Sc} and prion infectivity (*38*,*39*). Infectivity has been demonstrated also in feces from deer orally infected by CWD (*40*). In hamsters and mice, metabolic cages were used for urine collection, a method in which cross-contamination by feces may actually occur. However, prion infectivity was also demonstrated in urine from CWD-infected deer from which urine collection could easily be performed by catheterization (although this procedure was not mentioned by the authors) (*13*).

Additional studies are still needed to determine whether minute amounts of prion infectivity or PrP^{Sc} are present in urine from patients with sCJD and to assess the presence of infectious prion in urine from patients with every other form and subtype of human prion diseases. Our study shows that urine from patients with sCJDMM1, the most common subtype of sCJD, does not contain prion infectivity detectable by our bioassay and suggests that no significant prionuria occurs in this common subtype of human prion disease.

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High Prevalence of Multidrug-Resistant Tuberculosis, Swaziland, 2009–2010

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In Africa, although emergence of multidrug-resistant (MDR) tuberculosis (TB) represents a serious threat in countries severely affected by the HIV epidemic, most countries lack drug-resistant TB data. This finding was particularly true in the Kingdom of Swaziland, which has the world's highest HIV and TB prevalences. Therefore, we conducted a national survey in 2009–2010 to measure prevalence of drug-resistant TB. Of 988 patients screened, 420 new case-patients and 420 previously treated case-patients met the study criteria. Among culture-positive patients, 15.3% new case-patients and 49.5% previously treated case-patients harbored drug-resistant strains. MDR TB prevalence was 7.7% and 33.8% among new case-patients and previously treated case-patients, respectively. HIV infection and past TB treatment were independently associated with MDR TB. The findings assert the need for wide-scale intervention in resource-limited contexts such as Swaziland, where diagnostic and treatment facilities and health personnel are lacking.

Despite efforts to control the tuberculosis (TB) epidemic, there were an estimated 9.4 million incident cases of TB worldwide in 2009 (*1*). The HIV epidemic and the emergence of anti-TB drug resistance represent serious threats for achieving the Stop TB Partnership's goal of eliminating TB as a public health problem by 2050 (*2*). HIV co-infected patients are more likely to show development of active TB. Even though antiretroviral therapy for HIV reduces this risk, TB remains 5× more frequent in persons living with HIV/AIDS (*3*). Indeed, 30% of the patients in whom TB was diagnosed in 2008 worldwide were in Africa, possibly because of the HIV epidemic affecting the continent (*1*).

Patients infected with a *Mycobacterium* spp. strain resistant to rifampin and isoniazid, which defines a multidrug-resistant (MDR) TB strain, do not respond to World Health Organization (WHO) standardized directly observed short-course chemotherapy and require longer, more toxic, and more expensive treatment. Timely identification of patients with MDR TB enables rapid initiation of adequate treatment, thus preventing the patient from spreading the disease and from acquiring further resistance.

Ideally, routine drug susceptibility testing (DST) should be conducted before initiation of treatment in all patients with TB, but this is not achievable in most high-prevalence countries because of poor access to bacterial culture and DST tools. For the same reasons, in most countries with a high prevalence of TB, no surveillance of anti-TB drug resistance is conducted. Periodic surveys of a representative sample of patients with TB often constitute the only available sources of information on the prevalence of drug resistance (*4*). In the last WHO report on resistance to anti-TB drugs, data from periodic surveys with relatively recent data were available for only 21 of 46 African countries (*5*).

The Kingdom of Swaziland, in southern Africa, is the country with the world's highest HIV prevalence (26% among adults in 2007) and TB incidence rate per capita (1,257 cases per 100,000 population in 2009) (*6*,*7*). In 2007, in collaboration with the Ministry of Health and

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Social Welfare of Swaziland, Médecins Sans Frontières started an integrated HIV/TB project in Shiselweni in southern Swaziland.

In Swaziland, the last national anti-TB drug resistance survey had been conducted in 1995 and reported an MDR TB prevalence of 0.9% among new TB case-patients and 9.1% among previously treated case-patients (*5*). Several factors could have potentially resulted in an increase of MDR TB in the country in recent years and in emergence of extensively drug-resistant (XDR) TB, which is defined as MDR TB resistant to ≥1 injectable second-line drug and any fluoroquinolone. The National TB Control Programme in Swaziland had reported relatively poor TB treatment success rates (68% and 48% for new and retreatment smear-positive TB case-patients, respectively, in 2008), with high failure rates (7% in new case-patients and 11% in retreated case-patients) (*1*). Additionally, Swaziland borders the province of KwaZulu-Natal in South Africa, where an outbreak of XDR TB was reported in 2005 among HIV co-infected patients (*8*); many citizens of Swaziland regularly cross the border to work in South African mines. In 2007, the Ministry of Health and Social Welfare conducted a rapid survey among high-risk patients to detect the occurrence of XDR TB and reported 4 patients with XDR TB and an 18.5% MDR TB prevalence among previously treated case-patients (*9*). These findings justified the need for a new national anti-TB drug resistance survey that measured the current prevalence of MDR TB among new and previously treated patients with TB in Swaziland.

Methods

Design and Study Population

A cross-sectional survey was designed based on the most recent WHO guidelines for surveillance of drug resistance in TB (*4*). The 15 TB diagnosis centers of the 4 regions of Swaziland participated in the study. These included the Manzini TB center, 7 general hospitals, 5 health centers, and 2 clinics (Figure 1).

Consecutive smear-positive patients >14 years of age who were given a new diagnosis of TB were invited to participate in the survey. Patients were considered as new case-patients (NCs) if they had never received treatment for TB or had taken anti-TB drugs for <1 month in the past or as previously treated case-patients (PTCs) if they had ever received anti-TB drugs for ≥1 month. PTCs included patients who returned after defaulting treatment, experienced TB relapses, or had TB treatment failure according to WHO case definitions (*10*). To be consistent with the guidelines of the National TB Control Programme, a treatment failure case-patient was defined as a person who remained smear positive after 3 months of treatment and not 5 months as recommended by WHO (*10*).

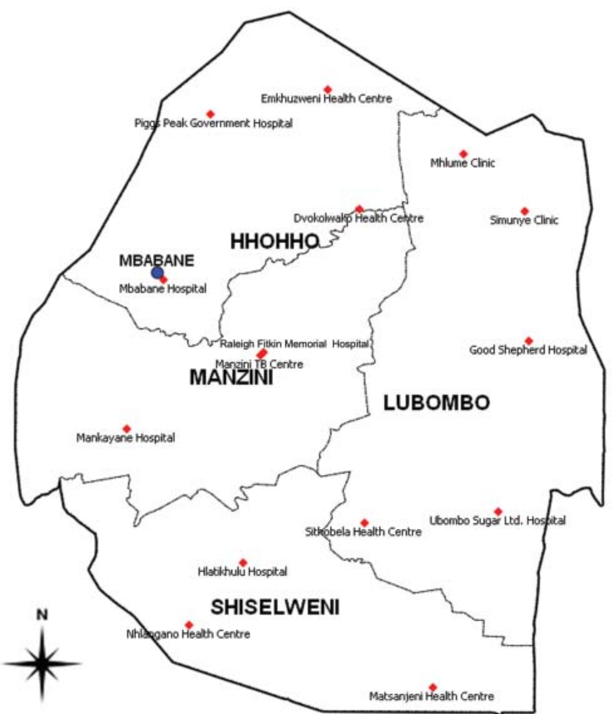

Figure 1. Tuberculosis diagnosis facilities (red diamonds), Swaziland.

Procedures

Data from screened patients were collected on a standardized form that included demographics (e.g., age, sex, region of residence), duration of illness, smear microscopy results, history of TB treatment (number of previous anti-TB treatment courses and outcomes), and HIV status. A 1-day training program was organized for all personnel participating in administering the survey during the month before the beginning of the study. Patient recruitment started after a pilot phase of 2 weeks where study procedures, including the transport of specimens, were assessed. Weekly site-monitoring visits were organized to support each site during patient recruitment.

Sputum smear examination was performed at the TB diagnostic centers on 2 sputum specimens from each patient, collected during 2 consecutive days, by using the hot Ziehl-Neelsen method. A smear-positive case-patient was defined by ≥1 positive smear result with ≥1 acid-fast bacilli per 100 high-power microscopic fields, as recommended by WHO (*11*). Smear-positive case-patients were asked to produce an extra on-the-spot sputum sample, which was stored in a refrigerator (4°C) until shipment to the Supranational Reference Centre for Mycobacteria in Borstel, Germany, for culture and DST. Specimens were shipped weekly.

For cultures, samples were placed into liquid medium by using the BACTEC MGIT 960 system (Becton

Dickinson, Franklin Lakes, NJ, USA) and on 2 solid media (Löwenstein-Jensen and Stonebrink). *M. tuberculosis* was identified by using the GenoType MTBC test (HAIN Lifescience GmbH, Nehren, Germany). For mycobacteria other than *M. tuberculosis*, the GenoType Mycobacterium CM/AS test (HAIN Lifescience GmbH) or DNA sequencing was performed. DST of first-line anti-TB drugs (i.e., rifampin, isoniazid, streptomycin, and ethambutol) was performed on all positive culture samples. Susceptibility to pyrazinamide was tested on strains resistant to rifampin, isoniazid, ethambutol, or streptomycin. DST of second-line anti-TB drugs, which are defined as a group of drugs active against TB used in case of resistance or intolerance to first-line drugs, was performed on strains resistant to rifampin, isoniazid, or both. Second-line drugs tested were amikacin, capreomycin, ofloxacin, 4-aminosalicylic acid, and ethionamide. Moxifloxacin susceptibility was tested in case of ofloxacin resistance. The indirect proportion method on Löwenstein-Jensen medium was used except for pyrazinamide, ethionamide, and moxifloxacin, which were tested on special acid media in the MGIT 960 (Becton Dickinson).

Free access to voluntary counseling and testing for HIV were offered to any patient given a new diagnosis of TB at each study site. According to national guidelines, positive results for 2 rapid HIV tests were required to define a patient as HIV positive. In the event of discordant results between the 2 tests, DNA PCR by using dried blood spot was performed at the National Reference Laboratory.

Sample Size and Statistical Analysis

Independent sample sizes were calculated for NCs and PTCs on the basis of the expected prevalence of rifampin resistance per group (5% for NCs and 15% for PTCs), maximal acceptable absolute error of 2.5% for NCs and 4.0% for PTCs, and 95% CI. Sample sizes were increased by 20% to account for expected losses (e.g., contaminated samples, nongrowing cultures, missing DST results).

Data were entered into EpiData version 3.1 software (EpiData Association, Odense, Denmark) and cleaned and analyzed with Stata for Windows version 10.1 (StataCorp LP, College Station, TX, USA). Distributions of categorical variables between 2 groups were compared by using the Fisher exact test. Comparisons of continuous variables were performed by using a 2-sample *t*-test when the variable showed a normal distribution and with a nonparametric Wilcoxon rank-sum test otherwise. Prevalence ratios and 95% CIs were calculated to measure the degree of association between independent variables and MDR TB through generalized linear models for the binomial family. We used an α error of 5% for all statistical tests.

Data quality was assessed through double entry of 5% of the case report forms and regular cross checks between case report forms and data entered in the data. Identified errors were removed before analysis. Missing data was not imputed or replaced.

Ethical Approval

The study was approved by the Ministry of Health and Social Welfare Scientific Ethical Committee of Swaziland and the Ethics Review Board of Médecins Sans Frontières. Written informed consent to participate to the survey was obtained from patients or from parents or caregivers for adolescents.

Results

Patient recruitment started in May 2009 and was completed in July 2009 for NCs and February 2010 for PTCs. Of 988 screened patients, 840 (85%) met the study inclusion criteria (Figure 2). During the survey period, the national TB program registered 1,175 TB smear-positive case-patients (618 NCs and 557 PTCs). Therefore, 84% of all registered patients were screened for the survey. Reasons given by the health personnel for not screening were heavy workload in the health center and staff turnover. No significant differences in age and sex between screened and nonscreened patients were observed.

Of 822 samples shipped to the mycobacteriology laboratory in Germany, 814 were cultured (99%). Of these samples, 74 (9.1%) were negative and 74 (9.1%) were contaminated. Half the negative culture results (n = 37) were from the PTC subgroup of treatment failure patients.

Fifteen mycobacteria other than *M. tuberculosis* were isolated: *M. avium* (n = 6), *M. avium* complex (n = 5), *M. fortuitum* (n = 2), and *M. kansaii* (n = 2). In 13 cultures, >1 type of mycobacteria grew.

For baseline characteristics of the included patients (Table 1), overall male-to-female ratio was 0.9 and median age was 33 years (interquartile range 27–41 years). The patients were equally distributed among the 4 regions of Swaziland. Regarding socioeconomic aspects, 80.6% of the patients had either not attended school or had not reached high school, and 68.8% had no permanent job.

Among PTCs, most had received only 1 previous category I treatment course; 55.7% had a successful outcome (cured or treatment completed) in their last treatment, and 28.6% failed treatment. HIV status was known for 758 (90.2%) patients. Of these patients, 606 were HIV positive (79.9%).

Of 352 NCs tested, 54 (15.3%) had TB strains resistant to ≥1 first-line drug, and 47 (13.4%) had strains resistant to isoniazid (Table 2). An MDR strain was isolated in 27 NCs, resulting in MDR TB prevalence of 7.7% (95% CI 4.9%–10.5%). Almost half (45.2%) of the 281 PTCs had a TB strain resistant to ≥1 drug; 127 (45.2%) had a strain resistant to isoniazid, and 95 (33.8%) had MDR TB (95%

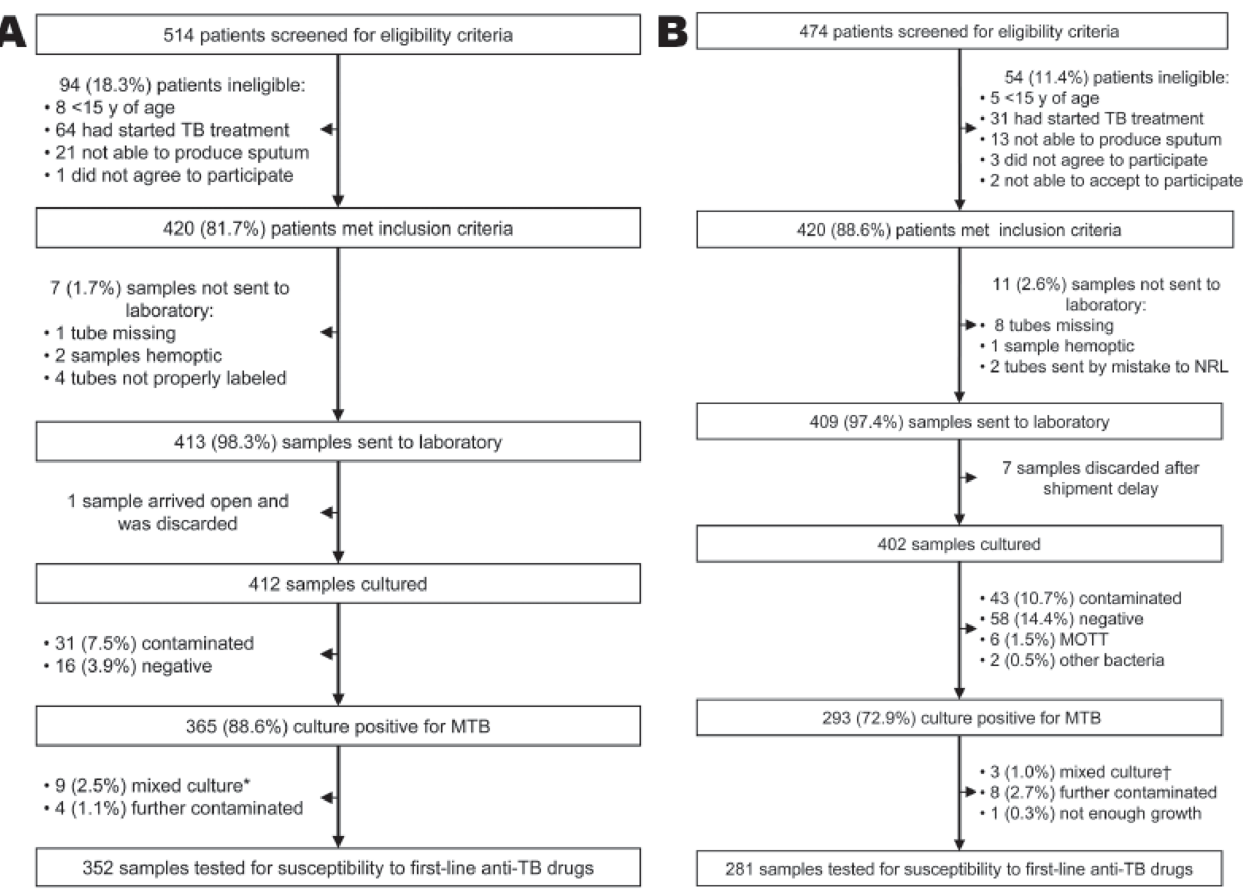

Figure 2. Study profile, national drug-resistant tuberculosis (TB) survey, Swaziland, 2009–2010. A) New patients; B) previously treated patients. NRL, National Reference Laboratory; MTB, *Mycobacterium tuberculosis*; MOTT, mycobacteria other than tuberculosis. *MTB + MOTT. †1 MTB + MOTT, 2 MTB + other bacteria (nonmycobacteria).

CI 28.3%–39.3%). Among PTCs, all who had failures of category II treatment (6/6), 83.5% (40/48) who had failures of category I treatment, 23.2% (42/181) relapses, and 13.4% (4/30) who had default treatment were infected with an MDR strain. The proportion of resistance to pyrazinamide among MDR TB strains was 74.1% (20/27) for NCs and 67.4% (64/95) for PTCs.

Among strains resistant to isoniazid or rifampin, second-line drug resistance was most frequently seen with ethionamide (Table 3). Of the 10 strains resistant to ofloxacin, 8 were also resistant to moxifloxacin (3 in NCs, 5 in PTCs). Only 1 XDR TB strain was isolated; it was resistant to moxifloxacin.

In univariate analysis, past TB treatment (PTCs), female sex, HIV infection, and age 28–40 years were significantly associated with MDR TB (Table 4). In multivariate analysis, PTCs and HIV-infected patients were 4× and ≈2× more likely to be infected with an MDR TB strain, respectively, compared with NCs and HIV-noninfected patients. The youngest age group was close to being significantly associated with MDR TB regardless of HIV status and history of previous treatment.

Discussion

In Swaziland, 7.7% and 33.8% of TB smear-positive NCs and PTCs, respectively, had MDR. This represents an 8.5-fold and 3.7-fold increase compared with MDR prevalence among NCs and PTCs, respectively, from the previous DST survey in 1995. This prevalence appears to be the highest MDR TB prevalence reported in an African country thus far. In neighboring countries, as in South Africa, the estimated MDR TB prevalence was 1.8% (95% CI 1.5–2.3) in NCs and 6.7% (95% CI 5.5–8.1) in PTCs in 2002 (*5*). In Mozambique, 3.5% (95% CI 2.5–4.7) of NCs and 11.2% (95% CI 4.2–30.0) of PTCs had MDR TB in 2006 (*5*). The next closest value of prevalence of MDR in new case-patients in an African country is nearly half the one observed in Swaziland (Rwanda, 3.9%) (*5*). If we consider the extremely high incidence of TB in the population, the prevalence of resistance observed would

result in a high MDR TB population rate, comparable to that observed in former Soviet Union countries. Resistance to fluoroquinolones and second-line anti-TB injectable drugs was surprisingly low, however, with only 1 XDR TB patient seen.

Several factors may have contributed to the increase of MDR TB in Swaziland. First, health services are overwhelmed by a huge increase in TB patients due to the HIV epidemic and a lack of health personnel, which could result in poor TB case management and subsequent development of drug resistance. Second, many citizens from Swaziland regularly cross the border to work in mines in South Africa, where the prevalence of MDR TB is high (*12*). Third, the small size of the country (17,364 km^2) enables the mobility of the population between regions.

High HIV rates might also play a role. In our study, HIV co-infection was independently associated with MDR TB. The correlation between HIV infection and anti-TB drug resistance remains controversial; there were more frequent associations reported in studies in North America than in studies in Africa (*13*–*15*). Several factors have been proposed to explain such an association. Malabsorption of anti-TB drugs has been documented for HIV-positive patients, which could increase the risk for acquired rifampin resistance (*13*). In settings where HIV infection is linked to socioeconomically vulnerable populations, poor treatment adherence and lack of access to proper treatment may contribute to the development of drug resistance (*14*). Also, persons with HIV/AIDS may be more exposed to patients with MDR TB during hospitalizations or consultations in

Table 1. Baseline characteristics of case-patients in a study of MDR TB, Swaziland, 2009–2010*

Characteristic	New, n = 420	Previously treated, n = 420	Total, n = 840
Median age, y	32 (IQR 26–40)	33 (IQR 28–43)	33 (IQR 27–41)
Sex			
M	206 (49.0)	196 (46.7)	402 (47.9)
F	214 (51.0)	224 (53.3)	438 (52.1)
Region of residence			
Shiselweni	106 (25.2)	96 (22.9)	202 (24.0)
Manzini	106 (25.2)	119 (28.3)	225 (26.8)
HhoHho	120 (28.6)	109 (26.0)	229 (27.3)
Lubombo	86 (20.5)	96 (22.9)	182 (21.7)
Unknown	2 (0.5)	0	2 (0.2)
Education			
None	97 (23.1)	75 (17.9)	172 (20.5)
Primary school	144 (34.3)	145 (34.5)	289 (34.4)
Secondary school	104 (24.8)	112 (26.7)	216 (25.7)
High school	62 (14.8)	56 (13.3)	118 (14.0)
Tertiary	10 (2.4)	19 (4.5)	29 (3.5)
Unknown	3 (0.7)	13 (3.1)	16 (1.9)
Employment (permanent job)			
No	285 (67.9)	293 (69.8)	578 (68.8)
Yes	127 (30.2)	115 (27.4)	242 (28.8)
Unknown	8 (1.9)	12 (2.9)	20 (2.4)
HIV-positive status known	279/316 (77.3)	327/397 (82.4)	606/758 (79.9)
Previous anti-TB treatment courses			
1		352 (83.8)	
2		39 (9.3)	
>2		14 (3.3)	
Unknown		15 (3.6)	
Previous TB treatment regimen			
Category I		349 (83.1)	
Category II		60 (14.3)	
MDR		3 (0.7)	
Other		8 (1.9)	
Outcome of most recent TB treatment			
Cured		76 (18.1)	
Completed		158 (37.6)	
Failed category I		109 (26.0)	
Failed category II		11 (2.6)	
Defaulted		45 (10.7)	
Unknown		21 (5.0)	

*Values are no. (%) or no. positive/no. tested (%) unless otherwise indicated. MDR, multidrug resistant; TB, tuberculosis; IQR, interquartile range.

Table 2. Patterns of first-line drug resistance in new and previously treated case-patients with TB, Swaziland 2009–2010*

Resistance pattern	New, n = 352		Previously treated, n = 281	
	No. (%)	95% CI	No. (%)	95% CI
Susceptible to all first-line drugs	298 (84.7)	80.9–88.4	142 (50.5)	44.7–56.4
Any resistance	54 (15.3)	11.6–19.1	139 (49.5)	43.6–55.3
Isoniazid	47 (13.4)	9.8–16.9	127 (45.2)	39.4–51.0
Rifampin	28 (8.0)	5.1–10.8	102 (36.3)	30.7–41.9
Ethambutol	29 (8.2)	5.4–11.1	94 (33.5)	27.9–39.0
Streptomycin	47 (13.4)	9.8–16.9	115 (40.9)	35.2–46.7
Monoresistance	12 (3.4)	1.5–5.3	24 (8.6)	5.3–11.8
Isoniazid	5 (1.4)	0.2–2.7	12 (4.3)	1.9–6.6
Rifampin	1 (0.3)	0.0–0.8	7 (2.5)	0.7–4.3
Ethambutol	0		0	
Streptomycin	6 (1.7)	0.4–3.1	5 (1.8)	0.2–3.3
MDR	27 (7.7)	4.9–10.5	95 (33.8)	28.3–39.3
Isoniazid + rifampin	1 (0.3)	0.0–0.8	3 (1.1)	0.0–2.3
Isoniazid + rifampin + ethambutol	0		2 (0.7)	0.0–1.7
Isoniazid + rifampin + streptomycin	6 (1.7)	0.4–3.1	12 (4.3)	1.9–6.6
Isoniazid + rifampin + streptomycin + ethambutol	20 (5.7)	3.3–8.1	78 (27.8)	22.5–33.0
Other first-line drug-resistance patterns different from MDR	15 (4.3)	2.2–6.4	20 (7.1)	4.1–10.1
Isoniazid + streptomycin	6 (1.7)	0.4–3.1	6 (2.1)	0.4–3.8
Isoniazid + streptomycin + ethambutol	9 (2.6)	0.9–4.2	14 (5.0)	2.4–7.5

*New case-patients were those not previously treated for TB or who had taken anti-TB drugs in the past for <1 month. TB, tuberculosis; MDR, multidrug resistant.

health structures with insufficient infection control because the complexity of management of MDR TB patients entails frequent visits to health facilities, which also increases the risk for MDR TB nosocomial outbreaks among HIV-infected persons (*16–18*). Finally, TB progresses rapidly in HIV-infected patients, who are likely to reactivate an infection acquired recently, compared with HIV-negative patients, who usually reactivate latent infection acquired a long time ago.

Therefore, we could speculate that TB strains harbored by HIV-infected patients are more likely to reflect the strains currently circulating in the community. This suggestion could explain the more frequent association observed between HIV infection and primary MDR TB compared with acquired MDR TB (*14*). In our study, the adjusted prevalence ratio was higher for HIV-infected NCs than PTCs (2.14 versus 1.74), although estimates were not significantly different because of the small sample size (data not shown). We also observed an association between the youngest age group, which is more likely to be recently infected, and MDR TB after correcting for HIV status and history of previous TB treatment. Unfortunately, because of

Table 3. Patterns of second-line drug resistance in rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* isolates, Swaziland, 2009–2010*

Resistance pattern	No. (%) isolates resistant to isoniazid or rifampin,† n = 60	No. (%) isolates from case-patients with MDR TB, n = 122
No resistance to second-line drugs	54 (90.0)	72 (49.2)
Any resistance	6 (10.0)	62 (50.8)
Ethionamide	6 (10.0)	58 (47.5)
Ofloxacin	0	10 (8.2)
p-aminosalicylic acid	0	0
Cycloserine	0	0
Amikacin	0	2 (1.6)
Capreomycin	0	3 (2.5)
Specific resistance patterns		
Ethionamide	6 (10.0)	50 (41.0)
Ethionamide + ofloxacin	0	6 (4.9)
Ethionamide + amikacin + capreomycin	0	1 (0.8)
Ethionamide + ofloxacin + amikacin + capreomycin	0	1 (0.8)
Ofloxacin	0	3 (2.5)
Capreomycin	0	1 (0.8)
XDR	0	1 (0.8)

*MDR, multidrug resistant; TB, tuberculosis; XDR, extensively drug resistant.
†Isolates from MDR TB patients excluded.

Table 4. Patient characteristics associated with risk for MDR TB, Swaziland, 2009–2010*

Variable	No. positive/no. tested (%)	Univariate analysis		Multivariate analysis	
		PR (95% CI)	p value	Adjusted PR (95% CI)	p value
Sex					
M	46/309 (14.9)	Ref		Ref	
F	76/324 (23.5)	1.58 (1.13–2.19)	0.007	1.30 (0.92–1.82)	0.139
Age, y†					
>40	21/156 (13.5)	Ref		Ref	
32–40	41/182 (22.5)	1.67 (1.03–2.71)	0.036	1. 43 (0.90–2.28)	0.132
27–31	27/136 (19.9)	1.47 (0.88–2.49)	0.145	1.34 (0.81–2.23)	0.253
15–26	32/158 (20.3)	1.50 (0.91–2.49)	0.112	1.61 (0.98–2.65)	0.058
Region of residence					
Shiselweni	26/147 (17.7)	Ref			
Manzini	30/169 (17.8)	1.00 (0.62–1.62)	0.988		
HhoHho	27/168 (16.1)	0.91 (0.56–1.48)	0.702		
Lubombo	39/148 (26.4)	1.49 (0.96–2.31)	0.076		
Education					
None	20/139 (14.4)	Ref			
Primary school	45/208 (21.6)	1.50 (0.93–2.43)	0.097		
Secondary school	31/166 (18.7)	1.30 (0.78–2.17)	0.321		
High school	17/89 (19.1)	1.33 (0.74–2.39)	0.346		
Tertiary	5/20 (25.0)	1.74 (0.73–4.11)	0.208		
Employment (permanent job)					
No	83/351 (19.1)	Ref			
Yes	183/37 (20.2)	1.06 (0.74–1.50)	0.753		
HIV status					
Negative	12/114 (10.5)	Ref		Ref	
Positive	102/451 (22.6)	2.15 (1.23–3.77)	0.008	1.78 (1.02–3.10)	0.043
TB patient type					
New case-patient	27/352 (7.7)	Ref		Ref	
Previously treated case-patient	95/281 (33.8)	4.41 (2.96–6.56)	<0.001	4.25 (2.78–6.50)	<0.001

*MDR, multidrug-resistant; TB, tuberculosis; PR, prevalence ratio; Ref, referent.
†Age categorized into quartiles.

the small sample size, we were not able to further analyze this association by stratified analysis. Nonetheless, the role of HIV infection on the transmission of MDR TB could be further assessed in a cluster analysis of the MTB strains by using DNA fingerprinting data.

In eastern European countries, the specific phylogenetic lineage Beijing *M. tuberculosis* genotype has been identified as a particularly prevalent strain independently associated with MDR TB and transmission, indicating a potential role of this pathogen in the epidemiology of drug resistance in these regions (*19*,*20*). The Beijing strain has also been isolated in several African countries (*21–26*), and an association between this strain and the emergence of drug resistance has been reported in South Africa (*27*). Such molecular epidemiologic studies would be recommended in Swaziland.

This study had several limitations. First, the survey population only represented the population of patients diagnosed through the health system. Therefore, not much is known about patients lacking access to health services. The 15 clinics providing TB diagnosis and treatment in the country were included in the survey, but, despite free TB diagnosis and treatment in Swaziland, transport costs and user fees for health facility registration are factors that can limit access to care. Also, almost 16% of the registered smear-positive patients during the survey period were not screened. Although, basic demographic data between assessed and nonassessed patients were not statistically different, we cannot fully exclude the possibility of a selection bias. The proportion of patients with unknown HIV status could have also biased the association seen with MDR prevalence. Therefore, we conducted a sensitivity analysis under a pessimistic assumption that all patients with unknown HIV status were HIV positive and under an optimistic assumption that they were all HIV negative. Figures did not diverge from the original result.

Current WHO recommendations for countries with high anti-TB drug resistance rates and high HIV prevalence are to systematically perform DST at the time of initiation of anti-TB therapy to avoid deaths caused by unrecognized MDR TB (*28*,*29*). The use of rapid drug-resistance testing methods (i.e., PCR) is also recommended to enable a quick start to empirical treatment in potential case-patients while waiting for DST results (*28*). Such strategies require a major effort from national TB programs in terms of laboratory capacity.

Line probe assays were recently introduced at the National Reference Laboratory in Swaziland. New technologies, such as the fully automated PCR method of Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) would be useful in Swaziland to detect *M. tuberculosis* and resistance to rifampin at lower levels of health care services (*30*).

The national TB program in Swaziland should also consider implementing a surveillance system for anti-TB drug resistance to follow drug resistance trends over time, detect outbreaks in a timely manner, and monitor achievements in infection control and treatment measures (*31*,*32*). Periodic surveys may be a costly alternative in terms of logistics, human resources, and shipment costs (≈€137,000 for this survey). Similarly, the implementation of a treatment strategy able to effectively treat all case-patients with drug-resistant TB in contexts of limited health services capacity, lack of health personnel, and high risk of nosocomial drug resistance transmission is another challenge. The National MDR TB Program of Swaziland was approved in 2009 by the WHO Green Light Committee for a MDR TB pilot project, and organizations such as Médecins Sans Frontières provide support to the program in certain regions. However, the overall response for MDR TB and HIV treatment for co-infected patients still needs to be scaled up.

In conclusion, this study reports a very high prevalence of MDR TB in Swaziland, which currently appears to have the highest prevalence in Africa, and shows a rapid increase in the prevalence of MDR TB in the space of slightly more than a decade. The experience from Swaziland calls for further investigation into the effect of the HIV epidemic on TB drug resistance. These results also highlight the inappropriateness of drug resistance surveys to detect early increase of drug resistance in a country when the surveys are not performed regularly. The lack of recent representative data in many African countries probably underestimates the prevalence of drug-resistant TB in this region (*5*,*33*). The high prevalence of drug resistance in a country already facing a huge epidemic of TB and HIV shows an urgent need for major interventions in terms of detection, treatment, and infection control.

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Release date: December 23, 2011; Expiration date: December 23, 2012

Learning Objectives

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

- Describe the current pediatric vaccination schedule against pertussis in England and Wales
- Analyze trends in the epidemiology of pertussis in England and Wales
- Distinguish the most common contact source of pertussis in the current study
- Evaluate the efficacy of the pertussis vaccine.

Editor

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Results of an accelerated pertussis vaccination schedule introduced in 1990 for infants in England and Wales were examined. Earlier scheduling and sustained high vaccine coverage resulted in fewer reported cases of pertussis among infants, reinforcing the World Health Organization drive for on-time completion of the infant vaccination schedule. As determined by using the screening method, the first dose of vaccine was 61.7% effective in infants <6 months of age, and effectiveness increased with subsequent doses. Three doses of a good whole-cell pertussis vaccine were 83.7% effective in children 10–16 years of age; a preschool booster vaccination further reduced pertussis incidence in children <10 years of age. As in other industrialized countries, surveillance data during 1998–2009 showed that pertussis in England and Wales mainly persists in young infants (i.e., <3 months of age), teenagers, and adults. Future vaccine program changes may be beneficial, but additional detail is required to inform such decisions.

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Pertussis incidence in England and Wales declined from the mid-1980s as confidence in whole-cell pertussis (wP) vaccine recovered from safety scares that began in the mid-1970s (*1*). By 1992, vaccine coverage reached 91% by the second birthday, aided by the change to an accelerated 2-, 3-, and 4-month primary schedule in 1990 (Figure 1).

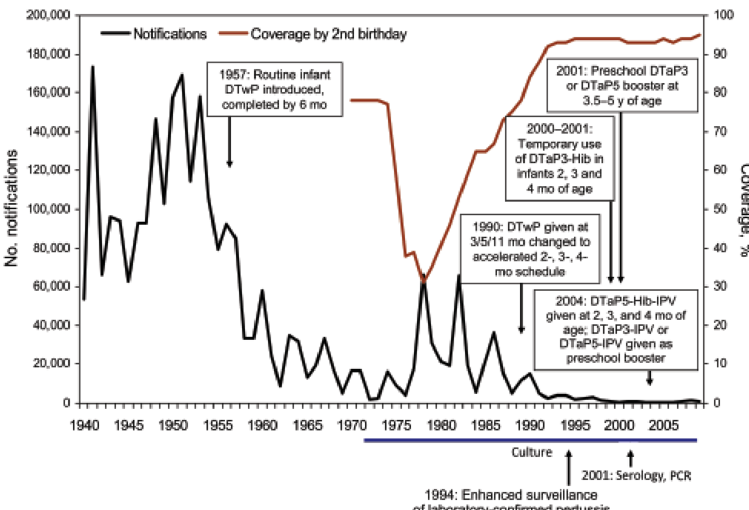

Figure 1. Changes to routine childhood pertussis immunization programs and notifications of pertussis disease (all ages) and vaccine coverage among children <2 years of age, England and Wales, 1940–2009. DTwP, diphtheria/tetanus/whole-cell pertussis vaccine; DTaP3, diphtheria/tetanus/3-component acellular pertussis vaccine; DTaP5, diphtheria/tetanus/5-component aP vaccine; DTaP3-Hib, diphtheria/tetanus/3-component acellular pertussis/*Haemophilus influenzae* type b vaccine; DTaP3-Hib-IPV, diphtheria/tetanus/3-component acellular pertussis/*Haemophilus influenzae* type b/inactivated polio vaccine; DTaP3-IPV, diphtheria/tetanus/3-component acellular pertussis/inactivated polio vaccine; DTaP5-IPV, diphtheria/tetanus/5-component acellular pertussis/inactivated polio vaccine.

From October 2001, pertussis was included in the preschool booster dose as diphtheria/tetanus (DT)/3- or 5-component acellular pertussis (aP3 or aP5) vaccine. In October 2004, aP5 vaccine replaced wP vaccine in the primary schedule and is given as part of the diphtheria, tetanus, *Haemophilus influenzae* type b (Hib), and inactivated polio (IPV) vaccine. This vaccine is as efficacious as the wP vaccine, but the pertussis component is less reactogenic (*2*,*3*). High primary coverage has been sustained; during October–December 2009, DTaP/Hib/IPV coverage in England was 95.3% (*4*).

Pertussis cases conventionally were confirmed by culture only, with testing available in diagnostic laboratories across England/Wales. However, since 2001, the Health Protection Agency (HPA) Respiratory and Systemic Infection Laboratory (RSIL), a *Bordetella pertussis* reference laboratory, has offered serologic testing for IgG against pertussis toxin. This testing is used predominantly for single serum samples obtained from older children and adults ≥2 weeks after cough onset, when culture and PCR are less likely to yield positive results (*5*). Since October 2001, RSIL has also offered PCR testing (*5*,*6*) for hospitalized infants with suspected pertussis. PCR is more sensitive than culture because a viable organism is not required (*6*).

In the 1990s, the number of reported pertussis cases began rising, predominantly among adolescents and adults, in some industrialized countries, including the United States, Canada, and Australia (*7*–*9*). This apparent reemergence has been ascribed to factors that likely differ by country (*10*,*11*). Pertussis surveillance is invariably incomplete and can be affected by enhanced awareness or improved diagnostic methods, leading to perceived changes in epidemiology. Pertussis epidemiology should therefore be considered in the context of any such changes. Debate continues on how to optimize protection for unvaccinated infants, who are at greatest risk for severe disease. We present data on pertussis epidemiology and vaccine effectiveness (VE) in England/Wales to assess the current state of disease control and implications for future national and international immunization strategies.

Methods

Data Source and Compilation

Pertussis notifications are clinically diagnosed cases with no predefined case definition; they have been reported on a statutory basis since 1940 and are collated by HPA. In England/Wales, notification data are supplemented by laboratory confirmations of *B. pertussis* by culture, PCR (began November 2001), and serologic testing (began July 2001); HPA also collates these reports nationally. Details on deaths registered in England/Wales with pertussis recorded as an underlying cause are routinely provided to HPA by the Office for National Statistics (*12*).

National pertussis epidemiology in England/Wales was last reviewed for 1995–1997 (*13*). For this study, pertussis cases during 1998–2009 were identified by using 4 sources of national surveillance data: case notifications, death registrations, laboratory confirmations, and hospital episode statistics. Titers of IgG against pertussis toxin above a predefined level were considered indicative of recent infection (*5*); thus, serology-confirmed case-patients with documented pertussis vaccination in the preceding year were excluded because of potentially raised IgG titers. Culture testing requested by hospitals and general practitioners was performed by local laboratories and collated by the HPA. Laboratories are encouraged to submit positive samples to RSIL for confirmation and surveillance puposes. Serologic testing and PCR were not routinely undertaken outside RSIL. In addition, person-specific ordinary hospital admissions in England from the Hospital Episode Statistics dataset (*14*) with International Classification of Diseases, 10th Revision, codes beginning

A37 (denoting whooping cough) in primary or other diagnoses were analyzed.

Age-specific rates of pertussis/100,000 population were calculated by using midyear population estimates from the Office for National Statistics (*15*). The average annual incidences for 1998–2001 and the 2 subsequent 4-year periods (each including a peak year) were calculated to examine the effect of the preschool booster introduced in October 2001. By using Stata version 9 (StataCorp LP, College Station, TX, USA), we estimated the percentage change in average annual incidence during 1998–2009 by linear regression analysis of the log of the annual incidence rate by age.

Details were collected on immunization status, contacts, hospitalizations, and complications through established enhanced surveillance of laboratory-confirmed pertussis cases (*13*). Age-specific descriptive analysis of these data was undertaken for 2002–2009, after the introduction of the preschool booster and routine serologic and PCR testing.

Vaccine Effectiveness

VE for England was calculated by using the screening method, which compares the probability of a pertussis case-patient being vaccinated with estimated population coverage for persons of comparable ages. This calculation is expressed by the following equation, in which PCV is the proportion of case-patients vaccinated (of those fully or not vaccinated) and PPV is the corrected population coverage (excluding those partially immunized): $VE = 1 - [PCV/(1 - PCV)]/[PPV/(1 - PPV)]$. Methods have been described (*16*).

Derivation of Coverage Data

We used annual Department of Health data (*17*) or, if annual data were not yet published, quarterly HPA COVER (cover of vaccination evaluated rapidly) estimates to apply coverage to each case-patient ≥6 months of age (*4*). Three-dose COVER data are collected at first, second, and fifth birthdays; 4-dose coverage is collected at the fifth birthday. We used the General Practice Research Database to estimate the proportion of partially vaccinated case-patients and to estimate coverage by individual month of age (*18*).

General Practice Research Database data were not suitable for infants ages 9 weeks–5 months because breakdown by days of age was required. The timing of each dose for these infants was obtained from the population-based Child Health Database systems (*19*). Coverage was then estimated by applying the timing of each dose to 12-month COVER data.

Effectiveness of Primary Vaccination Course

For 1998–2009, we included data for culture- or PCR-confirmed pertussis in patients ages 6 months–16 years. For 2002–2009, we separately analyzed data for serology-confirmed pertussis in patients ages 18–39 months and ≥5 years. All nonimmunized, serology-confirmed case-patients whose age was within 1 year of the scheduled pertussis vaccination were thereby excluded to obtain a consistent dataset; case-patients confirmed positive by serologic testing who were tested within 1 year of pertussis vaccination had already been excluded.

Case-patients' sex, date of birth, date of diagnosis, and vaccination history were available. Only data for fully vaccinated (defined as 3 or 4 doses) or completely nonimmunized patients were included. Cohorts were characterized according to primary immunization schedule (extended or accelerated) and pertussis vaccine (wP, aP3, or aP5) used when each child was eligible for vaccination.

Effectiveness of Preschool Booster

To ensure adequate numbers, we included case-patients with culture-, PCR-, and serology-confirmed pertussis during 2002–2009 who were eligible for the preschool booster and who had received 3 or 4 vaccine doses. Additional protection from the booster was calculated by using the proportion of those who received 4 doses and comparing that with the population coverage of 3 and 4 doses.

Effectiveness among Young Infants

The preschool booster and all test methods were available throughout 2002–2009; thus, to determine VE among young infants, we included cases from this period. Using patient age at illness minus 10 or 14 days (allowing time for protection), we mapped the derived population coverage for 1 dose to infants ages 9 weeks to <6 months. One-dose VE was estimated for infants who received 0 or 1 dose, and coverage was corrected on this basis and mapped to these children. Thus, the corrected 1-dose coverage was the estimated proportion of the population who received 1 dose among those who received 0 or 1 dose. The same method was used to calculate 2- and 3-dose VE.

Results

Effect of Accelerated Primary Schedule

Notifications of pertussis among infants continued to decline after peaking in 1990, with peaks recurring at lower levels every 3–4 years. The proportionate distribution of cases among infants changed notably (Figure 2); for example, in 6- to 11-month-old infants, the proportion of cases declined from 50% (1989) to 26% (2008), indicating earlier protection.

Pertussis Epidemiology (1998–2009)

During 1998–2009, pertussis rates from all sources continued to be highest among infants <3 months of age and

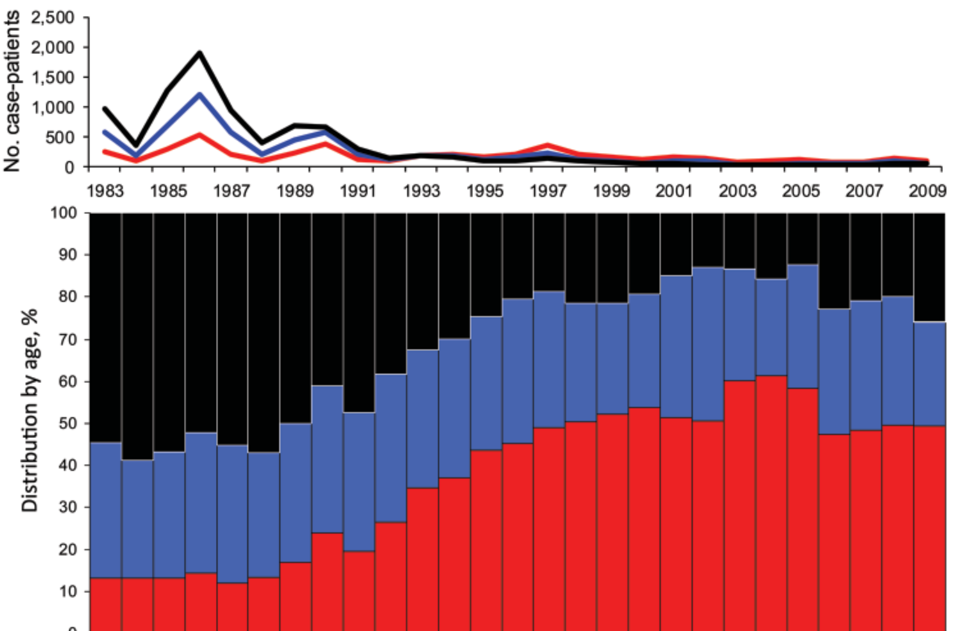

Figure 2. Age-group distribution of case-patients <12 months of age in pertussis case-notification reports, by total case-patients (A) and proportion by age (B), England and Wales, 1983–2009. Red, <3 months of age; blue, 3–5 months of age; black, 6–11 months of age.

to peak every 3–4 years (Figure 3). There was, however, an underlying downward trend in hospitalization rates among these infants, with an overall 9% annual decrease (p<0.001). Over the same time, annual notification rates declined by 7% (p = 0.001), and the incidence of laboratory-confirmed cases declined by 4% (p = 0.1) (Table 1).

Hospitalization rates among children <10 years of age declined considerably during 1998–2009 (Table 1). The greatest overall average annual reductions (27%; p<0.001) were among children ages 5–9 years. There were also overall downward trends in laboratory confirmations and notification rates for this age group. However, notifications increased for children ages 6–11 months and 1–4 years during 2002–2005 and 2006–2009.

During 1998–2009, laboratory-confirmed cases and notifications, but not hospitalizations, increased dramatically for patients >9 years of age (Table 1). From 2006 through 2009, an average of ≈370 pertussis cases was confirmed annually in persons ≥15 years of age; 95% were confirmed by serologic testing only. During 1998–2001, before serologic testing, an annual average of 9 laboratory-confirmed cases were found in this age group.

Deaths

Death registrations, laboratory confirmations, and hospital episode statistics data indicated that 39 pertussis-related deaths occurred during 2002–2009; 30 were in patients with laboratory-confirmed pertussis. Of the 39 deaths, 36 (92%) were among infants, 29 (72%) of whom were <3 months of age; 19 (49%) were among males. During this period, there was no clear pattern and no decrease in deaths (p = 0.8 by test for annual trend by Poisson regression).

Pertussis vaccination >10 days before death was not documented for any of the 30 case-patients who died with laboratory-confirmed pertussis. The overall case-fatality rate (CFR) among infants with laboratory-confirmed pertussis was 24 deaths/1,000 cases. At disease onset, 2 infants were <28 days of age (CFR 22/1,000), 17 were 28–55 days of age (CFR 43/1,000), 6 were 56–83 days of age (CFR 16/1,000), and 4 were >83 days of age (CFR 12/1,000). Pertussis was also confirmed for a 79-year-old patient whose cause of death was recorded as possible endocarditis.

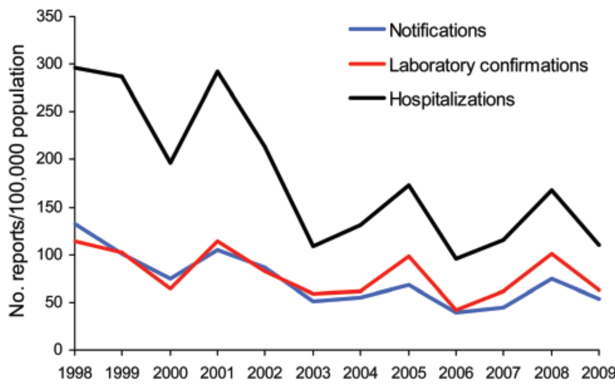

Figure 3. Pertussis disease notifications, laboratory confirmations, and hospital episodes in infants <3 months of age, England and Wales, 1998–2009.

Table 1. Changes in reported incidence of pertussis during 4-year periods before and after introduction of a fourth dose of pertussis vaccine, by age group, England and Wales, 1998–2009*

Age group and reporting mechanism	Reported incidence, by year†			% Change per year, 1998–2009 (95% CI)‡
	1998–2001	2002–2005	2006–2009	
<3 mo				
Laboratory reports	98.59	75.23	67.25	–4 (–9 to +1)
Notifications	103.65	64.83	53.77	–7 (–11 to –3)
Hospitalizations§	268.16	156.20	123.14	–9 (–13 to –4)
3–5 mo				
Laboratory reports	26.12	17.06	15.09	–6 (–11 to –2)
Notifications	57.94	34.44	31.94	–7 (–13 to –1)
Hospitalizations§	84.57	41.74	34.56	–10 (–14 to –6)
6–11 mo				
Laboratory reports	4.73	2.60	1.69	–11 (–17 to –5)
Notifications	19.59	7.72	12.09	–6 (–12 to +1)
Hospitalizations§	16.53	9.32	5.63	–12 (–9 to –16)
1–4 y				
Laboratory reports	1.65	1.05	0.87	–7 (–12 to –2)
Notifications	13.16	5.13	6.00	–9 (–16 to –2)
Hospitalizations§	5.62	2.32	0.88	–19 (–23 to –16)
5–9 y				
Laboratory reports	0.98	0.98	0.81	–3 (–7 to +2)
Notifications	8.02	3.67	3.44	–10 (–16 to –3)
Hospitalizations§	2.07	0.79	0.13	–27 (–34 to –20)
10–14 y				
Laboratory reports	0.33	0.69	3.10	+30 (+17 to +44)
Notifications	2.19	1.79	4.90	+9 (+1 to +19)
Hospitalizations§	0.49	0.27	0.39	–3 (–11 to +4)
≥15 y				
Laboratory	0.02	0.18	0.82	+53 (+43 to +63)
Notifications	0.23	0.26	1.04	+18 (+9 to +28)
Hospitalizations§	0.02	0.01	0.01	–1 (–7 to +6)

*Fourth dose introduced in October 2001.
†Rates per 100,000 population per year.
‡Calculated by linear regression analysis of the log of the annual incidence rate.
§Rates for England only.

Infection Source

Of reports on 3,890 cases confirmed during 2002–2009, only 1,255 (32%) had details regarding exposure to a suspected or known case of pertussis in the month before disease onset: 686 (18%) had no known contact, and 569 (15%) had a known source. The contact's age was provided for 274 (7%) case-patients; irrespective of patient age, the home was the most commonly cited location of transmission (75% of cases). Home contact was cited as the location for 81 (95%) of 85 infants <3 months of age; of 34 contacts, 16 (47%) were 1–9 and 18 (38%) were 15–44 years of age. Among 33 case-patients 1–9 years of age, 23 (70%) cited other children <10 years of age as the source. School contact occurred for 32% (38/119) of children 5–14 years of age. Work or school contact occurred for 11% (33/308) of case-patients ≥15 years of age.

Description of Laboratory-Conf rmed Cases

The median age of 1,185 infants with pertussis was 63 days; 9% were <28 days and 34% were 28–55 days of age, which is too young to have received vaccine. Ninety percent of all infants were hospitalized (Table 2), and 93% of those <3 months of age were hospitalized; infants <3 months of age were hospitalized the longest (Table 3). Thirty-nine percent had ≥1 specified complication: 31% apneic attack, 8% pneumonia, 1% convulsions, and 1% conjunctival hemorrhage. It was unclear what was being reported as apnea in patients >1 year of age; however, it was considered likely to indicate more serious disease. Thus, Table 2 shows total complications including and excluding reported apnea.

Vaccine Effectiveness

Effectiveness of Primary Course

During 1998–2009, culture- or PCR-confirmed pertussis was reported for 608 children 6 months–16 years of age. Patients with unknown or partial vaccination status were removed from analysis, leaving 460 patients for analysis. During 2001–2009, a total of 200 cases of

Table 2. Details of case-patients followed up through enhanced pertussis surveillance, England and Wales, 2002–2009

Variable	No. (%) case-patients, by age group*				
	<1 y, n = 1,185†	1–4 y, n = 190‡	5–9 y, n = 225§	10–14 y, n = 498¶	≥15 y, n = 1,781#
No. vaccine doses administered before symptom onset					
0	840 (77)	76 (44)	51 (24)	38 (8)	652 (59)
1	196 (18)	9 (5)	8 (4)	6 (1)	24 (2)
2	36 (3)	1 (1)	4 (2)	7 (1)	26 (2)
3	24 (2)	85 (49)	98 (47)	419 (86)	396 (36)
4	0	3 (2)	48 (23)	15 (3)	0
Total hospitalized	927 (90)	66 (42)	35 (19)	44 (10)	81 (5)
Any complications**					
Yes	394 (39)	30 (19)	22 (12)	29 (7)	176 (11)
Yes, excluding apnea in those ≥1 y old	394 (39)	11 (8)	19 (11)	18 (4)	69 (5)

*Proportions are based on total cases where these details were provided.
†Median age at onset 53 d; 48% male.
‡Median age at onset 2.5 y; 42% male.
§Median age at onset 7.8 y; 50% male.
¶Median age at onset 12.5 y; 49%male.
#Median age at onset 41.8 y; 44% male.
**Specifically reported pneumonia, convulsion, apnea, conjunctival hemorrhage, or death.

pertussis were confirmed by culture or PCR, and 772 were confirmed by serologic testing; of these, data for 175 and 707, respectively, were retained for analysis after excluding those with unknown or partial vaccination status.

During 1998–2009, there was no difference in VE between patients ages 6–11 months (97.6%, 95% CI 95.9%–98.6%) and 12–39 months (98.1%, 95% CI 97.2%–98.7%) with culture- or PCR-confirmed pertussis. VE significantly declined from 97.6% among infants 6–11 months of age to 83.7% among children 12–16 years of age (95% CI 69.5%–90.8%; $p<0.001$). Age-specific VE was similar across all vaccine cohorts (Table 4). VE estimates determined on the basis of serologic testing results were lower, but numbers were small (Table 5). These differences precluded a combined analysis with culture- or PCR-confirmed cases.

Effectiveness of Preschool Booster

Sixty-seven case-patients eligible for a preschool booster had received 0 or 4 vaccine doses; 42 (62.7%) received 4 vaccine doses. National 4-dose coverage was 79%, giving an adjusted population booster coverage of 97.3%, based on those who had received 0 or 4 doses only. Estimated VE for 4 doses was 95.3% (95% CI 91.9%–97.2%).

Fifty-six case-patients received 3 or 4 vaccine doses before disease onset. Of these patients, 42 (75%) received a fourth dose, compared with a 4-dose population coverage of 84.8%, giving a VE of 46% (–7% to 71%).

Effectiveness among Young Infants

There were 505 case-patients 9 weeks to <6 months of age. Vaccination status was known for 455 patients with culture- or PCR-confirmed pertussis and for 15 with serology-confirmed pertussis. Results were similar across vaccine cohorts, and VE increased with dose and age (Table 6). Assuming protection from 14 days after vaccination (rather than 10) gave similar results (data not shown).

Discussion

Improved vaccine coverage and earlier completion of the primary pertussis vaccine schedule reduced pertussis notifications and provided earlier protection against disease in England/Wales from 1990 onward. The preschool booster was introduced when coverage was high and disease incidence relatively low. The main aim was to reduce disease in older age groups, thereby decreasing transmission to unprotected young infants who are at highest risk for severe disease. The effect of the preschool booster was apparent in the continued overall reduction in pertussis incidence among children <10 years of age. This effect was particularly marked in hospitalization data, which are likely to be most consistent through time, with evidence of an indirect protective effect in infants <3 months of age. Calculated 4-dose VE was ≈95.3% with ≤7 years of follow-up, although numbers were small. Persistence of immunity 6–9 years after an aP3 booster dose in the second year of

Table 3. Details of hospitalized pertussis case-patients, England and Wales, 2002–2009*

Age group	No. case-patients (% male)	Mean duration of stay, d
Infants <1 y	2,338 (48)	5.6
<3 mo	1,695 (48)	6.5
3–5 mo	464 (50)	3.6
6–11 mo	179 (41)	2.2
1–4 y	298 (43)	1.5
5–9 y	113 (47)	1.0
10–14 y	82 (46)	0.9
≥15 y	43 (37)	5.8

*Case-patients with a primary or subsidiary diagnosis coded as pertussis in Hospital Episode Statistics data.

Table 4. Estimated effectiveness of a 3-dose pertussis vaccine schedule among case-patients with culture- or PCR-confirmed pertussis, by vaccine cohort and age group, England, 1998–2009*

Vaccine cohort†	Age group of pertussis case-patients							
	12–39 mo		40–59 mo		5–9 y		10–16 y	
	No. vaccinated/ no. total	% VE (95% CI)	No. vaccinated/ no. total	% VE (95% CI)	No. vaccinated/ no. total	% VE (95% CI)	No. vaccinated/ no. total	% VE (95% CI)
1	–	–	–	–	5/8	92 (48.8–98.5)	15/26	87.2 (69.2–94.5)
2	25/52	97.7 (95.9–98.7)	38/57	95.7‡ (92.2–97.6)	94/125	92.8‡ (88.8–95.2)	39/44	82.0‡ (41.4–92.9)
3	12/37	98.7 (97.4–99.4)	2/5	98.4§ (85.9–99.9)	1/4	99.2§ (89.9–100.0)	–	–
4	8/21	98.4 (95.9–99.4)	1/2	–	0/1	–	–	–
5	11/19	96.6 (90.2–98.7)	–	–	–	–	–	–
Overall total	56/129	98.1 (97.2–98.7)	41/64	96.1 (93.3–97.7)	100/138	93.4 (90.1–95.5)	54/70	83.7 (69.5–90.8)

*VE, vaccine effectiveness; –, no cases that met the criteria were identified during 1998–2009 and, therefore, VE could not be calculated, or case nos. were too small for calculation purposes.
†Vaccine cohorts: cohort 1, received diphtheria/tetanus/whole-cell pertussis vaccine (DTwP) at 3, 5, and 11 mo of age; cohort 2, received DTwP at 2, 3, and 4 mo of age; cohort 3, received DTwP or DTaP3 (DT/3-component acellular pertussis vaccine) at 2, 3, and 4 mo of age; cohort 4, received DTwP at 2, 3, 4 mo of age; cohort 5, received DTaP5 at 2, 3, and 4 mo of age.
‡VE mainly reflects 3 doses; however, some eligible children had received 4 doses.
§Some children were eligible for a booster vaccination; thus, VE is for 3 or 4 doses.

life (*20*) and no evidence of waning immunity 4 years after a preschool booster (*21*,*22*) have been reported.

Marked increases in pertussis notifications and laboratory confirmations, but not hospitalizations, were observed for adolescents and adults in England/Wales during 1998–2009. Different explanations have been proposed for the rising pertussis incidence in these age groups in countries with high vaccine coverage. The relevance of apparent increased pertussis among persons of these ages, in whom illness tends to be milder, atypical, and underdiagnosed (*7*,*23*), relates to whether the increases are real or driven by improved case ascertainment. A concomitant rise in pertussis in unprotected young infants would be consistent with a true increase at older ages. In England/Wales serologic testing, together with publications promoting higher awareness (*24*), improved case ascertainment. Testing of oral fluid samples from notified case-patients whose infection was not laboratory confirmed was also piloted nationally during June 2007–August 2009. These data are excluded from national datasets, but the pilot may have influenced testing and notification practice for noninfant case-patients.

Despite a reduction in pertussis among younger children and infants, rates of pertussis-related sickness and death remain high compared with rates for other vaccine-preventable diseases. In England, ≈300 hospitalized infants each year receive a diagnosis of pertussis. Relatively high hospitalization rates for patients with laboratory-confirmed cases suggest that national surveillance in England/Wales is proficient at ascertaining serious cases in young children. One US study reported that 67% of infants with pertussis were hospitalized (*25*), compared with 90% of infants in our study in England/Wales. The US study reported CFRs of 1% for infants 0–1 month of age; in our study, the CFR was 39/1,000 for infants <56 days of age. An earlier HPA study found incomplete pertussis diagnoses even in severely ill, hospitalized infants (*26*); routine PCR testing for hospitalized infants was therefore introduced. Differences between rates of laboratory-confirmed cases and hospitalization among infants have since been reduced (Table 1). Studies highlighting unrecognized pertussis-related deaths (*27*,*28*) may also have increased awareness and improved recording accuracy, but underreporting persists.

The United States, New Zealand, and Australia have reported increased pertussis incidence among infants despite use of an aP vaccine booster (*25*,*29*,*30*). In Australia, hospitalization rates of >400 hospitalizations/100,000 infants were reported during 2008– 2009 (*30*). The Netherlands reported pertussis epidemiology similar to that for England/Wales after a preschool booster was introduced. Infant hospitalization rates were comparable: 134 hospitalizations/100,000 infants <6 months of age in the Netherlands during 2002–2005, compared with 156 hospitalizations/100,000 infants <3 months of age during 2002–2005 in England/Wales (*22*). However, direct comparisons between countries are problematic because of differences in case definitions, vaccine schedules, and VE.

Consistent with findings in a previous study (*2*), we found high short-term effectiveness for the wP and aP5 vaccines used in England/Wales. As in other studies (*20*,*31*), there was evidence of waning protection 10–15

Table 5. Estimated effectiveness of a 3-dose pertussis vaccine schedule among case-patients with serology-confirmed pertussis, by vaccine cohort and age group, England, 2002–2009*

	Age group of pertussis case-patients					
	12–39 mo		5–9 y		10–16 y	
Vaccine cohort†	No. vaccinated/ no. total	% VE (95% CI)	No. vaccinated/ no. total	% VE (95% CI)	No. vaccinated/ no. total	% VE (95% CI)
1	–	–	–	–	28/29	–41.3 (–5,679 to 76.6)
2	1/1	–	88/108	90.4‡ (83.4 to 94.1)	463/500	72.1‡ (59.9 to 80.1)
3	2/2	–	26/33	91.0§ (75.4 to 96.2)	–	–
4	2/4	97.7 (67.6 to 99.8)	4/5	90.9§ (–348 to 99.1)	–	–
5	21/25	69 (–24.2 to 89.5)	–	–	–	–
Overall total	26/32	77.8 (34.2 to 91.1)	118/146	90.5 (85.1 to 93.8)	491/529	69.3 (56.1 to 78.0)

*VE, vaccine effectiveness; –, no cases that met the criteria were identified during 2002–2009 and, therefore, VE could not be calculated, or case nos. were too small for calculation purposes.
†Vaccine cohorts: cohort 1, received diphtheria/tetanus/whole-cell pertussis vaccine (DTwP) at 3, 5, and 11 mo of age; cohort 2, received DTwP at 2, 3, and 4 mo of age; cohort 3, received DTwP or DTaP3 (DT/3-component acellular pertussis vaccine) at 2, 3, and 4 mo of age; cohort 4, received DTwP at 2, 3, 4 mo of age; cohort 5, received DTaP5 at 2, 3, and 4 mo of age.
‡VE mainly reflects 3 doses; however, some eligible children had received 4 doses.
§Some children were eligible for a booster vaccination; thus, VE is for 3 or 4 doses.

years after completing a wP primary vaccine course. Greater waning after aP vaccine has been reported (*20*), underlining the need for continued surveillance. In a study seeking laboratory evidence of pertussis in all school-aged children who visited their primary care doctor for cough, 86% of children with and 97% without evidence of pertussis infection had been immunized (*24*), giving an estimated VE of 82%, a result consistent with our findings. However, patients who seek health care might have pertussis that represents the more severe end of the clinical spectrum of disease, for which VE is likely to be higher (*24*,*32*,*33*). An earlier HPA study showed higher VE estimates in nonepidemic (93%) than in epidemic (87%) years, which suggests that when awareness of the disease is high, the threshold for investigation and diagnosis of milder cases may be lower and thus reduce estimates of VE (*32*).

When coverage is exceptionally high and VE estimates are <90%, small coverage variations can markedly change VE calculated by the screening method. For example, in this study, VE for patients 10–16 years of age who received the DTwP vaccine under the accelerated schedule would increase from 82% to 90% if coverage increased from 97.7% to 98.7%. When VE is high, as with most estimates in this study, there is little effect from coverage inaccuracies; population booster dose coverage of 50% and 79% give 4-dose VE estimates of 93.3% and 95.3%, respectively.

Continued improvements in pertussis control in children <10 years of age, including disease reduction in young infants, have been observed in England/Wales since the preschool booster was introduced. Inclusion of the booster in the preschool vaccination schedule endorses the flexible World Health Organization (WHO) recommendation for a booster at age 1–6 years, with timing guided by local factors (*34*). Pertussis control is far from optimal, however, and disease continues predominantly in young infants and in teenagers and adults, in whom immunity may have waned. This presents 2 major policy issues: how to better protect vulnerable infants and whether pertussis in teenagers and adults warrants targeted prevention. Potential strategies to protect young babies have recently been reviewed by the WHO Strategic Advisory Group of Experts (SAGE) on Immunization: selective immunization of close family members of neonates (cocooning) and universal adolescent, maternal, and neonatal immunization (*34*).

Table 6. Effectiveness of pertussis vaccine among pertussis-infected infants 9 weeks to <6 months of age, England, 2002–2009*

No. doses received before disease onset	No. vaccinated case-patients/total no. (%)†	Average % coverage in comparable population†	% VE (95% CI)
1	174/428 (41)	64	62 (53–69)
2	36/91 (40)	82	85 (77–91)
3	5/24 (21)	84	95 (86–99)

*Data are for culture-, PCR-, and serology-confirmed cases with onset during 2002–2009. Case-patients received 1 of the following 3 vaccine regimens: 1) DTwP (diphtheria/tetanus/whole-cell pertussis) or DTaP3 (DT/3-component acellular pertussis vaccine) at 2, 3, and 4 mo of age; 2) DTwP at 2, 3, and 4 mo of age; or 3) DTaP5 at 2, 3, and 4 mo of age. VE, vaccine effectiveness.
†Coverage for 1, 2, and 3 doses was calculated by using the number of doses received or 0 doses. Note that case-patients with 0 doses may be counted in >1 group.

Household contacts are often the source of pertussis exposure for young infants (*26*,*35*). Consequently, some countries have adopted the cocooning strategy, a resource-intensive approach with little evidence of clinical effectiveness and one not recommended by SAGE (*34*). Our data suggest that household contacts were a key part of disease transmission, but a high proportion of responses indicated no known exposure; thus, other unrecognized transmission sources may also be relevant.

Studies suggest that pregnant women mount a good immune response to wP vaccines, and this response should provide protection to neonates (*34*). The duration of protection in infants and the degree of interference with primary vaccine responses remains unknown. Although this strategy is the only potential way to protect children against pertussis from birth, SAGE found insufficient evidence to propose this strategy (*34*). In the United States, the Centers for Disease Control and Prevention Advisory Committee on Immunization Practices recently recommended that pregnant women who have not been vaccinated against pertussis receive the tetanus-diphtheria-pertussis vaccine after their 20th week of pregnancy rather than delaying to the postpartum period (*36*). No other country currently recommends this vaccine during pregnancy.

Pertussis immunization at birth has been proposed after the demonstration of immune responses in neonates (*37*,*38*). However, immune interference to different antigens in the primary course remains a concern (*39*,*40*). No monovalent pertussis vaccine is licensed for use in neonates. Our data suggest that pertussis infection among infants can be reduced with an accelerated primary immunization schedule and that protection lasts well into school age. Although 43% of pertussis cases in infants in England/Wales occurred among those <2 months of age, even 1 dose of pertussis vaccine confers some protection in infants 9 weeks to <6 months of age, and high VE is conferred by 3 doses. This finding highlights the need for high coverage and timely administration of each pertussis vaccine dose as advocated by WHO.

Universal adolescent vaccination may be considered in countries in which it has been shown to be cost-effective, primarily to reduce adolescent disease rather than indirectly protect infants (*34*). In England/Wales it is not clear that the rise in pertussis incidence in adolescents and adults observed during 1998–2009 reflects a true increase. Better understanding of the disease at older ages is needed to assess the potential effect of additional vaccine doses and the role of adolescents in disease transmission to infants. Although vaccine boosting of adolescents may reduce disease within targeted age groups, the potential for this policy to shift disease to the main childbearing population needs to be better understood.

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The 1918–19 Influenza Pandemic in Boyacá, Colombia

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To quantify age-specific excess-mortality rates and transmissibility patterns for the 1918–20 influenza pandemic in Boyacá, Colombia, we reviewed archival mortality records. We identified a severe pandemic wave during October 1918–January1919 associated with 40 excess deaths per 10,000 population. The age profile for excess deaths was W shaped; highest mortality rates were among infants (<5 y of age), followed by elderly persons (≥60 y) and young adults (25–29 y). Mean reproduction number was estimated at 1.4–1.7, assuming 3- or 4-day generation intervals. Boyacá, unlike cities in Europe, the United States, or Mexico, experienced neither a herald pandemic wave of deaths early in 1918 nor a recrudescent wave in 1920. In agreement with reports from Mexico, our study found no death-sparing effect for elderly persons in Colombia. We found regional disparities in prior immunity and timing of introduction of the 1918 pandemic virus across populations.

Quantitative analyses of age-specific death rates, transmissibility, and dissemination patterns of the 1918 influenza pandemic in the United States (*1*,*2*), Mexico (*3*), Peru (*4*), Japan (*5*), Europe (*6*,*7*), Taiwan (*8*), and Singapore (*9*) have shed light on the epidemiology of the most devastating pandemic in recent history (*10*). These studies revealed the pandemic's unusual severity in young adults, occurrence in multiple waves, and higher transmission potential than that of seasonal epidemics (*11*).

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However, quantitative historical studies remain scarce for Latin America, Africa, and Asia, where our understanding of influenza disease patterns remains particularly weak.

The emergence of the pandemic influenza A (H1N1) 2009 virus in Mexico (*12*,*13*) reinforced the need to understand the epidemiology of past pandemics in the Americas to inform preparedness plans. We therefore analyzed death patterns for the 1918 influenza pandemic in Boyacá, a rural area in central Colombia, where influenza seasonality is less defined than in temperate regions (*14*). By using archival records, we quantified the age-specific excess-death rates and transmission potential of the 1918–19 pandemic in Boyacá and compared these findings with those reported for other locations, especially Mexico City, Mexico.

Materials and Methods

Study Location

Boyacá is located in the central part of Colombia within the Andes Mountains at latitude ≈5.5°N (Figure 1). In 1918, the population of Boyacá was 659,947 and <50% of the area was occupied. Hygienic conditions were poor. A centralized disease notification system was lacking; however, death records were maintained by parishes.

The climate in Boyacá varies from high humidity and high mean temperature (≈40°C) in low areas near the Magdalena River (altitude 600 m) to cold mean temperature (<6°C) and permanent snow in the Cocuy Mountains (altitutde 5,500 m). The 2 rainy seasons, April–May and October–November, produce ≈1,000 mm^3/rainfall/year.

Data Sources

Historical Death Records

A total of 32,843 death records, written mostly by Catholic priests and corresponding to January 1917–

Figure 1. Colombia, showing Boyacá (in red) and other departments. Inset, location of Colombia within South America. Boyacá is located in the central part of Colombia within the Andes Mountain range and has a surface area of 8,630 km^2. Insets show San Andrés Island (left) and Providencia Island. Figure adapted from http://en.wikipedia.org/wiki/Boyac%C3%A1_Department.

December 1920, were manually retrieved from the parish archives of 78 municipalities in the department of Boyacá. From these archival records, we extracted age, cause, and exact date of death. To estimate mortality rates, we compiled weekly numbers of deaths from all causes and from respiratory illness, stratified into 5-year age groups (Figures 2, 3). To obtain precise estimates of the transmission potential, we compiled daily death time series, combining all age groups.

Census Data

We obtained age-specific estimates of population size for the department of Boyacá from a 1918 census report (*15*). In 1918, ≈70% of Boyacá's population was located in rural areas. During 1912–1918, the average annual population growth rate in Boyacá was 1.7%; during 1918–1920, it was 3.8%.

Estimation of Excess Deaths

For characterization of mortality rates for the Boyacá pandemic, influenza-associated mortality rates must be separated from background mortality rates (deaths from respiratory illness other than influenza) and considered separately for each age group and cause of death (respiratory or all causes). To estimate pandemic mortality rate, we can define a discrete period of pandemic influenza activity and estimate the number of deaths in excess of background deaths that occurred during the pandemic period. Because mortality rates tend to vary seasonally throughout the year, our background estimate must also vary seasonally. To find the best estimate for baseline mortality rate in the absence of influenza activity, we applied regression methods, using harmonic terms and time trends, to mortality rate data (*6*,*16*,*17*) (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/10-1969-Techapp.pdf).

The regression model determines the extent to which observed weekly mortality rate fit the expectation of background mortality rate. Periods of poor fit indicate that observed mortality rate exceeds typical baseline levels, presumably because of increased influenza activity.

We defined pandemic periods as the weeks when deaths from respiratory illness exceeded the upper limit of the 95% CI of the background model. To estimate the mortality rate during the pandemic, for each age group we

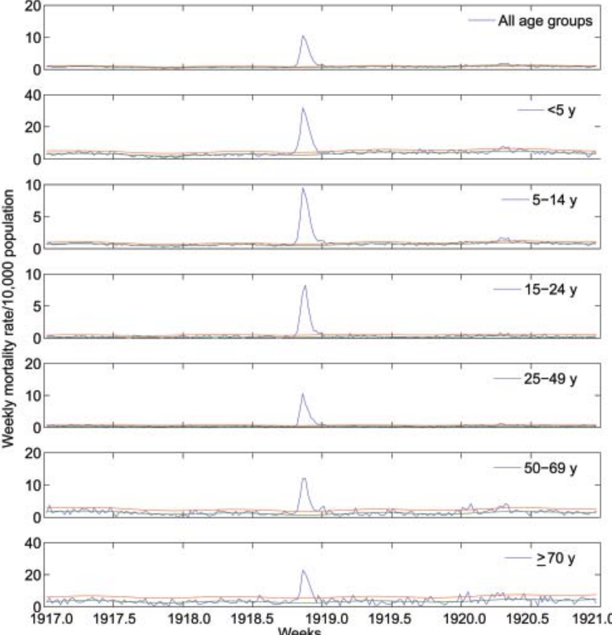

Figure 2. Age-stratified weekly respiratory mortality rates per 10,000 population in Boyacá, Colombia, 1917–1920. Background mortality rate derived from a seasonal regression model (blue); corresponding 95% CI curves are shown (red and green). Deaths in excess of the upper limit of the background mortality curve are deemed attributable to the 1918–19 influenza pandemic.

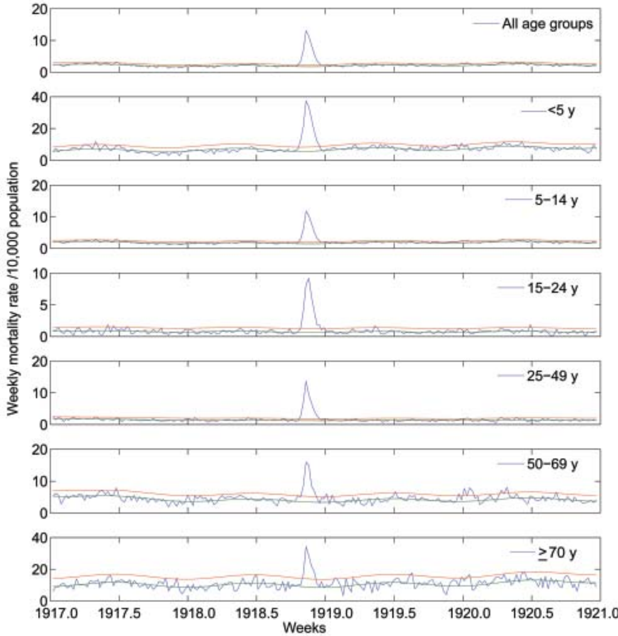

Figure 3. Age-stratified weekly all-cause mortality rates per 10,000 population in Boyacá, Colombia, 1917–1920. Background mortality rate derived from a seasonal regression model (blue); corresponding 95% CI curves are shown (red and green). Deaths in excess of the upper limit of the background mortality rate curve are deemed attributable to the 1918–19 influenza pandemic.

summed the weekly number of deaths from respiratory illness and from all causes that exceeded model baseline rates during each pandemic period during 1918–20.

To ensure that our estimates were not sensitive to modeling assumptions, we also estimated excess deaths by using an alternative approach to calculate background deaths. In this approach, background mortality rates for a given week are obtained by averaging mortality rates during the same week in previous years (online Technical Appendix).

Finally, we estimated a relative measure of the effects of pandemic-associated deaths for each age group, which considers the typical mortality rate experienced by that age group. We calculated relative risk for pandemic-associated death, defined as the ratio of excess deaths during pandemic periods to expected baseline deaths. Relative risk has been shown to facilitate comparison between age groups or countries, which have different background risks for death (*17*,*18*).

Comparing Patterns of Age-specif c Deaths

We compared patterns of age-specific excess deaths from the 1918–19 Boyacá pandemic with those recently published for Mexico City (*3*). The estimates for Mexico City were based on excess-death rates obtained from monthly pneumonia and influenza records (1916–1920), stratified by 6 age groups (<5, 5–19, 20–29, 30–49, 50–69, and ≥70 years). Excess-death rates for Mexico City were calculated with a method similar to that used in this study.

We also reviewed key epidemiologic features of the pandemic in various locations as recently reported (*1*,*3*,*4*,*6*–*9*,*19*,*20*), focusing on comparisons of overall excess-death rates associated with the pandemic. We also reviewed epidemiologic evidence for early (herald) waves occurring before September 1918 and for death sparing among elderly persons. We limited the review to studies that provided monthly or weekly historical death data because such data enable identification of herald waves and precise estimation of excess-death rates.

Estimation of Transmission Potential

Transmissibility of an infectious pathogen is measured by the basic reproduction number (R_0), which is the average number of secondary infections generated by an infectious person in an entirely susceptible population (*21*). A related quantity is the reproduction number, R, which can be used for partially immune populations who have been vaccinated or previously exposed to similar pathogens (*21*).

We estimated R for the 1918 pandemic virus in Boyacá by using a simple method that relies on the epidemic growth rate, a measure of how fast the number of cases increases over time (online Technical Appendix). Briefly, in the early ascending phase of an epidemic, the daily number of cases (or deaths) should follow an exponential function. By taking the log of daily deaths in the ascending phase, a straight line can be fit to the data. R can be derived from the growth rate estimate r by a simple equation involving the duration of the latency and infectious periods (*22*) (online Technical Appendix).

Because of the uncertainty associated with duration of the latency and infectious periods for influenza, we considered periods of 1.5 and 2 days each (*23*,*24*). Latency and infectious periods can be summed into a single statistic called the generation interval, which measures the interval between disease onset in 2 successive cases. The generation intervals considered in this study were 3 and 4 days (*23*,*24*).

We defined the ascending phase as the period between the day of pandemic onset (defined as the first day of the period of steadily increasing deaths) and the day immediately before the epidemic peak. We tested the robustness of R estimates to the choice of death indicator (deaths from respiratory illness or from all causes). We also compared estimates derived from crude numbers of deaths and excess deaths from respiratory illness that were above background rates.

The same approach and assumptions have been used to quantify Rs associated with the 1918 pandemic in

Copenhagen, Denmark, and Mexico City, and hence the Boyacá estimates are directly comparable to estimates from these studies (*3*,*6*). For comparison with Boyacá, we also reviewed the literature for published estimates of R associated with the 1918 pandemic in the Americas (*2*–*4*).

Results

Timing of Pandemic Waves and Age-specif c Patterns of Death

The age-stratified time series of deaths from respiratory illness or all causes in Boyacá indicated that a severe pandemic wave occurred during a 15-week period, October 20, 1918–January 26, 1919 (Figures 2, 3). The profile of age-specific excess deaths from respiratory illness associated with the pandemic period formed a W-shaped pattern; peak mortality rates among infants (<5 years of age) were followed by peak rates among elderly persons (>60 years) and young adults (25–29 years) (Table 1). Excess deaths were lowest among children 5–14 years of age and adults 50–59 years of age. Similar age patterns were found for all-cause deaths (Figure 4); the correlation coefficient between respiratory and all-cause excess-death rates was >0.99 ($p<0.01$). Excess deaths from respiratory illness captured most influenza-related all-cause excess deaths across all age groups (95% on average, range 81%–100%). Confidence intervals were larger for the most extreme age groups.

To facilitate the comparison between population age groups with different background risks for death, we calculated the risk for excess-death rates relative to baseline rates (Table 1). Although absolute excess-death rates were highest for young children (0–4 years of age) and elderly persons (≥60 years), during the pandemic the relative risks were lowest for these age groups. Relative risk was highest for young adults 25–29 years of age; excess-death rates increased 51-fold above background death rates for respiratory causes and 6-fold for all causes.

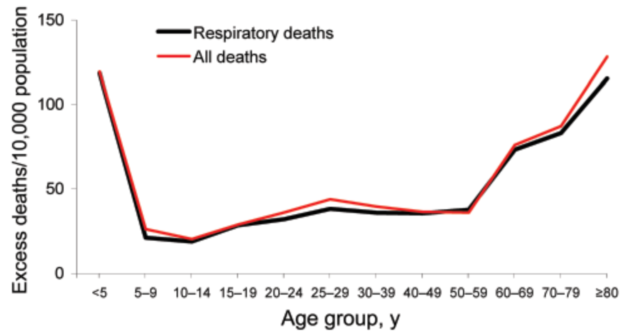

Figure 4. Age-specific excess-death rates per 10,000 population associated with the 1918–19 pandemic wave in Boyacá, Colombia, October 20, 1918, to January 26, 1919, based on deaths from respiratory illness and all causes.

Comparison of Boyacá and Mexico City shows that age-specific excess-death rates produced a W-shaped pattern for both locations (Figure 5). However, excess-death rates among young adults (20–29 years) were substantially higher for Mexico City than for Boyacá. By contrast, excess-death rates among infants were 2-fold lower for Mexico City than for Boyacá. Excess-death rates for elderly persons were similar for both cities. Overall, we estimate that the October 1918–January 1919 pandemic period was associated with 47 and 40 excess respiratory deaths per 10,000 population in Mexico City and Boyacá, respectively.

Table 1. Age-specific excess-death rates associated with the October 1918 –January 1919 influenza pandemic wave in Boyacá, Colombia*

	Deaths from respiratory illness		Deaths from all causes	
Age group, y	Excess mortality rate/10,000 population (95% CI)	Relative risk/ background mortality rate†	Excess mortality rate/10,000 population (95% CI)	Relative risk/ background mortality rate†
All ages	40.1 (39.1–41.1)	5.2	42.1 (39.1–44.1)	1.7
0–4	118.1 (111.1–125.1)	3.0	118.1 (109.1–127.1)	1.3
5–9	21.1 (20.1–23.1)	13.5	26.1 (23.1–29.1)	3.6
10–14	19.1 (18.1–20.1)	11.4	18.1 (16.1–20.1)	3.2
15–19	28.1 (27.1–30.1)	13.4	27.1 (24.1–30.1)	3.3
20–24	32.1 (30.1–33.1)	12.6	35.1 (31.1–38.1)	3.5
25–29	36.1 (34.1–37.1)	51.3	42.1 (39.1–45.1)	5.7
30–39	37.1 (35.1–38.1)	6.9	39.1 (36.1–42.1)	2.2
40–49	36.1 (33.1–39.1)	11.8	36.1 (31.1–41.1)	1.6
50–59	35.1 (31.1–40.1)	11.5	27.1 (19.1–35.1)	1.3
60–69	73.1 (67.1–80.1)	4.1	69.1 (55.1–82.1)	1.1
70–79	83.1 (69.1–98.1)	3.4	82.1 (59.1–106.1)	0.9
>80	100.1 (81.1–120.1)	3.5	124.0 (87.2–160.8)	0.9

*Excess death estimates are based on observed mortality rates during pandemic weeks occurring in excess of background mortality rates derived from a seasonal regression model.
†Ratio of excess deaths divided by background deaths during influenza pandemic weeks, facilitating comparisons across age groups with different background risks for death.

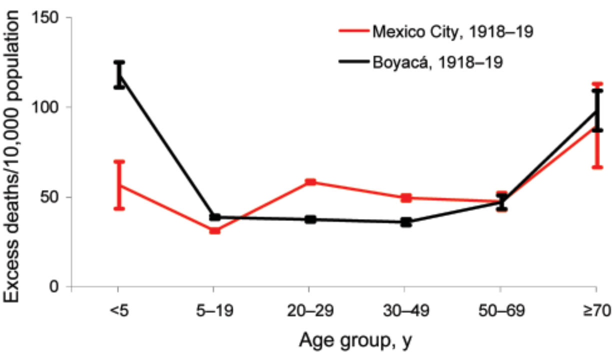

Figure 5. Comparison of age-specific excess-death rates for respiratory diseases during the main wave of the 1918–19 influenza pandemic in Mexico City, Mexico, and Boyacá, Colombia. Error bars represent 95% CIs, Mexico.

A broader comparison of epidemiologic patterns associated with the pandemic at 12 locations on different continents highlights substantial variations in the timing, number of pandemic waves, and age-specific death rates (Table 2). Europe and the United States generally experienced herald waves in during March–August 1918 (except for Paris) and low excess-death rates among elderly populations. In contrast, there was no evidence of death sparing among elderly populations in Latin America or Asia, and herald waves occurred at 4 of the 7 locations studied in these regions. Excess-death rates from respiratory illness were high for Iquitos, Peru; Toluca, Mexico; and Basque Provinces, Spain (121–288 deaths/10,000 population); intermediate in Taiwan (78–180 deaths/10,000 population); and lower elsewhere, including in Boyacá (29–67 deaths/10,000 population).

Reproduction Number Estimates

Table 3 provides summary estimates for the R for the 1918 influenza pandemic in Boyacá, based on growth in daily rates for death from respiratory illness. R was estimated to be 1.4, assuming a short generation interval of 3 days, and 1.5–1.6, assuming a longer interval of 4 days. A sensitivity analysis, based on excess deaths from respiratory illness occurring above a background of expected deaths, generated slightly higher R estimates (1.4–1.5 for a generation interval of 3 days and 1.6–1.7 for a generation interval of 4 days). Different approaches for estimating background deaths resulted in R estimate differences of <0.06 (4%).

Comparison of estimates derived from different locations in the Americas revealed some geographic variations in the transmission potential of the 1918–19 pandemic wave (Table 4). Although R estimates were 1.3–1.8 in most locations in the Americas, assuming a 3-day generation interval, the transmissibility of influenza during the autumn wave might have been particularly high in Toluca, Mexico (estimated R = 2.0–2.5).

Discussion

Our study makes use of extensive archival death records covering before and during the 1918–19 influenza pandemic in Boyacá, Colombia, and confirms the substantial number of deaths caused by the pandemic in this region. The main epidemiologic features of the pandemic in Boyacá include a single wave of excess deaths during October 1918–January 1919; high excess-death rates among infants and elderly

Table 2. Main epidemiologic features of the 1918–1920 influenza pandemic*

Location	Herald wave in 1918	Excess mortality rate from respiratory illness/10,000 population, main 1918–19 wave (mo of peak pandemic deaths, 1918)	Death-sparing effect among elderly persons	Reference
Americas				
New York, USA	Yes (Mar–Apr)	52 (Oct–Nov)	Yes	(*1*)
Mexico City, Mexico	Yes (May)	47 (Nov)	No	(*3*)
Toluca, Mexico	Yes (May)	162 (Nov)	No	(*3*)
Boyacá, Colombia	No	40 (Nov)	No	This study
Lima, Peru	Yes (Sep–Oct)†	29 (Nov)	No†	(*4*)
Iquitos, Peru	No	288 (Nov)	ND	(*4*)
Europe				
Copenhagen, Denmark	Yes (Jul–Aug)	39 (Nov)	Yes	(*6*)
Paris, France	No	61 (Oct)	ND	(*7*)
Basque Provinces, Spain	Yes (Jun)	121 (Oct)	ND	(*19*)
Madrid, Spain	Yes (Jun)	53 (Oct)	Yes	(*7*)
Asia				
Taiwan	No	67 (Nov)	No	(*8,20*)
Singapore	Yes (Jul)	78–180 (Oct)	ND	(*9,20*)

*Data from quantitative studies across different locations around the world. Locations are organized by continent (America, Europe, Asia) and latitude. ND, not determined.
†Cannot conclude because of lack of age-specific population data.

Table 3. Estimates of the growth rate and reproduction number associated with the 1918–19 influenza pandemic in Boyacá, Colombia*

Mortality outcome	Early growth phase period, 1918	Daily growth rate, mean (95% CI)	R estimate, mean (95% CI): 3-d generation interval, Exp dist.	3-d generation interval, Delta dist.	4-d generation interval, Exp dist.	4-d generation interval, Delta dist.
Deaths from respiratory illness	Oct 13–Nov 15	0.121 (0.120–0.122)	1.40 (1.39–1.40)	1.44 (1.43–1.44)	1.54 (1.54–1.54)	1.62 (1.62–1.63)
Excess deaths from respiratory illness	Oct 27–Nov 15	0.137 (0.136–0.139)	1.45 (1.45–1.46)	1.51 (1.51–1.52)	1.62 (1.62–1.63)	1.73 (1.72–1.74)

*Estimates are based on daily data. A generation interval of 3 or 4 d is assumed, with an exponential (exp) or a fixed (delta) distribution (dist.). R, reproduction number.

persons; and a moderate R (estimated at 1.4–1.5, assuming a 3-day generation interval).

We did not identify a herald wave of deaths from pandemic influenza in the early part of 1918 in Boyacá. According to epidemiologic data, herald waves of mild pandemic activity have been reported for the spring and summer of 1918 in other regions of the world, including New York City (*1*), Mexico (*3*), Lima (*4*) Geneva (*25*,*26*), Copenhagen (*6*), military camps in the United States (*6*), the United Kingdom (*27*), and Singapore (*9*). The absence of a herald wave in Boyacá could be explained by late introduction of the pandemic influenza virus; alternatively, a mild first wave may have occurred without causing many deaths. Thus, we cannot rule out early pandemic activity, which might have been associated with mild illnesses, before October 1918 in Boyacá. For instance, the summer pandemic wave of 1918 in Denmark was clearly evident only from time-series case data (*6*). These epidemiologic findings suggesting early pandemic virus activity have recently been confirmed by sequencing of pandemic influenza virus specimens isolated from Army camp populations in the United States as early as May 1918 (*28*).

Although substantial postpandemic waves have been reported for 1919–20 in New York City (*1*), Mexico City (*3*), Lima (*4*), Japan (*5*), and Taiwan (*8*), we could not identify a clear recrudescent pandemic wave in 1920 in Boyacá. A 3-week period in January 1920 and a 4-week period in April–May 1920 were associated with a small increase in deaths from respiratory illness, mostly affecting elderly persons, but we cannot with certainty attribute these deaths to pandemic influenza. Early public health warnings and effective implementation of control interventions in large cities such as New York City, Mexico City, Lima, and Taiwan, could have contributed to maintaining a large pool of susceptible persons, which could fuel subsequent pandemic waves (*29*). In Japan, postpandemic waves were somewhat limited to regions that escaped earlier waves (*5*). Given that Boyacá was a relatively small rural area, pandemic activity in 1918 might have proceeded unabated, with no particular interventions, medical or nonmedical. Alternatively, Boyacá could have escaped the recrudescent pandemic wave in 1920 because of its remote location. Overall, the main wave of deaths from pandemic influenza that occurred during October 1918–January 1919 in Boyacá is reminiscent of the single wave of pandemic influenza A (H1N1) 2009 wave that occurred in the Southern Hemisphere during the winter of 2009 (e.g., Chile [*30*], Australia [*31*], and New Zealand [*31*]). Additional data from the 1918 pandemic in other Southern Hemisphere locations are warranted before these findings can be generalized.

The W-shaped age-specific pattern of deaths during the 1918–19 pandemic wave in Boyacá is in agreement with recent reports from the Mexico City area (*3*) and Peru (*4*). These reports suggest a lack of death sparing among elderly populations of urban and rural areas of Latin America, although data from additional locations would be useful for generalizing these conclusions. This pattern is also in agreement with anecdotal evidence from aboriginal populations in Alaska in 1918 (32). In contrast to reports for Latin America and Alaska, reports for the United States and Europe suggest that elderly populations were substantially protected from influenza-associated death in 1918 (*1*,*5*,*6*). Previous studies have hypothesized that childhood exposure to influenza A (H1N1) viruses before 1870 might account for prior immunity among elderly persons during the 1918 pandemic. A similar phenomenon has been noted for pandemic (H1N1) 2009, during which risk for clinical infection and death was lower during the pandemic than during seasonal epidemics for persons >60 years of age (*13*,*33*).

Regional differences in prior immunity to influenza might result from heterogeneous circulation of influenza viruses during the 19th century, when long-distance travel was much less common than it is today (*3*). In 1918, Colombia's population of 5.8 million was heterogeneously distributed and relatively isolated from the rest of the world (*34*); this isolation could explain the lack of exposure to influenza viruses during the middle of the 19th century. Also in 1918, transportation was underdeveloped in Colombia, consisting mostly of horse- or mule-drawn street cars, waterways, and sparse railroads that did not connect with Boyacá (*34*). Remoteness could have affected the probability of introduction and of local dissemination of influenza viruses in the Boyacá region. A similar phenomenon could also explain the apparent lack of a herald pandemic wave

Table 4. Estimates of the reproduction number across influenza pandemic locations in the Americas, 1918–19*

Location, north to south	Time of pandemic wave	R estimate		Source
		3-d serial interval	6-d serial interval	
45 US cities†	1918 autumn†	1.7–1.8	2.5–3.3	(*2,22*)
Toluca	1918 spring	1.6–1.8	2.4–3.1	(*3*)
	1918 autumn	2.0–2.5	3.2–6.1	(*3*)
Mexico City	1918 spring	1.3–1.3	1.7–1.8	(*3*)
	1918 autumn	1.3–1.3	1.6–1.7	(*3*)
Boyacá, Colombia	1918 Oct–Nov	1.4–1.5	1.8–2.3	This study
Lima, Peru	1918 Nov–1919 Feb	1.3–1.4	1.6–2.0	(*4*)

*Values are based on a range of estimates provided by considering different distributions of the generation interval (exponentially distributed latent and infectious periods or fixed generation interval). R, reproduction number.
†R estimates are based on the mean of the initial growth rates across 45 US cities.

in the spring of 1918, when pandemic virus activity was not yet globally widespread. Of note, the capital city, Bogota, was the first area in Colombia to report increased influenza activity in October 1918; the virus quickly spread to other Colombia locations (*34*).

Excess-death rates among young adults were lower in Boyacá than in Mexico City (*3*). The reasons for this difference are unclear but could be associated with a more sporadic distribution of the population in Boyacá, resulting in lower overall influenza attack rates; however, we do not have epidemiologic evidence to support this assumption. Alternatively, the unidentified factors that made young adults particularly susceptible to influenza-related death in Europe, the United States, and Mexico in 1918 (*1*,*5*,*6*) might have been less common among young adults in Colombia. Despite these geographic differences in absolute risk for death from pandemic influenza, in all locations with sufficient data the relative risk for death consistently peaked among adults 20–29 years of age when compared with baseline death rates during nonpandemic years. Hence, our study confirms the universal atypical severity of this virus in young adults, as previously reported for the United States (*1*), Mexico (*3*), Europe (*6*,*7*), and Taiwan (*8*). We also note that data from Boyacá and Mexico City do not support the pessimistic hypothesis that populations lacking prior immunity to the 1918 virus would experience a V-shaped age-associated risk for death, in which risk would rapidly and continuously rise past teenage years (*35*).

Our excess-deaths approach warrants some caveats. The regression model used to estimate background deaths poorly fit the Boyacá data during the nonpandemic period, probably because of weak seasonality. However, our estimates of excess deaths from pandemic influenza based on deaths from respiratory illness and all causes were highly correlated, similar to those from other temperate countries, where baseline death rates are more seasonal (*1*,*3*,*6*). The sensitivity analysis that we conducted by using an alternative approach to estimate background deaths did not make assumptions about seasonality (*20*). This analysis produced excess-death estimates highly correlated with those derived from the regression approach (correlation = 0.97; p<0.01; mean difference 4%–7%).

Transmissibility estimates derived from 1918–20 pandemic illness and death data are 1.5–5.4 for community-based settings in several regions of the world (*2*,*6*,*36*,*37*) (Table 4). Our transmissibility estimates for Boyacá, Colombia, assuming a generation interval of 3 days, are in close agreement with those reported for the wave in autumn in Mexico City (*3*), Lima (*4*), England and Wales (*27*), and Copenhagen (*6*) and slightly lower than estimates reported for the city of Toluca, Mexico (*3*), and US cities (*2*,*38*). Boyacá's sparsely distributed population could explain why the estimated disease transmissibility is relatively low. It remains unclear whether differences in reproduction number estimates across locations and pandemic waves reflect true differences attributable to variation in attack rates or local factors affecting transmission or merely illustrate difficulties in measuring this parameter with precision (*38*). In previous studies focused on reproduction number estimates in which we used similar data and approaches, we have shown that inclusion of a delay between disease onset and death has little effect on the estimates (*39*).

In conclusion, historical studies from understudied areas are especially helpful for documenting the global death rates and transmission patterns of the 1918 pandemic and for revealing substantial variations among locations. In particular, the lack of death sparing for elderly persons in Colombia and Mexico differs markedly from contemporaneous observations in the United States and Europe. During the 19th century, the Latin American region was relatively isolated (and still is today) (*40*), which would affect the circulation of historical influenza viruses and baseline population immunity to influenza. We believe that this finding suggests recycling of influenza viruses as the best explanation for death sparing among elderly persons in the United States and Europe in 1918. Preservation and interpretation of archival epidemiologic data are crucial for a better understanding of past pandemics and for better preparedness against future pandemics.

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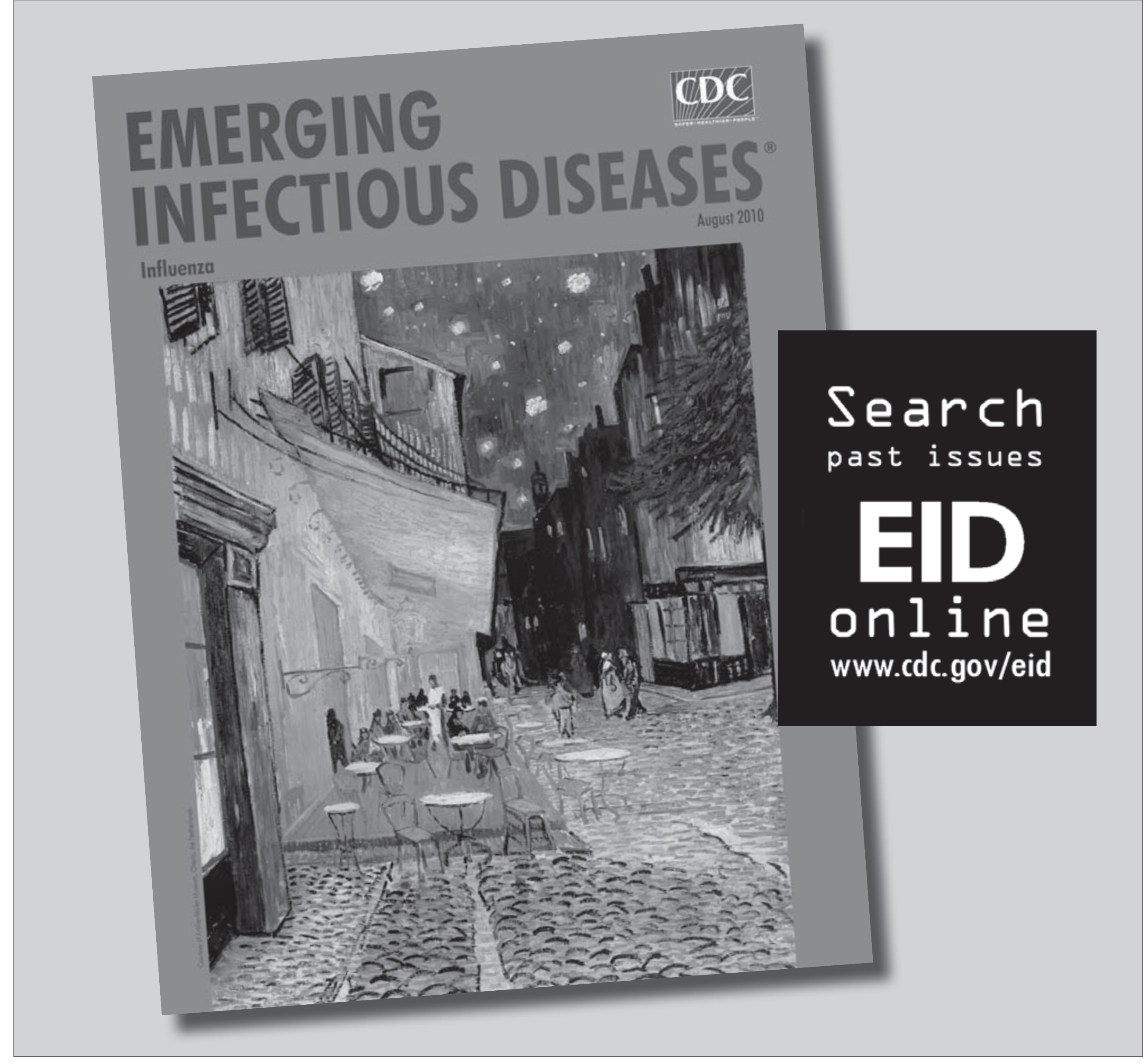

Use of Lean Response to Improve Pandemic Influenza Surge in Public Health Laboratories

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A novel influenza A (H1N1) virus detected in April 2009 rapidly spread around the world. North American provincial and state laboratories have well-defined roles and responsibilities, including providing accurate, timely test results for patients and information for regional public health and other decision makers. We used the multidisciplinary response and rapid implementation of process changes based on Lean methods at the provincial public health laboratory in British Columbia, Canada, to improve laboratory surge capacity in the 2009 influenza pandemic. Observed and computer simulating evaluation results from rapid processes changes showed that use of Lean tools successfully expanded surge capacity, which enabled response to the 10-fold increase in testing demands.

A novel influenza A (H1N1) virus was detected in Mexico and the southwestern United States in early April 2009 (*1*). Within days after confirmation that this virus was circulating in the western Canadian province of British Columbia, the number of requests for influenza diagnostic tests rapidly increased. It became evident that current operations would not enable the British Columbia Public Health Microbiology & Reference Laboratory (PHMRL), the major provider of influenza diagnosis for this province, to meet testing demands. We describe Lean processes that were implemented to rapidly expand surge capacity.

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Methods

Prepandemic Testing for Inf uenza

The PHMRL serves the entire health care system for the western Canadian province of British Columbia (population 4.45 million). Pandemic planning lead by the Canadian Public Health Laboratory Network included implementation of a reverse transcription PCR (RT-PCR) platform. Before the pandemic, sample data were entered into the Laboratory Information System (LIS) and barcoded in the Central Processing & Receiving section; the accessioned respiratory samples were then transported to the Virology Laboratory, located 3 floors away. In the Virology Laboratory, 1 laboratory assistant organized the samples and transferred aliquots into labeled tubes.

Testing was conducted by 1 medical laboratory technologist; tasks included nucleic acid extraction, RT-PCR, analysis of results, and report of results into the LIS. One easyMag extractor (bioMérieux, Marcy l'Etoile, France) (capacity 22 patient samples) and 1 ABI 7900 RT-PCR machine (Applied Biosystems, Foster City, CA, USA) (capacity 92 patient samples) were used. These processes were conducted 10.5 h/d, 6 d/wk during the normal British Columbia influenza season (September–March) by 1 laboratory assistant and 2 medical laboratory technologists (1 technologist on each of 2 shifts). These assignments enabled PHMRL to meet prepandemic demand for influenza testing. Test results were available on the same day as arrival in PHMRL, except for weekends. Volumes seldom exceeded 50 samples/d.

Initial Pandemic Response

Once the pandemic was confirmed, the need for an RT-PCR to reliably detect the novel subtype was the highest priority. Because of Canadian Public Health Laboratory Network leadership (*2*), followed by application of the recommendations by the provincial public health laboratory, an RT-PCR for influenza targeting the conserved matrix gene had been used in our Virology Laboratory for several years. However, it was able to identify only the new virus as influenza A; the subtyping RT-PCR (for seasonal subtype H1N1 virus) was not able to identify the pandemic virus. Using genomic information provided in GenBank by the Centers for Disease Control and Prevention (Atlanta, GA, USA), we addressed this priority by sequencing the M gene amplicons generated by the existing RT-PCR (*3*), which enabled initial testing response. Further improved assays were introduced later and validated against an RT-PCR provided by Canada's National Microbiology Laboratory. Commercially available point-of-care devices were not recommended in Canada except for use in defined small and remote communities.

Response to Surge by Using Lean Methods

With an assay that could detect the novel pathogen, expansion of the overall surge capacity of the PHMRL was the critical next step. Medical and operations directors met with a multidisciplinary team of scientific and senior technical staff to determine how to address this challenge. Although additional equipment and reagents were immediately approved for purchase, hiring of additional staff was not. Improving efficiencies by changing workflow processes was necessary. Before the onset of the pandemic, PHMRL staff had been trained in Lean methods (*4*,*5*), and many had participated in activities to enhance laboratory processes. Lean principles have been used extensively in manufacturing industries and more recently applied to various health care domains, including facility design (*6*) and redesign (*7*,*8*), patient flow (*9*), infection control (*10*), and clinical pathology laboratories (*11–13*). A key principle of Lean thinking is that work can be done more efficiently by identifying and eliminating waste to optimize the workflow. Lean also focuses on meeting client needs and involving support staff in making ongoing improvements to their work processes.

A Lean Team comprising medical, scientific, senior, and bench-level technical staff and some administrative and support personnel was set up to apply Lean methods (Table) to the influenza laboratory workflow. The team first created a flowchart of baseline laboratory tasks to assess processes, staff assignments, and sample batch sizes. This visually displayed value stream (Table) included all discrete steps in the laboratory workflow (Figure 1) for influenza detection and subtyping with the resources and time required for each step. Process information from the separate traditional laboratory work areas (Central Processing & Receiving on the ground floor and the Virology Laboratory on the fourth floor) were included. Review showed that most waste occurred between each discrete process step (wait times, setup time) as the 1 medical laboratory technologist in the Virology Laboratory took each batch of samples through the steps of extraction, RT-PCR, and reporting. The addition of 2 ABI 7900 RT-PCR analyzers and a high-throughput nucleic acid ABI MagMAX extractor (Applied Biosystems) with capacity of 92 patient samples highlighted current workflow process inefficiencies.

Table. Lean methods used to improve laboratory workflow

Method	Definition
Kaizen	The incremental, ongoing improvement process aimed at creating efficiencies and improving all functions. Productivity increases are attributed to eliminating waste in the system and having all staff participate in the process.
Value stream mapping	A technique used to identify the materials and information required in each step of a process to deliver a product to a consumer
Andon	A visual cue used to monitor functions and to notify workers of a quality or process issue
5S	The process of sorting, storing, sweeping, sustaining, and standardizing, which results in a visually managed environment that allows staff to perform tasks more efficiently

A kaizen session (Table) working with staff in the Virology Laboratory originated the concept of flow cells in which, instead of all steps in the influenza detection process being performed by 1 medical laboratory technologist, each task in the work flow was separated into individual flow cells. Separate staff members were dedicated to each cell. Within the new process (Figure 2), the new visually linked work units created more repetitive, standardized procedures. Some of the flow cells with shorter cycle times (such as data entry) could then be performed more times per day by the new flow cell teams. Their outputs were balanced with other flow cells with longer cycle times (such as RT-PCR). Flow cells, each with a new standard operating procedure and training checklist, were implemented with the support of laboratory staff and included a data entry cell; a labeling cell; a pipetting/aliquoting cell; and extraction, RT-PCR, and reporting cells. Put in place in the appropriate value stream sequence within the Virology Laboratory, they formed a new rapidly scalable and standardized work process.

The Lean Team also implemented changes in staffing to match improvements in workflow processes so that laboratory assistants were assigned the tasks of sample receiving, sorting, and triaging; data entry (accessioning into the LIS); and other support duties, and medical laboratory technologists were assigned to sample preparation, DNA

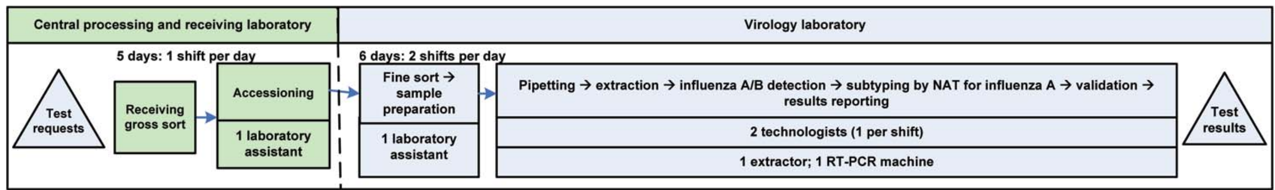

Figure 1. Seasonal influenza testing processes (pre-kaizen value stream). NAT, nucleic acid amplification techniques; RT-PCR, reverse transcription PCR.

extraction, RT-PCR, result validation and result reporting. Samples were batched into groups of 92 to optimize the plate platform.

Other efficiencies identified by the Lean Team included redeploying laboratory assistants, who were responsible for respiratory sample triage and data entry but worked 3 floors away, to work in the Virology Laboratory. This change also ensured that questions related to samples could be immediately addressed. Three additional laboratory assistants now joined the existing Virology Laboratory assistant in 2 shifts (2 laboratory assistants per shift). To accommodate the new workflow and the unprecedented testing demand, medical laboratory technologists from other laboratories in the PHMRL were cross-trained. Reducing the influenza detection process into discrete flow cell units meant that these additional medical laboratory technologists could quickly be trained and gain documented competency in the procedures within each flow cell. The new simplified, specific task focused workflow now enabled 8 medical laboratory technologists in 2 shifts (4 medical laboratory technologists per shift in 1 flow cell each) that could draw from a developing reserve pool of cross-trained staff.

Applying another Lean principle, the 5S approach (Table), we reorganized and clearly labeled bench tops and other work areas to demarcate discrete workflow steps. This change enabled visual management of the samples now being processed in a single site (Virology Laboratory).

Evaluation by Modeling Analysis

As part of a national laboratory initiative, PHMRL collaborated with the consulting firm Booz Allen Hamilton (McLean, VA, USA) to assess pandemic surge capacity. Model inputs, including information equipment, sample volumes, tests, work shifts, task times, and staff number and type, were collected, and prepandemic influenza data from the previous year's peak month (January 2008) were compared with peak pandemic month (October 2009). Data were used in a discrete event model in the ExtendSim Process Modeling Environment (Imagine That, Inc., San Jose, CA, USA), a customized process model replicating the PHMRL pandemic processes. FluLabSurge (*14*), an Excel-based software program (Microsoft, Redmond, WA, USA), was also used to provide a theoretical estimate of test volumes from a 1968-level pandemic (moderate severity) when corrected for current British Columbia population. Other model assumptions were 1) staff absenteeism was constant at 5%; 2) staff were considered fully used at 85% (accounting for breaks, administrative duties, and consultations with co-workers); 3) the simulation was run for a 180-day pandemic; and 4) reagents were not considered a limiting factor.

Results

The new Lean-based flow cell configuration with additional equipment, 3 additional laboratory assistants, 3 additional medical laboratory technologists per shift (by using newly cross-trained staff from other PHMRL laboratories), and an extended workday (13.5 h), enabled large numbers of samples to be tested each day. Implementation of the new flow cells for staffing balanced the additional equipment capacity, creating a smooth workflow of repetitive tasks that resulted in decreased sample waiting times and more efficient throughput. This

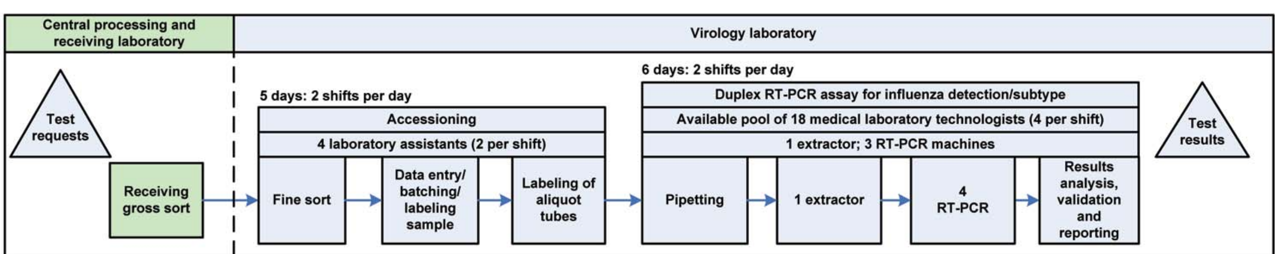

Figure 2. Pandemic (H1N1) 2009 testing processes (post-kaizen with the flow cells depicted as processes occurring within a box). RT-PCR, reverse transcription PCR.

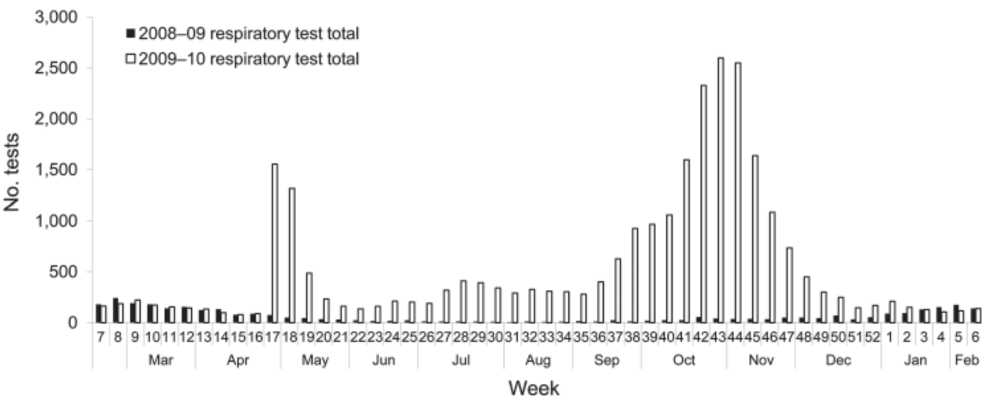

Figure 3. Number of respiratory samples tested by the British Columbia (Canada) Public Health Microbiology & Reference Laboratory in the 2008–09 influenza season compared with the pandemic (H1N1) 2009.

rapid response, implemented within a few days after the initial Lean team meeting, enabled the laboratory to meet the surge in demand; the number of tests and percentage of samples positive for influenza during the pandemic were beyond anything the laboratory had ever experienced (Figure 3). Compared with a maximum daily test volume of 53 in the 2008 routine influenza season, the corresponding maximum in the 2009 pandemic was 573 (November 3, in the second wave).

Results of modeling the surge that used the 2 different operational processes (prepandemic and pandemic modes) showed that an average of 231 samples per day theoretically could have been managed (with same-day testing) by using traditional seasonal influenza processes and resources. Although the actual maximum sample volume was only 53 samples per day in our experience, modeling was thus able to quantitate the potential capacity by using standard prepandemic resources and processes. Modeling again was able to quantitate the capacity increase after improvements that used Lean methods, calculating the testing capacity to be 528 samples per day (Figure 4) based on flow cell processes. Modeling also showed that, had the laboratory actually been faced with a 1968-level pandemic with its much higher sample surge, demand would have far exceeded output throughout the pandemic wave by using seasonal resources (Figure 5).

Daily reports based on another Lean concept, andon (Table), relayed the pandemic status to management. Information on test volumes; influenza virus subtypes; positivity rates; turnaround times; and status of equipment, reagents, and staffing were made available. Successful implementation of process improvements by using Lean methods rely heavily on acceptance and uptake by staff; after an initial discussion and decision to apply Lean principles, kaizen sessions were held with staff on the work floor. Other internal communications included staff training (and technical competency assessment) sessions, staff meetings, acknowledgment of contributions on bulletin boards, and regular internal newsletters. All PHMRL staff were invested in the pandemic because contributions came from all laboratory sections to support the Virology Laboratory.

Discussion

Although medical laboratories, including public health laboratories, are critical to the health care system, their

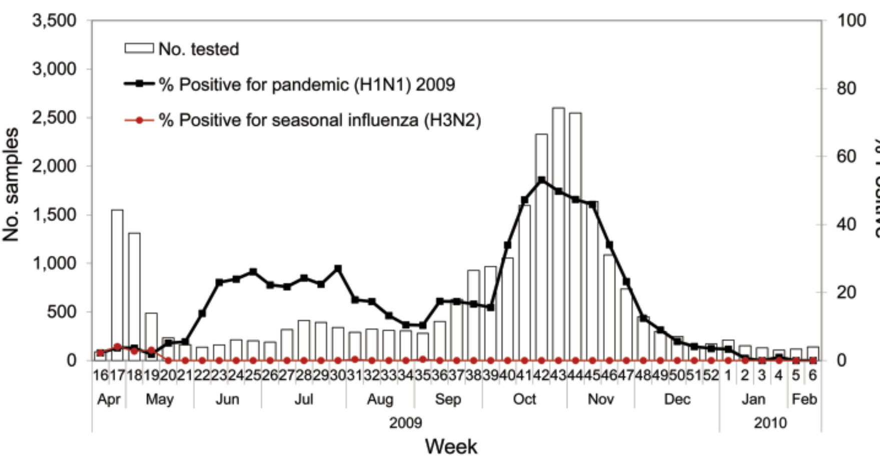

Figure 4. Simulated pandemic FluSurge level of daily test volume demand showing British Columbia (Canada) Public Health Microbiology & Reference Laboratory seasonal capacity estimated to be 231 samples per day and postemergency (kaizen) pandemic capacity 528 samples per day.

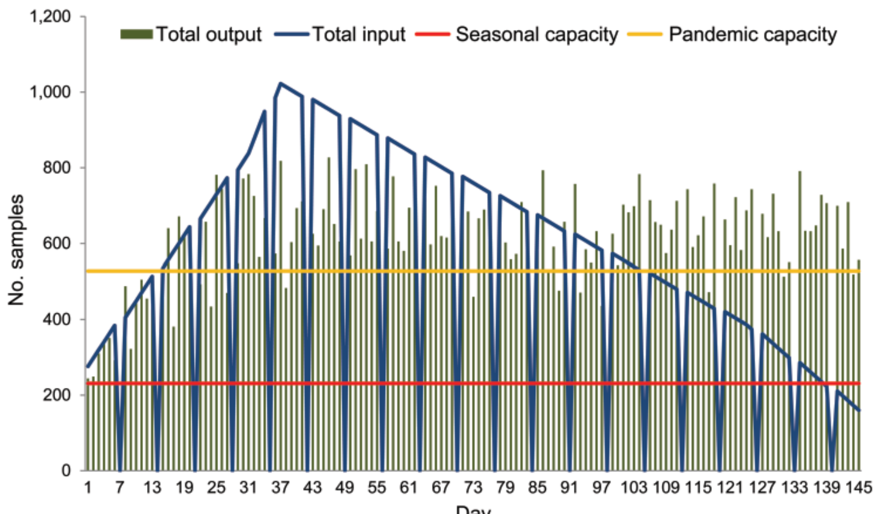

Figure 5. Simulated back-log using seasonal and postemergency (kaizen) pandemic processes with a FluLabSurge (1968 pandemic) level of expected test demand for population, British Columbia, Canada.

role is often not fully recognized. In addition to affecting >80% of clinical decisions (*15*), laboratories provide vital functions related to community-level health care. The 2009 pandemic underscores the contributions of laboratories and the need for continuous improvements by using methods such as Lean. Such preparedness provides staff with the ability to make rapid improvements, such as in surge capacity, and to share innovations and improvements (*16*). Although the initial response was development and implementation of the new RT-PCR by scientific staff and although prior preparation as a network was fundamental to the pandemic response, training local laboratory staff in Lean methods enabled these tools to be applied for rapid improvements in the PHMRL influenza detection process. In particular, implementation of flow cells to standardize repetitive tasks and to balance the additional equipment acquired resulted in meeting acceptable turnaround times despite the unprecedented influx of samples. Results of the rapid response were shown by computer process modeling to have been critical. Without making the changes noted above, the PHMRL would have been unable to meet the testing demands in the 2009 pandemic.

As fiscal constraints in health care continue to grow, the system needs to recognize the contributions—and the needs—of the complex laboratory system. We demonstrated how the application of Lean tools can rapidly improve processes required for surge capacity. They can be used between events for finding ongoing required system efficiencies. Computer process modeling that confirmed the Lean Team's work also appears to be a tool that, with further refinements, could be used to predict ways of improving other laboratory processes and to guide further change.

Acknowledgments

We thank all PHMRL staff who made this successful pandemic response possible. We also appreciate the timely support provided by Provincial Health Services Authority and the support of public health and clinical leaders who worked on the pandemic response across Canada.

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Dr Isaac-Renton is director of the PHMRL and a professor of medical microbiology at the University of British Columbia. Her primary research interest is the safety of drinking water.

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The International Conference on Emerging Infectious Diseases was f rst convened in 1998; ICEID 2012 marks its eighth occurence. The conference brings together public health professionals to encourage the exchange of scientif c and public health information on global emerging infectious disease issues. The program will include plenary and panel sessions with invited speakers as well as oral and poster presentations on emerging infections. Major topics to be included are current work on surveillance, epidemiology, research, communication and training, bioterrorism, and prevention and control of emerging infectious diseases, both in the United States and abroad.

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Invasive Meningococcal Capsular Group Y Disease, England and Wales, 2007–2009

Shamez N. Ladhani, Jay Lucidarme, Lynne S. Newbold, Stephen J. Gray, Anthony D. Carr, Jamie Findlow, Mary E. Ramsay, Edward B. Kaczmarski, and Raymond Borrow

Enhanced national surveillance for invasive meningococcal disease in England and Wales identified an increase in laboratory-confirmed capsular group Y (MenY) disease from 34 cases in 2007 to 44 in 2008 and 65 in 2009. For cases diagnosed in 2009, patient median age at disease onset was 60 years; 39% of patients had underlying medical conditions, and 19% died. MenY isolates causing invasive disease during 2007–2009 belonged mainly to 1 of 4 clonal complexes (cc), cc23 (56% of isolates), cc174 (21%), cc167 (11%), and cc22 (8%). The 2009 increase resulted primarily from sequence type 1655 (cc23) (22 cases in 2009, compared with 4 cases each in 2007 and 2008). cc23 was associated with *lpxL1* mutations and meningitis in younger age groups (<25 years); cc174 was associated with nonmeningitis, particularly pneumonia, in older age groups (≥65 years). The increase in MenY disease requires careful epidemiologic and molecular monitoring.

Invasive meningococcal disease is associated with substantial rates of illness and death worldwide (*1*), with most infections caused by 5 capsular groups, namely A, B, C, W135, and Y (*2*). In the United Kingdom, where capsular group C (MenC) conjugate vaccines have been routinely used since 1999 (*3*,*4*), capsular group B (MenB) causes >80% of confirmed cases, particularly among infants and adolescents (*5*). Invasive infections caused by other capsular groups are infrequent and sporadic (*6,7*).

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Invasive capsular group Y (MenY) has historically been uncommon in the United Kingdom and until recently accounted for <30 cases annually (*5*). After 2006, however, enhanced surveillance by the Health Protection Agency (HPA) identified an increase in invasive MenY cases in England and Wales. In addition, recent surveys suggest that MenY carriage in England increased substantially during the past decade (*8*,*9*). Consequently, the HPA investigated the clinical, epidemiologic, and microbiologic characteristics of invasive MenY disease in England and Wales during 2007–2009.

Methods

The HPA conducts meningococcal surveillance in England and Wales through a combination of clinical and laboratory reporting schemes and receives all meningococcal death registrations from the Office for National Statistics (www.statistics.gov.uk). In addition, the HPA Meningococcal Reference Unit provides a national service for species confirmation and grouping/typing of invasive *Neisseria meningitidis* and offers free nonculture confirmation by using PCR directly from clinical specimens routinely submitted by National Health Service hospital laboratories (*5*). Data from all sources are reconciled in a single database and deduplicated regularly. Invasive meningococcal disease was defined as isolation of the organism or identification of meningococcal DNA from a normally sterile body site. MenY was confirmed by sero/genogrouping (*5*).

Clinical Isolates

Isolates were preserved at –80°C on Microbank cryovials (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). Overnight cultures were performed on Columbia

agar plus 5% (vol/vol) horse blood (Oxoid, Basingstoke, UK) at 37°C in an atmosphere containing 5% CO_2.

Bacterial Genomic DNA Extraction

DNA was extracted by using the DNeasy blood and tissue kit (QIAGEN, Crawley, UK). Briefly, 1 mL meningococcal suspension in physiologic saline (adjusted to an optical density equal to 0.1 at 650 nm) was heat-killed at 60°C for 70 min and then pelleted at 6,000 × *g* for 10 min. After aspiration of the supernatant, DNA was extracted by using the manufacturer's gram-negative protocol (QIAGEN) (*10*).

Molecular Typing

Multilocus sequence typing and genotypic analysis of PorA variable regions (VRs) (*porA* VR1 and VR2), ferric enterochelin receptor VR (*fetA*), neisserial heparin-binding antigen (*nhba*), neisserial adhesin A (*nadA*), and factor H binding protein (*fhbp*) encoding regions were performed by using described protocols (*11*,*12*). Molecular typing results are reported by using the following recommended format: capsular group: porA (P1) subtype.VR1,VR2: fetA VR: ST- (cc); for example, Y: P1.5-1,10-4: F4-1: ST-23 (cc23) (*13*). Sequence data have been deposited in GenBank under the following accession numbers (corresponding peptide subvariant identifiers in parentheses): for *nhba*, JN166971 and JN166972 (P0006), JN166973 (P0007), JN166974 (P0008), JN166975 (P0009), JN166976 (P0020), JN166977 (P0024), and JN166978 (P0242); and for *nadA*, JN166979 (P0008). *fhbp* subvariants are assigned in the format "Novartis variant family.peptide" according to www.pubmlst.org.

The lauroyl acyltransferase gene, *lpxL1*, was genetically characterized by using primers listed in Table 1 (*14*). *lpxL1* PCRs were performed by using the HotStarTaq DNA polymerase kit (QIAGEN), with an initial activation step of 96°C for 15 min and a final step of 72°C for 7 min. The default PCR used primers *lpxL1-F* and *lpxL1-R* in which a single reaction comprised 2.5 µL of 10× PCR buffer (provides 1.5 mmol/L MgCl), 2.5 µL of each primer (5 µmol/L stock), 0.5 µL dNTP mixture (10 mmol/L [per dNTP] stock), 0.125 µL HotStar Taq (0.625 units), 14.875 µL molecular-grade water, and 2 µL of DNA template. Thermocycling conditions comprised 35 cycles of 96°C for 30 s, 63°C for 30 s, and extension at 72°C for 60 s. Where the default PCR was unsuccessful, further PCR was performed by using the alternative reverse primer, *lpxL1-rR4*, in conjunction with a final $MgCl_2$ concentration of 1.875 mmol/L and an extension step of 200 s. Sequence analysis was performed by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). A single sequencing reaction comprised 1.75 µL 5× sequencing buffer, 0.5 µL BigDye master mix, 0.66 µL primer (5 mmol/L stock), 6.09 µL molecular-grade water, and 1 µL purified PCR product.

Clinical Follow-up

Clinical follow-up was conducted for all MenY cases in 2009 confirmed by culture, PCR, or both. The general practitioner, hospital physician, or local health protection unit was contacted for information about underlying medical conditions, recent travel, clinical presentation, sequelae, and outcome.

Statistical Methods

Data were analyzed by using Stata version 11.0 (StataCorp LP, College Station, TX, USA). Annual age-specific population estimates were obtained from the Office for National Statistics. Data that did not follow a normal distribution were described as medians with interquartile ranges (IQRs) and compared by using the Mann-Whitney U test. Proportions were compared by using the χ^2 test or Fisher exact test, as appropriate. Logistic regression was used to calculate odds ratios (ORs) with 95% CIs after adjustment for potentially confounding variables and to identify independent risk factors for death.

Ethical Approval

HPA has approval under the Health and Social Care Act 2001 to process confidential patient information for

Table 1. Primers used for genotypic analysis of *lpxL1* of meningococcal capsular group Y, England and Wales, 2007–2009*

PCR/sequence	Primer identification no.	Direction	Sequence, 5′ → 3′	Reference
PCR/sequence	lpxL1-F†‡§	Forward	TGCAGGTCAAACAGGCGGTAGT	(*14*)
PCR/sequence	lpxL1-R†¶#	Reverse	TTCAT(A/G)GGTTTGCGGTATTTCTTCCA	(*14*)
PCR	lpxL1-rR4‡	Reverse	TCCACTTGAAATCGCGGCTGTC	NA
Sequence	lpxL1-s1C#	Forward	GTTCGAGATGGCGGTGTAC	NA
Sequence	lpxL1-s2#	Reverse	GAATCGTTGCGTCCGAAATCCTG	NA
Sequence	lpxL1-rR3§	Reverse	AATACAGGCTTTCGCCTGCG	NA
Sequence	lpxL1-Rnew§	Reverse	GTCAGTAAAAATCGGGGCTGCC	NA

*NA, not applicable.
†Default PCR primer.
‡Alternative PCR primer.
§Used for sequence analysis of alternative PCR products (*14*).
¶Degenerate base added for this study.
#Used for sequence analysis of default PCR products.

public health purposes. Details are available at www.legislation.hmso.gov.uk/si/si2002/20021438.htm.

Results

Epidemiology

Invasive meningococcal disease in England and Wales declined from 1,283 cases in 2007 to 1,228 in 2008 and 1,031 in 2009, with 54%, 53%, and 57% of cases, respectively, confirmed by PCR alone. MenB cases accounted for 1,096 (85%), 1,102 (90%), and 917 (89%) cases during this period, and MenY cases increased from 34 (3%) to 44 (4%) and 65 (6%), higher than at any time during the previous 30 years (*5*,*15*) (Figure 1, panel A). Surveillance and diagnostic methods that might have contributed to this increase did not change.

Clinical Follow-up

In 2009, there were 65 invasive MenY cases (46 culture-confirmed and 19 PCR-confirmed), equivalent to 1.2 cases per million population (Table 2). The incidence was >10-fold higher for persons ≥85 years of age (10.5/1,000,000) than for persons <85 years of age (0.99/1,000,000). Median age at disease onset was 59.9 years (IQR 19.2–82.5 years). Most cases (58 [89%]) occurred among whites; the remainder occurred among Indians (4 [6%]) and black Afro-Caribbeans (3 [5%]). Clinical features varied by age group (Table 2; Figure 1, panel B). More than one third (25 [38%]) had at least 1 underlying medical condition; 12 (18%; 95% CI 9.9%–30.0%) died. Likelihood of underlying medical conditions and death increased with age. Case-fatality rate (CFR) was significantly higher for persons with underlying medical conditions (11 [44%] of 25 vs. 1 [3%] of 40; χ^2 = 17.6, p<0.001) and varied by clinical feature, being substantially higher for pneumonia (9 [47%] of 19) than for meningitis (2 [9%] of 22), septicemia (1 [6%] of 17), or other diagnoses (no deaths). Median age at disease onset was higher for persons with pneumonia than for those with other conditions (86.1 years [IQR 69.9–90.0] vs. 42.8 years [IQR 14.4–71.1]; p<0.0001), and persons with pneumonia were also more likely to have underlying medical conditions (13 [68%] of 19 vs. 12 [26%] of 46; χ^2 =10.2, p = 0.001). After adjustment for age, underlying medical condition (OR 19.2, 95% CI 2.1–174, p = 0.009) and pneumonia (OR 7.0, 95% CI 1.4–36.4, p = 0.020) remained independently associated with death.

Molecular Typing

All 114 invasive MenY isolates for 2007–2009 were subjected to molecular typing. Age distribution was as follows: 21 (18%) cases among persons <5 years, 42 (37%) among persons 5–64 years, and 51 (45%) among persons ≥65 years of age. Clinical diagnosis was available for 60 cases (46 culture-positive cases from 2009 and 14 cases from the previous 2 years for which clinical details were provided with the isolate) and included meningitis, septicaemia, and pneumonia (18 [30%] each) and other presentations (6 [10%]). Almost all (110 [96%]) isolates belonged to 1 of 4 ccs: cc23 (64 [56%]), cc174 (24 [21%]), cc167 (12 [11%]), and cc22 (9 [8%]) (online Appendix Table, wwwnc.cdc.gov/EID/article/18/1/11-0901-TA1.htm). The only notable increase was observed for cc23 isolates, which more than doubled from 14 and 16 in 2007 and 2008, respectively, to 34 in 2009.

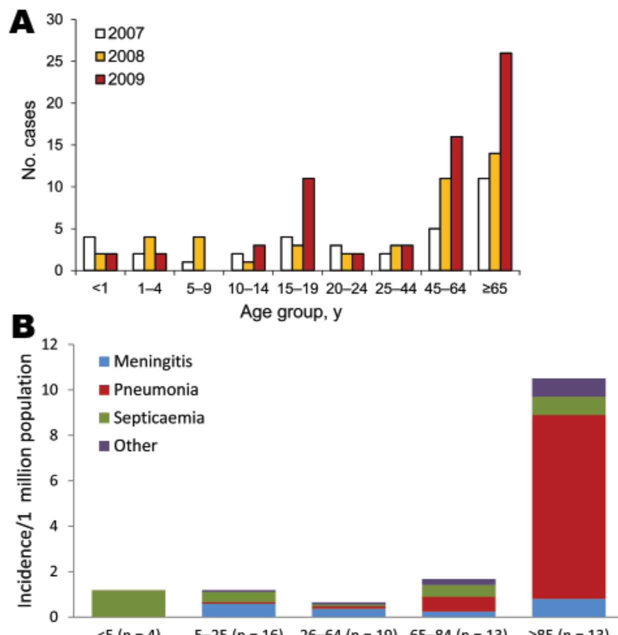

Figure 1. Number of persons with invasive meningococcal capsular group Y (MenY), by age group and year (A) and incidence with clinical features of MenY disease, by age group, in 2009 (B), England and Wales.

One cc23 isolate was not fully characterized by sequence type (ST) because existing primers failed to amplify the glucose-6-phosphate dehydrogenase gene (*gdh*) locus. The remaining 6 loci were successfully characterized and sufficient to assign the isolate to cc23. Of the 63 remaining cc23 isolates, 52 (83%) constituted ST-1655 (30 [48%]) or ST-23 (22 [35%]), and 20 (83%) of 24 cc174 isolates belonged to ST-1466 (online Appendix Table). The 2009 increase resulted almost exclusively from expansion of ST-1655, from 4 (13%) and 4 (12%) cases in 2007 and 2008, respectively, to 22 (44%) in 2009. Other STs accounted for 27, 29, and 28 cases during these 3 years. We found no evidence of temporospatial clustering of ST-1655 cases. All ST-1655 isolates had *porA* VR1 P1.5-1 and *fetA* F4-1 and varied only in their *porA* VR2 region; clone

Table 2. Clinical presentation of, risk factors for, and outcome of patients with invasive meningococcal capsular group Y disease, England and Wales, 2007–2009

Variable	Age group, y, no. (%) patients				Total, n = 65
	<25, n = 20	25–64, n = 19	65–84, n = 13	≥85, n = 13	
Female sex	11 (55)	14 (74)	10 (77)	12 (92)	47 (72)
Travel*	2 (10)	2 (11)	1 (8)	0	5 (8)
Clinical feature					
Meningitis	8 (40)	11 (58)	2 (15)	1 (8)	23 (35)
Pneumonia	1 (5)	3 (16)	5 (39)	10 (77)	19 (29)
Septicemia	10 (50)	2 (11)	4 (31)	1 (8)	17 (26)
Other	1 (5)	3 (16)	2 (15)	1 (8)	7 (11)
Underlying conditions†	3 (15)	5 (26)	7 (54)	10 (77)	25 (39)
Immune deficiency‡	2 (10)	1 (5)	2 (15)	3 (23)	8 (12)
Sequelae§	1 (5)	1 (5)	0	1 (8)	3 (5)
Deaths	2 (10)	1 (5)	2 (15)	7 (54)	12 (19)

*Travel-associated infection included 2 visitors from Australia and South America and 3 residents who had traveled to Jamaica and Thailand and taken a Mediterranean cruise in the preceding 4 weeks.

†Underlying medical conditions included complement deficiency (1 person), diabetes mellitus (1), and systemic lupus erythematosus among persons <25 years of age; chronic liver disease (3), diabetes mellitus (1), and rheumatoid arthritis on immunosuppressive therapy (1) among persons 25–64 years of age; malignancy undergoing chemotherapy (2), diabetes mellitus (2), chronic heart disease (2), and chronic respiratory disease (1) among persons 65–84 years of age; and chronic heart disease (7), malignancy undergoing chemotherapy (2), chronic respiratory disease (1), and rheumatoid arthritis on immunosuppressive therapy (1) among persons ≥85 years of age.

‡Immunodeficiency included malignancy receiving chemotherapy (4 persons), autoimmune disease requiring immunosuppressant therapy (3), and complement deficiency (1).

§Sequelae included hemiparesis after meningitis in a toddler, bilateral sensorineural deafness requiring cochlear implant after meningitis in an adult, and chronic renal insufficiency in an elderly person with septicemia.

Y:P1.5-1,10-1:F4-1:ST1655 (cc23) was the most prevalent (21 [70%] of 30) and responsible for 3 cases each in 2007 and 2008 and 15 cases in 2009. Cases from Y:P1.5-1,10-4:F4-1:ST1655 (cc23) increased to a lesser extent from 0 and 1 to 5 cases during the 3 years, respectively.

PorA

Genotypic analysis of *porA* VR1 showed that almost two thirds of the 114 strains belonged to P1.5-1 (72 [63%]), followed by P1.21 (16 [14%]) and P1.5-2 (12 [11%]). *porA* VR2 variants included mainly P1.10-1 (31 [27%]), P1.10-4 (20 [18%]), P1.2-2 (19 [17%]), and P1.16 (16 [14]%) (online Appendix Table).

FetA

We found 3 *fetA* types—F4-1 (48 [42%]), F3-7 (23 [20%]), and F5-8 (16 [14%])—in >75% of strains (online Appendix Table). F4-1 belonged mainly to cc23 (45 [94%] of 48 cases), and F3-7 variants all belonged to cc174.

NadA

The *nadA* gene was found exclusively in cc174 isolates, all 24 of which harbored the gene. Sequence analysis of *nadA* was performed among a subset of cc174 isolates (collected during epidemiologic year 2007–08 [7 isolates]) all harbored identical alleles encoding NadA peptide subvariant 3.P0008.

lpxL1

lpxL1 genotype was indeterminate in 1 cc174 strain because the existing primers failed to amplify the gene and flanking regions, possibly because of variation in the primer target sites. Mutations in the *lpxL1* gene were identified in all but 2 cc23 isolates and none of the other ccs. Of the 62 cc23 isolates with an *lpxL1* mutation, all 30 ST-1655 isolates harbored a previously unpublished single-base deletion at nt A_4 (designated mutation XVI) (Figure 2), which resulted in a frame shift and premature stop codon. This mutation also was observed in 1 ST-8414 (cc23) and 3 ST-23 (cc23) isolates. Fifteen of the remaining 19 ST-23 isolates harbored frame-shifted alleles (1 mutation IV, 13 mutation V, and 1 mutation VI [*15*]), whereas another was interrupted by the insertion sequence *IS1301* (mutation I) (*16*). The remaining 3 ST-23 isolates harbored an *lpxL1* allele in which 3 adenines occupied the prototype (strain MC58) stop codon. This configuration was designated mutation XV. The resultant allele was extended by 18 bp. Of the remaining 9 cc23 isolates (1 not fully characterized for ST and the remainder belonging to other less well-represented STs), another 2 harbored mutation XV, 6 harbored mutation V (*16*), and 1 harbored a previously unpublished deletion at nt T_{813} (designated mutation XVII). The frame-shifted allele was extended by 47 bp.

Approximately half (n = 56) of the isolates had mutations, which potentially resulted in an underacylated lipid A moiety of lipooligosaccharide and belonged mainly to either ST-1655 (30 [54%]) or ST-23 (18 [32%]). The number of mutant *lpxL1* isolates increased from 12 each in 2007 and 2008 to 32 in 2009, mainly because of the increase in ST-1655.

Mutation XV is predicted to extend the encoded peptide product by 6 aa. However, it does not alter the

upstream amino acid sequence, which remains complete in comparison with the putative wild-type peptide. It is possible, therefore, that the enzyme encoded remains functional. Of the affected isolates, 3 of 5 occurred in children <15 years of age, none of whom died; clinical diagnosis was available for 4 case-patients, including 3 with septicemia and 1 with meningitis; case numbers did not increase with time (1 in 2007, 2 in 2008, and 2 in 2009).

Factor H–Binding Protein

Almost all isolates (108 [95%]) harbored *fhbp* variant 2 (subfamily A) alleles. Three (3%) isolates harbored *fhbp* variant 1 (subfamily B) alleles encoding fhbp peptide variants 1.13 (cc174), 1.258 (cc174), and 1.335 (cc23); all 3 isolates caused disease in adults with no fatalities). Three (3%) isolates harbored *fhbp* variant 3 (subfamily A) alleles (encoding fhbp peptide variants 3.293 [2 isolates] and 3.300 [1 isolate]; all isolates belonged to cc23 and caused disease in children with no fatalities) (online Appendix Table).

Neisserial Heparin–Binding Antigen

All MenY isolates harbored *nhba* alleles (online Appendix Table). In terms of the NHBA peptide subvariants encoded, cc22 and cc167 were exclusively represented by P0020 and P0009, respectively. cc23 was predominantly represented by P0007 (43 [67%] of 64) as well as P0008 (14 [22%]) and P0006 (7 [11%]); cc174 was represented predominantly by P0006 (23 [96%] of 24 isolates).

MenY Clones

The 5 most prevalent MenY clones were Y:P1.5-1,10-1: F4-1:ST1655 (cc23) (21 [18%]); Y:P1.21,16:F3-7: ST-1466 (cc174) (13 [11%]); Y:P1.5-1,2-2:F5-8:ST23 (cc23) (11 10%]); Y:P1.5-1,10-4:F4-1: ST-1655 (cc23) (6 [5%]); and Y:P1.5-1,10-4:F4-1: ST-23 (cc23) (4 [4%]). All 5 deaths in persons infected with the 3 most prevalent clones occurred among those with pneumonia. No deaths occurred among persons infected with the remaining 2 clones.

Association with Age, Clinical Features, and Outcome

cc23, which harbored all the *lpxL1* mutations, was associated with younger age (<25 years) at disease onset (30 [47%] of 64 cc23 isolates vs. 9 [18%] of 50 other isolates; χ^2 = 10.4, p = 0.001) and with meningitis (16 [40%] of 40 vs. 2 [10%] of 20; χ^2 = 5.7, p =0.017) but not with death (9 [14%] of 64 vs. 6 [12%] of 50; χ^2 = 0.10, p = 0.75). In contrast, cc174, all of which had the *nadA* gene, was associated with older age (≥65 years) at onset (17 [71%] of 24 cc174 isolates vs. 34 [38%] of 90 other isolates; χ^2 = 8.4, p = 0.004) and lack of meningitis (9 [100%] of 9 vs. 33 [65%] of 51; χ^2 = 4.5, p = 0.033), with a trend toward presence of pneumonia (5 [56%] of 9 vs. 13 [25%] of 51; χ^2 = 3.3, p = 0.070) but not death (3 [13%] of 24 vs. 12 [13%] of 90; χ^2 = 0.011, p = 0.92). Of the 114 genotypically characterized isolates, CFR was 13% (15 deaths) and was not associated with any particular cc: 14% (9 deaths) for cc23; 13% (3 deaths) for cc174; and 8%, 11%, and 20% (1 death each) for cc167, cc22, and the remaining ccs, respectively.

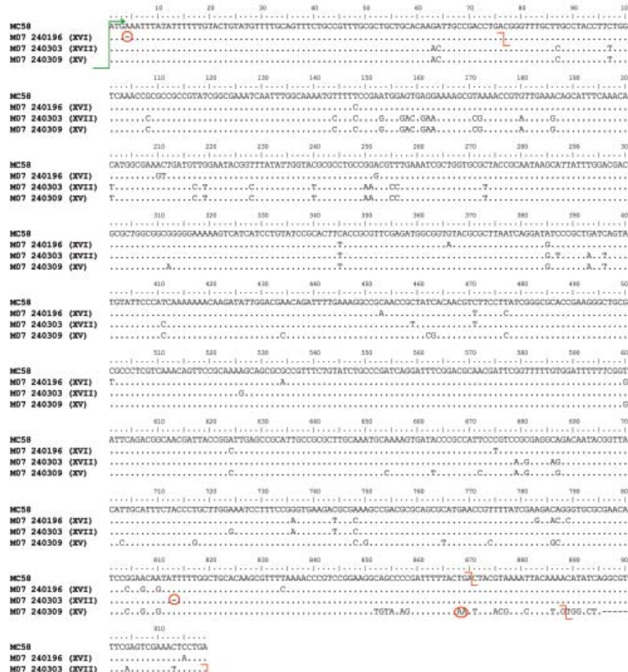

Figure 2. Newly identified *lpxL1* mutations XV, XVI, and XVII. *lpxL1* sequence data from isolates harboring each of the corresponding meningococcal capsular group Y mutations (in parentheses), England and Wales. Mutations are aligned with the full-length gene from strain MC58. All of the alleles share a common start codon (green arrow). Mutations and stop codons are denoted by red circles and red lines, respectively. Mutation XVI is a single-base deletion at nt A_4 that causes a frame shift resulting in a premature stop codon at nt 74. Mutation XVII is a single base deletion at nt T_{813}. This causes a frame shift resulting in a late stop codon at nt 919. Mutation XV is a polymorphism at the region of the prototypical stop codon (that of strain MC58). In affected isolates, 3 adenines occupy the site that comprises the stop codon, TGA, in MC58. The next available stop codon occurs 15 bp downstream. The encoded peptide has an additional 6 aa.

Discussion

Routine provision of a national reference service by HPA for meningococcal species confirmation, capsular grouping and typing, and regular integration of multiple data sources provides a robust and stable enhanced national surveillance for invasive meningococcal disease covering the 55 million persons in England and Wales. Our surveillance

indicates an increase in invasive MenY cases in England and Wales since 2007. A similar increase was observed a decade ago in the United States, where MenY currently accounts for one third of all meningococcal infections (*17*). Clinical follow-up of cases diagnosed in 2009 indicated that MenY affects mainly older adults with underlying medical conditions (*18,19*). Almost one third of MenY case-patients had pneumonia. MenY is the most prevalent cause of meningococcal pneumonia (*18*), although capsular group W135 pneumonia also has been described (*20–22*), whereas MenB and MenC rarely cause pneumonia (*18*). In 1 study, pneumonia was 4× more common among MenY cases (12%) than among cases with other capsular groups (3%), even after adjustment for patient age (*23*). Similarly, a literature review of 58 published cases of meningococcal pneumonia during 1974–1998 found MenY as the most common pathogen (44%) and caused disease mainly among older adults with underlying medical conditions (*18*). CFR for MenY pneumonia was 8.6% (*18*), which was similar to other MenY reports (*5,23–28*). The higher CFR in our surveillance (18% overall and 47% for pneumonia) may be explained by the older age of pneumonia case-patients and higher prevalence of underlying medical conditions. CFR was also higher than that reported for MenB (5.1%) or MenC (11.6%) in England and Wales (*5*).

Molecular typing showed that ST-1655 (cc23) was responsible for the increase in MenY cases. We found no evidence of clustering of cases to suggest an outbreak. A recent meningococcal carriage study involving first-year university students in Nottingham, United Kingdom, during 2008–09 reported rates of 42% at the initial time-point, which increased to 62% during the study, with more than half the carriage isolates belonging to MenY (*8*). These rates are substantially higher than those in a previous multicentre study among 15–19-year-old UK school students during 1999–2001, where MenY carriage was only 5%–6% (*9*). In the Nottingham study, more than half of the carriage isolates belonged to 1 of 4 *porA* types (P1.5-1,10; P1.21,16; P1.5,2; and P1.21-7,16) and 4 ccs (cc23, cc60, cc167, and cc174) (*8*), which confirms that most meningococcal strains causing invasive disease—particularly ST-1655 (cc23)—also are circulating in England.

The association between MenY ccs and specific clinical features in different age groups is intriguing. Certain strains might have acquired specific virulence factors that facilitate invasion of particular organs, such as the lungs or the brain. In our study, for example, infection with MenY strains harboring *lpxL1* mutations was associated with meningitis, particularly in younger persons. In contrast, cc174 strains, which all harbored *NadA*, were more likely to cause pneumonia in older adults.

Meningococcal lipid A is the bioactive component of lipooligosaccharide (or endotoxin) that induces a proinflammatory cytokine response through the toll-like receptor 4 (TLR4) innate immunity pathway (*29*). Up to 9% of invasive clinical meningococcal strains (including MenY) have 5 instead of 6 acyl chains because of inactivating mutations in the acyl-transferase gene, *lpxL1* (*16*). Such mutant strains induce a lower proinflammatory cytokine response, which may enable them to evade the host innate immune system (*16,30*). They were also more likely to cause meningitis in younger persons and less likely to have features associated with activation of the coagulation system in meningococcal septicemia (rash, septic shock, or thrombocytopenia) (*16*). A recent study in the Netherlands reported a high proportion (61%) of *lpxL1* mutations among 71 cc23 carriage isolates (predominantly MenY) compared with 0.3% among 751 non-cc23 carriage isolates and 8.6% among 464 non-cc23 invasive disease isolates (*31*). No cc23 strains existed among invasive clinical isolates collected in the Netherlands during 2001–2006 (*31*).

In our study, *lpxL1* mutations were identified in 97% of invasive cc23 MenY isolates and occurred infrequently among non-cc23 MenY isolates. The 2009 increase in ST-1655 (cc23), which all harbored *lpxL1* mutations, is concerning because of its preponderance for causing meningitis in healthy young persons. Many of the *lpxL1* mutations result from potentially reversible alterations in homopolymeric tracts, and phase variation of *lpxL1* has been speculated to occur within the host to avoid enhanced recognition by the immune system after crossing the nasopharygeal epithelium (*16*). The uniquely high frequency of mutants among invasive disease and carriage cc23 isolates suggest that these have an enhanced propensity to the mutant state for reasons as yet unknown. The diversity of the mutations within closely related lineages suggest that they have occurred in independent events. The most widely distributed mutation among cc23 (mutation V) occurs in a relative long poly-A tract, which would be more likely to occur independently at relatively high frequencies. Mutation XVI occurs in a relatively short poly-A tract, as reflected in its relatively narrow distribution. That it is the sole mutation among ST-1655 isolates probably reflects the recent, rapid expansion of this particular lineage. We also have identified new *lpxL1* mutations that could potentially result in underacylated lipid A, but whether the mutations result in underacetylation will require confirmation in future studies.

The *nadA* gene was present only in cc174 strains, representing 21% of MenY isolates. A similar proportion (23%) was reported among invasive MenB isolates in England and Wales (*12*), but may be present in up to 100% of certain hypervirulent lineages (*32,33*). NadA is a surface-exposed neisserial adhesin that binds human β1-intergrins (*34*) and is involved in epithelial cell adhesion

and invasion (*32,33*). Interactions between bacterial adhesins and β1 integrins have been described for several pathogens that invade the respiratory or gastrointestinal mucosa and play a major role in mucosal translocation and triggering the release of chemokines (*34*). NadA may thus enable MenY strains to invade locally and cause respiratory tract infections, particularly among elderly persons, who often have multiple underlying medical conditions. We have also reported on other meningococcal antigens that are relatively conserved within certain ccs. Some of these antigens are included in investigational meningococcal vaccines currently undergoing clinical trials. Licensed meningococcal quadrivalent conjugate vaccines are available, but given the small number and age distribution of patients, routine vaccination against these capsular groups is unlikely to be cost effective at this time.

Invasive MenY disease has increased in England and Wales, mainly because of ST-1655 (cc23). This cc was significantly more likely to cause meningitis, mainly in young adults, and was associated with the presence of *lpxL1* mutations. The increase in invasive MenY disease, particularly as accompanied by recent reports of substantially increased carriage of MenY, will require careful epidemiologic and molecular monitoring.

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Dr Ladhani is a pediatric infectious disease consultant with an interest in vaccine-preventable diseases. His primary research interests include infectious disease epidemiology and prevention of childhood infection–related morbidity and mortality.

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Differential Mortality Rates by Ethnicity in 3 Influenza Pandemics Over a Century, New Zealand

Nick Wilson, Lucy Telfar Barnard, Jennifer A. Summers, G. Dennis Shanks, and Michael G. Baker

Evidence suggests that indigenous populations have suffered disproportionately from past influenza pandemics. To examine any such patterns for Māori in New Zealand, we searched the literature and performed new analyses by using additional datasets. The Māori death rate in the 1918 pandemic (4,230/100,000 population) was 7.3× the European rate. In the 1957 pandemic, the Māori death rate (40/100,000) was 6.2× the European rate. In the 2009 pandemic, the Māori rate was higher than the European rate (rate ratio 2.6, 95% confidence interval 1.3–5.3). These findings suggest some decline in pandemic-related ethnic inequalities in death rates over the past century. Nevertheless, the persistent excess in adverse outcomes for Māori, and for Pacific persons residing in New Zealand, highlights the need for improved public health responses.

Evidence suggests that indigenous populations have been disproportionately affected more by influenza pandemics than other population groups. In the most detailed review to date for the Spanish influenza pandemic (1918–1920), Mamelund (*1*) reported elevated mortality rate ratios (RRs) for indigenous populations relative to European populations in North America: the continental United States (RR 3.2); Alaska (RR range 6.8–191.5); all of Canada (RR 4.8); Labrador, Canada (RR range 8.3–129.0) and Greenland (RR 4.9). This pattern was also apparent for the Sami in Nordic countries of Norway (RR 4.8), Sweden (RR 8.2), and Finland (RR 16.9). Indigenous Australians were particularly affected (RR 172.4) but so were indigenous Pacific persons in Guam (RR 3.2), Fiji (RR 4.8), Tonga (RR range 2.6–5.3), Samoa (RR 16.5), Nauru (RR 11.2), Tahiti (RR 10.9), and Hawaii (RR 4.1).

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In contrast, little is known about ethnic gradients in outcomes for other influenza pandemics of the 20th century, such as the 1957 pandemic. More recently, many studies have considered the 2009 influenza pandemic, and there are reports of increased risk for either hospitalization or death for indigenous persons from Canada, the United States, Brazil, Australia, New Zealand, and New Caledonia (*2,3*). Other work involving 12 US states indicated elevated mortality RRs for American Indian/Alaska Natives (RR 4.0, 95% CI 2.9–5.6) (*4*). Canadian research also identified First Nations (indigenous) ethnicity as an independent risk factor for increased disease severity, with the multivariable model accounting for age, sex, medical comorbidity, interval from onset of symptoms to initiation of antiviral therapy, rurality, and income (*5*). That is, First Nations ethnicity was associated with increased likelihood of being admitted to an intensive care unit (odds ratio 6.52, 95% CI 2.04–20.8), but this pattern was not seen for low-income persons or those residing in rural areas.

Despite this historical and more recent work, little evidence exists concerning how the ethnic mortality differential of pandemic influenza may have changed over time. Therefore, in this study we considered such data for Māori (the indigenous population of New Zealand) and to some extent for Pacific populations residing in this country.

Methods

We searched the literature (Medline and Google Scholar) for relevant publications relating to New Zealand up to December 1, 2010. Search terms used were combinations of influenza and Māori/Pacific and New Zealand. The bibliography of a key text (*6*) was used to help identify this literature.

1918–19 Pandemic

Mortality rate data for military personnel in the New Zealand Expeditionary Force (NZEF) were obtained from an electronic dataset (Roll-of-Honor) covering all deaths in these personnel during World War I (WWI) (Figure 1). This dataset was obtained courtesy of the compiler, Professor Peter Dennis (University of New South Wales at the Australian Defence Force Academy). Those reported as dying of disease or from no specified cause were studied further, and if dying during known pandemic periods, they were considered to have died from pandemic influenza (i.e., after excluding deaths that had a specific alternative diagnosis in the archival records). These pandemic periods were 1) August 27–December 31, 1918, for the second wave in the Northern Hemisphere); 2) January 1–March 31, 1919, for the third wave in the Northern Hemisphere; and 3) November 1–December 31, 1918, for the second wave in the Southern Hemisphere. There was no evidence for excess mortality rates in military personnel from any first or third pandemic wave in the Southern Hemisphere.

Military personnel were classified as having Māori ethnicity if they: had a first, second, or surname in the Māori language; had a parent with a Māori language name; were buried in a Māori cemetery or had a memorial in such a cemetery; or had a iwi (tribal) affiliation listed in the Cenotaph database covering NZEF personnel (purchased from the Auckland War Memorial Museum [*7*]). Personnel were classified as having Pacific person ethnicity if they came from a South Pacific island providing military personnel for the NZEF (i.e., Fiji, Gilbert and Ellis Islands, Niue, Samoa, Tonga, and the Cook Islands) and had a Pacific name or a parent with a Pacific name or came from a named village. The approach of using the language of a name for considering ethnicity has been used elsewhere in historical work in New Zealand (*8*). It is also used for identifying from the electoral roll potential Pacific persons as respondents to public health surveys in New Zealand (the Lexicon method used by Massey University) (*9*).

Figure 1. Māori military personnel (Pioneer Battalion) performing the haka for New Zealand Cabinet Minister Sir Joseph Ward (at Bois de Warnimont, France, June 30, 1918). Photograph taken by Henry Armytage Sanders; from Alexander Turnbull Library, Timeframes: New Zealand and the Pacific through images; reference no. 1/2-013283-G (http://timeframes.natlib.govt.nz).

For denominator data, we extracted a random sample of 1,000 persons (≈1%) who served in the NZEF as detailed in the Cenotaph database. This denominator sample was then adjusted further to replace (with additional random selection) those who died in the prepandemic period. Ethnicity coding was then performed as for the numerator data.

A validation study was performed for the method of ethnicity coding for Māori; this study involved a University of Otago colleague with local history expertise (Dr George Thomson) who independently classified the ethnicity of WWI participants in a rural area in which he had performed historical research. The results indicated that the coding system we have used was underascertaining Māori ethnicity (sensitivity 73%, i.e., n = 11/15). Of note, however, is that the rural locality used in this validation study had a relatively high Māori population in the pre-WWI era, and intermarriage between Māori and New Zealand European persons was relatively common. As such, the underascertainment found would be a worst-case assessment if applied to New Zealand in general. In contrast, all of those classified as Māori by our coding system were also classified as Māori by Dr Thomson (specificity 100%, n = 17/17).

1957 Pandemic

In addition to considering Māori mortality rates in official data identified in our literature search, we undertook an additional analysis of an online national database for deaths in New Zealand (*10*). Because ethnicity coding at this time in New Zealand history was poor, we again adopted the approach of using the language of the surname. We examined surnames in the Māori language by using the most common 50 surnames in the 2006 Māori electoral roll. Similarly, this examination was performed for the most common 10 surnames from the general electoral roll of 2006 (all of these were surnames of European origin). The periods considered were for August–September in 1956, 1957, and 1958; each of these months had maximum pandemic impact in 1957 (*11*). The 1956 and 1958 periods were used for comparison with the known pandemic period in 1957. This method was also used to identify the increased mortality rate for the 1890s pandemic for the European population because registration of Māori deaths was not routine in the 19th century. We did not study the Hong Kong influenza pandemic of 1968–1969 because we could find no published studies in New Zealand and no evidence for increased mortality rates in the annual mortality data (*12*).

A limitation with this surname method was that the number of Māori deaths involved was small, e.g., 23 and

28 deaths in August–September in 1956 and 1958 respectively, compared with 38 deaths in August–September in 1957 (using the top 50 surnames in Māori language). Furthermore, some persons with Māori surnames may not have been Māori (e.g., non-Māori women who married Māori men with Māori surnames). Also, a proportion of the total New Zealand mortality rate would have included Māori deaths because the most common 7 surnames on the Māori electoral roll (2006) are actually names of European origin that Māori have commonly adopted over the past 150 years (e.g., Smith, Williams, Brown, Wilson; Kingi, the first name in te reo Māori, is listed eighth). The latter factor is likely to dominate. Thus, our analysis is likely to have underestimated the true pandemic-related Māori mortality rate.

2009 Pandemic

Anonymized mortality data were obtained from an official mortality review group that was charged by the New Zealand government with identifying deaths caused by pandemic (H1N1) 2009 (*13*). All but 1 of the 49 deaths included in our analysis occurred during June 1–September 30, 2009. The review group used prioritized ethnicity (i.e., where there are multiple ethnicities assigned, Māori ethnicity takes precedence, followed by Pacific persons, with the remaining persons included as European and others). Rates and RRs were calculated and age-standardized to the 2006 New Zealand Census with Māori as the standard population (the Māori population has a younger age structure than the European population and is more similar to Segi's world standard population).

Results

1918–19 Pandemic

During the 1918 pandemic, the Māori mortality rate (4,230/100,000 population) was 7.3× the rate for the rest of the population (*6*), which was nearly all composed of settlers of European origin (Table; Figure 2). This work by Rice (*14*) was far more comprehensive than previous work that compared rates by ethnicity (*15*). Nevertheless, Rice noted limitations with data quality and considered that the Māori rate was still an underestimate of the true rate because some Māori deaths were not registered (*14*). This underestimation of Māori pandemic-related deaths was also a finding in a study of the pandemic in New Zealand's largest city, Auckland (*16*).

The analysis of the effect of 2 pandemic waves among New Zealand military personnel found a substantially higher mortality rate among Māori personnel (2,501/100,000) than for European military personnel (Table). The rate for Māori soldiers was 2.3× higher than that for European soldiers.

1957 Pandemic

In this pandemic, the Māori mortality rate (39.6/100,000) was 6.2× the European rate (Table; Figure 2). In our new analysis using selected surnames, an equivalent ratio 2.4× higher for Māori was found for 2 months when the pandemic was most active in New Zealand in 1957 (Table).

2009 Pandemic

A national serosurvey after wave 1 of the pandemic found that Māori had evidence of higher seroprevalence of antibodies to the pandemic (H1N1) 2009 virus but not at statistically significant levels (Māori 36.3%, 95% CI 28.0–44.6, vs. other [mainly European] 25.9%, 95% CI 22.4–29.4) (*18*). However, multivariate analysis identified significantly higher seroprevalence rates for Pacific populations (49.5%; adjusted odds ratio 2.2, 95% CI 1.5–3.4) compared with the other ethnic group population (i.e., mainly European New Zealanders).

2009 Pandemic Outcomes

During the 2009 pandemic, Māori had relatively higher notification rates for pandemic influenza (age-standardized relative risk [aRR] 2.0, 95% CI 1.9–2.1) compared with Europeans and others (and similarly for Pacific persons, aRR 4.0, 95% CI 3.8–4.3) (*19*). The cumulative age-standardized hospitalization rate for Māori was 43.0/100,000 compared with Europeans and others at 14.1/100,000 (Māori aRR 3.0, 95% CI 2.9–3.2; Pacific persons aRR 6.7, 95% CI 6.2–7.1) (*19*). A local study also found higher hospitalization rates for Māori in the Wellington region, at 5× the rate for other ethnic groups (excluding Pacific persons) (*20*).

Intensive care unit (ICU) admissions were also significantly higher for Māori and Pacific persons compared with Europeans (*13*). More specifically, Māori and Pacific women who were pregnant were more likely to have an ICU admission compared with other pregnant women (RR 2.3, 95% CI 1.4–3.7) (*21*).

Our analysis of national mortality data found that the Māori rate was significantly higher (2.6×) than the rate for other New Zealanders (non-Māori and non-Pacific New Zealanders, largely European) (Table). Similarly, for Pacific persons, the rate was 5.8× higher than that for other New Zealanders (Table). An official post-pandemic review also reported elevated rates for both ethnic groups, but it did not perform age standardization of rates (*13*).

Risk Factors (All Pandemics)

None of the work on these 3 pandemics systematically analyzed risk factors for adverse outcomes of pandemic influenza on Māori. Nevertheless, in 1918 the high rate of illness and death among adult caregivers is thought to have

Table. Comparison of mortality rates for Māori versus non-Māori/European residents of New Zealand during multiple influenza pandemics*

Pandemic and data source	Mortality rate, Māori	Mortality rate, Non-Māori	Ratio†	Comments (see Methods for details)
1890s pandemic				
Individual mortality data in BDM database (*10*)	Unknown (deaths not registered)	9.1% increase in deaths for 1890–94 compared with 1885–89	–	Based on comparison of no. deaths for top 10 surnames (see method used for 1957 pandemic). Official data also suggest increased influenza deaths for the 1890s beginning in 1890 (*17*).
1918–19 pandemic				
National mortality data, second wave (*6*), n = 8,573 deaths	4,230/100,000 population	580/100,000 population) (European)	7.3	See limitations with data quality described in the main text. Comparison was not age-standardized.
Mortality in New Zealand military personnel, second and third waves,‡ n = 1,113	2,501/100,000 population	1,103/100,000 population (European/ other)	2.3 (1.6–3.1)§	New Zealand military personnel of Pacific peoples ethnicity also had a raised mortality rate, but absolute number of deaths was small (n = 12) and difference was not significant.
1957 pandemic				
National mortality data for Asian influenza pandemic, official report (*11*), n = 179 deaths	39.6/100,000 population	6.4 per 100,000 population (European)	6.2	Of note, at this time surveillance systems were crude, and attention to quality ethnicity coding was not robust. There was no widespread use of vaccination in response to this pandemic in New Zealand.
Individual mortality data in BDM database for selected surnames (*10*), n = 38 deaths in Aug/Sep 1957	49.0% increase for Aug/Sep 1957 compared with same period in 1956 and 1958	Whole New Zealand population: 20.3% increase	2.4 (Māori vs. total population)	See Discussion for limitations with this method.
2009 pandemic¶				
All cases with pandemic (H1N1) 2009 as primary cause of death,# n = 49	2.0 (0.8–3.1)§	0.8 (0.5–1.1)§	2.6 (1.3–5.3)§	For Pacific peoples in New Zealand, rate = 4.6 (2.0–7.2)§

*BDM, Births, Deaths & Marriages.
†Māori:non-Māori except as indicated.
‡Age standardization was not possible with available data; 1 study reported that Māori and European soldiers had similar median ages of 24 and 26 years, respectively (*8*).
§Values in parentheses are 95% confidence intervals.
¶Jun–Sep 2009. Cumulative age-standardized mortality rates per 100,000 population, age-standardized to Māori population. Ratio is Māori versus other New Zealanders (non-Māori and non-Pacific, mainly European).
#Identified by Mortality Review Group.

limited capacity to provide basic care for others, contributing to relatively high total Māori mortality overall (*6,15*). Furthermore, various supportive care services in towns and cities may have favored better health outcomes for the affected European population relative to Māori (*6*). Māori were largely rurally based in 1918 (*22*) and rural health services were limited at that time.

For the 2009 pandemic, it was reported that 86% of those who died had ≥1 concurrent or associated condition, particularly obesity (74%), morbid obesity (56%), and respiratory disease (49%) (*13*). Furthermore, 39% of those who died had a deprivation score of either 9 or 10 (most deprived) compared with the expected 20% of the population (using a New Zealand–specific small area deprivation index). Similarly, the ICU study identified risk factors for pandemic influenza–related admission, including pregnancy, body mass index >35, and preexisting asthma or other chronic pulmonary disease (*23*).

Discussion

The mortality rates from pandemic influenza for Māori and European New Zealanders declined markedly over the 3 pandemics. Nevertheless, this finding may only partly represent improved public health controls and health care, given marked variation in virulence of difference pandemic influenza viruses over this period.

In terms of relative inequalities, the excess in Māori mortality rates (compared with those of European New Zealanders) appears to have declined over the 3 pandemics. However, the persistently poorer health outcomes of Māori during the 2009 pandemic is of continuing concern and is compatible with other evidence for persisting inequalities in major health risk factors (*24*) and in health outcomes when comparing Māori with non-Māori (*25*). In particular, total Māori hospitalization rates for infectious diseases have been consistently double those seen for the European/other population over the 20-year period of 1989–2008 (*26*). Māori also have markedly elevated rates for several

specific infectious diseases for which household crowding has been a risk factor in the New Zealand setting, i.e., tuberculosis (*27*), meningococcal disease (*28*), and rheumatic fever (*29*).

Other possible factors for poorer pandemic influenza outcomes in indigenous populations (*2*) include 1) a higher prevalence of infection (but this was not found to be significant in the New Zealand seroprevalence study); 2) higher prevalence of concurrent conditions (which is relevant for Māori, particularly in terms of diabetes and chronic respiratory diseases [*25*]); and 3) possibly poorer access to health services. Differential use of seasonal influenza vaccination by ethnic group affiliation might also be relevant, but the literature regarding the benefit of prepandemic seasonal vaccination for pandemic (H1N1) 2009 outcomes remains unclear (*18*).

The results for Māori are compatible with the findings for Pacific persons serving in the military in 1918 and living in New Zealand in 2009 (Table). Like Māori, these persons are largely of Polynesian ethnicity and are less socioeconomically advantaged relative to the European New Zealander ethnic group. Furthermore, for other Pacific persons such as those living in New Caledonia, there appear to have been increased adverse outcomes from the 2009 pandemic (*2,30*).

The relatively higher mortality rate from pandemic influenza for Māori military personnel in 1918 indicates that the excess pandemic effect was even seen among relatively fit young men. One possible explanation for this pattern comes from a large study of Australian soldiers in 1918, which suggests that one's previous experience with respiratory pathogens was an important protective factor in the pre–antimicrobial drug era (*31*). Preexisting immunity was also a probable important risk factor in a study of 1918 mortality among the Sami of Norway in a multivariate analysis that considered such factors as wealth, poverty, crowding, and occupational structure (*32*). Other similar work, but on an international scale, has suggested that geographic remoteness (associated with less frequent exposure to other forms of influenza) may have contributed to high mortality rates in indigenous persons (*1*). This work also suggested plausible roles for high concurrent disease load, crowding, and a lack of basic care (i.e., for dependents when many younger adults died suddenly).

Concurrent disease incidence is a plausible risk factor in the 1918 pandemic for Māori soldiers and the civilians in 1918, given the much higher rates of other infectious diseases such as tuberculosis (*33*). Relative poor nutritional status may also have mattered for the civilian population of Māori, but for the military population no evidence (*34,35*) of any poorer provision of supplies for Māori soldiers compared with European soldiers was found (and similarly for the quality of accommodation or health care services).

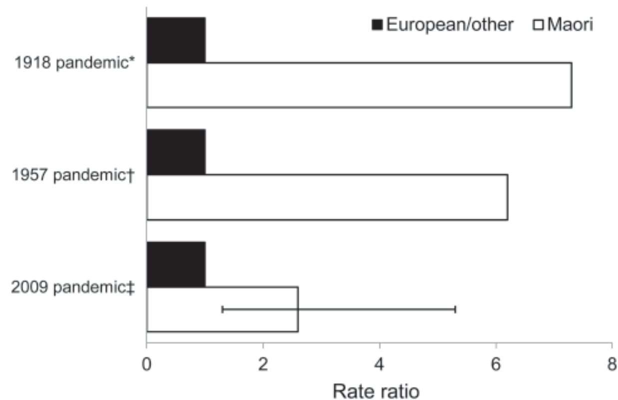

Figure 2. Mortality rate ratios (age-standardized on the basis of 2009 data) for Māori versus European/other New Zealanders (non-Māori/non-Pacific) during 3 influenza pandemics in New Zealand. *Data from (*6*); †official mortality rate data; ‡age-standardized to the Māori population. Error bar represents 95% CI.

Also, 1 study reported no differences in stature and only small differences in median weight for Māori compared with European military recruits during WWI (160 vs. 150 lbs., respectively) (*8*). Little information is available on the 1957 pandemic, but concurrent conditions, e.g., higher tuberculosis rates (*33*), poorer housing, and poorer access to health services, are plausible risk factors.

New Zealand data made it possible to describe health outcomes by ethnicity for pandemics from 1918 onwards. Integrated national-level data collection systems for notifications, hospitalizations and deaths also facilitated such comparisons for the 2009 pandemic.

A limitation of the 1918 data identified was the incomplete ascertainment of pandemic-related deaths among Māori, caused partly by incomplete death registrations in this population (*6,16*). Similarly, a limitation with our analysis of the military personnel for 1918–19 was that the ethnicity classification system used is likely to have underestimated Māori.

Māori deaths occurring during 1957 may have also been underestimated because ethnicity classification at this time was based on funeral director assessments. We acknowledge the limitations of using the language of surnames as part of ethnicity coding and the likelihood of underestimating the mortality rates for Māori; practical alternatives for analyzing such historical data are lacking.

To identify risk factors for these ethnic disparities, analytic epidemiologic methods are needed, such as case–control studies of the 1918 military personnel, for which there are detailed archival data and hospitalization records for 2009. Such studies need to include risk factors for influenza infection as well as possible risk factors for adverse outcomes, e.g., low socioeconomic position, household

crowding, smoking, obesity, preexisting chronic diseases, the lack of prior seasonal influenza vaccination or pneumococcal vaccination, and poorer access to healthcare. Indeed, further detailed work is underway on the ethnic gradient for influenza for 2009 in New Zealand by some of us with Māori health colleagues.

Although further research is desirable, enough is now known about health inequalities in nations with indigenous peoples for government agencies and health care workers to pursue specific interventions. For example, in New Zealand, interventions should continue to raise the social and economic well-being of Māori, i.e., improve housing, reduce smoking, control obesity, improve management of diabetes, increase immunization rates, and improve access to health care services. Fortunately, many such interventions are part of New Zealand health sector activity and range from national-level smoking cessation campaigns to more local community-level programs such as a Let's Beat Diabetes program. Other policy development is occurring, with new and substantive tobacco control measures recommended by a Māori Affairs Select Committee in late 2010 (*36*). These responses are also relevant to improving health protection for Pacific persons, albeit in different ways that are also culturally appropriate. In addition, improvements are needed for influenza vaccination coverage in more vulnerable populations and more pandemic planning on how to reduce inequalities in influenza outcomes for indigenous persons (highlighted in work to protect Australian Aboriginal peoples [*37,38*]; indigenous persons in the United States [*39*], and other ethnic groups in the United States [*40*]).

Analysis of multiple health data sources indicates large reductions in absolute mortality rates from pandemic influenza for Māori and European New Zealanders and is suggestive of some decline in relative ethnic health inequalities for pandemic mortality over the past century. However, the persistent Māori excess in hospitalizations and deaths for the 2009 pandemic highlights the need for additional research to clarify contributing factors. There remains an ongoing need for societal and public health action to reduce known risk factors for influenza infection and adverse health outcomes for indigenous populations such as Māori.

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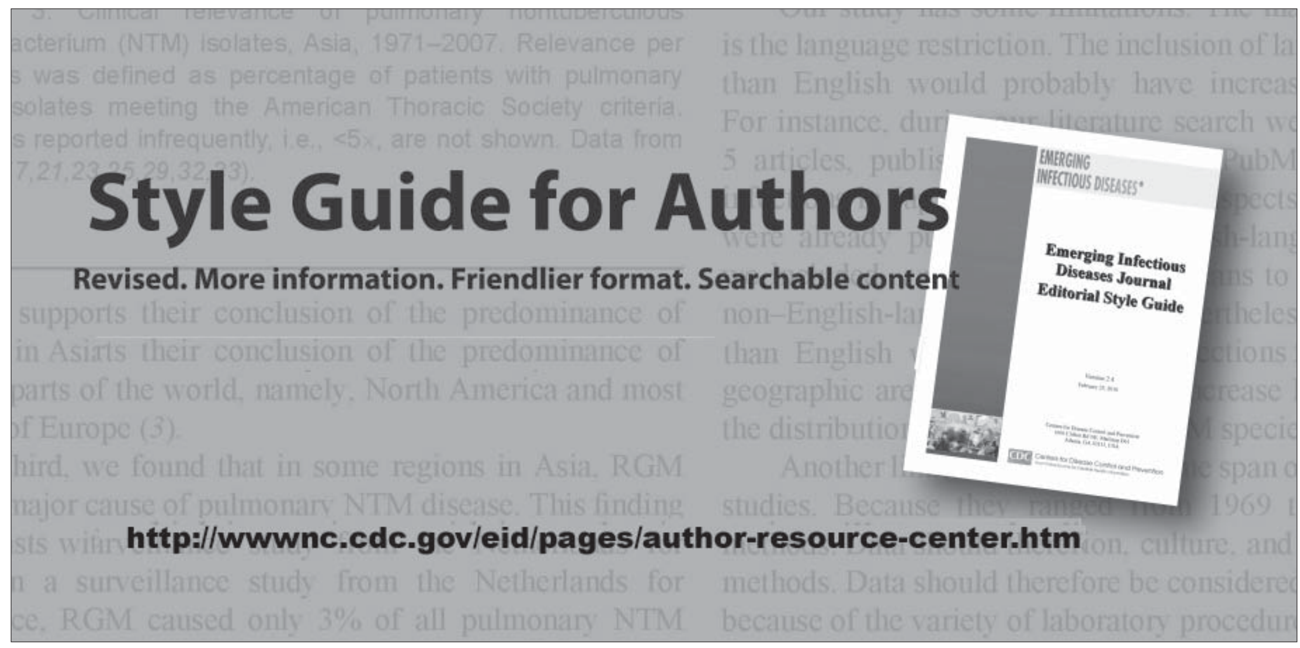

Daily Newspaper View of Dengue Fever Epidemic, Athens, Greece, 1927–1931

Christos Louis

During the late summers of 1927 and 1928, a biphasic dengue epidemic affected the Athens, Greece, metropolitan area; >90% of the population became sick, and >1,000 persons (1,553 in the entire country) died. This epidemic was the most recent and most serious dengue fever epidemic in Europe. Review of all articles published by one of the most influential Greek daily newspapers (I Kathimerini) during the epidemic and the years that followed it did not shed light on the controversy about whether the high number of deaths resulted from dengue hemorrhagic fever after sequential infections with dengue virus types 1 and 2 or to a particularly virulent type 1 virus. Nevertheless, study of the old reports is crucial considering the relatively recent introduction of *Aedes albopictus* mosquitoes and the frequent warnings of a possible reemergence of dengue fever in Europe.

The dengue fever (DF) epidemic of 1927–1928 in Greece, which first affected the Athens area, was the most recent dengue epidemic in Europe. Transmitted by *Aedes aegypti* mosquitoes, the epidemic probably involved dengue virus type 1 (DENV-1) and type 2 (DENV-2) (*1*–*4*). The rapid economic and social development of the continent has since led to dramatically reduced habitats of the vector and, most likely, to its elimination and is the main reason the likelihood of a similar event is small. Yet, the recent invasion of Europe by *Ae. albopictus* mosquitoes (*5*) and the marked ecology of this mosquito make emergence of a new DF epidemic possible, even if the impact of such an event is not expected to be of the same dimension as the epidemic described here.

Greece in 1927 lagged behind other countries in development. For example, most roads in Athens were unpaved; electric service was intermittent; a citywide sewage system was nonexistent; and the potable water supply was rudimentary, often forcing residents to store water in containers. Moreover, the population of the metropolitan Athens area, like the remainder of the country, had increased markedly because of the exchange of populations between Turkey and Greece after the war between the 2 countries and the defeat of Greece in 1922 (*6*). The Hellenic National Statistical Agency reported the arrival to Greece of ≈1 million refugees, leading to a 23.68% increase of the population during 1920–1928. In the Athens metropolitan area, the increase was ≈68%, and most of the newcomers were destitute and lived in extremely poor housing conditions (*7*).

In addition to these problems, the country was deeply divided politically into royalists and liberals, a division initiated early in 1915 by the rift between King Konstantin I and Prime Minister E. Venizelos about whether Greece should enter World War I. This division, exacerbated by the defeat of Greece in the Asia Minor campaign of 1921–1922, contributed to the lack of political solutions for the country's major problems (*8*).

During this period of hardship, the DF epidemic struck Greece. The epidemic was then often called the dengue pandemic because almost the entire population of Athens (population ≈600,000) was affected. On the basis of the current definition of pandemic, the term epidemic is used here to describe the events of the summers of 1927 and 1928.

By focusing exclusively on all 561 items published in 1 daily newspaper during the epidemic, this review compares reports from 83 years ago with today's knowledge. I chose the fully digitized archives of the Athens newspaper H Καθημερινή (I Kathimerini) (*9*). This newspaper was, and still is, one of the major newspapers in Greece (currently first in daily circulation). During the time of the DF

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epidemic, the newspaper held a steady antiliberal and proroyalist position at a time when the liberal party ruled Greece. This position is often indicated in commentaries about the government's handling of the epidemic, which will only briefly be mentioned here. Unless specifically referenced, all sources of original statements listed below (translated into English and appearing in quotes) are based on the pieces published by I Kathimerini.

1927

I Kathimerini first mentioned DF on November 1, 1927, not as news but rather as a short joke about financial matters, followed several days later by an advertisement for "FLY FUME" (ΦΛΑΪ ΦΙΟΥΜ), which "...protects you from the awful Dengue Fever, because it eradicates immediately the mosquitoes, which transmit it...." While publishing more jokes during the next few weeks and further advertisements for FLY FUME, I Kathimerini did not mention the DF epidemic until August 1928, when an article reported that the epidemic during the fall of 1927 had resulted in 3,000 cases with 0 fatalities; a few days later, the number of ill persons was reported as 75,000. Moreover, the second article suggested that many of these DF cases were initially mistaken for the yearly recurrence of "3-day fever," which was known to be transmitted by phlebotomine sandflies. In August 1928, citing an unnamed physician, I Kathimerini reported that the previous year's epidemic had started in mid-August in a middle-class household in the center of Athens through a young woman in whom DF symptoms developed 2 days after she arrived from Alexandria, Egypt.

1928

On August 2, 1928, when the second epidemic had been ongoing for several days, I Kathimerini began publishing frequent articles mentioning DF. An article in early September mentioned that the first 3 DF cases in 1928 had appeared in mid-July in the center of Athens among construction workers. This article also criticized the medical authorities for not issuing directions and for not taking any palliative and prophylactic measures despite the fact that "whole families stay in bed" or "big factories are forced to stop work." The first real medical facts were published several weeks later in late August.

The headline of a long article appearing on August 15 was "25,000 Athenians are burnt by DF" and continued "... science remains speechless in front of the mysterious disease." It described the symptoms of DF and mentioned a common prescription ("one spoon per hour of Riviera Potion 250 gr, Pyramidon 1 gr, Aspirin 1 gr"). The article concluded that "all measures and drugs suggested by [medical] science are symptomatic and not therapeutic." It also reported that "those [persons] injected with 606 and Bismuth did not get DF" (606 was the synonym for arsphenamine/salvarsan).

The Table indicates the numbers of cases in the DF epidemic according to figures published by I Kathimerini (no figures were published after August 30, 1928). The Athens city center was hardest hit. An article on August 22 reported 30 deaths on the previous day, the first time that I Kathimerini reported deaths resulting from the epidemic (Figure). On that same day, another article noted that the fatal cases resulted from a "[coexistent] different organ affection" [*sic*]. Isolation of the DF "microbe" was unsuccessful, leading to the opinion that the transmitting mosquito might be poisonous. The vector was identified in different articles as *Stegomyia fasciata* mosquitoes, an old name for *Ae. aegypti* mosquitoes.

On August 24, some of the effects of the epidemic on society were reported for the first time. For example, the Athens–Piraeus Railroad used fewer trains, half the judges of the Athens Lower Court were absent, and the armed forces draft was suspended. Although more fatal cases were reported, hemorrhagic DF-like illness was not described, and deaths were attributed to older age or preexisting diseases. Some articles suggested that residents

Table. Accumulated number of dengue fever cases as reported in the newspaper I Kathimerini during the 1928 epidemic, Athens, Greece, August 1928*

Date of report	No. cases: Athens	No. cases: Metropolitan area†	No. cases: Greece	Notes
	3,000–75,000			Mentioned in articles in 1928
Aug 15	25,000			
Aug 19	43,000			
Aug 23	80,000			
Aug 25	100,000			
Aug 27	150,000			
Aug 29	433,000	649,000		Indicated as 75% of population
Aug 30	461,000	693,000		Indicated as 80% of population
			959,884	Government statistics cited by Mavrogordatos (*8*)

*Blank cells indicate no information.
†Includes the neighboring city of Piraeus (population ≈250,000) and the remaining suburban area that had a small population in the 1920s.

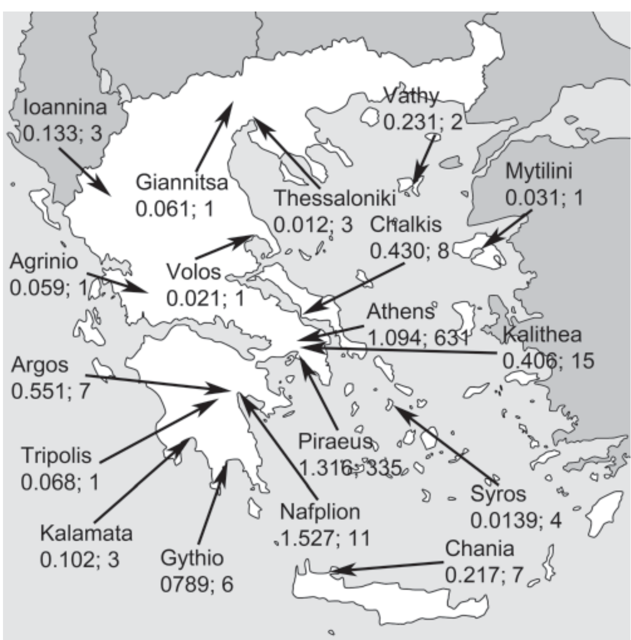

Figure. Map of Greece and deaths in the dengue fever epidemic, 1927–1928. The numbers are from the September 22, 1928, issue of the newspaper, I Kathimerini, and are ≈30%–40% lower than the official final death total. The first numbers indicate deaths per 1,000 inhabitants of each affected city (arrows); the number after the semicolon shows the total number of fatal cases from the epidemic in the respective location.

were afraid the epidemic might be yellow fever, but medical authorities explicitly denied this concern because "no patient ever showed any symptoms of hemorrhage" (the term σύμπτωμα in Greek covers both symptoms and signs). However, Cardamatis (*10*) described the course of the epidemic on the island of Aegina, located ≈25 km from the mainland, and stated that hemorrhage in all organ systems was the most common complication and cause of death. Furthermore, on August 25, I Kathimerini stated that "the present epidemic is malignant" and that neurologic disorders or cardiomyopathies often developed in the DF patients. To prevent complications, articles suggested that "intravenous salysalic [*sic*] acid, well sterilized [*sic*] should prevent cardiac diseases, especially myocarditis, ensuing from DF."

Experts from Greece and abroad were asked for their views, but often the same expert cited in different articles in the same issue expressed contradictory opinions (e.g., possibility of DF transmission by house animals, excluded a few pages later). A common theme was that DF was associated with environmental degradation, and general measures for other diseases, such as cholera, should successfully curb the epidemic. The British power company performing public works and digging in Athens was accused of "providing breeding sites" for mosquitoes. Authorities seemed to be aware that only control of mosquitoes would help alleviate the epidemic and suggested that any "ditch should be covered as soon as possible and petroleum and/or lime should be used in order to prevent mosquitoes from breeding." City authorities were advised to "wash the streets regularly and swamps should be dried the soonest possible." These proposed measures were accompanied by proposals to "brush one's teeth regularly" and take the drug "emostyrol." Finally, several clergymen's suggestion that "prayers and litanies would help end the epidemic" was made public.

On August 25, I Kathimerini reported the first cases outside the Athens area. Persons who had traveled to their birthplaces to vote in the general elections a few days earlier were held responsible for transporting the disease. On August 28, an article appeared that described what was then generally known about DF without citing the source of the information. Until that date, this article was 1 of only 2 scientifically sound articles describing the disease and its treatment. Additional articles contained amateur advice about how to cure DF (e.g., by "drinking milk and lemonade"). Development of an efficient drug also was reported (an "iodine solution per os" without further details), later followed by a letter from the chemist who developed it, cautioning that success achieved might have been purely coincidental.

More scientifically sound articles appeared in September. For example, the Minister of Health, an established pediatrician, explained how the "microbe" remained in Greece throughout the year; he had seen 1 DF case himself, 3 months before the new outbreak. He also explained that, after the acute phase, patients were expected to have lifelong immunity against dengue. Dr. Georges Blanc, head of the Hellenic Pasteur Institute, who was in France at the beginning of the epidemic, returned to Athens and granted an interview in which he tried to correct several statements. Moreover, a report signed by him and the health authorities also described facts about DF and its mode of transmission. I Kathimerini published a summary: the vector was *Stegomyia* mosquitoes, and the "microbe" had no relation to *Spirochaeta* (an association between DF and syphilis was considered possible by many physicians) or *Plasmodium laverani*.

One article cited a different newspaper claiming that the epidemic was not in fact DF but, rather, yellow fever. Two unnamed physicians asked for their opinion were cited as saying that if the disease were yellow fever, mortality rates would have been much higher. Initial reports on the epidemic in mid-August claimed that, compared with 1927, the new DF outbreak was much more malignant, on the basis of the lack of deaths reported during the previous year. I Kathimerini acknowledged the possibility that

some deaths might have occurred in 1927 but, because of the previous ill health of the patients, could have been attributed to other causes.

The second month of the outbreak saw qualitatively different articles. Small news pieces about the everyday effects of the epidemic appeared frequently: entrance examinations to the universities were postponed, the army draft was deferred, theaters were closed, spouses and children were sent to resorts far from Athens, courts were closed because of the lack of judges and jurors, and the mode of taxation was changed to address lower income. Brief articles reported numbers of DF cases from Athens and elsewhere, including non-Greek cities such as Smyrna (now Ismir), Copenhagen, and Berlin, attributed to travelers from Greece. In mid-September, I Kathimerini reported that the epidemic was clearly weaker: "... the crowd at the horse races was again high after several weeks of low attendance." On the same day, a 12-word item bore the headline, "ONLY TWO DEATHS!" On September 22, I Kathimerini published a report of the Ministries of National Economy and Health about the total number of deaths caused by the epidemic, aimed at calming foreigners wishing to travel to Greece. The numbers were corrected slightly a few days later, but the overall picture did not change (Figure). I Kathimerini did not subsequently publish new numbers of fatal cases.

The end of the epidemic saw a new kind of article about a potential reappearance in the following year. In a letter to I Kathimerini, a physician claimed that several patients bitten by infected mosquitoes late in the fall would become sick only in early spring. Therefore, in addition to mosquito control measures, an absolute isolation (i.e., quarantine) of the first patients of 1929 should prevent a new spread.

On October 29, I Kathimerini published a final report of the Nomiatros, the highest medical authority in the Νομός (i.e., county) of Attica; he claimed that DF could become endemic to Athens, but the fact that most persons living in the metropolitan area got sick and became immune, combined with the enhanced mosquito control measures that would be initiated, led him to conclude that DF would not be a real threat during the following years. That same day, I Kathimerini claimed that efficient measures against *Stegomyia* mosquitoes should suffice: covering any kind of water container and sewage-connected pipe or container and removing stagnant water from houses, kitchens, washing places, and flower pots.

1929–1931

Throughout the winter of 1928–29, sporadic articles citing DF, usually about unrelated matters, appeared in I Kathimerini. An often-recurring advertisement stated that many persons developed a hernia because of the "awful dengue disease," and true relief from it could be found only in the "incomparable invention" of a Mr. Em. Rousos. Additionally, a short advertisement said, "Against Dengue, Life Savers [candies] triumphed; they act the same way against the flu."

In May 1929, a series of articles in I Kathimerini described several findings and conclusions of investigations led by the Hellenic Pasteur Institute. In addition to confirmation of *Stegomyia* mosquitoes as the vector and lifelong immunity against DF, the scientists claimed that they also had produced an efficient vaccine; however, it was not to be used commercially. The vaccine had been tested, according to the newspaper, on members of the leper colony on the small island of Spinalonga and on patients of a public psychiatric hospital near Athens.

On July 7, 1929, I Kathimerini cited Ministry of Health officials as saying that no new DF case had appeared in Athens; only 2 days later, I Kathimerini reported that a person suspected to have DF was brought to the hospital. On July 21, three presumed DF cases were reported from Thessaloniki, the second largest city in Greece, ≈300 km north of Athens. Isolated cases of diseases resembling DF were reported sporadically throughout Greece but were never confirmed. Of those, the most important ones are ≈30 cases on the island of Syros in August 1931, of which a few supposedly were fatal. Yellow fever and an unknown spirochete infection were again discussed by the public (and discarded by health professionals); the unknown pathogen was declared to be transmitted by unidentified mosquitoes. To positively diagnose the disease, 3 psychiatric patients in Athens were inoculated with infected blood. One of the 3 who had not acquired DF 3 years earlier became sick with signs and symptoms of DF; the other 2, who had acquired DF during the epidemic, remained healthy. (Relatives of the first patient later sued the leader of the Hellenic Pasteur Institute team for attempted homicide. I Kathimerini did not report the outcome of this lawsuit.) Four months after these experiments, Institut Pasteur in Paris stated that the disease cases on Syros were caused by a spirochete.

Discussion

Reading the old news items sparks 3 issues. First, did the high number of fatal cases in 1928 result from dengue hemorrhagic fever? Two reports support this notion on the basis of the limited serologic study of Athenians born around the time of the epidemic (*2*,*3*). The data are consistent with a sequential infection in some of the population with dengue viruses of different serotypes, a *conditio sine qua non* for the appearance of dengue hemorrhagic fever (here DENV-1 and DENV-2). This notion was contradicted by another serologic analysis that indicated only DENV-1 was involved in the epidemic (*11*). According to statements by health officials, the newspaper articles alleged that no hemorrhagic illnesses were observed. However, this

information contradicts the findings from Aegina (*10*). Could the statement of the health officials have been false simply to calm the public?

Finally, several of the news items in 1928 cite physicians' assertions that DF patients from the previous year never became re-infected. This fact does not exclude the possibility of an asymptomatic or a misdiagnosed first infection. The high death rate could have resulted from the incidence of 2 independent circumstances: highly virulent DENV-1 combined with the limited appearance of DENV-2, which could account for the hemorrhagic disease on Aegina and presumably elsewhere. The essence is that the question about the high death rate cannot be answered, and only a vast serologic study of the population, perhaps involving residents of the other areas in Greece where rates of death were high, might provide clues. However, 83 years after the epidemic, the pool of potential prospects continues to diminish.

The second issue regards the presence of *Ae. aegypti* mosquitoes in Greece. Except for infrequent sampling of specimens, the species seems to have disappeared from the country, as it has from most of the Mediterranean region (*12*,*13*). Two main reasons seem to account for its disappearance: 1) the widespread urban and rural antimalaria campaign based on insecticides and environmental management that lasted into the late 1950s (*14*); and 2) economic development that brought along measures and changes, such as running water, which helped reduce the number of containers in which mosquitoes reproduced. As in other places, malaria vectors, although found in much smaller numbers, have not disappeared from Greece and still can be identified in entomologic collections (*13*). As in other European countries, *Ae. albopictus* mosquitoes are now found in several areas in Greece (*15*; J. Vontas, pers. comm.).

The third issue is, of course, whether such a devastating epidemic could recur. The social conditions, including medical sciences and health care, now differ substantially from the late 1920s, and recurrence of an epidemic of such an extent and magnitude is unlikely in Greece or elsewhere in Europe. On the basis of present standards, in the absence of *Ae. aegypti* mosquitoes, even a major *Ae. albopictus*–transmitted DF epidemic is extremely unlikely, although minor events (in terms of numbers), such as the recent chikungunya epidemic in northern Italy (*16*), cannot be excluded. Recent cases of dengue (*17*,*18*) and chikungunya (*19*) in Europe resulting from international travel also support this notion (*4*).

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Unexpected Result of Hendra Virus Outbreaks for Veterinarians, Queensland, Australia

Diana H. Mendez, Jenni Judd, and Rick Speare

A qualitative study of equine veterinarians and allied staff from Queensland, Australia, showed that veterinarians are ceasing equine practice because of fears related to Hendra virus. Their decisions were motivated by personal safety and legal liability concerns.

In the mid-1990s, Hendra virus (HeV) emerged as a new pathogen that spilled over from bats to horses to humans (*1*,*2*). All 7 cases of HeV infection among humans in Australia occurred in Queensland. Five of these cases involved equine veterinary personnel who conducted routine necropsies or endoscopies; 3 of the 5 cases were fatal (*2–6*). In Australia, equine clinical services are mostly delivered by veterinarians working in private practice. The 3 deaths prompted government and veterinary professional agencies to promote the overhaul of infection-control measures in veterinary practice (*3*,*4*) and increase auditing of veterinary infection-control strategies in private equine practice by Workplace Health and Safety Queensland (*7*). In 2011, HeV outbreaks multiplied throughout Queensland and New South Wales, and samples from a dog were positive for HeV (*8*,*9*).

With the approval of the James Cook University Human Ethics Committee (permit H3513), we interviewed veterinarians and allied staff from veterinary practices with the aim of capturing the HeV-related infection-control and workplace health and safety issues faced by equine practices. We report on 1 unexpected emerging issue: the departure of veterinarians from equine practice as a result of HeV outbreaks.

The Study

During 2009–2010, we conducted face-to-face, in-depth interviews with 21 veterinarians and allied staff from 14 equine and mixed private veterinary practices from a range of urban and rural areas between Cairns and Brisbane, Queensland, Australia (Table 1) (*10*). We asked a series of open-ended questions to determine what HeV-related infection-control and workplace health and safety issues confront equine practices (Table 2). Interviews were recorded, transcribed, and analyzed for themes.

Of the 20 veterinary professionals interviewed, 12 (60%) had dealt with ≥1 suspected cases of HeV, and 7 (35%) had dealt with a confirmed case of HeV. Of the 18 veterinarians interviewed, 4 (22%) reported having ceased equine practice, and as many as 8 (44%) knew of ≥1 colleagues who had done so in the previous 12 months. The decisions to quit were mostly motivated by the HeV-related fear for personal safety and legal liability.

Under the current Queensland legislation governing private businesses, private veterinarians are responsible for the safety of all persons in their workplace, both in the clinic and the field (*11*). Ten (47.6%) of the study participants were principal veterinarians (Table 1) who carried the highest degree of legal responsibility within their veterinary practice; they were quite concerned about their HeV-related legal liability. Four of these principal veterinarians reported ceasing equine practice because of the difficulty in enforcing infection control–related workplace health and safety compliance among their staff, because the logistical outlay of bringing change to their practice was too costly, or both. One participant declared, "The HeV situation was the last straw that made us stop equine practice…. We put it in the too hard basket." Their fear of prosecution became too big a threat for their business. However, ceasing this high-risk activity does not result in improved infection-control standards.

Principal veterinarians from other practices preferred to personally deal with all equine patients, thus taking the highest risk themselves rather than putting their staff at risk or not providing the service. In some instances, staff and principal veterinarians resorted to working in suboptimal personal safety conditions to fulfill their legal

Table 1. Location of participants in a study of Hendra virus–related safety issues faced by equine practices, Queensland, Australia, 2009–2010*

Zone, category	No. (%) participants
Metropolitan zone	
Capital cities	3 (14.30)
Population >100,000	6 (28.55)
Rural zone	
Population 25,000–99,999	6 (28.55)
Population 10,000–24,999	0
Population <10,000	3 (14.30)
Remote zone	
Population >4,999	3 (14.30)
Population <5,000	0

*Location zones and categories are according to the Australian Rural, Remote and Metropolitan Areas classification system (*10*).

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Table 2. Demographic characteristic of participants in a study of Hendra virus–related safety issues faced by equine practitioners, Queensland, Australia, 2009–2010

Study participants	No. (%)	Age, y (range)*	Years since graduation* (range)†	% Time spent doing equine work* (range)‡	Distribution by job title, no. (%)			
					Principal veterinarian	Partner/associate or employee veterinarian	Veterinary nurse	Practice manager
Female	8 (38.1)	35.8 (31–48)	13.1 (4–27)	30.4 (2–95)§	1 (4.8)	5 (23.8)	2 (9.5)	0
Male	13 (61.9)	48.5 (28–63)	22.9 (4–40)	52.1 (2–100)¶	9 (42.8)	3 (14.3)	0	1 (4.8)
Total	21 (100.0)	42.2 (28–63)	19.0 (4–40)	47.3 (2–100)	10 (47.6)	8 (38.1)	2 (9.5)	1 (4.8)

*Average.
†The practice manager interviewed was not a veterinarian and did not wish to supply this information.
‡Self-reported.
§One female participant did not provide this information.
¶The 1 participant who was a practice manager but not a veterinarian had not spent any time with animals and therefore was not included.

and ethical responsibility to their patients and clients, thus jeopardizing the legal situation. As one participant pointed out, "Veterinarians usually end up with less authority… taking the risk out of concern for the welfare of the horse." Veterinarians have a legal right to refuse service if safety is compromised; however, this would mean forfeiting immediate and future income through the loss of a client(s) and, possibly, reputation. In such instances, the staff and the business remain safe, but the principal veterinarian may not, and the overall standards of infection control within the practice do not improve.

Up to 6 (60%) of the interviewed principal veterinarians had embraced the need for improvement of infection-control practices and had made major changes to their protocols and premises, but they felt that the best level of compliance would not be legally protective because of the unpredictable character of the veterinary work environment. Another participant expressed concern over this legal uncertainty: "You still have to worry about what might occur out of the blue…. With workplace health and safety we are very aware that complying is often not enough if an incident occurs." In this scenario, although safety improvement is achieved, the legal risk remains.

Those participants still in equine practice also expressed concern over the consequences that the loss of skilled equine veterinarians would have on the profession and their practice. One participant said, "… this might introduce problems of gaps in the welfare of animals. Vets will need to refer animals." The lack of equine specialists would increase demands on the remaining equine veterinarians, who would have to further extend their already overstretched time and resources: they would work longer hours, travel farther to provide services, and be unable to reach sick horses in remote locations or to have them tested in a timely fashion. Participants still in equine practice considered that all these factors made working with horses less safe. Indeed, several studies showed that across a wide range of sectors, working >60 hours/week increased the risks for occupational injury and illness (*12–14*). Furthermore, several study participants reported that some colleagues now choose to only provide services to healthy animals and refuse to treat sick horses. A participant described this as choosing the "easy safe money" over the "hard dangerous money." This choice was creating resentment among members of an otherwise tight-knit veterinary community. Over time, resentment could jeopardize professional networking, which seems to play an essential role in disseminating clinical and safety information among veterinarians.

Although this study did not measure the overall effect of the decreased number of veterinarians who treat equids in Queensland, participants viewed the decrease as a major source of increased occupational risk for the remaining equine practitioners. If this trend is sustained, more private veterinarians may cease equine practice. Other participants no longer regarded themselves as equine practitioners and declared that they had ceased equine practice; however, they later admitted to still regularly treating horses. Their "official" departure from equine practice would increase their safety and legal risks because they might miss program updates on equine health information or infection-control improvement. It is also possible that the perception of increased risk may adversely influence the decision by younger veterinarians to pursue work in equine practice, thereby jeopardizing the normal replacement of the existing pool of aging equine practitioners. One parallel was the effect of severe acute respiratory syndrome. Overall, 35% of severe acute respiratory syndrome–related deaths were in health care workers. Some workers refused to go to work and others adopted a heroic stance and continued to work, resulting in substantial medium-term psychological effects on the healthcare professionals (*15*).

Conclusions

HeV remains a threat to the veterinary profession and public health in Australia. The experimental success of an HeV vaccine for horses was recently announced; if a vaccine becomes available, it may re-instill confidence in existing and future equine practitioners (*9*). However, the potential that emerging infectious diseases might dismantle the veterinary workforce should be considered when developing official strategies for the management of

HeV outbreaks. Infection-control management guidelines and workplace health and safety regulations must consider the context in which services are feasibly delivered to the public and should be devised in consultation with the private veterinary professionals on the frontline of outbreaks.

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Candida spp. with Acquired Echinocandin Resistance, France, 2004–2010[1]

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We report 20 episodes of infection caused by acquired echinocandin-resistant *Candida* spp. harboring diverse and new Fksp mutations. For 12 patients, initial isolates (low MIC, wild-type Fksp sequence) and subsequent isolates (after caspofungin treatment, high MIC, mutated Fksp) were genetically related.

Echinocandins are effective in patients with invasive candidiasis and recommended as first-line therapy, especially for patients with severe sepsis or those previously exposed to azoles or infected with *Candida glabrata* (*1*). Fewer than 50 persons infected with echinocandin-resistant species that are usually susceptible, such as *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei*, have been described in limited series or case reports (*2–4*). All species were found in patients preexposed to echinocandins. The major mechanism of resistance is related to mutations in *FKS* genes coding for β-1,3-glucan-synthase (*5*), with almost 20 known *FKS* mutations. We describe the characteristics of infections from caspofungin-resistant *Candida* spp. isolates belonging to usually susceptible species recorded in France (2004–2010) and analyze their *FKS* mutations and effect on echinocandin susceptibility.

The Study

Isolates received at the French National Reference Center for Mycoses and Antifungals (NRCMA) are identified to the species level by standard mycologic procedures and routinely tested for susceptibility to caspofungin, micafungin, and anidulafungin by using European Committee for Antimicrobial Susceptibility Testing (EUCAST) methods (*6*) and AM3 medium (*7*). In addition, RPMI 1640 medium was used here for selected isolates and reference strains. For the clinical isolates with caspofungin MIC ≥0.5 µg/mL in AM3, nucleotide sequences of hot spot (HS) 1 and 2 regions of the *FKS1* gene for *C. albicans* and *C. krusei* and of HS1 region of *FKS1*, *FKS2*, and *FKS3* genes for *C. glabrata* were determined (*7*,*8*).

The resulting protein sequences were aligned with the BioloMics software (BioloMics, BioAware SA, Hannut, Belgium) and compared with reference strains (*C. albicans*, ATCC32354; *C. krusei*, ATCC6258; and *C. glabrata*, ATCC2001). Genetic relatedness of *C. albicans* and *C. glabrata* paired isolates was studied by using microsatellite-length polymorphism analysis (*9–11*). The Wilcoxon signed-rank test was used to compare echinocandin MICs of paired isolates. Surveillance for mycoses by the NRCMA has been approved by the Institut Pasteur Internal Review Board and by the Commission Nationale de l'Informatique et des Libertés.

During September 2004–April 2010, twenty proven infections caused by *C. albicans* (n = 10), *C. glabrata* (n = 8), or *C. krusei* (n = 2) with caspofungin MIC ≥0.5 µg/mL and a mutation in the target enzyme were reported to the NRCMA (Table 1). Nineteen of the isolates were recovered after caspofungin treatment for a median duration of 27 days (range 10–270 days; 13 of 19 patients received caspofungin at the time the resistant isolate was recovered). Caspofungin was prescribed for 14 patients with proven *Candida* spp. infection, 1 patient with proven invasive aspergillosis, and 2 patients with febrile neutropenia; for 2 persons with hematologic malignancies, caspofungin was prescribed prophylactically.

The geometric mean MIC for *C. glabrata* and *C. albicans* were 2.8 and 1.7 µg/mL for caspofungin, 0.4 and 0.7 µg/mL for micafungin, and 0.2 and 0.09 µg/mL for anidulafungin, respectively (Table 2). Of the 20 mutated isolates found resistant to caspofungin in AM3 by using the EUCAST method, 19 also were resistant to caspofungin (1 intermediate), 18 to micafungin (1 intermediate and 1 susceptible), and 9 to anidulafungin (5 intermediate and 6 susceptible) according to Clinical Laboratory Standards Institute (CLSI) breakpoints and RPMI 1640 medium (Table

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[3]Additional members of the French Mycoses Study Group who contributed data are listed at the end of this article.

Table 1. Characteristics of 20 patients with infections caused by a non–*parapsilosis/guilliermondii Candida* spp. Fks mutation, France, 2004–2010*

Patient no.	Age, y/sex	Underlying condition	Neutropenia	Species	Site of infection	Duration of caspofungin exposure, d†	Outcome at 30 d‡
1	34/M	HIV positive	No	*C. albicans*	Esophagus	21	Alive
2	20/M	Hematologic malignancy: familial lymphohistiocytosis	Yes	*C. albicans*	Blood	17	Dead
3	77/M	Hematologic malignancy: AML	Yes	*C. albicans*	Blood	25	Alive
4	46/M	Hematologic malignancy: AML	Yes	*C. albicans*	Blood, peritoneum, pleural fluid	26	Dead
5	34/F	Liver transplant: cirrhosis	No	*C. albicans*	Hepatic abscess, peritoneum	60	Alive
6	64/F	Hematologic malignancy: AML; breast cancer	No	*C. albicans*	Blood	25	Alive at 17 d
7	59/M	Teratocarcinoma	No	*C. albicans*	Pharynx	35	Dead
8	28/M	Chronic mucocutaneous candidiasis	No	*C. albicans*	Pharynx, nails	270	Alive
9	14/F	Hematologic malignancy: ALL	Yes	*C. krusei*	Lung	45	Alive
10	79/M	Hematologic malignancy: non-Hodgkin lymphoma	Yes	*C. krusei*	Blood	10	Dead
11	46/M	Hematologic malignancy: Burkitt lymphoma; HSCT	Yes	*C. glabrata*	Blood	None	Dead
12	85/M	Gastric ulcer; CVC	No	*C. glabrata*	Blood	32	Alive
13	28/M	Hematologic malignancy: non-Hodgkin lymphoma; HSCT	No	*C. glabrata*	Palate§	135	Alive
14	48/M	Esophageal cancer	No	*C. glabrata*	Blood	12	Alive
15	41/M	Liver transplant: fulminant hepatitis	No	*C. glabrata*	Blood, peritoneum	37	Dead
16	38/F	Hematologic malignancy; AML; HSCT	Yes	*C. glabrata*	Blood	51	Dead
17	60/M	Acute pancreatitis; GI tract surgery	No	*C. glabrata*	Bile	34	Alive
18	39/M	Hematologic malignancy: AML; HSCT	No	*C. glabrata*	Sinus§	15	Alive
19	55/F	Lock-in syndrome; neurogenic bladder	No	*C. glabrata*	Urine¶	27	Alive
20	63/M	Colon cancer	Yes	*C. glabrata*	Blood	14	Alive

*AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; HSCT, hematopoietic stem cell transplantation; CVC, central venous catheter; GI, gastrointestinal.
†Duration of caspofungin exposure before isolation of the first resistant *Candida* isolate.
‡Outcome 30 d after isolation of the first resistant *Candida* isolate.
§From a biopsy specimen.
¶With sepsis.

2). According to EUCAST breakpoints, 19 isolates also were resistant to anidulafungin, and 1 isolate was almost resistant (MIC 0.03 μg/mL). We thus showed discrepancies between CLSI and EUCAST regarding anidulafungin susceptibility (www.srga.org/eucastwt/MICTAB/EUCAST%20clinical%20MIC%20breakpoints%20-%20antimicrobials%20for%20Candida%20infections.htm [V 3.0 2011–4-27]) (*12,13*).

Of the 10 caspofungin-resistant *C. glabrata* isolates, 8 harbored a mutation in Fks2p only, 1 isolate had a mutation in Fks1p, and 1 had mutations in Fks1p and Fks2p (Table 2). Of the 8 caspofungin-resistant *C. albicans* isolates, 1 had a missense mutation in HS2, and 1 had a combination of 2 heterozygous mutations in HS1. The other 6 isolates harbored 4 different mutations in HS1 (Table 2). Finally, the 2 *C. krusei* isolates had 2 different mutations in HS1 region. Of the 20 mutated isolates, 6 harbored 7 mutations not yet described in the literature (Table 2) (*13*).

Prior initial isolates available for 12 patients had the wild-type sequence for the HS regions that were mutated in the paired resistant isolate. All initial isolates were susceptible to anidulafungin and to micafungin and anidulafungin according to EUCAST and CLSI, respectively (data not shown). According to CLSI caspofungin breakpoints, 5 of 6 initial isolates of *C. albicans* were susceptible, and 1 was intermediate; 4 of 5 *C. glabrata* isolates were resistant (0.5 μg/mL), and 1 was intermediate; and the *C. krusei* isolate was resistant (1 μg/mL). For each of the 12 pairs, MICs increased significantly (from 3 to 8 dilutions for caspofungin and micafungin and from 1 to 8 dilutions for anidulafungin) between the wild-type and the mutant isolate (Figure; $p<0.001$). Genetic

Table 2. In vitro susceptibility and Fksp mutations of 20 echinocandin-resistant *Candida* spp. isolates, France, 2004–2010

Patient no.	Strain	Species	MIC, µg/m, AM3/RPMI 1640 medium			Fksp mutation	
			Caspofungin	Micafungin	Anidulafungin	Gene	Mutation
1*	05BL1-38	*C. albicans*	1/2	0.25/1	0.06/0.125	*FKS1* (HS1)	F641S
2*	ODL13-1254	*C. albicans*	1/2	1/1	0.5/0.5	*FKS1* (HS1)	S645Y
3†	06BL2-127	*C. albicans*	2/2	1/0.5	0.125/0.125	*FKS1* (HS1)	F641S‡ + S645P‡
4	ODL19-1894	*C. albicans*	4/2	2/2	0.125/0.25	*FKS1* (HS1)	S645P
5*	08BL1-94	*C. albicans*	2/4	0.25/1	0.06/0.5	*FKS1* (HS2)	R1361G§
6*	08BL2-143	*C. albicans*	8/4	4/2	0.25/0.5	*FKS1* (HS1)	S645P
7*	09BL1-43	*C. albicans*	1/2	0.25/1	0.06/0.25	*FKS1* (HS1)	F641S
8*	09BL1-77	*C. albicans*	0.5/0.5	0.5/0.25	0.015/0.03	*FKS1* (HS1)	R647G§
9	06BL1-34	*C. krusei*	4/8	2/4	1/2	*FKS1* (HS1)	L648W§,¶
10*	10BL1-50	*C. krusei*	2/4	1/2	0.06/1	*FKS1* (HS1)	F645L§,¶
11	ODL7-647	*C. glabrata*	8/8	0.5/1	0.25/0.125	*FKS2*	DelF658#
12*	07BL2-157	*C. glabrata*	4/1	1/0.5	0.25/0.5	*FKS2*	DelF658#
13*	06BL1-33	*C. glabrata*	8/8	4/8	2/2	*FKS2*	S663P
14*	ODL21-2028	*C. glabrata*	1/1	0.25/0.25	0.25/0.25	*FKS1*	S629P
15*	ODL22-2183	*C. glabrata*	8/2	0.25/0.25	0.25/1	*FKS2*	S663P
16	ODL23-2221	*C. glabrata*	1/4	0.06/2	0.06/0.25	*FKS1* + *FKS2*	F625I§ (*FKS1*) + P667T§ (*FKS2*)
17*	08BL2-142	*C. glabrata*	1/4	0.25/2	0.25/2	*FKS2*	S663P
18	09BL1-55	*C. glabrata*	8/4	2/4	0.5/0.5	*FKS2*	S663P
19	10BL1-19	*C. glabrata*	0.5/4	0.06/0.5	0.06/1	*FKS2*	F659S + L664V§
20	10BL1-67	*C. glabrata*	4/4	0.5/1	0.125/1	*FKS2*	DelF658#

*Parentage of initial isolate available.
†In this patient, another isolate with reduced susceptibility to echinocandin was retrieved. This isolate harbored an S645P mutation in *FKS1*.
‡Heterozygous mutation.
§Mutations not already described (*13*).
¶Strains had also an L701M mutation.
#Deletion.

relatedness was demonstrated for all *C. albicans* and *C. glabrata* paired isolates.

Conclusions

We demonstrated that recent exposure to caspofungin altered the distribution of species causing *Candida* bloodstream infections (*14*), and that caspofungin exposure was independently associated with fungemia associated with intrinsically less-susceptible species in hematology (*15*). Echinocandin resistance in *Candida* spp. is still uncommon (*4*,*13*). Through our surveillance program, we estimated the incidence of decreased susceptibility to caspofungin associated with *FKS* mutations among *C. albicans, C. glabrata*, and *C. krusei* isolates responsible for candidemia in children and adults in Paris at 6 (0.4%) of 1,643 (NRCMA, unpub. data). We report proven caspofungin-resistant *Candida* spp. infections with none of the isolates belonging to the intrinsically less-susceptible species *C. parapsilosis* or *C. guilliermondii*.

We determined antifungal susceptibility testing by the EUCAST technique using AM3 because it enables better discrimination between susceptible wild-type and resistant mutant isolates (*7*). All isolates with high caspofungin MIC (≥0.5 µg/mL) had mutation in the HS1 and/or HS2 region of *FKS* genes. The mutations were not restricted to a given position but were diverse, especially for *C. albicans* with 6 different mutations among the 8 resistant isolates; 5 different mutations were observed among the 10 *C. glabrata* resistant isolates. Most mutations in *C. glabrata* isolates were in Fks2p. Two mutations in *C. albicans*, 2 patterns of mutation in *C. glabrata*, and 1 mutation in *C. krusei* had not been reported before, highlighting the great mutation diversity that could be responsible for echinocandin resistance (*13*).

All but 1 patient had received caspofungin (70 mg on day 1, then 50 mg/d) before recovery of the resistant isolate, with a variable duration of exposure (<10 days to >8 months), in agreement with the literature (5 [*3*] to 420 days). In addition, 13 of 19 patients received caspofungin at the time of recovery of the resistant isolate. Most patients had malignancy, but 7 intensive care unit hospitalizations also were recorded. Echinocandins MICs between the wild-type parent and the subsequent mutant isolate increased by up to 8 $\log_2$ dilutions (Figure). The source of the resistant isolate is not unequivocal; it was acquired from the environment as an already resistant isolate or from the patient's own flora under drug pressure. Our genotyping results favor the second hypothesis. This study suggests in France the emergence of infections from acquired echinocandin resistance in usually susceptible *Candida* spp. in patients preexposed to caspofungin, which highlights the need for careful species identification, antifungal drug susceptibility testing, and evaluation of prior drug exposure before antifungal drug prescription.

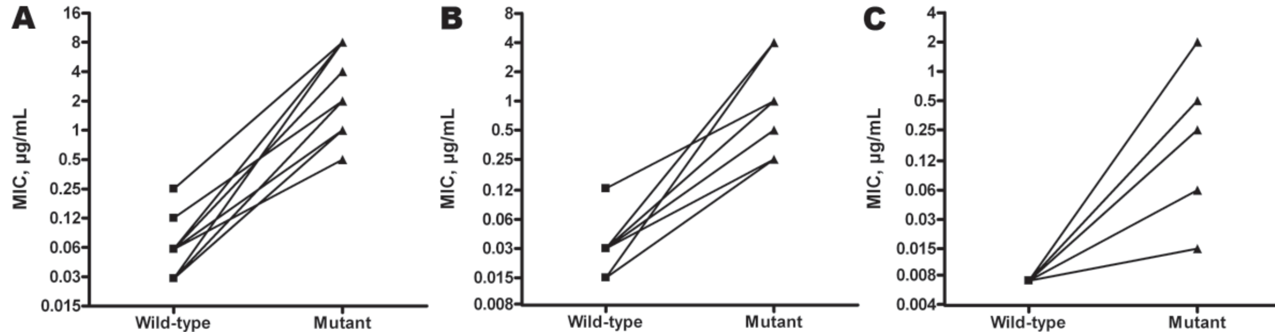

Figure. Corresponding caspofungin (A), micafungin (B), and anidulafungin (C) MICs in 12 Fksp mutant *Candida* spp. isolates and their wild-type parent isolates, France, 2004–2010. Susceptibility testing was performed by using the European Committee for Antimicrobial Susceptibility Testing method (*6*) and AM3 medium (*7*).

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Outbreak of Leptospirosis after Flood, the Philippines, 2009

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After a typhoon in September 2009, an outbreak of leptospirosis occurred in Metro Manila, the Philippines; 471 patients were hospitalized and 51 (10.8%) died. A hospital-based investigation found risk factors associated with fatal infection to be older age, hemoptysis, anuria, jaundice, and delayed treatment with antimicrobial drugs.

Leptospirosis is highly endemic to the Philippines. Outbreaks usually occur during the typhoon season (July–October) (*1–3*). On September 26, 2009, a typhoon caused serious flooding in Metro Manila (*4*). Starting in the first week of October, the number of patients with suspected signs and symptoms of leptospirosis increased sharply. Until mid-November, 2,299 patients, including 178 who died (case-fatality ratio [CFR] 8%), in 15 hospitals in Metro Manila were reported to the Department of Health (*4*,*5*). For this outbreak, we conducted a hospital-based investigation to describe the characteristics of hospitalized patients, investigate risk factors for death, and identify the causative *Leptospira* species and serogroups.

The Study

We conducted our investigation at San Lazaro Hospital, a national 500-bed referral infectious disease center for Metro Manila and neighboring provinces, which mostly serves economically disadvantaged persons. On October 11, 2009, this hospital initiated prospective surveillance and retrospective data collection. The study ended on October 31, when cases had decreased to baseline level (<1 case/d). The list of patients was obtained from the inpatient database with International Classification of Diseases, 10th revision, coding. Vital sign information and laboratory findings at admission were collected from medical charts.

Eligible were all hospitalized patients who had 1) fever plus at least 2 other signs and symptoms of leptospirosis (headache, myalgia, eye pain, nausea, vomiting, abdominal pain, diarrhea, conjunctival suffusion, jaundice, tea-colored urine, oliguria, anuria, or unusual bleeding) (*6*); 2) history of wading in floodwater; and 3) symptoms that started September 26–October 31. We evaluated prognostic factors and the effect of therapeutic factors by using univariate and multivariate Poisson regression analyses with robust SEs (*7*).

A total of 486 cases met clinical criteria for leptospirosis; 15 were excluded because of insufficient data. Patients were predominantly young and male (Table 1). The most common clinical features were conjunctival suffusion and myalgia, followed by abdominal pain and oliguria. Among 471 patients, 51 died (CFR 10.8%); 12 (2.6%) were discharged before improvement; and 7 (1.5%) were transferred to other hospitals, mainly for dialysis. Primary causes of death were pulmonary hemorrhage (18 [35%]) and acute respiratory distress syndrome/severe respiratory failure (12 [24%]), followed by acute renal failure (10 [20%]) and multiple organ failure/disseminated intravascular coagulation (8 [16%]). Mean time ± SD from illness onset to death was 7.7 ± 5 days; 36 (71%) died within 2 days of admission.

Univariate analysis showed the following to be associated with death: older age, jaundice, anuria, and hemoptysis (Table 1). Of the initial laboratory findings, neutrophilia, thrombocytopenia, increased blood urea nitrogen, and increased creatinine levels were associated with death. Most patients received antimicrobial drugs (mainly penicillin G) as first-line treatment. Delayed initiation of treatment increased risk for death (Table 2). No patient received prophylactic antimicrobial therapy. Administration of rapid volume replacement therapy and diuretics was associated with death but probably reflected severe renal disease. Only 1 patient received peritoneal dialysis and 1 received mechanical ventilation.

During the outbreak, 2 kinds of rapid diagnostic tests for leptospirosis—Leptospira Serology Kit (Bio-Rad, Marnes-la-Coquette, France) and PanBio IgM ELISA (Panbio Diagnostics, Brisbane, Queensland, Australia)—were available in the hospital, although the number of kits was limited. Plasma collection for additional laboratory confirmation started October 11. Samples were initially stored at –4°C in the hospital laboratory and then frozen at –40°C in the National Reference Laboratory, STD/AIDS

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Table 1. Characteristics of 471 leptospirosis patients, San Lazaro Hospital, Manila, the Philippines, 2009*

Characteristic	No. (%) patients total, n = 471	No. (%) patients who died, n = 51	Univariate analysis	
			Risk ratio	95% CI
Sex				
M	424 (90)	45 (88.2)	Reference	
F	47 (10)	6 (11.8)	1.2	0.54–2.67
Age ≥30 y†	235 (49.9)	37 (72.6)	2.65	1.47–4.78
Low BMI, <20, n = 398	129 (32.4)	16 (35.6)	1.15	0.65– 2.04
Clinical features, n = 471				
Headache	246 (52.2)	19 (37.3)	0.54	0.32–0.93
Myalgia	361 (76.7)	36 (70.6)	0.73	0.42–1.29
Cough	83 (17.6)	3 (5.6)	0.29	0.09–0.92
Malaise	208 (44.2)	23 (45.1)	1.04	0.62–1.75
Vomiting	272 (57.8)	22 (43.1)	0.56	0.33–0.94
Abdominal pain	288 (61.2)	28 (54.9)	0.77	0.46–1.3
Diarrhea	192 (40.8)	24 (47.1)	1.29	0.77–2.17
Conjunctival suffusion	368 (78.1)	41 (80.4)	1.15	0.6–2.21
Jaundice	225 (47.8)	38 (74.5)	3.2	1.74–5.84
Tea-colored urine	156 (33.1)	17 (33.3)	1.01	0.58–1.75
Oliguria	286 (60.7)	27 (52.9)	0.73	0.43–1.22
Anuria	26 (5.5)	10 (19.6)	4.17	2.37–7.36
Hemoptysis	15 (3.2)	7 (13.7)	4.84	2.63–8.9
Skin hemorrhage	2 (0.4)	0	NA	
Convulsion	3 (0.6)	1 (2)	3.12	0.62–15.82
Dizziness	43 (9.1)	1 (2)	0.2	0.03–1.41
Vital status at admission				
Hypotension, <100 mm Hg, n = 455	177 (38.9)	14 (28.6)	0.63	0.35–1.13
Tachycardia, >100 beats/min, n = 461	84 (18.2)	14 (27.5)	1.7	0.96–3.0
Tachypnea, >20 breaths/min, n = 460	253 (55)	34 (66.7)	1.64	0.94–2.84
Initial laboratory findings				
Neutrophilia, >12 x 10^9 cells/L, n = 430	171 (39.8)	18 (58.1)	2.1	1.05–4.17
Thrombocytopenia, <50 x 10^3 cells/L, n = 426	50 (11.7)	10 (33.3)	3.76	1.87–7.57
AST >100 IU/L, n = 220	44 (20)	2 (28.6)	1.6	0.32–8.0
ALT >100 IU/L, n = 220	34 (15.5)	2 (28.6)	2.2	0.44–10.9
BUN >80 mg/dL, n = 385	185 (48.1)	12 (75)	3.24	1.06–9.89
Cr >3.0 mg/dL, n = 413	183 (44.3)	13 (72.2)	3.27	1.19–9.01

*BMI, body mass index; NA, not applicable; AST, aspartate aminotransferase; ALT, alanine transaminase; BUN, blood urea nitrogen; Cr, creatinine.
†Mean ± SD 31.3 ± 13.1 for total patients, 39.5 ± 13.6 for patients who died; risk ratio (95% CI) 1.04 (1.02–1.06).

Cooperative Central Laboratory, San Lazaro Hospital. In June 2010, microscopic agglutination test (MAT) and PCR were performed in the National Institute of Infectious Diseases, Tokyo, Japan, as described (*8*,*9*). A case was defined as laboratory confirmed if 1) specific antibodies were detected with titer ≥400 or at least a 4-fold increase in reciprocal MAT titer between paired samples, 2) PCR for *Leptospira flaB* gene was positive for at least 1 blood sample, or 3) rapid diagnostic test result (either test) was positive for at least 1 blood sample.

A total of 134 plasma samples were collected from 93 patients. From 25 patients, 2–4 samples were collected; mean time from first to last collection was 6.1 (range 1–18) days. Among the 93 patients, 15 had a single MAT titer ≥400 and 4 showed a 4-fold increase in the MAT titer in paired samples. Most antibodies were against *L. borgpetersenii* serovar Tarassovi, followed by serovars Poi and Sejroe and *L. interrogans* serovars Losbanos and Manilae. PCR produced positive results for 4 patients, 2 of whom had a negative MAT titer. PCR-detected *Leptospira* strains were phylogenetically characterized by the *flaB* sequence (*8*,*9*); 3 were identical to those of isolates from rats in Metro Manila, but 1 was distinct (Figure).

Rapid-test results were available for 85 case-patients; 48 of 78 samples were positive by Leptospira Serology Kit and 5 of 7 were positive by ELISA. Of 27 cases tested by MAT and Leptospira Serology Kit, 6 were positive by both tests. One sample was negative by MAT and ELISA. Taken together, 149 (32%) of all cases underwent any diagnostic testing for leptospirosis, and results for 67 (45%) of these were laboratory confirmed. Clinical and laboratory findings of laboratory-confirmed and suspected cases, respectively, were almost identical except for the presence of jaundice (61.2% vs. 45.5%; p = 0.018), convulsions (4.5% vs. 0%; p = 0.003), and death (3% vs. 12.1%; p = 0.03). No plasma sample test results have been validated; thus, deduced incidence should be considered with caution. To explore the possibility of hantavirus co-infection contributing to

Table 2. Effects of therapeutic approaches on death from leptospirosis, San Lazaro Hospital, Manila, the Philippines, 2009

Therapeutic factor	Total cases, n = 471*	Fatal cases, n = 51*	Univariate analysis		Multivariate analysis	
			Risk ratio	95% CI	Risk ratio	95% CI
Days from onset to first antimicrobial drug therapy, n = 466	4.9 ± 2.6	5.6 ± 2.8	1.09	1.01–1.18	1.09†	1.00–1.17
<7	351 (75.3)	31 (63.3)	Reference		Reference	
≥7	115 (24.7)	18 (36.7)	1.77	1.03–3.05	1.76†	1.03–3.01
First antimicrobial agent used, n = 469						
Penicillin G	434 (92.5)	47 (94)	Reference		Reference	
Ceftriaxone	10 (2.1)	2 (4)	0.79	0.26–2.42	0.62‡	0.16–2.38
Doxycycline	9 (1.9)	0 (0)				
Others	16 (3.4)	1 (2)				
Days from onset to admission†	5.1 ± 2.6	5.7 ± 3.2	1.08	1.0–1.16	1.08†	1.0–1.16
Rapid volume replacement therapy						
Performed	334 (70.9)	46 (90.2)	3.77	1.53–9.3	2.63§	0.6–11.4
Not performed	137 (29.1)	5 (9.8)	Reference		Reference	
Diuretics						
Used	356 (75.6)	46 (90.2)	2.97	1.21–7.31	1.15§	0.31–4.23
Not used	115 (24.4)	5 (9.8)	Reference		Reference	

*Values are no. (%) or mean ± SD.
†Adjusted for age group (<30 or ≥30 y).
‡Adjusted for age group and duration from symptom onset to antimicrobial therapy initiation.
§Adjusted for age group and creatinine level.

the high CFRs, we screened samples for antibodies against hantavirus (*10*), but all were negative.

Conclusions

Risk factors for fatal leptospirosis were jaundice, anuria, and hemoptysis at admission. These are typical signs of the severe form of leptospirosis called Weil disease (*11*), confirming earlier work (*12*,*13*). Hemoptysis with high CFR (7 [47%] of 15) may have represented leptospirosis-associated severe pulmonary hemorrhagic syndrome (*14*). Some clinical features, including cough, seemed to be associated with lower risk for death, but minor symptoms in dying patients might have been overlooked. High leukocyte counts, blood urea nitrogen and creatinine levels, and lower platelet counts were also associated with death.

Deaths could be reduced if these indicators are detected and appropriate management introduced early. Laboratory data such as potassium level might also be helpful, but these tests are often not available for all suspected cases in resource-poor settings, especially during outbreaks. The trend of most patients being male is compatible with previous reports and thought to reflect higher exposure to contaminated water (*6*,*12–14*).

Although all patients received antimicrobial therapy, a considerable proportion died of acute respiratory distress syndrome and acute renal failure within only 2 days of admission, indicating that most patients sought care too late. The World Health Organization recommends starting antimicrobial therapy before the fifth day of disease onset (*6*), but for half of the case-patients reported here, it was started after the fifth day. Our results support the benefits of early initiation of antimicrobial therapy.

Renal failure was clearly a cause of death, but only 1 patient received peritoneal dialysis and another 7 were transferred to other hospitals for hemodialysis after only 3.4 days of hospitalization. This lack or delay of dialysis

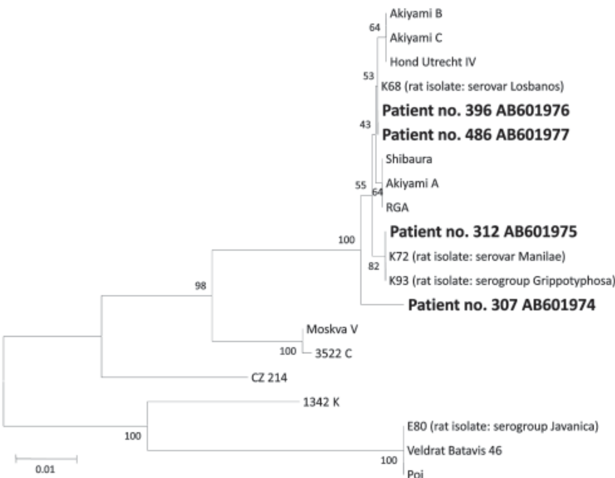

Figure. Phylogenetic tree based on the *Leptospira flaB* gene sequence. The sequences obtained in this study are indicated in **boldface** and have been deposited in DDBJ/GenBank/EMBL (accession numbers indicated). The sequences of rat isolates are derived from a previous study (*9*). Sequence alignments were conducted by using MEGA4 software and ClustalW (www.megasoftware.net), and phylogenetic distances were calculated by using MEGA4 software and the neighbor-joining method. All 4 PCR products belong to *L. interrogans*; 2 are closely related to serovar Losbanos, 1 to serovar Manilae, and 1 is not associated with strains or serovars included in our analysis. Microscopic agglutination test results indicate infecting serogroups. *L. borgpetersenii* serovar Tarassovi had the highest titer in patients 307 and 486, but this finding can be explained by a cross-reaction. Scale bar indicates nucleotide substitutions per site.

might have affected outcomes. San Lazaro Hospital is not sufficiently equipped for intensive care. Expanded access to peritoneal dialysis might reduce deaths from severe leptospirosis complicated by acute renal failure.

Leptospirosis is typically seen in resource-poor settings, where costly medical equipment such as dialyzers and ventilators are rarely accessible. For leptospirosis outbreak control and CFR reduction in leptospirosis-endemic regions, continuous case and environmental monitoring and early introduction of appropriate treatment for suspected cases are warranted.

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Legionella longbeachae and Endocarditis

Nicola Leggieri, Frédérique Gouriet, Frank Thuny, Gilbert Habib, Didier Raoult, and Jean-Paul Casalta

We report a case of infectious endocarditis attributable to *Legionella longbeachae*. *L. longbeachae* is usually associated with lung infections. It is commonly found in composted waste wood products. *L. longbeachae* should be regarded as an agent of infectious endocarditis, notably in the context of gardening involving handling of potting soils.

Legionella longbeachae is a facultative intracellular gram-negative bacillus commonly found in composted waste wood products used in potting mixes. It is usually associated with lung infections. We report a case of infectious endocarditis attributable to *L. longbeachae* 6 months after the patient had an aortic valve bioprothesis replacement.

The Patient

In July 2008, a 73-year-old man was admitted to La Timone Hospital, Marseille, France; it was suspected that an aortic bioprosthetic valve had become displaced. The patient had received an aortic bioprosthetic valve replacement in January 2008 for aortic insufficiency. After he returned home in March, he carried out gardening activities and used potting mixes to plant flowers. In April 2008, he was admitted to the emergency department of a general hospital for fever (38.9°C). At admission, his leukocyte count was 12 × 10^9 cells/L. He was empirically treated with amoxicillin/clavulanic acid plus ciprofloxacin. Results of clinical examination and all investigations (chest radiograph, transesophageal echocardiograph, 3 blood cultures, urine analysis, and *Legionella* urinary antigen test) did not identify an infectious agent, and antimicrobial drug treatment was stopped after 24 hours.

In May 2008, the patient had a new episode of fever (39°C), with a weight loss of 20 kg within 3 months. He was admitted to the cardiology department of La Timone Hospital. An endocarditis diagnostic kit was used. The endocarditis kit included, besides blood cultures, tubes to collect a serum sample, which was used to detect rheumatoid factor and estimate specific antibodies against *Coxiella burnetii, Bartonella* spp., *Brucella* spp., *Chlamydia* spp., *Mycoplasma pneumoniae*, *Legionella pneumophila, L. anisa*, and *Aspergillus* spp. (*1*). Results of all tests (including transesophageal echocardiograph) were negative. The modified Durack score (*2*) showed that only 2 minor criteria were met (and no major criteria), and the Richet score was 3 (fever = 1, male = 1, previous valvular pathology = 1), with a positive predictive value of 0.28 and a negative predictive value of 0.82 (*3*). He was discharged without receiving any antimicrobial drugs.

In July 2008, the patient was hospitalized at La Timone Hospital for heart failure. A transesophageal echocardiograph showed an aortic bioprothesis displacement with a false aneurysm and 9 mm of vegetation (Figure). At admission, leukocyte count was 9.4 × 10^9 cells/L, hemoglobin level was 9.8 gm/dL, erythrocyte sedimentation rate was 53 mm, and C-reactive protein level was 126 mg/L. A new endocarditis diagnostic kit was used. The blood cultures remained sterile. Results of serologic tests for *L. pneumophila* and the urinary antigen test for *Legionella* spp. were negative. Only the serologic test for *L. anisa* was positive (titer 256). The diagnosis of blood culture–negative endocarditis was established, and intravenous antibiotherapy was begun with vancomycin (30 mg/kg/d) plus gentamicin (3 mg/kg/d).

Cardiac surgery was performed after 30 days because of heart failure. The aortic bioprothesis was replaced. We conducted bacterial 16S rDNA amplification and sequencing on the valvular tissue as reported (*4*) and found a sequence 100% similar to that of *L. longbeachae* (GenBank accession no. AY444741). The valvular culture grew gram-negative bacilli that were catalase positive and oxidase negative after 10 days on buffered charcoal yeast extract medium (AES Chemunes, Bruz, France). The strain was identified as *L. longbeachae* by 16S rDNA (*5*) and *mip*

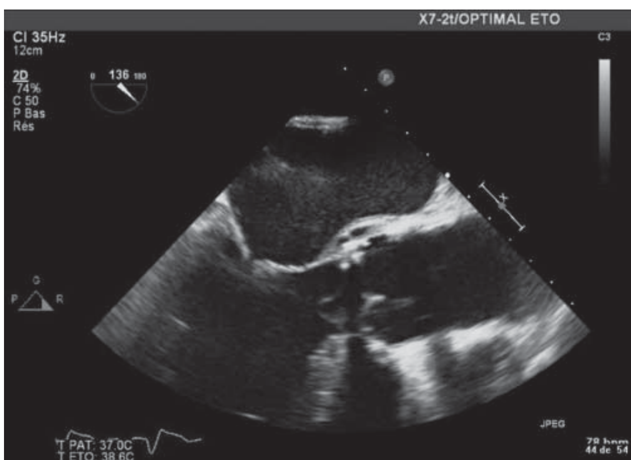

Figure. Transesophageal echocardiograph of patient with Legionnaires' disease, Marseille, France, July 2008.

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gene (*6*) amplification and sequencing (GenBank accession no. AJ810226).

Histologic examination of the removed valve was performed, and findings were compatible with the diagnosis of infective endocarditis. Results of immunohistochemical analysis performed on the valvular tissue were positive for *L. longbeachae*. Results of immunofluorescence and Western blot of the patient's blood were positive for the same strain.

The patient recently used only Australian potting mixes, which may contain *L. longbeachae*, although we were not able to cultivate *L. longbeachae* or other *Legionella* spp. from the potting mix. Vancomycin administration was stopped, and the patient's treatment was changed to erythromycin (3 g/d intravenously for 6 weeks) and ciprofloxacin (400 mg/d intravenously for 15 days and 1.5 g/d by mouth for 1 month). The patient recovered. At a 3-month follow-up visit, he had gained 6 kg, and no inflammatory syndrome was found. At 6 months, he had gained 13 kg and felt well.

Conclusions

L. longbeachae was first described as a new species in 1981 after it was isolated from a patient with pneumonia in Long Beach, California, USA (*7–9*). A second serogroup was described in 1981 (*10*). This species is common in composted wood products used in potting mixes in Australia and in Japan (*11,12*). Although the composition of potting soil used in Europe is different, Legionnaires' disease has also been described in Scotland in connection with the use of potting soil (*13*). A case of pneumonia caused by *L. longbeachae* associated with gardening was described in 2006 in the Netherlands. *L. longbeachae* was cultured from the patient's sputum and from the commercial potting soil he had used (*14*). The source of contamination for the patient described could be the Australian potting mixes he had used for 1 month before the first symptoms, although a culture of potting soil was negative. The patient did not experience cutaneous inoculation or a wound during gardening, although minor trauma is a possibility.

Noninvasive diagnosis for this patient was not successful: the results of serologic immunofluorescence testing for *L. pneumophila* and the urinary antigen test for *Legionella* spp. were negative. Only the serologic testing was positive for *L. anisa* (titer 256). Cross-reactivity between *L. anisa* and *L. longbeachae* should be considered.

In a previous report, a patient with aortic valve endocarditis showed an increase in titers of antibodies against *L. bozemanii*, *L. longbeachae*, and *L. jordanis*, but the infection was not confirmed by the isolation of bacteria (*15*). The patient was a 38-year-old woman with type 1 diabetes mellitus and bronchial asthma but no medical history of heart disease. She was hospitalized 2 months before acquiring infectious endocarditis for pneumonia and fever caused by *M. pneumoniae*. Transesophageal echocardiograph showed a thickened bicuspid aortic valve with 5–7-mm vegetation. The serologic test results were positive only for *L. bozemanii*, *L. longbeachae*, and *L. jordanis*. She was treated with erythromycin (1 g 4×/d intravenously, then orally, for 6 weeks, then 1 g 3×/d orally for 2 weeks). For the patient reported here, the diagnosis of infectious endocarditis was definitive because results of PCR, serologic testing, and valvular culture were positive for *L. longbeachae*.

L. longbeachae should be regarded as an agent of infectious endocarditis, notably in context of gardening involving handling potting soils. Definitive identification was possible only by using molecular biology–based methods on the removed valve and on culture. As mentioned by other investigators, *L. pneumophila* urinary antigen test should not be used to rule out *L. longbeachae* infection (*13*).

Dr Leggieri has completed internships in internal medicine and infectious diseases in Switzerland. He is completing a postgraduate specialization in endovascular infections at the Fédération de Microbiologie Clinique in Timone's University Hospital, Marseille, France. His research interests include legionellosis.

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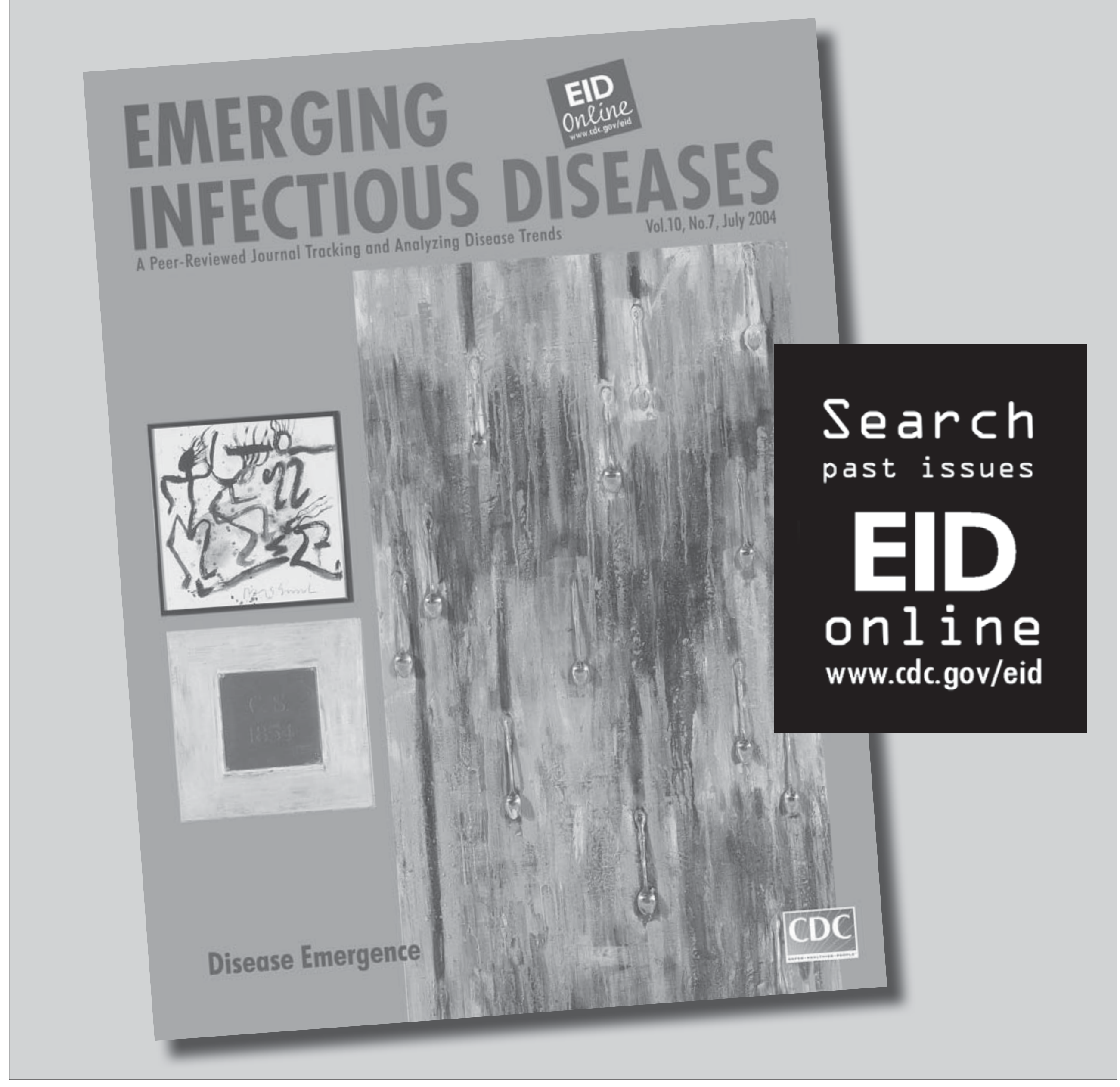

Emergence of *Blastoschizomyces capitatus* Yeast Infections, Central Europe

Tanja Birrenbach, Sonja Bertschy, Franziska Aebersold, Nicolas J. Mueller, Yvonne Achermann, Konrad Muehlethaler, and Stefan Zimmerli

We report 5 cases of disseminated infection caused by *Blastoschizomyces capitatus* yeast in central Switzerland. The emergence of this yeast in an area in which it is not known to be endemic should alert clinicians caring for immunocompromised patients outside the Mediterranean region to consider infections caused by unfamiliar fungal pathogens.

In recent years, the frequency of opportunistic fungal infections has increased in parallel with the growing number of patients receiving aggressive chemotherapy (*1,2*). Most fungal infections are caused by *Candida* spp. and *Aspergillus* spp., but reports of infections caused by rare molds and unusual yeasts are increasing. The widening range of fungal pathogens usually has been ascribed to the growing number of immunocompromised hosts and the use of antifungal prophylaxis (*1,3*). Another major contributor to the changing epidemiology of opportunistic fungal infections may be climatic changes (*4,5*), particularly for organisms for which geographic distribution appears to be restricted by climatic factors. We report the emergence of disseminated infections caused by *Blastoschizomyces capitatus* in the temperate zone of central Switzerland.

B. capitatus, a yeast formerly known as *Trichosporon* spp. and *Geotrichum capitatum*, can be isolated from the environment and may be a constituent of the microflora of the skin and the mucosa of the respiratory and digestive tracts (*6*). Colonization of human mucosa may precede invasion and hematogenous dissemination, resulting in invasive tissue infection of mainly the lung, liver, and skin (*7–9*). The dominant predisposing factor in >90% of reported cases is prolonged and profound neutropenia in patients with acute leukemia (*6,7,9*). Infections in neutropenic hosts share similarities with invasive candidiasis, but with *B. capitatus*, the rate of recovery from the bloodstream (≈80%), frequency of invasive tissue infections (≈60%), and rate of death (≈60%) are higher (*6–9*).

Climatic factors seem to play a selective role in the epidemiology of infections caused by *B. capitatus*. The geographic distribution of reported cases is restricted; most cases (87%) are observed in Europe, particularly in the Mediterranean region during the hottest period of the year. Italy, Spain, and France report 87% of the cases in Europe (*7,8*). Of the 99 *B. capitatus* infections reported worldwide through 2004, a total of 38 occurred in Italy (all but 1 in the central and southern regions), 30 in Spain, and 7 in France. Thus, approximately two thirds of all cases occurred in locations below 44° northern latitude, characterized by a Mediterranean climate with hot, dry summers and mild, wet winters. Central European countries, which have a temperate climate, contributed only 11 cases, and only 13 cases were reported from other continents (*7*). These findings are confirmed by recent data collected by the ARTEMIS DISK Surveillance Study, a longitudinal fungal surveillance program including *Candida* and non-*Candida* spp. yeasts isolated globally (*10,11*). To our knowledge, the only known case of invasive blastoschizomycosis in Switzerland was reported in 1983 (*12*).

The Patients

Five patients with *B. capitatus* infections were observed at 3 tertiary care hospitals in Switzerland during June 2009–June 2011 (Tables 1, 2). These hospitals provide services to a population of ≈3 million persons living in the temperate zone north of the Alps in an area ≈5,000 km^2. A structured chart review was conducted for each patient, and further information was obtained from the involved infectious diseases specialist. The yeast was identified by using standard culture methods (Sabouraud dextrose agar with chloramphenicol and gentamicin and corn meal agar Tween 80; both from Oxoid, Cambridge, UK) and ID 32 C (bioMérieux, Marcy l'Etoile, France) (*13*). Identification of isolates was confirmed by DNA sequence analysis of the intergenic spacer (ITS) region (primers ITS1 + ITS4, GenBank accession nos. HQ014711.1 and HQ014712.1). For antifungal drug susceptibility testing, a microtiter broth dilution method based on the Clinical and Laboratory Standards Institute M27-A2 standard (Sensititre YeastOne; TREK Diagnostic Systems, East Grimstead, UK) was performed. Disk diffusion testing of fluconazole and voriconazole was performed as described (*10*). A review

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Table 1. Clinical characteristics of 5 patients with *Blastoschizomyces capitatus* yeast infection, Switzerland, June 2009–June 2010*

Patient no.	1	2	3	4	5
Age, y/sex	66/M	68/F	58/M	60/M	56/F
Underlying disease	AML	AML	AML	CAPD for renal failure after renal transplant	Diabetes mellitus, renal/pancreas transplant
Immunosuppression	Induction chemotherapy; reinduction 2×	Induction chemotherapy	Induction chemotherapy	CAPD; CycA, PDN	Tacrolimus, mycophenolate, PDN, thymoglobuline
Galactomannan assay	Negative	Negative	BAL positive; serum not done	Not done	Negative
Day of chemotherapy cycle at first isolation	14	13	22	NA	NA
Neutrophil count at diagnosis, $\times 10^9$ l^{-1}	<0.1	<0.1	0.25	7.49	4.74
Days of neutropenia at diagnosis	16	8	18	NA	NA
Days of persistent fungemia/funguria	6	4	3	NA	114
Sites involved clinically	Lungs, skin, liver, spleen, brain	Lungs	Lungs, brain	Peritoneal fluid	Bladder, right kidney
Isolation of *B. capitatus*	Blood, skin	Blood	Blood, urine, DTA	Peritoneal fluid, peritoneal dialysis catheter, deep tissue biopsy	Urine
Sequential treatment after isolation (duration, d)	1. L-AMB (7) 2. L-AMB + VRC (8) 3. L-AMB (25) 4. VRC (145)	1. L-AMB (2) 2. VRC (3)	1. L-AMB (1) 2. L-AMB + VRC (1) 3. FLC (11)	1. VRC (28)	1. VRC (17) 2. FLC (8) 3. AMB (4) 4. VRC (110, ongoing)
Days from collection of first positive sample to start of adequate treatment	0	1	3	4	3
Outcome (cause of death)	Death 185 d after diagnosis (relapsing leukemia and MOF)	Death 9 d after diagnosis (MOF)	Death 15 d after diagnosis (MOF)	Alive	Alive
Contribution of *B. capitatus* to death	–	+	+	NA	NA
Travel history during previous year	Trip to southern France 3 months before diagnosis	None	Unknown	None	None

*Diagnosis is defined as the day of collection of first sample positive for *B. capitatus*. No patients received antifungal prophylaxis. AML, acute myeloid leukemia; CAPD, continuous ambulatory peritoneal dialysis; CycA, cyclosporine A; PDN, prednisone; BAL, bronchoalveolar lavage; NA, not applicable; DTA, deep tracheal aspiration; L-AMB, liposomal amphotericin B; VRC, voriconazole; FLC, fluconazole; AMB, deoxycholate amphotericin B; MOF, multiorgan failure.

of the microbiology laboratory records of the 5 Swiss university centers yielded no additional isolations of *B. capitatus* in the past 5 years.

Patient 1 was a 66-year-old man with secondary acute myeloid basophilic leukemia. He became febrile, and pulmonary nodules and pustular skin lesions developed after induction chemotherapy. Blood cultures and cultures from a skin lesion yielded *B. capitatus*. The patient was treated with amphotericin B and voriconazole, both of which had low MICs. Skin lesions improved within days, and pulmonary nodules resolved within weeks. Eventually, relapsing leukemia proved refractory, and the patient died of multiorgan failure.

Patient 2 was a 68-year-old woman with acute myeloid leukemia. Neutropenic fever developed after induction chemotherapy. Blood cultures yielded *B. capitatus*. She was treated with liposomal amphotericin B and voriconazole. However, she died of multiorgan failure 9 days after diagnosis.

Patient 3 was a 58-year-old man receiving induction chemotherapy for acute myeloid leukemia, who was treated for polybacterial neutropenic sepsis. Caspofungin was added because of persistent fever. *B. capitatus* was isolated from blood cultures, urine, and tracheobronchial fluid while he was being treated. Antifungal therapy was switched to liposomal amphotericin B and voriconazole. When a preliminary report indicated an amphotericin B MIC >2 µg/mL (Etest; bioMérieux) for the isolate, treatment was changed to fluconazole. Multiorgan failure occurred, and he died 15 days after diagnosis. Autopsy findings confirmed extensive fungal pulmonary infiltrates with angioinvasion and multiple foci of *B. capitatus*

Table 2. In vitro antifungal susceptibility profile according to microtiter broth dilution for patients with *Blastoschizomyces capitatus* yeast infections, Switzerland, June 2009–June 2010*

	MIC, μg/mL						
Patient no.	AMB	5-FC	FLC	ITC	VRC	POS	CAS
1	0.5	0.03	2	0.06	0.016	0.25	4
2	0.5	0.03	8	0.12	0.06	0.25	4
3	0.5 (3)†	0.06	4	0.06	0.06	0.25	8
4	0.5	16	1	0.032	0.032	0.064	4
5	0.5	0.12	16	0.25	0.25	0.5	>16

*AMB, deoxycholate amphotericin B; 5-FC, 5-fluorocytosine; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; CAS, caspofungin.
†By Etest (bioMérieux, Marcy l'Etoile, France), the isolate had an AMB MIC of 3 μg/mL.

infection in the liver, spleen, kidneys, bone marrow, myocardium, and brain.

Patient 4 was a 60-year-old man who was receiving continuous ambulatory peritoneal dialysis for renal failure 15 years after kidney transplantation for chronic glomerulonephritis; he sought care for acute peritonitis. Upon cultivation of yeasts in the dialysis fluid, caspofungin was started and changed to voriconazole when *B. capitatus* was identified. The patient's symptoms rapidly improved, and voriconazole was discontinued after 4 weeks.

Patient 5 was a 56-year-old woman who underwent combined pancreas–kidney transplantation because of diabetic nephropathy and pancreatic insufficiency in December 2010. Two months later, *B. capitatus* was cultivated in urine while she was being treated empirically with caspofungin for possible fungal infection. Emphysematous cystitis was detected by computed tomographic scan, and *B. capitatus* was cultured from urine collected from the right kidney. Despite treatment with voriconazole, *B. capitatus* was cultured from urine for several weeks; however, the patient recovered.

Conclusions

The repeated isolation of *B. capitatus* in a temperate climate zone was unexpected because of the well-documented restriction of *B. capitatus* to areas with a Mediterranean climate ≥450 km south in Italy, Spain, and France. Because of the high recovery rate of the organism from blood cultures of patients with disseminated infection and the ease of culturing *B. capitatus* on standard media, we believe it unlikely that a large number of cases could have been missed in the past.

Central Switzerland has witnessed a steady rise in temperatures since the mid-1980s. Mean annual temperatures for the past 25 years were all above the mean for 1961–1990 and now exceed those of 1980 by >1°C. The rise in average temperatures was more pronounced during spring and summer months. Since 1981, average spring and summer temperatures in central Switzerland have increased by 0.77°C and 0.48°C per decade, respectively (*14*).

The occurrence of 4 of the 5 infections reported here during the warm season indicates that the rising temperatures might have contributed to the expanded range of climatically restricted fungi to cooler areas and that the emergence of *B. capitatus* might be a consequence of the local effects of global warming (*5,15*). Alternatively, importation of the pathogen from areas to which it is endemic through increasing traffic of humans and goods might have led to establishment of a new endemic hotspot. Our observation should alert clinicians caring for severely immunocompromised patients in temperate areas to consider infections caused by unfamiliar fungal pathogens, notably *B. capitatus*.

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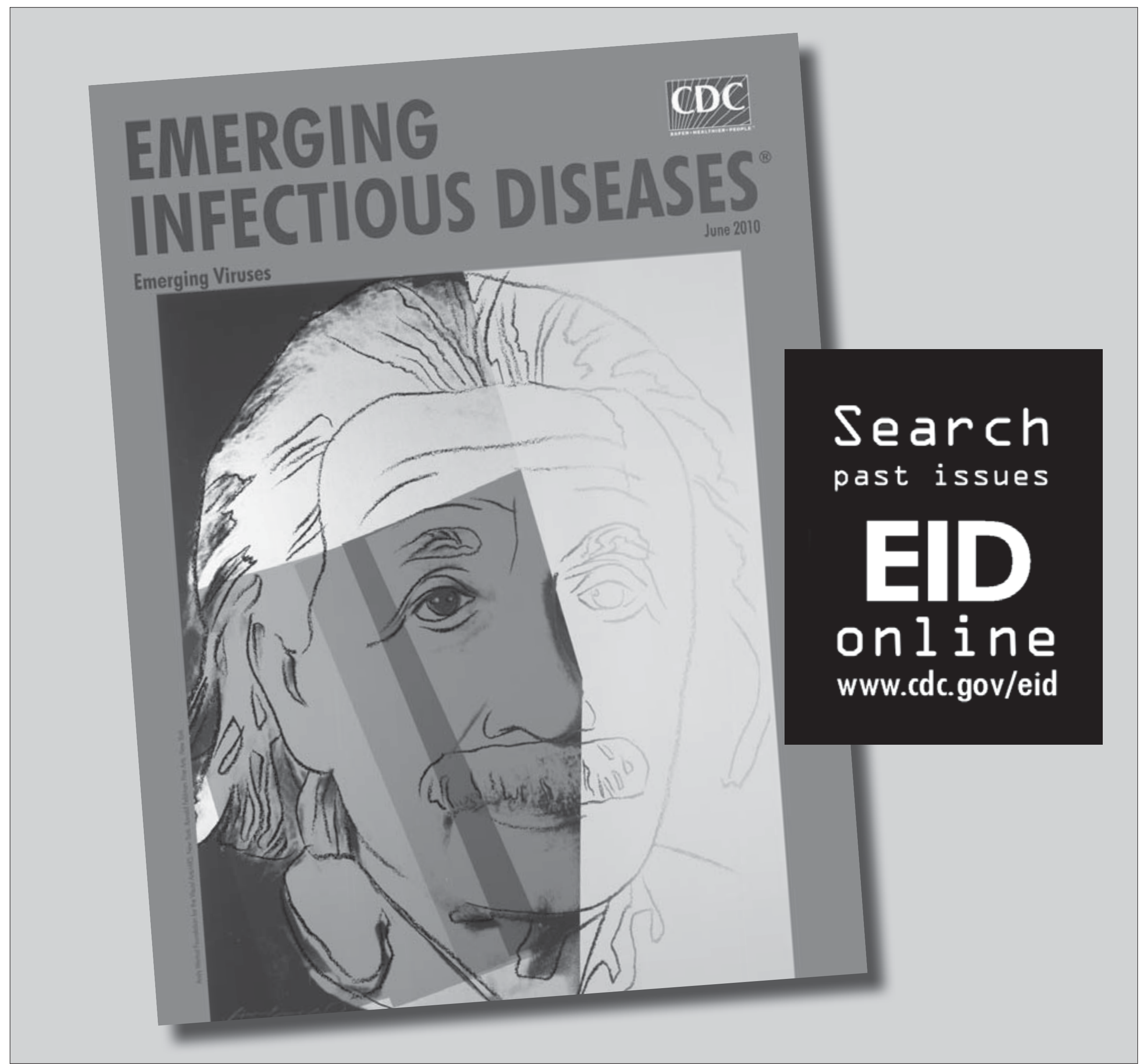

Asymmetric Type F Botulism with Cranial Nerve Demyelination

Alina Filozov,[1] Jessica A. Kattan,[1] Lavanya Jitendranath, C. Gregory Smith, Carolina Lúquez, Quyen N. Phan, and Ryan P. Fagan

We report a case of type F botulism in a patient with bilateral but asymmetric neurologic deficits. Cranial nerve demyelination was found during autopsy. Bilateral, asymmetric clinical signs, although rare, do not rule out botulism. Demyelination of cranial nerves might be underrecognized during autopsy of botulism patients.

Botulism is an illness caused by neurotoxin-producing *Clostridium* species (*1,2*). Botulinum neurotoxins are classified into 7 types, A–G. Toxin types A, B, and E cause most human botulism, and type F represents only 1% of reported cases (*1,2*). The source of intoxication with type F botulism among adults is often uncertain but might result from either intestinal colonization with clostridial spores and subsequent intraintestinal toxin production or from ingestion of preformed toxin in contaminated food (*1*). Antimicrobial drug use and functional or anatomic bowel abnormalities can facilitate colonization through alteration of normal gut flora (*1,3*).

The clinical signs of botulism typically consist of bilateral, symmetric cranial nerve palsies and descending, symmetric, flaccid paralysis (*2,3*). Illness from type F botulism is distinguished by a fulminant onset and short duration (*1,4*). We report atypical type F botulism associated with demyelination of cranial nerves.

The Patient

While traveling in Connecticut, a man from North Carolina in his mid-60s was admitted to a community hospital for new onset of diplopia, vertigo, truncal ataxia, and vomiting. Approximately 10 days before admission, the patient had been prescribed doxycycline for sinusitis. The physical examination at admission was notable for dilated, asymmetric (5 mm on the right and 4 mm on the left), sluggishly reactive pupils; cranial nerve IV palsy; bilateral proptosis (right more than left); bilateral peripheral facial weakness; and proximal left upper extremity weakness. Routine blood test results and imaging studies of head, brain, and orbits were unremarkable.

During hospital day 1, the patient experienced hypophonia, complete ophthalmoplegia, bilateral ptosis (right more than left), pupils unresponsive to light, dysphagia, and bilateral limb-girdle muscular weakness. His cognition remained intact, and no sensory deficits were documented. Cerebrospinal fluid contained 4 leukocytes/mm^3, 70 mg glucose/dL, and 43 mg protein/dL; bacterial cultures were negative. Serum was negative for IgG and IgM against *Borrelia burgdorferi*, and rapid plasma reagin test results were negative. Test results for antibody levels against *Campylobacter* spp. and gangliosides (anti-GQ1b IgG) were negative.

On hospital day 2, the patient had difficulty breathing and bulbar signs progressed, followed by descending extremity weakness (left more than right) and areflexia. A diagnosis of Miller Fisher syndrome (MFS), a variant of Guillain-Barré syndrome, was considered, and treatment with intravenous immunoglobulin was begun.

The Connecticut Department of Public Health and the Centers for Disease Control and Prevention (CDC) were contacted for a botulism consultation. Botulinum antitoxin was not administered at that time because asymmetric neurologic deficits and lack of exposure to injection-drug use or home-preserved foods made botulism unlikely. Respiratory paralysis progressed, and on hospital day 3 the patient required mechanical ventilation. A diagnosis of botulism was reconsidered; however, antitoxin was not administered because an alternative diagnosis (MFS) was still likely. Serum was collected on hospital day 5 and sent to CDC for botulism testing; a stool sample was collected on hospital day 14 after resolution of ileus. Neurologic improvement was first noted on hospital day 7, consisting of improved upper-extremity strength. On hospital day 14, weaning from mechanical ventilation was complete.

Botulinum toxin type F was confirmed in the serum sample on hospital day 16. Treating physicians and CDC agreed that administration of antitoxin might still be beneficial because of potential clostridial intestinal colonization. On hospital day 17, investigational heptavalent (A–G) botulinum antitoxin was administered (*5*). Stool sample was negative for botulinum toxin and botulinum toxin–producing *Clostridium* spp. On hospital day 28, the patient experienced self-limited serum sickness. He was discharged to a rehabilitation center later the same day.

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At the time of admission to the rehabilitation center, the patient was able to stand with assistance. At 6 weeks after symptom onset, he was able to keep his eyes open and walk with assistance, but dysphagia persisted. Subsequently, he continued to improve. Approximately 9 weeks after the illness had begun, the patient was found unresponsive; cardiopulmonary resuscitation was unsuccessful. Autopsy was limited to the brain and demonstrated inflammatory demyelination of cranial nerve tissue (Figure).

An epidemiologic investigation was conducted by state and local health departments in Connecticut and North Carolina. No contacts experiencing similar paralytic illness were identified. Two food items consumed by the patient were submitted for CDC analysis; both were negative for botulinum toxin and botulinum toxin–producing *Clostridium* spp.

Conclusions

This case illustrates the challenge of diagnosing a rare form of botulism in a patient with atypical clinical features. At initial examination, the patient had bilateral but asymmetric cranial nerve deficits and extremity weakness. Asymmetric clinical signs are unusual for botulism but have been documented previously with non–type F botulism (*6*). Additionally, truncal ataxia, an uncommon finding, was present. The time from symptom onset to intubation for this patient was 3 days, which is longer than previously recorded for type F patients (most are intubated within <24 hours) (*1*). Some clinical characteristics were typical for type F botulism, such as time to initial motor improvement and duration of ventilatory support (*1*). Also similar to reports of other type F cases, the mechanism of botulism intoxication in this patient was unclear (*1*). Intestinal colonization was suspected on the basis of recent antimicrobial drug use and absence of known risk factors for foodborne or wound botulism but was not thoroughly investigated because of the limited availability of stool samples.

A diagnosis of MFS was considered early in the clinical presentation but was eventually ruled out in favor of botulism; botulism sometimes is misdiagnosed as MFS (*7*). The triad of ophthalmoplegia, areflexia, and ataxia in this patient supported a diagnosis of MFS (*8*), although the former 2 findings also can be observed with botulism (*3*). Progression to descending paralysis was typical of botulism (*2,3*). The cerebrospinal fluid protein level and *Campylobacter* spp. and anti-GQ1b IgG ganglioside antibody test results did not support a diagnosis of MFS. Anti-GQ1b ganglioside antibodies are present among >90% of MFS patients (*8*). Given the descending pattern of paralysis, positive mouse bioassay for type F botulinum neurotoxin, and lack of supporting laboratory evidence for an MFS diagnosis, we believe that the patient's neurologic illness was caused by botulism alone.

The patient's cause of death is unclear. Death occurred after 2 months of sustained neurologic recovery; botulism relapse was not clinically apparent. Brain autopsy did not elucidate a cause of death; however, the cranial nerve demyelination is noteworthy. According to rare reports, neuropathologic features of botulism include normal histopathologic appearance of peripheral nerves and nonspecific, microscopic hemorrhage and vascular engorgement in the central nervous system (*9,10*). However, cranial nerve demyelination was reportedly found in 1 type A botulism patient who received type E antitoxin (*11*). The mechanisms that account for termination of botulinum toxin action and elimination of toxin from cranial nerves remain unidentified, and the possibility of toxin-induced demyelination cannot be excluded in the patient reported here. Alternatively, the abundant inflammatory cells in areas of demyelination might reflect the allergic response to investigational heptavalent botulinum antitoxin manifested by serum sickness reaction. Although we are unable to conclude which hypothesis is more likely, the fact that the patient's baseline neurologic function was within normal limits weighs against causes preceding his episode of botulism.

We conclude that a bilateral but asymmetric presentation of neurologic signs, although rare, does not rule out the possibility of botulism. In addition, demyelination of cranial nerves might be an underrecognized finding during autopsy of botulism patients, possibly resulting from either the effects of botulism or its treatment.

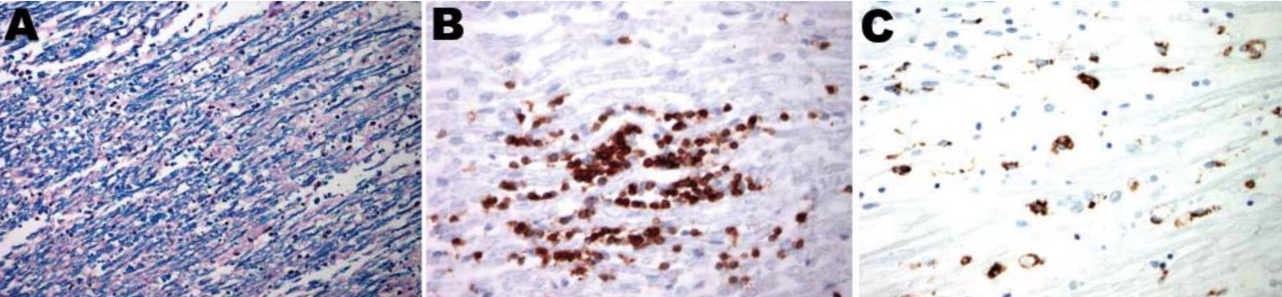

Figure. Postmortem cranial nerve tissue from a patient with botulism. A) Fragmentation of myelin sheaths and inflammatory infiltration of B and CD3$^+$ T-cells within the nerve tissue (original magnification ×200). B) B-cell infiltration of nerve tissue; C) CD68-positive myelinoklastic macrophages (original magnification ×400).

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MRSA USA300 at Alaska Native Medical Center, Anchorage, Alaska, USA, 2000–2006

Michael Z. David, Karen M. Rudolph, Thomas W. Hennessy, Diana L. Zychowski, Karthik Asthi, Susan Boyle-Vavra, and Robert S. Daum

To determine whether methicillin-resistant *Staphylococcus aureus* (MRSA) USA300 commonly caused infections among Alaska Natives, we examined clinical MRSA isolates from the Alaska Native Medical Center, Anchorage, during 2000–2006. Among Anchorage-region residents, USA300 was a minor constituent among MRSA isolates in 2000–2003 (11/68, 16%); by 2006, USA300 was the exclusive genotype identified (10/10).

Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, once concentrated among patients who had contact with the health care environment, have become epidemic among otherwise healthy populations in the United States. In the 48 contiguous states, community-associated MRSA skin and soft tissue infections (SSTIs) are predominantly caused by strain USA300 (*1*). In contrast, in 1996, 2000, and 2004–2006, in rural southwestern Alaska, we found that USA300 was rarely isolated, although community-associated MRSA SSTIs were common. Instead, sequence type (ST) 1, the type of USA400 isolates, was more common (*2*), as others have found in northern Canada (*3*). We wondered whether, over time, USA300 might replace USA400 among Alaska Natives as it has elsewhere in North America (*4*,*5*).

To investigate this possibility, we conducted surveillance at the Alaska Native Medical Center (ANMC). ANMC is the primary hospital for Alaska Natives residing in the Anchorage area and the statewide referral hospital for the Alaska Tribal Health System.

The Study

During 2000–2003, 695 clinical MRSA isolates were obtained by the Clinical Microbiology Laboratory of ANMC. A convenience sample of 567 isolates was collected and termed the retrospective collection. This collection was stratified by year of isolation and by 3 geographic regions of Alaska in which patients resided: 1) the Anchorage region (Anchorage, the Mat-Su region, and the Aleutian Islands); 2) the region of southwestern Alaska previously studied (*2*); and 3) all other regions. A randomly selected sample of 163 (28.7%) of the 567 isolates, stratified by year of isolation, was chosen for genotyping, including 20% of the isolates from Anchorage-region residents, 20% from residents of southwestern Alaska, and all isolates from residents of other regions (Table 1).

The prospective collection collected in 2004–2006 consisted of the first 5 clinical MRSA isolates obtained each month by the ANMC Clinical Microbiology Laboratory from different patients. Although 177 MRSA isolates had been collected, 2 were not available, and 2 lacked the *mecA* gene by PCR, leaving 173 isolates for further study. Genotyping was carried out on a random sample of 20% of isolates from this collection, stratified by year of isolation from the Anchorage-region patients, and on samples from all patients from all other regions (Table 1).

Clinical and demographic information was collected about the patients comprising the retrospective and prospective isolate groups. Site of care was recorded only for the prospective collection. Active surveillance for MRSA was not performed at ANMC during 2000–2006.

Isolates were genotyped by multilocus sequence typing (MLST), and clonal complexes (CCs) were assigned to closely related sequence types as described (*6*,*7*). Staphylococcal cassette chromosome *mec* (SCC*mec*) typing was performed (*8*), and the presence of Panton-Valentine leukocidin (PVL) genetic determinants was assessed as described (*9*). Additionally, to clarify the relationship between ST and typing by pulsed-field gel electrophoresis (PFGE), a random sample of strains that were ST8 and ST1 were tested by PFGE as described (*10*). Control strains were USA300-LAC for USA300 and strain 649, a clinical strain identical to MW2 by PFGE, for USA400. Antimicrobial drug susceptibilities were determined by using automated testing (bioMérieux Vitek, Durham, NC, USA). The D-zone test for inducible clindamycin resistance was performed for isolates resistant to erythromycin and susceptible to clindamycin by single-agent testing (*11*). Results were compared by χ^2 or Fisher exact tests using Stata version 11 (StataCorp LP, College Station, TX, USA).

The patients in the 20% Anchorage-region retrospective sample (n = 68), in the 20% retrospective sample from the region of southwestern Alaska (n = 33), and in the 20% Anchorage-region prospective sample (n = 29) did not differ significantly by demographic characteristics from the larger sampled groups (data not shown). Isolates in the combined

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Table 1. Overview of patient characteristics from retrospective (2000–2003) and prospective (2004–2006) collections from Alaska Native Medical Center, Anchorage, Alaska, USA, by location of patient residence*

	No. (%) samples					
	Anchorage region		Southwestern Alaska region		Other Alaska regions	
Characteristic	Retrospective collection, n = 68	Prospective collection, n = 29	Retrospective collection, n = 33	Prospective collection, n = 23	Retrospective collection, n = 62	Prospective collection, n = 9
Sex						
M	34 (50)	10 (34)	22 (67)	12 (52)	41 (66)	7 (78)
F	34 (50)	19 (66)	11 (33)	11 (48)	21 (34)	2 (22)
Age group, y						
0–2	1 (2)	3 (10)	4 (12)	1 (4)	4 (7)	0
3–12	7 (10)	2 (7)	4 (12)	6 (26)	4 (7)	0
13–20	9 (13)	2 (7)	3 (9)	1 (4)	3 (5)	1 (11)
21–39	24 (35)	10 (35)	11 (33)	7 (30)	7 (11)	0
40–59	24 (35)	11 (38)	5 (15)	4 (17)	24 (39)	4 (44)
≥60	3 (4)	1 (4)	6 (18)	4 (17)	20 (32)	4 (44)
Clinical specimen						
Blood	1 (2)	1 (4)	0	1 (4)	0	0
Bone or joint	1 (2)	0	0	1 (4)	1 (2)	1 (11)
Respiratory tract	3 (4)	1 (4)	6 (18)	2 (9)	22 (36)	2 (22)
Skin or soft tissue	56 (82)	25 (86)	26 (79)	15 (65)	33 (53)	3 (33)
Urine	1 (2)	1 (4)	0	0	1 (2)	0
Other†	6 (9)	1 (4)	1 (3)	4 (17)	4 (7)	3 (33)
Unknown	0	0	0	0	1 (2)	0
Site of care (prospective only)						
Inpatient	NA	2 (7)	NA	11 (48)	NA	5 (56)
Outpatient	NA	27 (93)	NA	12 (52)	NA	4 (44)
Emergency	NA	0	NA	0	NA	0

*NA, not available.
†Liver abscess, pleural fluid, tracheal culture after tracheostomy placement, surgical drain fluid culture, eye culture from eye with conjunctivitis, cultures from other eye specimens, culture from soft tissue of the neck, culture from ear with otitis media, and cultures from other ear specimens.

retrospective and prospective collections were distributed among 14 MLST types (Table 2). Nearly all CC1 (99%), CC8 (98%), and CC30 (100%) isolates were positive for Panton-Valentine leukocidin (PVL); all isolates in these 3 CCs carried SCC*mec* IV. No CC5 or CC45 isolates were PVL positive. Among CC5 isolates, 41/44 (93%) carried SCC*mec* II. All CC45 isolates carried SCC*mec* IV.

PFGE was performed on 30 ST8 and 14 ST1 isolates that were PVL positive and contained SCC*mec* IV. Of the ST8 isolates, 100% (30/30) were USA300, and of the ST1 isolates, 93% (13/14) were USA400.

When our sample was adjusted to account for the sampling strategy, we were able to estimate the genotypic spectrum of all isolates. In the retrospective collection, an estimated 12% (67/567) of MRSA isolates were ST8, and 42% (236/567) were ST1. Similarly, we estimated in the prospective collection that 61% (105/173) were ST8, and 25% (44/173) were ST1.

ST8 isolates were first identified among Anchorage-region patients in 2002 and accounted for 31% (5/16) of the genotyped isolates in that year; this increased to 100% (10/10) in 2006. When isolates from 2000–2003 were

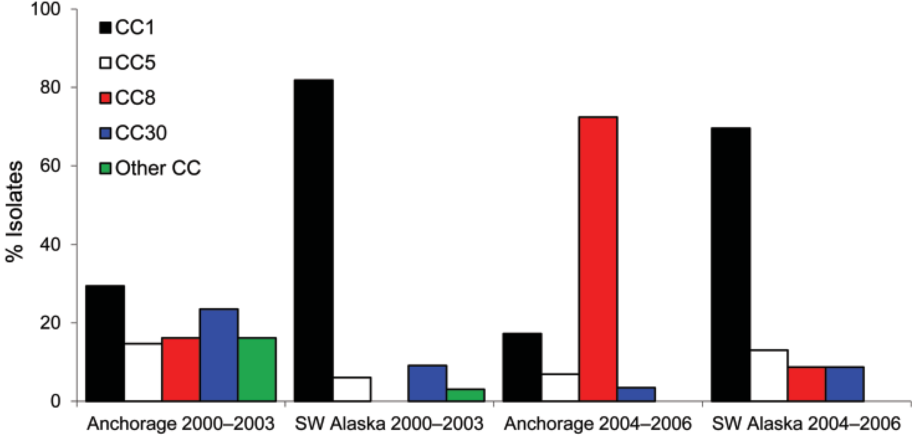

Figure. Percentage of clonal complex (CC) 1, CC5, CC8, CC30, and other CC methicillin-resistant *Staphylococcus aureus* isolates from residents of the Anchorage region and the region of southwestern Alaska, USA, 2000–2003 and 2004–2006.

Table 2. MRSA isolate characteristics, combined retrospective and prospective collection samples by region of patient residence, Alaska, USA, 2000–2006*†

CC and ST	No. (%) patients		
	Anchorage region, n = 97	Southwestern region, n = 56	Other regions, n = 71
CC1‡			
ST1	25 (26)	43 (77)	10 (14)
CC5			
ST5	11 (11)	2 (4)	16 (23)
ST105	1 (1)	2 (4)	9 (13)
ST225	0	0	1 (1)
ST231	0	1 (2)	1 (1)
CC8§			
ST8	32 (33)	2 (4)	9 (13)
CC30			
ST30	14 (14)	3 (5)	16 (23)
ST30slv	3 (3)	2 (4)	0
CC45			
ST45	1 (1)	0	0
ST45slv	1 (1)	0	0
ST54	1 (1)	0	0
CC59			
ST59	7 (7)	1 (2)	7 (10)
ST969	0	0	2 (3)
ST969slv	1 (1)	0	0
SCC*mec* type			
II	10 (10)	4 (7)	29 (41)
IV	88 (91)	52 (93)	39 (55)
c-2.5	1 (1)	0	3 (4)

*MRSA, methicillin-resistant *Staphyloccus aureus*; CC, clonal complex; ST, sequence type; slv, single locus variant; SCC*mec*, staphylococcal cassette chromosome *mec;* PFGE, pulsed-field gel electrophoresis.
†Among 61 isolates genotyped from the prospective cohort, 18 were from inpatients (ST1 [n = 9], ST105 [n = 1], ST231 [n = 1], ST5 [n = 3], and ST8 [n = 4]), and 43 were from outpatients (ST1 [n = 14], ST105 [n = 1], ST30 [n = 2], ST30slv [n = 1], ST5 [n = 3], and ST8 [n = 22]).
‡CC1 has recently been changed to CC15 by the administrators of the multilocus sequence typing system. A randomly selected sample of 14 ST1 isolates that carried the SCC*mec* IV element and were Panton-Valentine leukocidin positive underwent PFGE, and 93% (13/14) were USA400.
§42/43 ST8 strains identified in the study carried the SCC*mec* type IV element and were Panton-Valentine leukocidin positive. They are all considered to be USA300 because 30 of the 42 were randomly selected to undergo PFGE, and 100% (30/30) had the USA300 pulsotype.

compared with those from 2004–2006, the proportion of ST8 isolates obtained from Anchorage-region residents increased significantly (11/68, 16% vs. 21/29, 72%; $p<0.001$); among patients from southwestern Alaska ($p = 0.2$) and from other regions ($p = 0.8$), the percentage of ST8 isolates also increased, but the increase was not significant (Figure).

MRSA isolates from Anchorage-region patients were obtained more often from an SSTI (81/97, 84%) than those from other regions (77/127, 61%; $p<0.001$). In the prospective collection, isolates from the Anchorage region were more likely to be from outpatients (27/29, 93%) than were those from other regions (17/33, 52%) ($p<0.001$), and outpatients were more likely to have an SSTI (39/43, 91%) than were inpatients (5/18, 28%; $p<0.001$). In 2006, when all MRSA isolates obtained from Anchorage-region residents were ST8, 9/10 were from outpatients with SSTIs.

Among the Anchorage-region patients, SSTIs accounted for only 33% (4/12) of CC5 isolates, compared with 96% (24/25) of CC1, 91% (29/32) of CC8, 100% (17/17) of CC30, and 100% (3/3) of CC45 isolates. Also among these patients, CC8 (94%), CC30 (100%), and CC59 (100%) isolates were almost all susceptible to clindamycin. Of all CC1 isolates, 16/25 (64%) were resistant to clindamycin; 14/16 (88%) were not susceptible by virtue of a positive D-zone test result.

Conclusions

We documented the emergence and rapid dominance of USA300 among clinical MRSA isolates from Anchorage-region patients who received treatment at the ANMC in 2002–2006. This complete strain replacement by 2006 suggests that Anchorage-region patients were exposed to a growing reservoir of USA300 during this era. In contrast, in 2006, USA300 still remained a less common cause of MRSA infections among ANMC patients drawn from other regions of Alaska, perhaps because patients from other regions were less likely to have been referred to ANMC for uncomplicated SSTIs.

Of the tested PVL-positive ST8 MRSA isolates bearing SCC*mec* IV, we confirmed that 100% (30/30) were USA300 by PFGE. This finding has useful implications for comparing MLST and PFGE typing methods.

ST1 isolates frequently had inducible clindamycin resistance, whereas strains with PVL and SCC*mec* IV of other genetic backgrounds rarely did. This may explain the seemingly anomalous data from the USA400 era in Chicago that 31/33 (94%) of clindamycin-susceptible, erythromycin-resistant strains of MRSA were D-zone test positive (*12*).

Documented introduction of USA300 has not resulted in strain replacement in Europe (*13*), Asia (*14*), or Australia (*15*). That USA300 emerged and came to predominate among Anchorage-region residents, mirroring the process that occurred earlier in several cities in North America (*4*,*5*), suggests that some characteristic of USA300 provides a survival advantage, enhanced virulence, or both, relative to other MRSA pulsotypes in those regions. The rapid appearance and emergence of USA300 at ANMC in 2002–2006 were remarkable, but its limited global spread remains unexplained.

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Mutations I117V and I117M and Oseltamivir Sensitivity of Pandemic (H1N1) 2009 Viruses

Aeron C. Hurt, Sook Kwan Leang, David J. Speers, Ian G. Barr, and Sebastian Maurer-Stroh

Analysis of mutations I117V and I117M in the neuraminidase of influenza A pandemic (H1N1) 2009 viruses showed that I117V confers a mild reduction in oseltamivir sensitivity and has a synergistic effect of further increasing resistance when combined with H275Y. Contrary to recent reports, the I117M mutation does not alter oseltamivir sensitivity.

The neuraminidase inhibitors (NAIs) oseltamivir and zanamivir have been widely used in many countries to treat infection with influenza A pandemic (H1N1) 2009 virus. Since the start of the pandemic, >27,000 strains have been tested for oseltamivir resistance, of which 383 (1.4%) have contained the H275Y neuraminidase (NA) mutation, the same mutation that was found in oseltamivir-resistant prepandemic seasonal subtype H1N1 viruses that emerged in 2007–08. Apart from the H275Y mutation, a small number of other NAI resistance mutations have been detected in the NA of pandemic (H1N1) 2009 viruses, such as S247N (*1*), I223V (*2*), and I223R (*3*). Another NA mutation, I117M, has also recently been associated with oseltamivir resistance in pandemic (H1N1) 2009 viruses (*4*,*5*). The I117M NA mutation was detected in a virus from South Korea that was isolated from a patient before oseltamivir treatment and then was detected, with the H275Y mutation, in the same patient after oseltamivir treatment, although the dual mutations were not conclusively shown in the same virus (*4*,*5*). In both studies, neither the I117M variant nor the dual I117M + H275Y strain were specifically tested for oseltamivir resistance. Instead, the viruses were assumed to be resistant on the basis of previous studies that described a reduction in oseltamivir sensitivity in influenza A (H5N1) viruses because of an I117V amino acid substitution (*6*,*7*), rather than the I117M substitution detected in their studies.

Other studies have documented that different amino acid substitutions at key NA residues can cause considerably different effects on NAI susceptibility and that these mutations can have a variable effect in different NA backgrounds (*8*). Therefore, we tested the assumption that the I117M mutation confers oseltamivir resistance and investigate the role of the I117M and the I117V mutations with and without the H275Y mutation in pandemic (H1N1) 2009 viruses.

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The Study

By using site-directed mutagenesis and reverse genetics as described (*9*), we generated recombinant viruses with NA from the pandemic (H1N1) 2009 virus A/Auckland/1/2009 and the remaining genes from A/PR/8/34. Recombinant viruses were constructed with no NA mutations, with single I117V or I117M NA mutations, and with dual I117V + H275Y or I117M + H275Y NA mutations.

NAI sensitivity analysis with a fluorescence-based NA inhibition assay (*10*) found that compared with a recombinant with no mutations, the I117V mutation conferred a 5-fold increase in the oseltamivir concentration required to inhibit 50% (IC_{50}) of the NA activity, a 2-fold increase in zanamivir IC_{50}, and no change in peramivir IC_{50}. In comparison, the I117M mutation had no effect on sensitivity to any of the NAI drugs (Table 1).

The dual I117V + H275Y variant had oseltamivir and peramivir IC_{50} values that were 3× and 2× higher, respectively, than the IC_{50} of a virus with the H275Y mutation alone. In contrast, the IC_{50} of the I117M + H275Y variant was not substantially different from that of the H275Y mutant for all of the NAIs, further demonstrating the lack of effect of the I117M mutation on NAI sensitivity.

Analysis of 3,334 pandemic (H1N1) 2009 strains received at the World Health Organization (WHO) Collaborating Centre, Melbourne, Victoria, Australia, through the WHO Global Influenza Surveillance and Response System from April 2009 through June 2011, showed that 1 isolate had a I117V NA mutation, but no I117M variants were detected. The I117V variant, A/Perth/504/2010 (GenBank accession nos. HA:EPI279165 and NA:279164; www.gisaid.com), was isolated from a 5-year-old boy and had a 4-fold and 3-fold reduction in sensitivity to oseltamivir and zanamivir, respectively, similar to that of the RG-I117V strain (Table 1). Neither the

Table 1. NAI sensitivity of naturally occurringpandemic (H1N1) 2009 virus I117V mutant and I117V, I117M, I117V + H275Y, I117M + H275Y, and H275Y reverse genetics variants*

Pandemic (H1N1) 2009 viruses	Mutation	Zanamivir		Oseltamivir carboxylate		Peramivir	
		Mean IC_{50} ± SD, nmol/L	-fold difference†	Mean IC_{50} ± SD, nmol/L	-fold difference†	Mean IC_{50} ± SD, nmol/L	-fold difference†
Mean of NAI-sensitive viruses‡	–	0.28 ± 0.15	–	0.45 ± 0.35	–	0.20 ± 0.10§	–
A/Perth/504/2010	I117V	0.96 ± 0.28	3	1.63 ± 0.70	4	0.09 ± 0.01	1
RG-WT	–	0.24 ± 0.05	–	0.30 ± 0.20	–	0.09 ± 0.01	–
RG-I117V	I117V	0.54 ± 0.12	2	1.42 ± 0.52	5	0.08 ± 0.02	1
RG-I117M	I117M	0.32 ± 0.05	1	0.31 ± 0.06	1	0.11 ± 0.01	1
RG-I117V + H275Y	I117V + H275Y	0.44 ± 0.05	2	568.84 ± 54.89	1,896	47.08 ± 32.57	523
RG-I117M + H275Y	I117M + H275Y	0.29 ± 0.04	1	163.72 ± 17.76	546	16.12 ± 0.84	179
RG-H275Y	H275Y	0.26 ± 0.03	1	195.02 ± 21.05	650	19.72 ± 1.42	219

*NAI, neuraminidase inhibitor; IC_{50}, 50% inhibitory concentration; –, not applicable.
†-fold differences compared with IC_{50} value of RG-WT, except for A/Perth/504/2010, which was calculated for on the basis of comparison to the mean IC_{50} of circulating pandemic (H1N1) 2009 viruses.
‡n = 3,334, obtained through World Health Organization Global Influenza Surveillance and Response System, April 2009–June 2011.
§Mean and SD of peramivir IC_{50} values were based on analysis of 273 isolates. RG strains were derived by using site-directed mutagenesis and reverse genetics. Mean IC_{50} ± SD values of the A/Perth/504/2010 virus and the RG strains were calculated on the basis of values derived from 3 independent assays.

patient nor his family members or siblings were undergoing any NAI treatment.

Apart from the A/Perth/504/2010 strain, no other pandemic (H1N1) 2009 strains with an I117V NA mutation were reported on GenBank or public sequence databases, demonstrating the high degree of conservation at this residue. However, 45 NA sequences from highly pathogenic influenza A (H5N1) strains in the public sequence databases contained the I117V mutation. The I117V mutation in highly pathogenic influenza A (H5N1) viruses has previously been reported to confer a 5- to 16-fold reduction in oseltamivir sensitivity and up to a 4-fold reduction in zanamivir sensitivity (*6,7*).

Residue I117 is not in direct structural contact with oseltamivir, although it has neighboring residues that are known to affect drug susceptibility such as E119, R118, and V116. By using the predictive computational force field FoldX (*11*) in YASARA (*12*), we modeled the effects on structural stability of I117V, I117M, H275Y, I117V + H275Y, and I117M + H275Y mutations in the pandemic (H1N1) 2009 NA crystal structure (Protein Data Bank no. 3NSS; www.pdb.org) (*13*). The model estimated local destabilization effects for the known oseltamivir-resistance mutation H275Y, which served as a control for the approach, whereas substantially smaller effects for I117V were observed, and almost no stability change was predicted for the I117M mutation (Table 2). The estimated local destabilization effects for the dual mutations H275Y + I117M and H275Y + I117V were not substantially different from that predicted for the H275Y mutation alone. When the NA inhibition assay IC_{50} data (Table 1) were compared with the estimated local destabilization effects of the mutants (Table 2), a good correlation was demonstrated between the 2 methods, although functional testing showed a larger difference between the H275Y and the H275Y + I117V variants than that estimated in the computational model.

The destabilization effect of I117V appears to be mainly caused by the increase in an internal cavity (Figure 1), which could increase flexibility of neighboring residues that form part of the drug-binding framework. The H275Y and I117V mutations are at opposite sides of the binding pocket (Figure 2) and, although they are not expected to affect each other's side-chain environment directly, the simultaneous changes on both sides of the drug show more effects on oseltamivir binding than the single mutations alone.

Conclusions

Although the I117V mutation was detected in 1 isolate from Australia, analysis of sequences from public databases shows that it is extremely rare in pandemic (H1N1) 2009 viruses to date. Although the I117V mutation causes a mild reduction in oseltamivir sensitivity, on the basis of pharmacokinetic data, we expect that a variant carrying this mutation would not be clinically resistant

Table 2. Predicted local structure destabilization for the different NA mutations from pandemic (H1N1) 2009 viruses*

NA mutation	Mean ± SD level of destabilization, kcal/mol†
H275Y	4.7 ± 0.4
H275Y + I117V	5.2 ± 0.4
H275Y + I117M	5.0 ± 0.3
I117V	0.5 ± 0.02
I117M	0.1 ± 0.1

*NA, neuraminidase.
†Calculated with FoldX (*11*) on the basis of 5 repetitions.

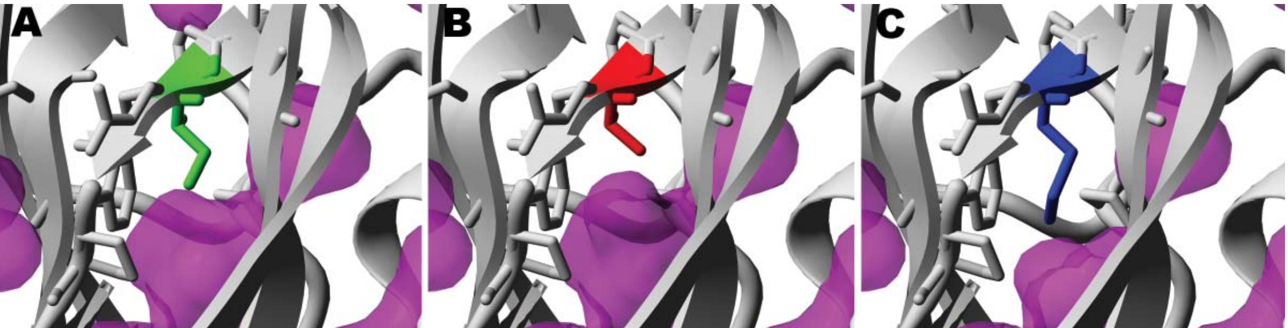

Figure 1. Structural details of neuraminidase mutations from pandemic (H1N1) 2009 viruses. A) Wildtype mutation I117 (green). B) mutation I117V (red). C) I117M (blue). All were modeled with FoldX (*11*) in YASARA (*12*) in the context of the pandemic (H1N1) 2009 virus neuraminidase crystal structure (Protein Data Bank: 3nss). Side chains of residues ≤3 Å of residue 117 are shown as sticks. Cavities within the structure (1.4 Å radius water probe) are shown in magenta.

(*14*,*15*). However, in combination with H275Y, the I117V mutation has a synergistic effect on oseltamivir resistance, raising the oseltamivir IC_{50} to 3× that caused by the H275Y mutation alone and to a level that is likely to be clinically important. Previous studies have reported that the I117M mutation may confer oseltamivir resistance (*4*,*5*), although in this study we have demonstrated that this is not the case. These results therefore highlight the importance of assaying functional drug resistance when reporting novel mutations because resistance cannot be assumed on the basis of data from other amino acid substitutions at the same residue.

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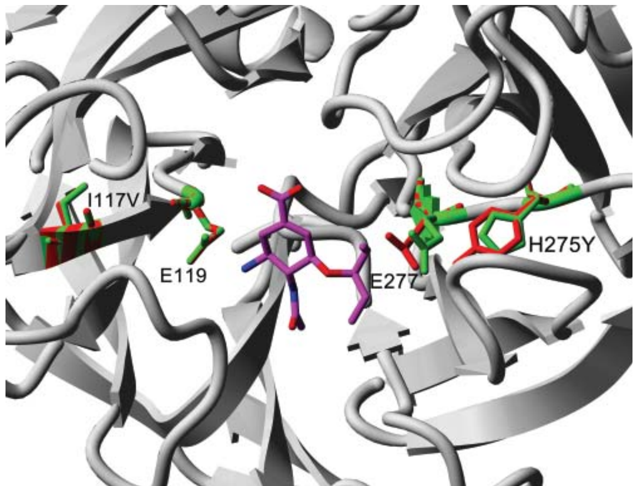

Figure 2. Comparison of wildtype I117 mutation from pandemic (H1N1) 2009 viruses (green residues; Protein Data Bank: 3nss) with FoldX/YASARA model of I117V + H275Y double mutant (red residues) (*11,12*). Residue numbering is according to pandemic (H1N1) 2009 neuraminadase. Oseltamivir is added as reference (Protein Data Bank: 3clO) and shown in magenta.

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Multistate Outbreak of MDR TB Identified by Genotype Cluster Investigation

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In 2008, diagnosis and investigation of 2 multidrug-resistant tuberculosis cases with matching genotypes led to identification of an outbreak among foreign-born persons who performed short-term seafood production work in Alaska during 2006. Tuberculosis control programs should consider the possibility of domestic transmission even among foreign-born patients.

In the United States, 60% of tuberculosis (TB) cases occur among foreign-born persons (*1*). Infection is often assumed to be acquired before immigration. However, many foreign-born persons have risk factors for acquiring TB domestically, such as living and working in crowded conditions with persons at higher risk for having TB (*2*). With the nationwide implementation of universal TB genotyping through the National TB Genotyping Service (NTGS) (*3*), previously unknown outbreaks can be identified. We describe an outbreak of multidrug-resistant (MDR) TB among foreign-born migrant workers that was identified by genotype cluster investigation.

The Study

During 2009, the California Department of Public Health became aware of MDR TB cases with a matching genotype and drug-resistance pattern (resistant to isoniazid, rifampin, ethionamide, and streptomycin) in 2 foreign-born patients (designated CA1 and CA4). The cases were diagnosed during 2008 in adjoining California counties; 1 patient was born in Asia, the other in Latin America. Review of the patients' activities and lists of contacts did not expose commonalities. For 2004–2009, the NTGS database contained 1 other case in the United States with a matching genotype. This case, in an Africa-born patient (WA1), was diagnosed in Washington, USA, in 2008. Sputum smear results were positive, and the drug-resistance pattern matched that of the other 2 cases-patients. Further investigation, including repeat interviews, showed that all 3 case-patients had a history of short-term seafood production work in Alaska.

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The California Department of Public Health notified the Alaska Division of Public Health (ADPH) about this suspected MDR TB transmission in Alaska. ADPH reviewed case records and identified an Africa-born patient (AK1) with MDR TB and positive sputum smear results who had been employed in seafood production at the time of his 2006 diagnosis. During contact investigation for the case, ADPH evaluated 3 roommates with previously positive tuberculin skin test results. ADPH did not expand the contact investigation because many workplace contacts were no longer employed at the facility and were unreachable. No persons identified in the initial contact investigation were subsequently identified as outbreak case-patients. A 2010 review of employer records confirmed that all 4 case-patients had been employed in the same facility during AK1's infectious period (Figure). Because contact information for other workers was unknown, no further investigation could be pursued.

Initial genotyping results (spoligotyping and 12-locus mycobacterial interspersed repetitive units–variable number of tandem repeats [MIRU-VNTR] analysis) demonstrated that AK1's isolate had a genotype that differed from those in California and Washington at 1 MIRU-VNTR locus (*4*). To confirm these results and further evaluate the relatedness of the isolates, 24-locus MIRU-VNTR (*5*) and IS*6110*-based restriction fragment length polymorphism analyses (*6*) were conducted and showed exact matches among the 4 cases by spoligotype, 24-locus MIRU-VNTR, and restriction fragment length polymorphism (Table 1). The single-locus difference in initial and subsequent genotype results of AK1 was determined to be a laboratory error.

The 4 case-patients worked in a seafood production facility in Alaska during the summer of 2006. The facility included multiple large buildings with high ceilings and open areas with production lines. Patients AK1, CA1, and CA4 completed follow-up interviews about their activities in Alaska. All 3 reported working in the same building; 2 also worked in a second building. They lived in 3 different apartments that were in 2 different buildings and ate and socialized primarily in each dormitory's cafeteria. Case-patients reported working up to 12 hours per day, 7 days per week; they did not report any common activities outside of work. No other links were identified among the case-patients.

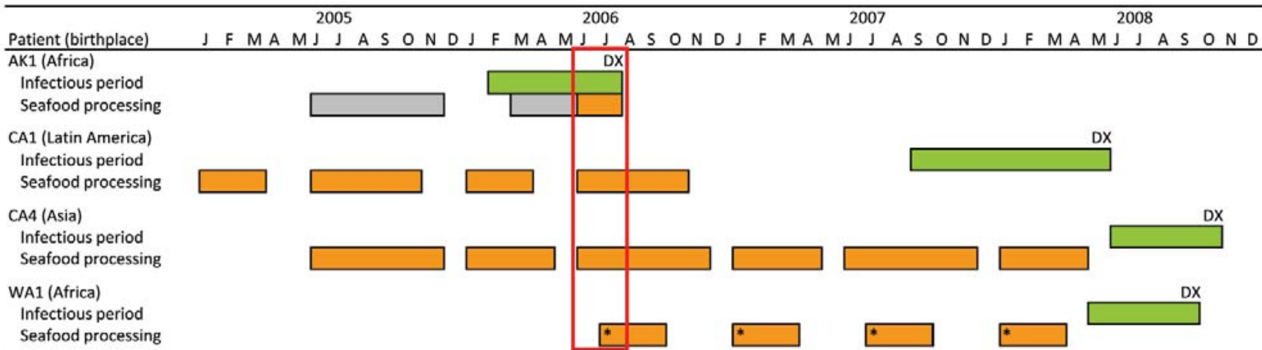

Figure. Infectious periods and work schedules for 4 multidrug-resistant tuberculosis–infected seafood production workers (AK1, CA1, CA4, and WA1) with matching mycobacterial genotypes, Alaska, USA, 2005–2008. Green, infectious period; orange, work at processing facility A; gray, work at another processing facility; red box, likely period of transmission. DX, date of diagnosis. *Approximate time periods.

Contact investigation of the secondary cases (CA1, CA4, and WA1) was conducted, and 47 (96%) of 49 contacts were fully evaluated. Of these 47 contacts, 2 had active TB disease (CA2 and CA3, US-born children with negative culture results), and 30 (64%) had latent TB infection (LTBI); 28 began treatment for MDR LTBI (Table 2). Review of these contact investigations determined that reopening and expanding the investigations would not be productive.

Table 1. Genotyping of MDR TB spoligotype 477777777720771 among case-patients linked to seafood production work, Alaska, USA, 2005–2008*

Locus	No. repeats
Miru02	2
Miru04	2
Miru10	7
Miru16	3
Miru20	2
Miru23	5
Miru24	1
Miru26	5
Miru27	3
Miru31	3
Miru39	2
Miru40	3
424	2
577	3
1955	3
2163b	5
2165	3
2347	4
2401	4
2461	2
3171	3
3690	3
4156	3
4052	5

*Determined by using 24-locus mycobacterial interspersed repetitive units–variable number of tandem repeats. MDR TB, multidrug-resistant tuberculosis.

To facilitate prompt investigation, the Centers for Disease Control and Prevention is now actively monitoring the NTGS database for new cases matching the outbreak genotype. NTGS surveillance identified 1 additional case in Washington State during 2010. This case-patient had never worked in seafood production and had not been identified during the contact investigation of WA1. However, his cousin was WA1's roommate, and he later moved into WA1's apartment after WA1 moved out, so unrecognized contact could have occurred.

Genotype cluster investigation showed previously unrecognized domestic transmission of MDR TB among foreign-born migrant workers. Investigation identified 7 MDR TB cases: the probable source case in Alaska, 3 secondary cases among co-workers in whom MDR TB subsequently was diagnosed elsewhere, and 3 tertiary cases among contacts of the secondary cases. Transmission probably occurred while the case-patients were working.

Conclusions

The initial contact investigation of the presumed source case was limited by the remote location and the short-term nature of the employment. In addition, a high rate of previously positive tuberculin skin test results among close contacts made the degree of transmission among that group impossible to assess. In similar circumstances, expanding contact investigations of patients with positive sputum smear results beyond the initial group of contacts might be productive. The use of interferon-γ release assays to test samples from contacts who have received *Mycobacterium bovis* BCG might help assess the degree of transmission (*7*).

This outbreak underscores the importance of considering TB transmission in nonresidential settings. Current TB-control guidelines emphasize the need to identify and assess the risk for transmission at all possible sites, including workplaces (*8*). Local health departments

Table 2. Results of initial contact investigations of MDR TB cases linked to seafood production work, Alaska, USA, 2006–2008*

Contacts	No. contacts, by case-patient				Total no. contacts
	AK1†	CA1‡	CA4§	WA1¶	
Total no. contacts identified	3	33	10	3#	49
Total no. contacts evaluated	3	33	10	1	47
Active TB	0	2**	0	0	2
LTBI	3	18	8	1	30
TST positive††	3	9	8	1	21
TST conversion‡‡	0	9	0	0	9
Started on LTBI treatment§§	0	17	10	1	28
TST negative	0	13	2	0	15

*MDR TB, multidrug-resistant tuberculosis; ID, identification; AFB, acid-fast bacilli; LTBI, latent TB infection; TST, tuberculin skin test.
†Source patient. Patient had positive sputum AFB smear results, and initial chest radiograph did not show cavitary disease.
‡Patient had positive sputum AFB smear results, and initial chest radiograph showed cavitary disease.
§Patient had negative sputum AFB smear results, and initial chest radiograph did not show cavitary disease.
¶Patient had positive sputum AFB smear results, and initial chest radiograph showed cavitary disease.
#An active TB case was later identified; the patient had a matching genotype and an epidemiologic link to WA1, but this tertiary case was not identified during the secondary case-patient's contact investigation.
**Contacts are children (CA2 and CA3) with negative culture results.
††Includes persons for whom time of conversion was not known (i.e., prior positive or newly positive TST).
‡‡Defined as a positive TST following a negative TST performed in preceding 2 y.
§§Includes 2 children <5 y of age with initial negative TSTs started on "window" LTBI treatment while awaiting follow-up TST.

should weigh the probable yield of expanded and worksite investigations relative to other TB-control activities. If worksite investigations are pursued, clarifying employers' responsibilities for funding and supporting those investigations might help mobilization of the substantial resources typically required.

Industries that employ large numbers of foreign-born workers from countries with a high TB incidence might encounter TB among their employees. In seafood production facilities, where those workers live and work together, transmission risk is likely increased. Interventions to identify TB cases more quickly include employee and employer education regarding TB symptoms and institution of a cough alert program to ensure access to clinical evaluation of a persistent cough (*9,10*). TB control programs should consider the possibility of domestic TB transmission even among foreign-born patients, particularly if the patients have lived or worked in crowded conditions with other persons at higher risk of having TB.

Because initial genotype results indicated an exact match among only 3 of the 4 cases, an additional conclusion of this outbreak is that epidemiologic links are possible among case-patients with closely related genotypes. However, resources to explore epidemiologic links among patients with nonmatching genotypes should be used judiciously. Discussion with the genotyping laboratory and retesting are important first steps when epidemiologic links are suspected among patients with closely related genotypes.

Although pre-employment TB screening and LTBI treatment is a strategy for preventing progression to TB among foreign-born persons, and the standard LTBI drug regimens used probably would have prevented an outbreak of drug-susceptible TB, that strategy would not have averted this MDR TB outbreak. A 1992 Centers for Disease Control and Prevention guideline on preventing and controlling TB among migrant farm workers prioritized screening asymptomatic workers for TB as an activity lower than diagnosing, treating, and performing contact investigation for cases of active TB (*2*). The costs and benefits of screening in this analogous population should be investigated.

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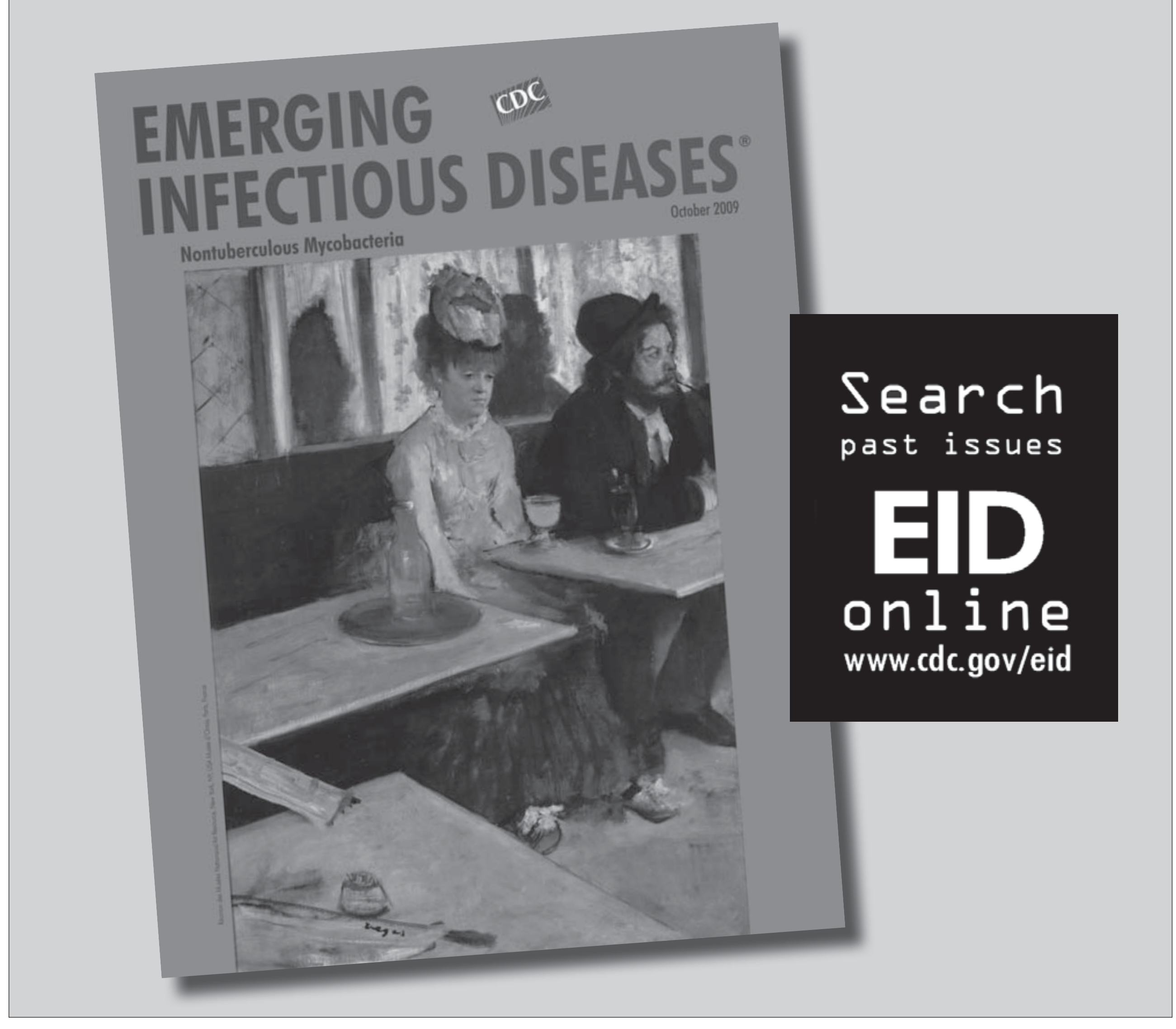

Spoligotyping of *Mycobacterium africanum*, Burkina Faso

Michel K. Gomgnimbou, Guislaine Refrégier, Serge P. Diagbouga, Sanou Adama, Antoinette Kaboré, Adama Ouiminga, and Christophe Sola

Using Ziehl-Neelsen–positive slides collected from tuberculosis diagnostic centers in Burkina Faso, we showed that 20% of 80 spoligotyping-positive DNA samples had a characteristic *Mycobacterium africanum*–specific genomic signature. This result suggests that *M. africanum* is still present in Burkina Faso at almost the same prevalence as 15–20 years ago.

Mycobacterium africanum remains a major pathogen in Africa (*1*). Recently, de Jong et al. estimated the prevalence of *M. africanum* to be 1.7% in Burkina Faso (*1*); their estimate was based on a 2007 study by Godreuil et al., who unexpectedly did not identify any *M. africanum* isolate within a collection of 120 *M. tuberculosis* complex clinical isolates in 2001 from 79 tuberculosis patients living in Ouagadougou and 41 living in Bobo Dioulasso, the 2 largest cities in the country (*2*). However, 2 patterns (isolates 94 and 90) can be recognized as *M. africanum* by their characteristic spoligotyping signature (deletion of spacers, 8, 9, and 39) and an association of mycobacterial interspersed repetitive unit (MIRU) 24 ≥ 2, MIRU31 ≥ 4, and MIRU40 < 3 signature (*2*,*3*).

In the neighboring country of Ghana (which has 200 km of common borders with Burkina Faso), another study suggested that the population structure of *M. tuberculosis* complex comprises 1) 34% spoligo-international type (SIT) 61 (named the Cameroon clade, also present in Burkina Faso); 2) 30% *M. africanum* (including *M. africanum* West African 1 and West African 2); and 3) 36% principal genetic group 2 and 3 modern strains (e.g., T, U [unknown], Haarlem, X, LAM [Latino-American and Mediterranean]), with minor prevalence of other principal genetic groups, i.e., the East-African Indian, Beijing, and *M. bovis* clades (*4*,*5*). These observations—and their congruence to estimates by Ledru et al. in 1996 of an 18.4% prevalence of *M. africanum* strains isolated from the 300 patients in whom tuberculosis was newly diagnosed in Burkina Faso during 1992–1994 (*5*)—prompted us to reexamine the conclusions of Godreuil et al. on the *M. africanum* prevalence in Burkina Faso.

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The Study

The study, which we conducted during March–September 2010, had 3 goals. First, we wanted to determine whether we could extract DNA and perform high-throughput spoligotyping on a Luminex 200 device (Luminex, Austin, TX, USA) on acid-fast bacillus–positive slides (*6*). Second, we wanted to reestimate the prevalence of *M. africanum* in Burkina Faso from a recent and random sample of slides. Third, we wanted to further analyze the relative proportion of *M. africanum* West African I and West African 2 strains in Burkina Faso because this country is part of central western Africa, where the 2 *M. africanum* West African 1 and 2 strains are present at various relative rates (*2*). We report on all the goals of this project, even though goal 3 remains to be confirmed because of the small sample size.

From within 14 geographically independent centers in Burkina Faso (Figure), we recruited a random sample of 186 Ziehl-Neelsen (ZN) slides that had been included in a national study on drug resistance, as approved by the ethical committee for health research in Burkina Faso (2007–031; June 28, 2009). Of 186 DNA samples extracted from as many ZN slides, 143 sputum samples had been scored 3+, 18 were scored 2+, 10 were scored 1+, 5 had 1–9 bacilli total (±), totaling 176 positive slides from as many sputum samples. In addition, test results were negative for 9 and unknown for 1.

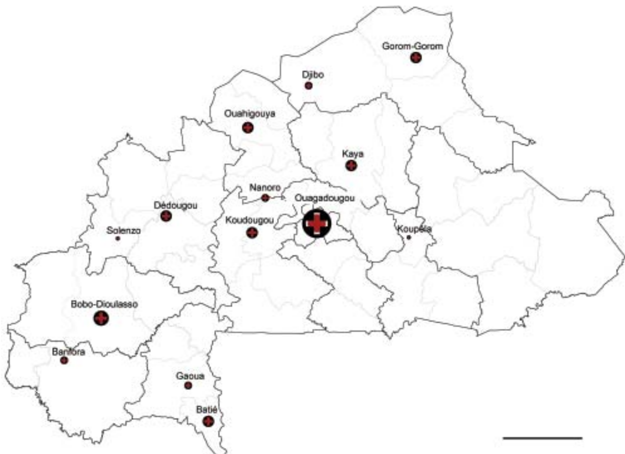

Figure. Origin of samples described in study of *Mycobacterium africanum* in Burkina Faso. Dark outlined borders indicate province; light outlined borders indicate regions. Scale bar = 100 km. Sources: Institut Géographique du Burkina Faso/Centre Muraz-PNT.

In a preliminary trial of 9 independent 3+ positive slides, DNA extraction was attempted by 2 methods: an enzymatic method (*7*) and a classical thermic lysis in a Chelex suspension (InstaGene; Bio-Rad, Hercules, CA, USA) (*8*). In our study, only the Chelex method produced good results, i.e., enabled us to obtain DNA that was successfully PCR amplified and produced a full spoligotyping pattern (results not shown). The quantity of DNA extracted was superior for all tests by the enzymatic lysis (n = 3) as by the Chelex (n = 6), as estimated by spectrophotometry (NanoDrop ND-1000; LabTech, Ringmer, UK). Thus, DNA can be successfully extracted by the enzymatic method for many human or bacterial cells but not for *M. tuberculosis* complex because no spoligotype could be obtained. We therefore analyzed the 176 experimental slides by using the Chelex extraction procedure.

The origins of all ZN slides assessed in this study are shown in the Figure, and genotyping results are shown in the Table (full results in the online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-0275-Techapp.pdf). As observed in a much larger set of samples from Ghana, the Cameroon family (SIT61) also prevails in Burkina Faso (18 [25%] isolates) (*4*). Whether the Cameroon strains from Burkina Faso are similar or identical to those from Ghana remains to be studied.

Our result confirms the observation by Godreuil et al. in 2007 on the prevalence of the SIT61/Cameroon strains in Burkina Faso (*2*). However, we detected 16 *M. africanum* spoligotypes (West African 1 and West African 2), i.e., a minimal *M. africanum* prevalence of 20%, close to 18.4% found by Ledru et al. in 1996 (*5*). Third, the T and Haarlem strains represented 16 (22%) and 10 (14%), respectively, of the patterns; other genotypes were rare (5 CAS [Central Asian], 4 X, 1 *M. bovis*, 1 Beijing, 1 LAM). Finally, the relative prevalence of *M. africanum* West African 1 from *M. africanum* West African 2 could first be assessed by the spoligotyping signature (2 vs. 14; online Technical Appendix). Specific single nucleotide polymorphism detection could constitute another classification tool for *M. africanum* sublineages (*10,11*). Unfortunately, detection of katG203 single nucleotide polymorphism failed on the slide-extracted DNAs (results not shown), and our study is limited by a suboptimal yield in positive spoligotyping results (80 [43%] of 186), an issue that should be improved.

Table. Distribution of classified genotypes of *Mycobacterium tuberculosis* complex, Burkina Faso*

Clade†	No. (%) isolates, n = 72‡
CAM	18 (25)
Including CAM_family prototype = SIT61	14 (19)
Other CAM	4 (6)
T	16 (22.2)
Including T1	10 (13.9)
Undefined T1-T2	2 (2.8)
T2	1 (1.4)
T3	1 (1.4)
T5_MAD2	2 (2.8)
Haarlem	10 (13.9)
Including H1	7 (9.7)
H3	3 (4.2)
X	4 (5.6)
Including X3	1 (1.4)
M. africanum I (WA I and WA 2)	16 (22.2)
M. bovis	1 (1.4)
CAS1_Delhi	5 (6.9)
LAM9	1 (1.4)
Beijing	1 (1.4)

*CAM, Cameroon; SIT, spoligo-international type; WA, West African; CAS, Central Asian; LAM, Latino American–Mediterranean.
†Described in (*9*).
‡Excludes 4 new and 4 unclassified genotypes.

Conclusions

The results of our study diverge on the *M. africanum* prevalence in Burkina Faso from results from Godreuil et al. (*2*) (1.9% vs. 20%). These authors were intrigued to not identify more *M. africanum* isolates and suggested that their finding might reflect "a decrease in *M. africanum* prevalence in these countries," referring to a similar decrease in Cameroon during 1971–2003 (*12,13*). We believe that in the study by Godreuil et al., an unintentional bias was introduced against *M. africanum*, given the difficulty of isolating this genotypic variant in routine practice in mycobacteriologic laboratories. Differences in *M. africanum* prevalence in culture-based and sputum-based studies might reflect the difficulties of growing and isolating *M. africanum* in some national TB reference laboratories in western Africa. *M. africanum*, which is closely related to *M. bovis*, has peculiar growing requirements that are not always satisfied. Supplementation of Löwenstein-Jensen medium with pyruvate is mandatory and not standardized (from 0.1% to 0.4%).

The pyruvate requirements of some members of the *M. tuberculosis* complex were recently shown to be caused by a mutation creating an inactive pyruvate kinase (*14*). This specific mutation of *M. africanum* has major implications for its metabolism and growth.

Implementation of adequate culture and molecular identification facilities in Burkina Faso are needed. A potential solution to avoiding the bias from culture and from DNA extraction from slides could be to extract DNA directly from sputum, e.g., by storing surplus sputum prospectively in 70% ethanol. Additional work also is needed to improve analytical methods for ZN slides to refine description of *M. tuberculosis* genetic diversity and eventually to provide predictive genetic drug susceptibility testing. Introduction of newer and faster TB diagnostic methods are urgently needed in this area of western Africa.

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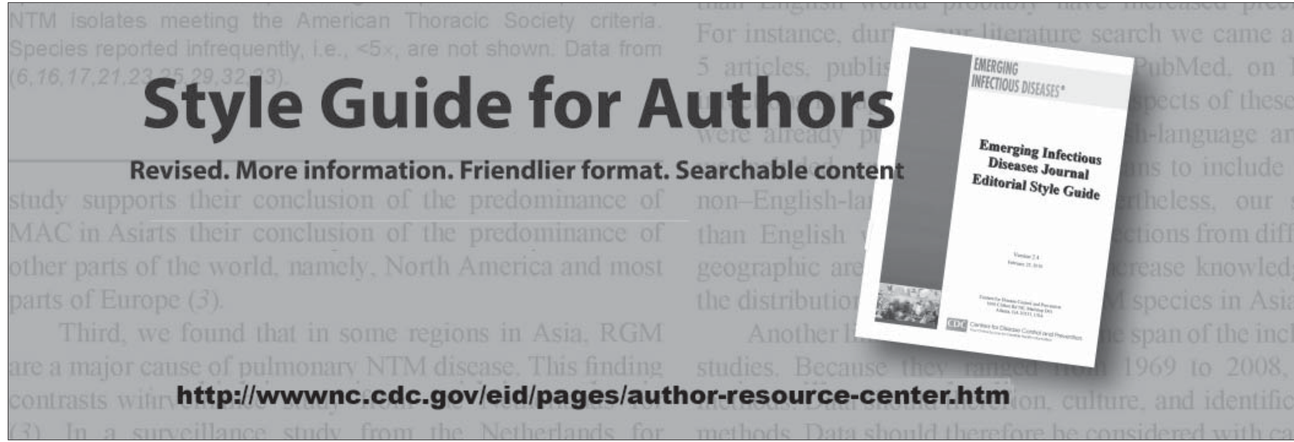

Molecular Evolution of Respiratory Syncytial Virus Fusion Gene, Canada, 2006–2010

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To assess molecular evolution of the respiratory syncytial virus (RSV) fusion gene, we analyzed RSV-positive specimens from 123 children in Canada who did or did not receive RSV immunoprophylaxis (palivizumab) during 2006–2010. Resistance-conferring mutations within the palivizumab binding site occurred in 8.7% of palivizumab recipients and none of the nonrecipients.

Human respiratory syncytial virus (RSV) is the most common cause of acute respiratory tract infections (RTIs) and a major cause of hospital admission and death among children <5 years of age worldwide (*1*). Risk for severe RSV-associated illness is highest among children born prematurely or with chronic medical disorders (*2*). Palivizumab immunoprophylaxis is the only available measure to prevent severe RSV disease.

The RSV fusion (RSV-F) surface glycoprotein mediates virus fusion to host cells. It is a major antigenic determinant that elicits neutralizing antibodies and cytotoxic T-lymphocyte immunity (*3*). Palivizumab (MedImmune, Gaithersburg, MD, USA) is a humanized mouse monoclonal antibody that inhibits RSV-F by binding to a defined epitope (residues 262–276) (*4*,*5*). Palivizumab immunoprophylaxis is recommended for the prevention of serious lower RTIs caused by RSV in children at high risk (*6*). RSV strains with mutations in key amino acid residues within the palivizumab binding site are resistant to this antibody (*7*–*9*); however, little is known about the prevalence of such mutations in clinical samples. Furthermore, despite its role in RSV pathogenesis, immunity, and prevention strategies, few data on RSV-F molecular evolution are available (*10*,*11*) because previous phylogenetic studies have focused on the RSV-G glycoprotein (*12*,*13*). Therefore, we monitored evolutionary changes in RSV-F, particularly potential resistance mutations in the palivizumab binding site, among strains from children who did and did not receive palivizumab.

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The Study

This cohort study was approved by the Centre Hospitalier Universitaire de Québec Research Ethics Board. Participants were <3 years of age and either received medical attention at an outpatient pediatric clinic or were hospitalized at a pediatric center for acute RTI during 4 winter seasons (2006–2010), in Québec City, Québec, Canada.

Clinical data were prospectively collected at study entry and after 1-month follow-up. For all patients, at the first visit a nasopharyngeal aspirate was collected. The aspirate was frozen at −80°C until subsequent testing by a multiplex PCR/DNA hybridization assay that detects RSV genotype-A (RSV-A), RSV-B, and 22 other respiratory viruses (Infiniti RVP assay; Autogenomics, Carlsbad, CA, USA) (*14*).

RSV infection was identified in aspirates from 467 (63.6%) of 734 hospitalized children (257 RSV-A, 210 RSV-B) and from 147 (48.2%) of 305 outpatient children (85 RSV-A, 62 RSV-B). During 2006–2010, a total of 724 children received palivizumab in the Québec City region (L. Cliche, pers. comm.). RSV-positive samples from all 12 study participants receiving palivizumab and from 100 not receiving palivizumab underwent RSV-F sequencing.

Additionally, F-gene analysis was performed on 11 RSV-positive clinical samples from palivizumab recipients retrospectively identified by using neonatal clinic registries at McMaster Children's Hospital (Hamilton, Ontario, Canada) and Montréal Children's Hospital (Montréal, Québec, Canada) during 2009–2010. Clinical data were collected by chart review.

RNA was extracted directly from nasopharyngeal samples by using a QIAmp Viral RNA Mini Kit (QIAGEN, Mississauga, Ontario, Canada). Random primers (Amersham, Piscataway, NJ, USA) and Superscript II RT Kit (Invitrogen, Carlsbad, CA, USA) were used for reverse transcription. PCR amplification was performed with QuantiFast Probe PCR+ROX Vial Kit (QIAGEN); primers and thermocycling conditions are available from G.B. upon request. RSV-F amplicons were sequenced by using an automated sequencer (Applied Biosystems, Foster City, CA, USA).

Along with the newly generated nucleotide sequences from the 123 children (23 palivizumab recipients; 100 nonrecipients), we analyzed 92 clinical RSV-F sequences provided by other investigators (*10*,*11*) or available from GenBank. Palivizumab exposure was unknown for these samples, and all originated outside Canada. We also included 10 unpublished RSV-F sequences from a 2004–2005 study of palivizumab recipients in Canada (*15*). Multiple-sequence alignment was performed with ClustalW in MEGA5 (www.megasoftware.net). A 1,524-bp region (positions 79–1602 in the prototype A2 and B1 RSV-F genes) was translated into amino acid sequences, and the palivizumab binding site (residues 262–276) was assessed for variations. We removed 55 redundant (identical) nucleotide sequences. The final dataset comprised 170 unique sequences, including 89 that were newly generated in this study (GenBank accession nos. JF776691–JF776779).

Phylogenetic reconstructions were computed in MEGA5 (Figure). Strains from Canada tended to group together. However, this segregation likely reflects temporal evolution rather than geographic influence because all RSV-F sequences originating from other countries were collected ≥10 years ago. Previous RSV-G studies have demonstrated concurrent circulation of related lineages in distant areas (*13*). Overall, RSV-F was highly conserved (Table 1). RSV-A exhibited more genetic diversity than RSV-B. This difference may reflect sampling bias because more RSV-A sequences originating from diverse locations and years were available; however, the greater genetic diversity of RSV-A compared with RSV-B has also been observed in a study conducted in South Korea (*10*).

Of the 23 RSV-F amino acid sequences from patients who received palivizumab (Table 2), 2 exhibited a mutation at residue 272 (K272Q, K272M), whereas no such mutations were identified in the other 100 strains obtained from those who did not receive palivizumab (8.7% versus 0%; $p = 0.03$, by Fisher exact test). A sublineage of RSV-A, noteworthy for an N276S substitution in the palivizumab binding site, emerged during 2008–2009 (8 [44%] of 18 RSV-A strains) and became the predominant RSV-A clade during 2009–2010 (25 [100%] of 25 strains) in palivizumab recipients and nonrecipients. This N276S lineage was detected in all 3 Canadian communities studied. No sequences from GenBank or other studies (*10*,*11*) harbored any palivizumab binding site mutation.

Microneutralization assays were performed as described elsewhere, with minor modifications (*9*). RSV was incubated (for 2 h at 37°C) with serially diluted palivizumab, then cultured in Vero cells (for 5 d). RSV replication and 50% inhibitory concentrations (IC_{50}) were subsequently determined by F protein quantification by using ELISA. The mean ± SD IC_{50} of C0910–1006A, a N276S strain, was 0.33 ± 0.04 µg/mL, similar to that of RSV-A2 wild type (IC_{50} 0.46 ± 0.04 µg/mL) and therefore was considered susceptible. Position-272 variants did not grow in culture and were not tested.

Conclusions

We report the prevalence of resistance-conferring mutations in RSV-F among children receiving or not receiving palivizumab. Although infrequent (8.7% of infections in palivizumab recipients), residue 272 mutations were significantly associated with palivizumab exposure and not observed at all in nonexposed patients.

We identified 2 new clinical specimens with position 272 mutations (K272Q and K272M). We cannot exclude the possibility that additional specimens contained mixed viral populations with minor proportions of position-272 mutants not detectable by conventional sequencing methods. Changes at this position (from lysine to asparagine, glutamine, glutamic acid, methionine, or threonine) have produced palivizumab resistance in vitro (*9*) and in a cotton rat model (*7*). As previously reported, 1 (10.0%) of 10 sequences from our 2004–2005 study of palivizumab patients carried a 272 mutation (K272E) (*15*). The K272E substitution is the only substitution also demonstrated to confer resistance to motavizumab, an enhanced-potency monoclonal antibody developed by affinity maturation of palivizumab (*9*). We could not perform neutralization assays on position-272 variants because they did not grow in culture. This finding suggests that such changes adversely affect viral replicative capacity (*7*,*9*).

Phylogenetic analysis demonstrated that mutations in the palivizumab binding site occur in diverse genetic backgrounds; all 3 strains with substitutions at residue 272 grouped to different clades (Figure). Furthermore, these mutant strains caused mild disease treatable in an outpatient clinic and severe illness requiring hospitalization.

From 3 Canadian communities we detected a lineage harboring an N276S mutation in 44.4% of RSV-A sequences

Table 1. Estimated mean nucleotide and amino acid identities of 170 unique respiratory syncytial virus fusion gene sequences*

Sequence	Mean nucleotide identity, % ± SD	Mean amino acid identity, % ± SD
Overall, n = 170	89.59 ± 8.38	95.99 ± 3.25
RSV-A (within group), n = 105	97.06 ± 1.33	98.85 ± 0.76
RSV-B (within group), n = 65	98.65 ± 0.69	99.48 ± 0.28
RSV-A vs. RSV-B (between groups), n = 170	80.85 ± 0.66	92.64 ± 0.56

*Mean nucleotide and amino acid identities were estimated by calculating pairwise distances in MEGA5 software (www.megasoftware.net) and using the maximum-composite likelihood and Poisson correction models, respectively. RSV, respiratory syncytial virus.

Table 2. Characteristics of 23 children in whom clinically significant respiratory syncytial virus respiratory tract infection developed while receiving palivizumab immunoprophylaxis, Canada, 2006–2010*

Location and patient ID	Age, mo/ sex†	GA at birth, wk + d	Underlying comorbidities	No. doses PZB‡	Delay, d§	Clinical diagnoses	H	Multiplex PCR/DNA results	Mutation
Québec City, Québec (2006–2010)									
C0607-1023	9/F	32 + 4	Prematurity, LBW	3	21	Bronchiolitis	No	RSV-A; enterovirus type A	K272Q
H0607-064	24/M	38 + 3	Congenital myopathy	3	15	Pneumonia; bronchospasm	Yes	RSV-B	NF
H0607-132	12/M	38 + 5	Pulmonary artery stenosis	5	7	Bronchiolitis	Yes	RSV-A	NF
H0708-199	4/M	30 + 4	Prematurity, VLBW	4	14	Bronchiolitis	Yes	RSV-B	NF
H0809-037	11/F	27 + 5	Prematurity, ELBW	3	14	Bronchiolitis	Yes	RSV-A	NF
C0809-1055	6/F	29 + 0	Prematurity, ELBW, triplet	4	27	Bronchiolitis	No	RSV-A	N276S
C0809-1056	6/M	29 + 0	Prematurity, ELBW, triplet	4	27	Bronchiolitis	No	RSV-A	N276S
C0809-1057	6/M	29 + 0	Prematurity, VLBW, triplet	4	27	Bronchiolitis	No	RSV-A	N276S
H0910-004	4/F	39 + 5	Choanal hypoplasia	1	16	Apnea; upper RTI	Yes	RSV-A	N276S
H0910-140	6/M	25 + 5	Prematurity, ELBW	4	29	Bronchiolitis	Yes	RSV-B	NF
H0910-144	13/F	26 + 5	Prematurity, VLBW	4	7	Pneumonia	Yes	RSV-A	K272M, N276S
H0910-150	9/M	28 + 3	Prematurity, VLBW	4	12	Upper RTI; acute otitis media	Yes	RSV-A; adenovirus type C	N276S
Montréal, Québec (2009–2010)									
MCH0910-001	15/M	40 + 4	Total anomalous pulmonary venous return	3	26	Pneumonia	Yes	RSV¶	N276S
MCH0910-002	6/F	39 + 0	Pulmonary valve stenosis, right aortic arch	2	7	Bronchiolitis	Yes	RSV¶	N276S
MCH0910-003	5/M	39 + 6	Cystic fibrosis	3	24	Bronchiolitis	No	RSV¶	N276S
MCH0910-004	7/M	36 + 2	Prematurity, BPD hypotonia	4	6	Bronchiolitis	Yes	RSV¶	N276S
MCH0910-005	15/M	40 + 4	Neuromuscular disorder, recurrent aspirations	4	13	Upper RTI; acute otitis media	No	RSV¶	N276S
MCH0910-006	2/M	34 + 6	Prematurity, LBW	1	14	Bronchiolitis	Yes	RSV¶	N276S
MCH0910-007	19/F	25 + 0	Prematurity, ELBW, BPD	3	19	Bronchiolitis, bronchospasm	Yes	RSV¶	N276S
MCH0910-008	2/F	38 + 1	Neuromuscular disorder, ventricular septal defect	2	12	Bronchiolitis	Yes	RSV¶	N276S
Hamilton, Ontario (2009–2010)									
MAC0910-001	1/F	34 + 5	Prematurity, LBW	2	3	Bronchiolitis	Yes	RSV¶	N276S
MAC0910-002	6/F	34 + 3	Prematurity, LBW, twin	1	25	Bronchiolitis	Yes	RSV¶	N276S
MAC0910-003	6/F	34 + 3	Prematurity, VLBW, IUGR, twin	1	27	Bronchiolitis	Yes	RSV¶	N276S

*Patient identification (ID) nomenclature: hospitalized (H) or clinic (C) prospective study participant, Montréal Children's Hospital (MCH) or McMaster Children's Hospital (MAC) patient. GA, gestational age; PZB, palivizumab; multiplex PCR/DNA, hybridization assay; mutation, mutation in respiratory syncytial virus fusion protein PZB binding site (residues 262–276); RSV, respiratory syncytial virus; LBW, low birthweight (1,500–2,500 g); NF, no mutation found in PZB binding site; VLBW, very low birthweight (1,000–1,499 g); ELBW, extremely low birthweight (<1,000 g); RTI, respiratory tract infection; BPD, bronchopulmonary dysplasia; IUGR, intrauterine growth restriction.
†Median patient age 6.0 mo (range 1–24 mo).
‡Mean ± SD no. palivizumab doses received that winter: 3.0 ± 1.2 doses.
§Median interval between last palivizumab dose and symptom onset: 15.0 d (range 3–27 d).
¶Retrospectively identified participants from Montréal Children's Hospital or McMaster Children's Hospital were RSV-positive by direct immunofluorescence assay (Chemicon International, Temecula, CA, USA) and were not tested by the multiplex PCR/DNA hybridization assay (*14*).

Figure. Phylogenetic analysis of 170 near–full-length unique respiratory syncitial virus fusion (RSV-F) gene sequences (nt 79–1602). Panels A and B are detailed phylograms of the RSV-A and RSV-B taxa analyzed, respectively. One bovine RSV-F sequence was added to the dataset (GenBank accession no. AF295543.1) as the outgroup (not shown) and used for rooting the phylograms. Topology was inferred by using the neighbor-joining method, and evolutionary distances were computed by using the maximum-composite likelihood method in MEGA5 software (www.megasoftware.net). The topologic accuracy of the tree was evaluated by using 1,000 bootstrap replicates. Only bootstrap values ≥50% are shown. Blue text and triangles represent RSV strains isolated in Canada and sequenced at the Centre de Recherche du Centre Hospitalier Universitaire de Québec; red branches indicates a sublineage of RSV-A with a N276S mutation; underlining indicates prototypical RSV A2 and RSV B1 strains. The clinical origin of strains from Canada (prospective study hospitalized patient [H] or clinic patient [C]; 2004–2005 palivizumab study patient [S]; retrospectively identified patient from the Montréal Children's Hospital [MCH] or McMaster Children's Hospital [MAC]) is indicated, followed by the year collected, the specimen identifier, and the result of RSV genogroup testing (RSV-A [A], RSV-B [B]) by multiplex PCR/DNA hybridization assay (*14*). Specimens with a nonsilent mutation at codon 272 have the amino acid substitution identified in brackets. When a taxon represents ≥1 identical sequences, the number of patients that it represents and the number of palivizumab recipients (PZB) among these patients are noted in parentheses. SA, South Africa; CHN, People's Republic of China; UK, United Kingdom, JPN, Japan; AUS, Australia; USA, United States; CDC, US Centers for Disease Control and Prevention; NY, New York; SL, St. Louis; WV, West Virginia; MN, Minnesota; MD, Maryland; WA, Washington; SEL, Seoul, South Korea. Scale bars represent substitutions per basepair per the indicated horizontal distance.

from 2008–2009 and 100% from 2009–2010, unrelated to palivizumab exposure. Adams et al. have proposed that N276S led to palivizumab resistance in a clinical specimen (*8*). However, that sample also comprised a K272E subpopulation. Our microneutralization assay results and unpublished neutralization data using recombinant viruses and clinical isolates (Q. Zhu, pers. comm.) suggest that N276S does not confer resistance.

Although serious RSV RTIs during palivizumab prophylaxis remain uncommon, we observed an 8.7% prevalence of known resistance mutations among 23 medically attended patients receiving palivizumab. These findings underscore the need for continued monitoring of RSV-F evolution.

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Dr Papenburg is a pediatric infectious disease specialist and medical microbiologist who performed this work during a fellowship at the Centre de Recherche en Infectiologie de l'Université Laval. His clinical practice is now at the Montréal Children's Hospital, McGill University. His research interests include the clinical epidemiology and molecular virology of childhood respiratory tract infections.

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Colpodella spp.–like Parasite Infection in Woman, China

Cong L. Yuan, Patrick J. Keeling, Peter J. Krause, Ales Horak, Stephen Bent, Lindsay Rollend, and Xiu G. Hua

The phylum Apicomplexa comprises intracellular protozoa that include many human pathogens. Their nearest relatives are chromerids and colpodellids. We report a case of a *Babesia* spp.–like relapsing infection caused by a newly described microorganism related to the Apicomplexa. This case is highly suggestive of a previously undescribed type of colpodellid that infects vertebrates.

The phylum Apicomplexa comprises intracellular protozoa that include human pathogens that cause diseases such as babesiosis, malaria, and toxoplasmosis (*1*). Apicomplexa evolved from algal ancestors, and their nearest relatives are algae (chromerids) and predatory flagellates (colpodellids) (*2*,*3*). We report a case of relapsing infection that has many characteristics in common with babesiosis. Amplification of DNA in blood and molecular phylogenetic characterization revealed a novel nucleotide sequence closely related to *Colpodella* and *Chromera* spp.

The Case

On March 20, 2008, a 57-year-old woman in the People's Republic of China sought care for a productive cough and malaise that she had had for 6 months; she was admitted to General Hospital in Kunming City, Yunnan Province. She had evidence of hemolytic anemia with decreased hematocrit and hemoglobin, an elevated reticulocyte count, and elevated lactate dehydrogenase levels. Evaluation of her immune status detected a low percentage of natural killer cells. A peripheral blood smear showed many erythrocytes that contained parasites (Figure 1, panel A). On the basis of its microscopic appearance, the infectious agent was thought to be an *Eperythrozoon* spp. organism, and the patient was treated with intravenous artemether and oral tetracycline for 17 days (*4*).

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She had a poor response to therapy and had 4 more episodes of relapsing illness despite a variety of empirically prescribed antimalarial therapies. Parasitemia decreased with each course of therapy but then increased a few days after antimicrobial therapy was stopped.

Blood samples were sent to the Laboratory of Zoonosis and Comparative Medicine, Shanghai Jiaotong University, where *Babesia* spp.–like parasites were identified microscopically. Therapy with atovaquone and azithromycin was initiated, and within a week the parasitemia decreased. The patient subsequently received atovaquone and azithromycin for 8 weeks, and the parasitemia and symptoms resolved completely. She remains asymptomatic 1 year after discontinuation of antimicrobial therapy.

Suspicious *Eperythrozoon* spp. and other bacterial infections were excluded by using a universal primer targeting the 16S rRNA gene (*4*). All published primers for amplification of the specific 18S rRNA region of *Babesia* spp. failed to produce amplification (*5*). To test a wider range of possible candidate species, we aligned all publicly available 18S rRNA sequences from *Babesia* spp. and designed primers to target a highly conserved fragment of the 18S rRNA gene (forward 5′-CCATGCATGTCTMAGTRTAAAC-3′ and reverse 5′-TTCCTCTAAYTGWTAAGGTTC-3′). With these primers, PCR product yielded a 1,653-bp fragment that was cloned and sequenced (triple repeats).

Unexpectedly, the sequence did not closely match any characterized *Babesia* species. Instead, the closest match in BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) analyses based on the entire sequence was *Colpodella tetrahymenae* (89% identity), a member of a genus that is closely related to Apicomplexa but that has never, to our knowledge, been found to infect animals or people (GenBank accession no. GQ411073).

Phylogenetic trees based on the 18S rRNA were inferred by using Bayesian and maximum-likelihood methods (*6*,*7*). Phylogenetic analyses that included a broad range of eukaryotes confirmed the organism's overall relationship to the apicomplexan lineage (data not shown). Further analyses included a greater diversity of apicomplexan sequences and showed that it branched at the base of Apicomplexa in the phylogenetic tree. More specifically, in all analyses it appeared to be the sister of a well-supported group consisting of members of the genera *Colpodella* and *Chromera* (Figure 2). On the basis of this position, we refer to the new parasite as colpodellid strain HEP (human erythrocyte parasite).

Convalescent-phase serum from the patient 3 months after the onset of infection was strongly reactive against *Colpodella* antigen; serum from a healthy control was nonreactive (Figure 1, panels B and C). Serum from the

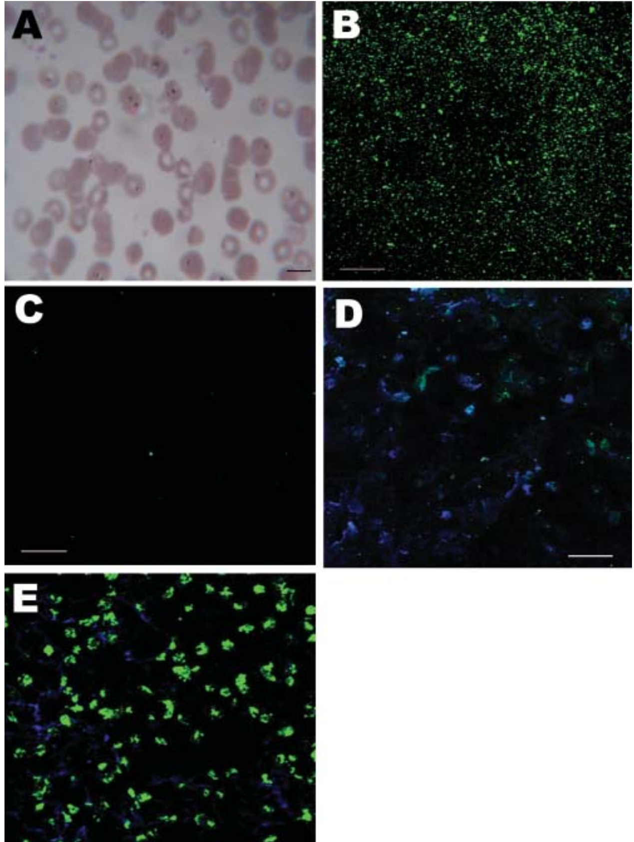

Figure 1. Morphologic appearance of infected erythrocytes of a 57-year-old woman in China and immunofluorescent antibody test results. A) Giemsa-stained thin blood smear showing erythrocyte infected with multiple ring forms (arrowhead). Scale bar = 10 μm; original magnification ×100. B) Patient serum reactive against *Colpodella* antigen. Scale bar = 20 μm; original magnification ×40. C) Healthy control serum not reactive against *Colpodella* antigen. Scale bar = 20 μm; original magnification ×40. D) Patient serum not reactive (green fluorescence) against *Babesia microti* antigen. Scale bar = 20 μm; original magnification ×40. E) *B. microti*–infected mouse serum reactive against *B. microti* antigen. Scale bar = 20 μm; original magnification ×40.

patient did not react against *Babesia microti* antigen (Figure 1, panels D and E). These results suggest that the patient's serum contained antibodies against a *Colpodella* species.

Although the exact diagnosis was not clear until amplified DNA of the intraerythrocytic organism had been genetically sequenced, the patient showed many typical signs and symptoms of babesiosis (*8–10*). The relapsing course of illness despite use of antimicrobial drugs that generally are effective against *Babesia* spp. is similar to the course of *B. microti* infection in highly immunocompromised hosts (*8*,*10*). Such patients often require prolonged (at least 6 weeks) antimicrobial therapy to clear infection (*8*).

Phylogenetic analysis of the molecular sequence data show that the patient's blood contained an organism distantly related to *Babesia* spp. and more closely related to *Colpodella* and *Chromera* spp. Although we cannot rule out the possibility that the intraerythrocytic organisms represent artifact and that the amplified DNA may have resulted from an environmental contaminant, other findings were consistent with the microscopy and PCR findings, i.e., clinical course, response to antiparasitic therapy, and demonstration of antibody against *Colpodella* antigen in the patient's serum.

Conclusions

To our knowledge, *Colpodella* spp. have not been shown to infect humans, and it is not surprising that this

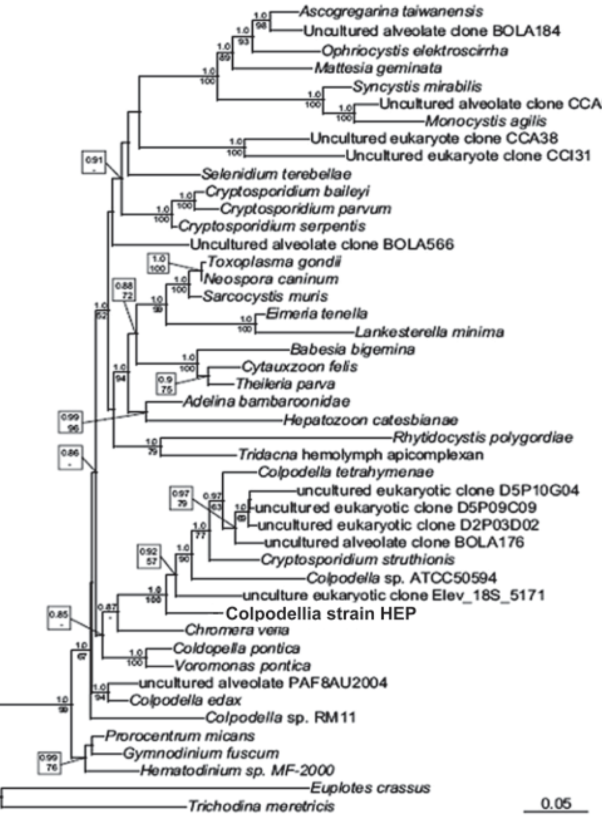

Figure 2. Maximum-likelihood small subunit rDNA phylogenetic tree showing the position of a novel human erythrocytic parasite (in box). Numbers above nodes represent Bayesian posterior probabilities computed by using MrBayes 3.1.2 (www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=mrbayes), with priors set to defaults and a Markov chain Monte Carlo run for 1,000,000 generations of which the first 100,000 were omitted from topology reconstruction. Numbers below nodes depict maximum-likelihood bootstrap support computed from 1,000 replications with the software and model described above. These numbers are bootstrap numbers and refer to the statistical confidence for that node as estimated by the different phylogenetic reconstruction methods. Scale bar indicates 1 base substitution/10 nt.

infection might emerge in an immunocompromised host. Although the patient reported here had no known immunodeficiency syndrome, she was >50 years of age and deficient in natural killer cells. Solitary natural killer cell deficiency is uncommon, and the clinical outcome ranges from no apparent immune deficiency to severe, recurrent, and fatal viral infections (*11*). The effect of natural killer cell deficiency on other pathogens is less clear, although they have been shown to protect against murine malaria (*12*). Colpodellids are microbial predators that feed on various algae and protozoa (*13*). Although they are not known to be parasites, finding a parasitic colpodellid is not entirely suprising. Their mode of predation by use of a primitive apicomplexan infective apparatus is similar to the mechanism by which Apicomplexa enter cells. Thus, the erythrocyte infection by colpodellid strain HEP implies that the feeding apparatus is adept at invading cells as well as extracting their contents.

According to available data, colpodellid strain HEP is best considered to be a new type of colpodellid, but little else is known about it. The manner in which this patient became infected is unknown. During this study, we were alerted to the existence of another new variety of colpodellid that had been isolated from fecal samples of calves with diarrhea in Turkey (A. Cilouglu, pers. comm.). The sequence of the organism isolated from these calves (GenBank accession no. JN245625) is the closest known relative to colpodellid strain HEP (data not shown).

In summary, we describe a case of apparent erythrocyte infection in a human, and the organism's sequence showed it to be related to colpodellids. Although our findings do not provide conclusive evidence that colpodellids can cause human disease (because the sequence and the infectious agent were not definitively linked and cannot be linked now that the infection has been cleared), no other obvious source for this sequence is apparent.

Colpodellids are not likely to be contaminants in blood samples because they are not common in nature, not known to be associated with humans, and actually rather difficult to maintain in the laboratory. Together with the recent finding of a closely related colpodellid sequence from calves with diarrhea, this case is highly suggestive of a previously undescribed type of colpodellid that infects vertebrates. New studies are needed to further describe this organism and confirm these findings in humans.

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Babesiosis among Elderly Medicare Beneficiaries, United States, 2006–2008

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We used administrative databases to assess babesiosis among elderly persons in the United States by year, sex, age, race, state of residence, and diagnosis months during 2006–2008. The highest babesiosis rates were in Connecticut, Rhode Island, New York, and Massachusetts, and findings suggested babesiosis expansion to other states.

Human babesiosis is a zoonotic disease caused by intraerythrocytic protozoan parasites of *Babesia* species. In the United States, *Babesia microti* is the primary etiologic agent of human babesiosis and is usually transmitted through the bite of *Ixodes scapularis*, the principal tick vector for this species (*1–3*). Human *B. microti* infections are regional, endemic to Northeastern (Connecticut, Rhode Island, Massachusetts, New York, New Jersey) and Midwestern (Minnesota, Wisconsin) states, and the geographic range is believed to be expanding (*1*,*2*,*4*,*5*). Babesiosis is characteristically seasonal, with peak transmission from May through September (*1*,*2*,*6*). In younger persons, babesiosis is more likely to be a mild or asymptomatic disease that may persist for months or even years undetected (*1*,*2*,*7*,*8*). Elderly, splenectomized, and other immunocompromised persons tend to be symptomatic (e.g., fever, chills, fatigue) and at risk for complications, including hemolytic anemia, acute respiratory failure, renal failure, and death (*1–3*,*9*).

Recently, there has been an increase in the number of reported clinical and transfusion-transmitted babesiosis cases in the United States (*1*,*2*,*10*). Efforts to mitigate transfusion-transmitted babesiosis risk include development of donor screening tests and testing strategies. Initiatives on babesiosis encompassed addition of *Babesia* spp. infections to the list of nationally notifiable diseases in 2011, a Food and Drug Administration sponsored workshop (*1*), a Blood Products Advisory Committee Meeting (*10*), and creation of the AABB *Babesia* Task Force. Because elderly persons are one of the most vulnerable at-risk populations and there are no published nationwide studies on babesiosis in that group, we used Centers for Medicare & Medicaid Services (Baltimore, MD, USA) administrative databases to assess babesiosis among elderly Medicare beneficiaries in the United States during 2006–2008.

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The Study

We used 100% inpatient, outpatient, skilled nursing facility, and carrier standard analytical as well as Medicare enrollment files for calendar years 2006–2008 to assess babesiosis among elderly Medicare beneficiaries ages ≥65 years of age. Standard analytical files are generated to capture medical services rendered and patient diagnoses, and enrollment files help to ascertain coverage eligibility. To be eligible for the study, beneficiaries had to be continuously enrolled in Medicare fee-for-service Parts A and B for ≥365 days before and including the latest month of continuous enrollment in the calendar year. We identified likely new babesiosis cases on the basis of the first recording of the International Classification of Diseases, 9th Revision, Clinical Modification diagnosis code 088.82 during the calendar year, with no recorded babesiosis infection in the preceding 365 days.

We assessed annual babesiosis rates by estimating the number of cases recorded per 100,000 beneficiaries per calendar year, overall and by sex, age, race, and state of residence. Seasonal occurrence was analyzed by using the number of cases in each month and number of beneficiaries continuously enrolled in Medicare fee-for-service within 365 days of each month. Cases were assigned age on the basis of diagnosis date, and persons without babesiosis had age assessed at the beginning of the latest enrollment month in the year. Beneficiaries with babesiosis were excluded from denominators of subsequent calendar years or diagnosis months. We performed χ^2 tests comparing babesiosis rates by using Epi Info version 3.5.1 (wwwn.cdc.gov/epiinfo). We conducted this institutional review board-approved study in coordination with the Centers for Medicare & Medicaid Services and within the SafeRx Project.

Among 27,278,865, 26,381,435, and 25,908,122 elderly Medicare beneficiaries in 2006, 2007, and

[1]These authors contributed equally to this article.

Table 1. Number of babesiosis cases and rates by sex and age among elderly Medicare beneficiaries, United States, 2006–2008

Sex and age group, y	No. beneficiaries with babesiosis (rate/100,000*)			Total no. enrolled beneficiaries		
	2006	2007	2008	2006	2007	2008
F						
≥65	509 (3)	441 (3)	647 (4)	15,919,094	15,356,954	15,032,121
65–74	283 (4)	240 (4)	365 (6)	6,794,195	6,547,813	6,487,120
75–84	171 (3)	153 (3)	214 (4)	6,075,497	5,781,146	5,527,531
≥85	55 (2)	48 (2)	68 (2)	3,049,402	3,027,995	3,017,470
M						
≥65	476 (4)	410 (4)	576 (5)	11,359,771	11,024,481	10,876,001
65–74	248 (4)	195 (3)	313 (6)	5,792,828	5,601,691	5,563,925
75–84	179 (4)	165 (4)	209 (5)	4,211,721	4,051,649	3,921,203
≥85	49 (4)	50 (4)	54 (4)	1,355,222	1,371,141	1,390,873
Both						
≥65	985 (4)	851 (3)	1,223 (5)	27,278,865	26,381,435	25,908,122
65–74	531 (4)	435 (4)	678 (6)	12,587,023	12,149,504	12,051,045
75–84	350 (3)	318 (3)	423 (4)	10,287,218	9,832,795	9,448,734
≥85	104 (2)	98 (2)	122 (3)	4,404,624	4,399,136	4,408,343

*Babesiosis rates were rounded to the nearest whole number.

2008, respectively, there were 985 (3.6/100,000), 851 (3.2/100,000), and 1,223 (4.7/100,000) babesiosis cases based on International Classification of Diseases, 9th Revision, Clinical Modification diagnosis code (Table 1). Annual babesiosis rates (per 100,000 beneficiaries) among the white elderly were 4.0, 3.6, and 5.2, and among nonwhite elderly the rates were 0.6, 0.9, and 1.4 for 2006–2008, respectively. Rate comparisons for whites versus nonwhites showed significant differences ($p<0.0001$) for each year. Babesiosis rates were significantly ($p<0.001$) higher for men versus women and younger elderly women (ages 65–84 years) versus older elderly women (≥85 years old) (Table 1). The Figure displays highest babesiosis rates in July and August of each year, with 74.4% of cases diagnosed in May through October.

Babesiosis varied by state, with the top 10 states and District of Columbia accounting for 834 (84.7%), 709 (83.3%), 1,024 (83.7%) of all cases in 2006–2008, respectively (Table 2). Connecticut, Rhode Island, New York, and Massachusetts had the highest babesiosis rates among the elderly (Table 2). Babesiosis cases were also recorded in other states, including California, Florida, Texas, Pennsylvania, Minnesota, and Wisconsin, with those 6 states accounting for 9.8%, 10.8%, and 12.3% of all cases in 2006, 2007, and 2008, respectively (data not shown).

Conclusions

We report a national population-based study of babesiosis among the US elderly, which used large administrative databases. The study found variations in the number of babesiosis cases by year, state, race, sex, age, and diagnosis month. Overall, our 3-year study suggests that there were more cases of babesiosis in 2008 compared with previous years. Northeastern and Mid-Atlantic States accounted for most newly diagnosed cases among the US elderly, with state-specific rates up to 10× higher than national annual rates. Our results show highest babesiosis rates in known babesiosis-endemic states of Connecticut, Rhode Island, New York, and Massachusetts and suggest possible expansion of human babesiosis to Maryland, Virginia, and other states. Human encroachment into tick and deer habitat, growth of deer population, climatic effects, and travel to disease-endemic areas may be responsible for variations in number of babesiosis cases and spread of the infection to non–disease-endemic states (*1–3*,*9*,*11*).

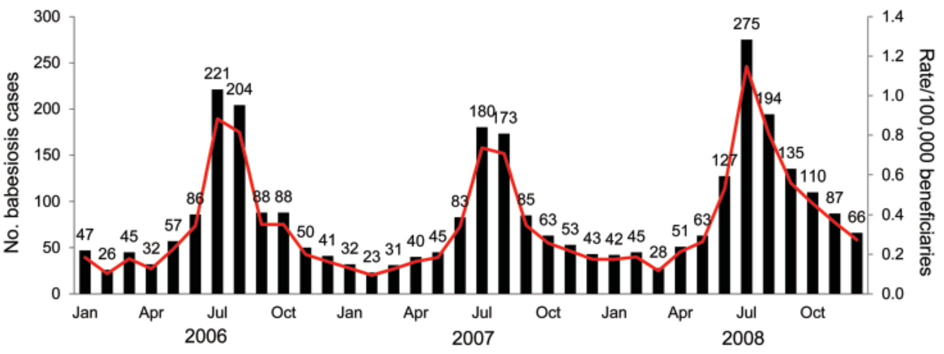

Figure. Number of babesiosis cases (black bars) and rates (red line) by month of diagnosis among elderly Medicare beneficiaries, United States, 2006–2008.

Table 2. Highest babesiosis rates among elderly Medicare beneficiaries, by state, United States, 2006–2008*

State or district	No. beneficiaries with babesiosis (rate/100,000†) 2006	2007	2008	Total no. enrolled beneficiaries 2006	2007	2008
US total	985 (4)	851 (3)	1,223 (5)	27,278,865	26,381,435	25,908,122
Connecticut	166 (42)	168 (44)	183 (50)	394,431	381,453	364,449
Rhode Island	34 (45)	13 (18)	30 (41)	74,951	73,387	72,430
New York	395 (24)	268 (17)	379 (25)	1,621,816	1,559,031	1,508,401
Massachusetts	76 (13)	128 (22)	185 (32)	596,349	590,318	586,278
Maryland	43 (8)	34 (7)	61 (12)	508,445	510,573	512,902
New Jersey	80 (9)	59 (7)	98 (11)	887,588	884,107	885,544
District of Columbia	3 (7)	1 (2)	3 (7)	44,419	43,843	42,986
Virginia	25 (3)	29 (4)	59 (8)	749,256	723,406	718,145
Maine	7 (4)	4 (2)	10 (6)	180,030	179,797	176,233
New Hampshire	3 (2)	4 (3)	9 (6)	149,654	151,120	150,567
Delaware	2 (2)	1 (1)	7 (7)	102,197	103,937	105,359

*States include District of Columbia. States are shown in descending order of average babesiosis rate during the 3-year period.
†Babesiosis rates were rounded to the nearest whole number.

Our findings show babesiosis trends similar to surveillance results in Rhode Island and New York State over the same period, with fewer cases reported in 2007, and more in 2008 (*12*,*13*). Data from the Connecticut Department of Public Health demonstrate broad annual variation in numbers of reported babesiosis cases in 2000–2008, with an increasing trend over time (*14*). Our finding of higher babesiosis rates among men versus women and among younger elderly women versus older elderly women are generally consistent with state surveillance data (*12*,*15*). Similarly to the literature (*1*,*2*,*6*,*12*,*15*), our study shows that most babesiosis cases are diagnosed during May through October. These findings are likely related to life cycle and activity of the tick vector and to activity of human and other mammalian hosts (*1*,*2*,*9*).

Study limitations are related to the use of administrative databases and include difficulty in identifying incident versus prevalent cases, possible misdiagnosis, and lack of clinical detail for diagnosis verification and transfusion-transmitted babesiosis case identification, as well as inability to differentiate *Babesia* species. Medical record review is needed to address above-mentioned limitations. Choosing a different continuous enrollment period could produce slightly different rates. Although Medicare data do not provide population-wide information on babesiosis among persons <65 years of age, younger persons are more likely to remain asymptomatic and less likely to get a diagnosis (*1*,*2*,*7*).

Our nationwide large medical database study is an additional tool to better understand regional, seasonal, and other babesiosis transmission patterns, by year and demographic characteristics, among the US elderly. Because the elderly are also known to use the majority of transfused blood, studies are needed to evaluate transfusion-transmitted babesiosis in this group. Overall, our study suggests that large administrative databases can be useful for assessing emerging infections in the United States.

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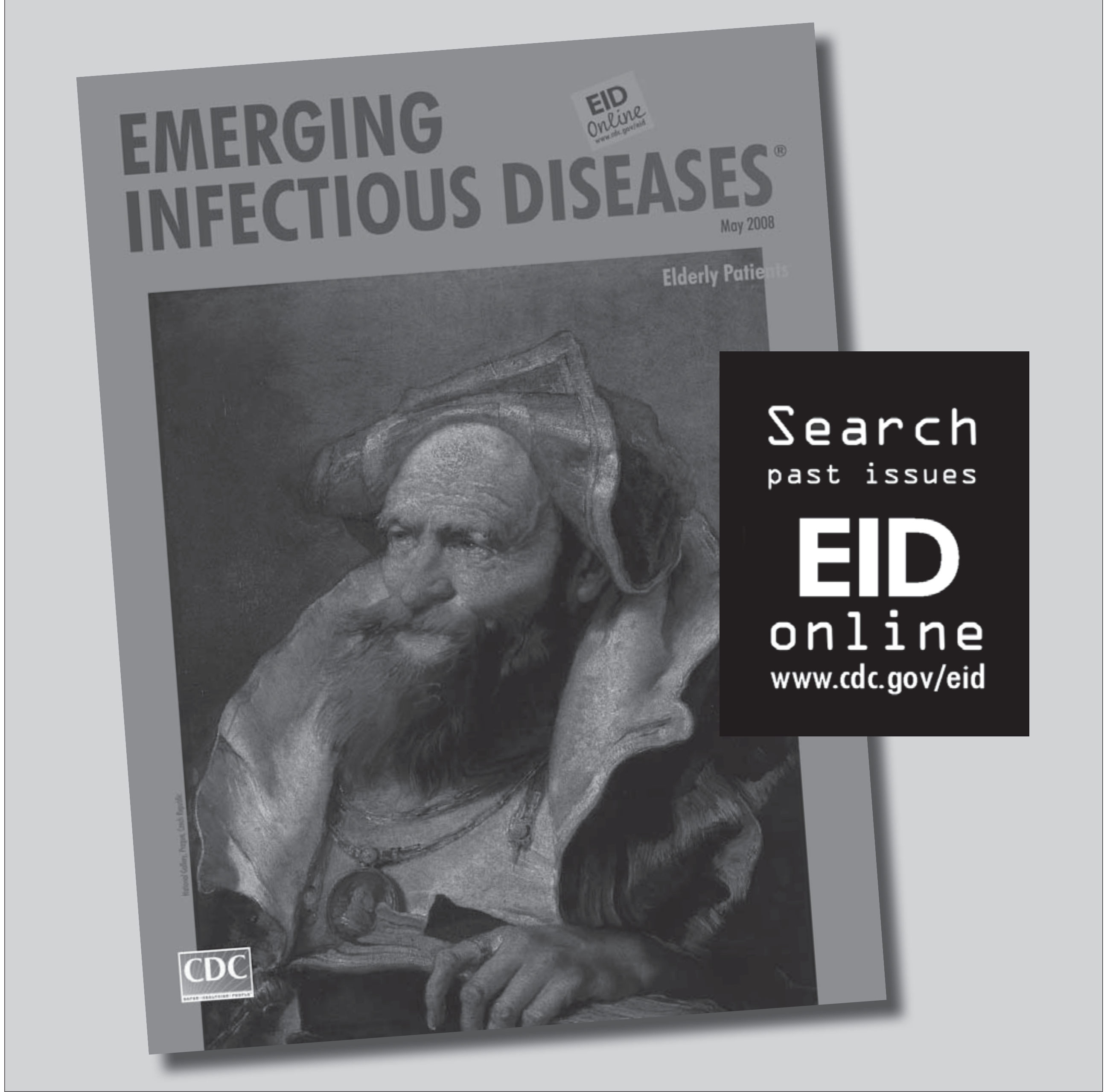

Foodborne Outbreak and Nonmotile *Salmonella enterica* Variant, France

Simon Le Hello, Anne Brisabois, Marie Accou-Demartin, Adeline Josse, Muriel Marault, Sylvie Francart, Nathalie Jourdan-Da Silva, and François-Xavier Weill

We report a food-related outbreak of salmonellosis in humans caused by a nonmotile variant of *Salmonella enterica* serotype Typhimurium in France in 2009. This nonmotile variant had been circulating in laying hens but was not considered as Typhimurium and consequently escaped European poultry flock regulations.

Identification of *Salmonella enterica* serotypes is based on flagellar and somatic antigens. Of the 2,610 serotypes distinguished in the Kauffmann-White-Le Minor Scheme (*1*,*2*), only Gallinarum are obligatory nonflagellate and hence, nonmotile. Nonmotile variants of diphasic *Salmonella* spp. are rarely reported in humans, food, or animals (*3*,*4*) as opposed to monophasic variants (*5*), in particular the monophasic variant of serotype Typhimurium (antigenic formula 1,4,[5],12:i:–) that has emerged worldwide (*6*–*8*). The French National Reference Center for *Salmonella* (FNRC-Salm) network is based upon voluntary reporting from ≈1,500 hospital and private clinical laboratories representing two thirds of all clinical *Salmonella* spp. strains isolated per year. In France, serotype Typhimurium is the most prevalent serotype and remains stable, with ≈4,000 human isolates per year; 1,4,[5],12:i:– strains have dramatically increased since 2005 (≈100 isolates) to reach ≈1,000 isolates in 2009.

We report a 2009 outbreak (8 cases) of salmonellosis in humans caused by a nonmotile strain of *Salmonella enterica* subsp. *enterica* with the antigenic formula 4,5,12:–:–. To estimate the extent of circulation and to determine the molecular subtypes of these nonmotile *Salmonella* strains, we performed a comprehensive, molecular epidemiologic study on human and nonhuman 1,4,[5],12:–:– strains isolated during 2000–2009 in France.

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The Study

In May 2009, diarrhea and fever developed in 8 persons living in southwestern France 1 day after they ate a homemade tiramisu prepared with raw eggs. Fecal analysis was performed on samples from 5 of the 8 persons. We also cultured a sample from the tiramisu. In medical laboratories, the isolation was performed by using standard procedures (i.e., use of conventional selective media). Isolation from the food sample was performed as required by the current International Organization for Standardization ISO 6579:2002 (i.e., by 2 selective enrichment media) (*9*). All cultures yielded *S. enterica* subsp. *enterica* 4,5,12:–:–.

An investigation at the suspected layer farm was conducted and showed the presence of 11 nonmotile *Salmonella* spp. isolates (with the same antigenic formula) in dust and feces collected from laying-hen houses. The layer farm, located in northwestern France, is a major farm that produces >32,000,000 eggs per year. All 17 isolates (5 from humans, 1 from the tiramisu, and 11 from the laying hens) were pan-susceptible to all antimicrobial drugs tested (*10*).

Analysis by Pulsenet (www.cdc.gov/pulsenet/protocols.htm) standardized *Xba*I pulsed-field gel electrophoresis (PFGE) showed an indistinguishable profile, XTYM-1, associated with the multidrug-resistant *S. enterica* serotype Typhimurium DT104 clone (*10*,*11*) (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/1/11-0450-FA1.htm). Multilocus variable number tandem repeat analysis (MLVA) typing (*12*) showed an unique type 3–14–7–21–311 (loci STTR9–5–6–10–3, respectively) in 16/17 isolates and a single-locus variant (differing by 1 repetition) for the remaining isolate, which was an environmental isolate collected 1 month after the control measures were implemented (slaughter of laying hens) (online Appendix Figure).

The tiramisu isolate 09CEB3100 was also characterized by multilocus sequence typing (MLST) and for the *fliC* and *fljB* genes encoding the flagellar antigens (*13*). The isolate belonged to MLST sequence type 19, the main sequence type of serotype Typhimurium (http://mlst.ucc.ie/mlst/dbs/Senterica). PCR and sequencing identified the *fliC* gene encoding the i antigen and the *fljB* gene encoding for the 1,2 antigen, confirming that this *Salmonella* spp. strain of the antigenic formula 4,5,12:–:– was a nonmotile variant of serotype Typhimurium. The investigation and molecular data concluded that a nonmotile variant strain

of serotype Typhimurium, genetically distinct from the emerging monophasic variant described worldwide, has been circulating in laying hens, whose contaminated eggs had likely caused food poisoning.

During 2000–2009, a total of 108,362 serotyped *Salmonella* spp. isolates from humans were registered at the FNRC-Salm, of which 374 (0.3%) were nonmotile. The 1,4,[5],12:–:– strains were the most prevalent (147/374, 39%). Such 1,4,[5],12:–:– strains have been rarely reported in food, environment or animals with 166 registered isolates collected by the French Food Safety Agency *Salmonella* network during 2001–2009 (compared with 21,214 serotype Typhimurium and 157,885 *Salmonella* spp. isolates registered).

To determine whether the population of nonmotile *Salmonella* was circulating, we studied 43 additional *S. enterica* spp. serotype 1,4,[5],12:–:– strains: 22 human and 21 nonhuman strains (5 from laying hens, 6 from cattle, 2 from sheep, 2 from partridges, 1 from chickens, 3 from the environment, and 3 from foodstuffs) isolated during 2001–2009. These strains were selected on the basis of their diversity (geographic area, year of isolation, and source). All the strains were characterized by antimicrobial drug susceptibility testing, PFGE, MLVA, and by *fliC* and *fljB* genes sequencing.

The 60 *S. enterica* serotype 1,4,[5],12:–:– isolates studied (17 isolates linked to the food poisoning and 43 additional isolates) displayed 22 different PFGE profiles. Only 10 profiles, from 49 isolates (81.6%), matched with profiles from the FNRC-Salm PFGE Typhimurium database (which includes 207 profiles generated in routine surveillance on 632 strains isolated during 1981–2010). All 49 strains were confirmed as nonmotile variants of Typhimurium by the identification of the *fliC* gene encoding the i antigen and the *fljB* gene encoding for the 1,2 antigen. The 11 strains whose PFGE pattern did not match any Typhimurium PFGE profile in our database belonged to serotypes Typhimurium (corresponding to 4 new PFGE profiles), Paratyphi B (n = 3), Agona, Derby, Indiana, and Saintpaul (1 each).

Among the 49 strains with a XTYM profile, 37 (75%) belonged to XTYM-1 (online Appendix Figure). The nonmotile XTYM-1 strains were divided into 2 groups regarding their susceptibility to antimicrobial agents: pan-susceptible for those (17) linked to the food poisoning plus 6 strains isolated before 2009 (3 human and 3 nonhuman isolates) and penta-resistant profile (resistant to amoxicillin, streptomycin/spectinomycin, sulfonamide, chloramphenicol, and tetracycline) for the 14 remaining strains. Notably, all 23 nonmotile XTYM-1 pan-susceptible strains had a single amino acid substitution in the *fliC* gene (Asp251 encoding an asparagine residue) compared with those available in public database, including serotype Typhimurium reference strain LT2. The search was made with BLASTN (www.ncbi.nlm.nih.gov). The 12 nonmotile strains that matched other non–XTYM-1 profiles exhibited 7 PFGE profiles, suggesting the loss of motility was acquired independently by 7 distinct serotype Typhimurium populations.

Thirty-eight MLVA types were found after testing the 60 isolates. The MLVA type 3–14–7–21–311 found in the food poisoning strains was not observed in the other strains tested. A minimum-spanning tree based on the MLVA types is shown in the Figure. The strains from the food poisoning clustered at the external extremity of a tree branch, whereas 3 human and 3 laying hen strains (from the same producer) isolated earlier (2005–2007) clustered at the internal extremity of the same branch. The 2 MLVA types differed by only 1 repetition difference at 2 loci. Two laying-hen strains isolated in 2009 (also from the same producer) were grouped in the interconnecting node. This finding, combined with antimicrobial drug susceptibility testing, PFGE, and *fliC* sequencing data, suggested that the 2009 strain causing the food poisoning is a derivative of the

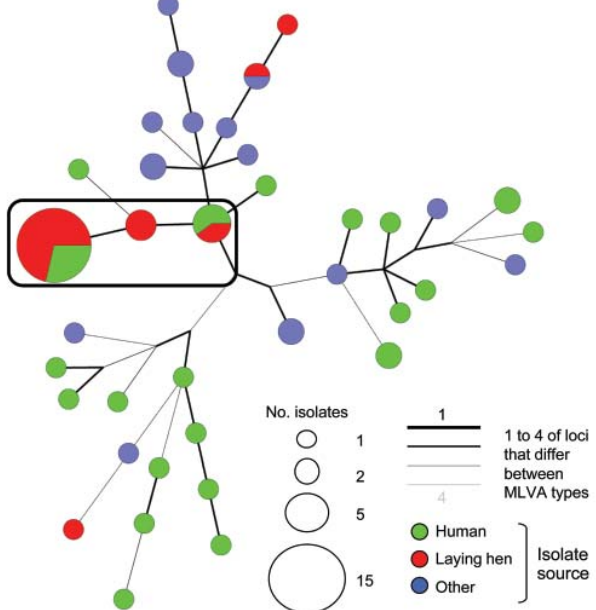

Figure. Minimum-spanning tree of multilocus variable number tandem repeat analysis (MLVA) of *Salmonella enterica* serotype 1,4,[5],12:–:– strains isolated from humans and nonhumans during 2001–2009, France. Each MLVA type is indicated by 1 node or branch tip, displayed as circles that are connected by branches of minimum-spanning tree. The length and the color of the branches represent genetic distances (changes in loci) between 2 neighboring types. The sizes of the different color circles depend on their population size. Wedges in circles indicate the proportion of isolates from respective sources with a particular MLVA type. A complex is shown in the black rectangle, based on maximum neighbor distance of changes at 2 loci and minimum size of 2 types. This specific complex linked the tiramisu food poisoning strains and other loci-derived strains for 2005–2009.

2005 strain; both strains were isolated from the same egg producer during a 4-year interval.

Conclusions

Regarding the European Directive and the Commission Regulation on the monitoring and reduction of zoonotic agents (*14*), the French Regulation has extended the target for reduction of prevalence of *Salmonella* spp. in poultry producers to include notification of monophasic (because of the recent emergence in humans) and nonmotile (because of this food poisoning) variants of Typhimurium after January 2010 (*15*). This food poisoning outbreak also highlighted the need for a second selective enrichment media for *Salmonella* spp. detection not based on the motility in complement to the modified semisolid Rappaport-Vassiladis medium recommended as a single medium by the European Directive.

We report a foodborne outbreak caused by a nonmotile *S. enterica* 4,5,12:–:– strain in France. This strain has been present in laying hens in France for the past decade. Despite continuous advances in food safety and disease surveillance, control, and prevention, atypical pathogenic *Salmonella* spp. strains that bypass existing procedures do emerge. Foodborne bacterial infections remain a major public health concern.

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Dengue Outbreak in Key West, Florida, USA, 2009

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After 3 dengue cases were acquired in Key West, Florida, we conducted a serosurvey to determine the scope of the outbreak. Thirteen residents showed recent infection (infection rate 5%; 90% CI 2%–8%), demonstrating the reemergence of dengue in Florida. Increased awareness of dengue among health care providers is needed.

Dengue is the most common mosquito-borne viral infection worldwide (*1*); however, it has been eliminated from the continental United States. No locally acquired cases have been reported outside the Texas–Mexico border (*2*,*3*) in >60 years, despite global increases in incidence and severity. Concern exists that dengue virus (DENV) may be reintroduced (*4*) because it is the most frequent cause of febrile illness among travelers returning from the Caribbean, South America, and Asia (*5*). Immigrants and visitors from dengue-endemic countries also provide opportunity for its reintroduction (*6*).

In September 2009, the Florida Department of Health (FDOH) was notified that a person with suspected dengue had recently traveled to Key West, Florida. The Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA) confirmed the diagnosis. Subsequently, dengue was confirmed in 2 Key West residents without a history of recent travel. FDOH and CDC conducted an investigation to determine size and scope of the outbreak. Cases identified through passive surveillance were reported (*7*).

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The Study

Key West is a tourist destination with >2 million visitors annually (*8*). Old Town, the area with reported cases, has a population of 19,846 (*9*). *Aedes aegypti* mosquitoes, the usual vectors of dengue, are widespread in Key West, but *Ae. albopictus* mosquitoes are uncommon.

In September 2009, we surveyed Old Town residents to estimate the infection rate and identify risk factors using stratified, 1-stage cluster sampling to randomly select 911 (15%) households within 1 km of the residence of the index case-patient. The area around the residences of the index case-patients was divided into 3 strata: strata 1 and 2 (within 200 m of each case-patient) and stratum 3 (201–1,000 m). Investigators asked household members (≥5 years of age) for a blood sample and information on recent illness, travel, foreign residence, and risk factors for dengue. One adult per household completed a questionnaire concerning the household. Investigators revisited unresponsive households 3 times unless the homes were empty.

Serum specimens were screened by ELISA for dengue-specific IgM and IgG (*10*,*11*). IgG-positive samples were tested by plaque reduction neutralization test with 90% cutoff ($PRNT_{90}$) against DENV serotypes 1–4 and West Nile virus (*12*). A >4-fold difference in titer between viruses was used to identify the infecting virus. Participants reporting febrile illness within a week were tested by reverse transcription PCR for DENV and West Nile virus and by nonstructural protein 1 ELISA for DENV antigen.

We classified participants with laboratory-positive DENV infection as follows: acute, if positive with reverse transcription PCR or nonstructural protein 1 ELISA; recent, if IgM-positive ELISA and $PRNT_{90}$ results were consistent with DENV infection; and presumptive recent, if they had dengue-like illness within 3 months, IgG-positive ELISA, and $PRNT_{90}$ results consistent with DENV infection. We classified participants as having previous DENV infection if they had IgG-positive ELISA and $PRNT_{90}$ results without recent febrile illness.

We weighted responses to account for sampling design using different probabilities of inclusion across strata and within-household participation rates, allowing for population inference (*13*). CIs accounted for sampling design and finite population correction factors. We used weighted logistic regression to assess risk factors for infection, and resulting inferences accounted for sampling design. Tests were performed in SAS version 9.2 (SAS Institute, Cary, NC, USA); p = 0.10 was significant.

Informed consent was obtained from all participants ≥18 years of age. Assent from the minor and informed consent from a parent were obtained for minors.

Of 911 selected households, 200 (22%) had been vacated, 387 (42%) did not have a resident at home, and 324 (36%) had a resident contacted; 170 (52%) households

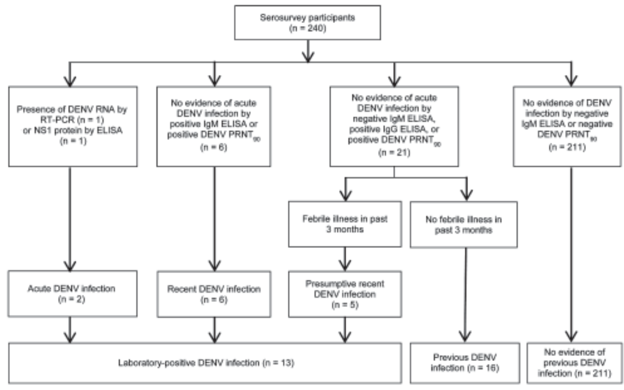

Figure 1. Case classification of 240 household survey participants Key West, Florida, USA, September 2009. DENV, dengue virus; RT-PCR, reverse transcription PCR; NS, nonstructural; $PRNT_{90}$, plaque reduction neutralization test with 90% cutoff.

and 240 persons participated. Median age was 53 years (range 15–95), slightly older than the median population (41 years) of Old Town (*14*). Most participants were non-Hispanic white (78%) and male (58%), similar to Old Town's population (*14*). Forty-three (18%) had lived in dengue-endemic countries, and most (148, 62%) had previously traveled to dengue-endemic areas.

Thirteen (5%; 90% CI 2%–8%) participants had laboratory-positive DENV infections (2 acute, 6 recent, 5 presumptive) (Figure 1). Without including presumptive infections, the rate was 3% (90% CI 1%–4%). Acute infections were confirmed as DENV-1. Two samples from those persons with presumptive infections had antibodies against DENV-1 by $PRNT_{90}$; samples from 3 persons were cross-reactive with multiple DENV serotypes. Infection rates were 4% in strata 1 and 3 (90% CI 0%–11% and 0%–6%, respectively) and 17% in stratum 2 (90% CI 0%–33%) (Figure 2). Sixteen (6%; 90% CI 3%–8%) participants had previous DENV infections, with antibodies against multiple serotypes (Figure 1). Seventy participants had IgG-positive ELISA results, but confirmatory testing did not show DENV infection.

Persons who kept windows open >50% of the time, lived in a household with >50% of the yard covered with vegetation, had a bird bath, or reported receiving mosquito bites at work were more likely to be infected with DENV (Table). Persons who used air conditioning >50% of the time, emptied containers of standing water weekly, and used *N,N*-diethyl-*m*-toluamide–containing repellents were less likely to be infected. African Americans were more likely (19%) to be infected than whites (4%; p = 0.09).

Conclusions

Approximately 3%–5% of Old Town residents were infected with DENV during July–September 2009, and surveillance identified new cases through October (*7*). Additionally, 63 cases, caused by the same strain of DENV-1, were reported there in 2010. All infections in Key West seem to have been locally acquired. Our findings that less frequent use of air conditioning, leaving windows open, and yard vegetation were risk factors for infection agree with results of previous dengue studies (*2*,*3*,*14*).

A specific route and time of introduction of dengue to Key West cannot be identified. The island has many tourists from dengue-endemic countries and a well-traveled population. Because the clinical features of dengue are often nonspecific, transmission was likely ongoing before the index case-patient's condition was diagnosed and FDOH was notified. Without diagnosis of this case, the outbreak may not have been recognized. Efforts to inform local healthcare providers about the identification, diagnosis, and clinical management of dengue are ongoing.

A useful discovery was the evidence of previous infections with other serotypes among residents, which indicates that a segment of the population is at risk for secondary infection, which is associated with severe dengue (*15*). The survey population was well traveled, placing them at risk of acquiring severe disease during travel to dengue-endemic countries or of different dengue serotypes being introduced into Key West. Other risk factors include the age, immune status, and genetic background of the infected person (*15*).

This investigation was performed quickly after outbreak identification while transmission was ongoing, which allowed acute infections to be identified. Study limitations included the circumstances that many households could not be contacted and many residents refused to participate.

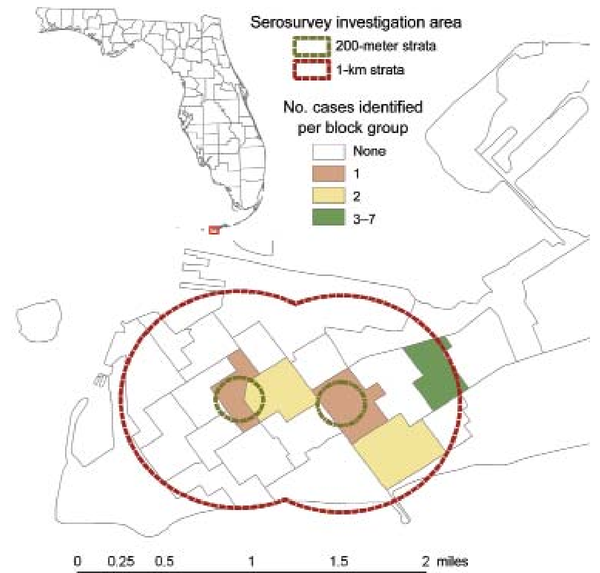

Figure 2. Locations of laboratory-positive cases of dengue in Key West, Florida, USA, in household survey, September 2009.

Table. Risk factors associated with laboratory-positive dengue virus infection among residents of Key West, Florida, USA, according to household survey, September 2009*

Variable	No. (%)† persons with infection, n = 13	No. (%) persons without recent infection, n = 227	Crude OR (90% CI)‡
Bird bath in yard	5 (41)	26 (11)	5.6 (1.5–21.3)
Windows open >50% of the time	5 (41)	37 (15)	3.9 (1.1–14.0)
Vegetation covers >50% of yard	8 (59)	61 (30)	3.4 (1.0–11.2)
Outside in evenings	11 (86)	149 (67)	3.1 (1.0–9.5)
Bitten by mosquito at work/school	5 (32)	28 (14)	3.0 (1.1–8.2)
Uses repellent containing DEET	3 (20)	98 (41)	0.4 (0.1– 0.9)
Uses mosquito bite prevention measures	4 (26)	119 (52)	0.3 (0.1–0.7)
Air conditioning on >50% of time	6 (37)	170 (75)	0.2 (0.1– 0.6)
Traveled outside Florida in past 3 mo	2 (12)	93 (38)	0.2 (0.1–0.9)
Empties water from containers regularly	1 (6)	79 (36)	0.1 (0.0–0.7)

*OR, odds ratio; DEET, *N,N*-diethyl-*m*-toluamide.
†Weighted percentages are reported, reflecting the stratified, 1-stage cluster sampling design. Responses were weighted to account for the different probabilities of household inclusion across strata, within-household participation rates, and interhousehold clustering of infections.
‡Significance level, p = 0.10. Weighted logistic regression models were used to assess risk factors for recent infection, and CIs were based on the modeling accounted for the sampling design.

We attempted to minimize participation bias through our sampling scheme. Also, presumptive infections may have been misclassified, which makes the 3% infection rate more likely to be accurate. Other misclassification because of prior flavivirus infection is possible, but was minimized by careful interpretation of laboratory results.

This outbreak and its continuation into 2010 demonstrate the potential for reemergence of dengue in subtropical areas of the United States where *Ae. aegypti* mosquitoes are present. Awareness among healthcare providers should be increased for optimal patient management and to limit outbreaks. Minimizing the effects of future dengue epidemics depends on personal protection against mosquitoes, mosquito control, early diagnosis, appropriate testing, and prompt reporting of suspected cases to public health authorities.

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Rabies in Captive Deer, Pennsylvania, USA, 2007–2010

Brett W. Petersen, Danielle M. Tack, Allison Longenberger, Aliza Simeone, Mària E. Moll, Marshall P. Deasy, Jesse D. Blanton, and Charles E. Rupprecht

Since January 2007, a total of 11 rabid deer from 4 deer farms have been identified in 2 neighboring Pennsylvania counties. Vaccination of deer against rabies, decreasing wildlife animal contact with deer, and education of deer farmers may prevent further cases of rabies in captive deer and exposures to humans.

Rabies is an acute progressive encephalitis caused by highly neurotropic zoonotic lyssaviruses. The disease is nearly always fatal in humans if postexposure prophylaxis (PEP) is not administered promptly after exposure (*1*,*2*). Although human cases of rabies are rare in the United States, each year nearly 7,000 rabid animals are reported and ≈35,000 humans receive PEP (*3*,*4*).

Since January 2007, a total of 11 rabid deer from 4 deer farms were identified in the neighboring counties of Lancaster and Chester, Pennsylvania, USA. These farms were located within a 45-mile radius in an area of high prevalence for rabies among all mammals (excluding bats) (Figure 1). We investigated this cluster to identify factors that might have contributed to disease transmission, assess the risk to humans, and provide recommendations for prevention.

The Study

The death of a rabid captive deer, an adult doe, was reported initially in August 2007 at farm A; during October 2007–January 2008, three buck fawns from this farm also died of rabies (Figure 2). In April 2008 and December 2009, two adult does died of rabies at farm B and farm C, respectively (Figure 2). Lastly, during July 2010, one adult doe, followed by 4 buck fawns, died at farm D (Figure 2). All reported cases were laboratory confirmed, and diagnostic testing detected a rabies virus variant associated with raccoons.

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We conducted a case–control study among deer farms in Pennsylvania. A case farm was defined as a registered deer farm with ≥1 laboratory-confirmed case of rabies in a deer. A control farm was defined as a registered deer farm that did not report any laboratory-confirmed rabies in deer. Ten control farms were chosen by referral from farmers and from the Pennsylvania Deer Farmers Association website. Control farms were limited to the affected counties and 1 noncontiguous county to account for local ecologic diversity. Four farms (farms A–D) met the case definition. Staff from the Centers for Disease Control and Prevention, state and local health departments, and the Pennsylvania Department of Agriculture conducted site visits to each affected farm. A standardized questionnaire was administered in person or by telephone to all study farms during August 2–5, 2010. Proportions were compared with Fisher exact test by using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

Farmers' ages, levels of education, and number of years farming did not differ between case and control farms (Table 1). Control farms tended to be larger than case farms (27.4 vs. 15.3 acres; odds ratio [OR] 0.99, 95% CI 0.95–1.03) with more deer per acre (36.8 vs. 34.7; OR 0.99, 95% CI 0.96–1.04), although neither difference was significant (Table 1). Farming practices did not differ between case and control farms (Table 1). Trough and bottle feeding of deer were common practices, and most (71%) farms reported using sweet deer feed containing molasses, which might attract rabies reservoir species, such as raccoons. Deer were moved infrequently between pens, and few outside deer were brought onto the farms (mean 2.5 deer/year); however, interstate travel of deer was reported. Most (71%) farms reported vaccinating deer against at least 1 disease, but deer were vaccinated on case farms only in response to previously rabid deer (Table 1). A low perceived risk for rabies was cited as

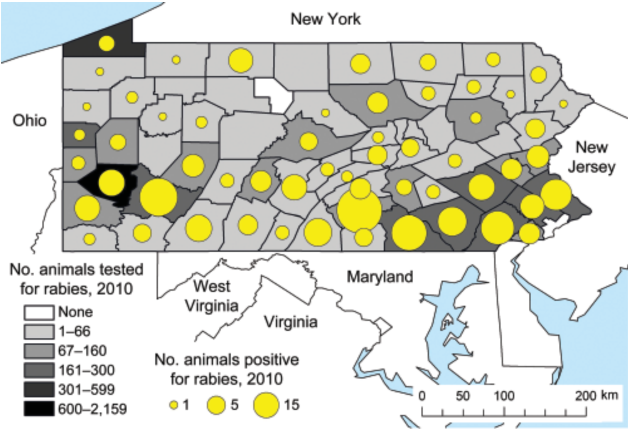

Figure 1. Reports of suspected and confirmed rabies among all animals (excluding bats) in Pennsylvania, 2010.

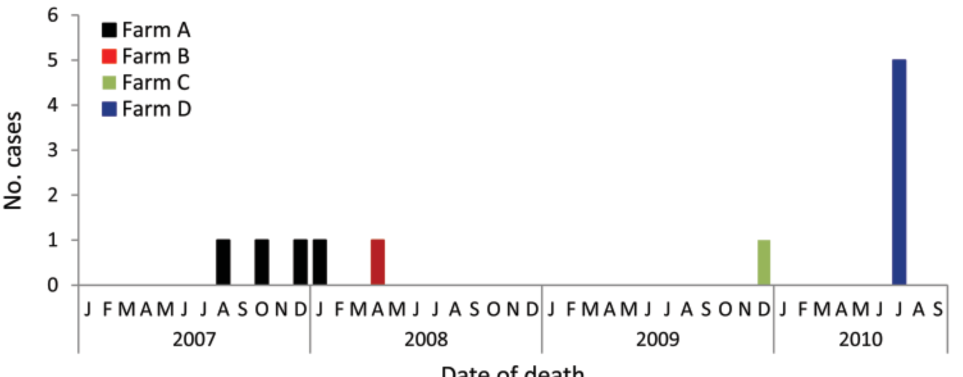

Figure 2. Rabies deaths among captive deer at 4 farms in Lancaster and Chester counties, Pennsylvania, USA, 2007–2010.

the primary barrier to rabies vaccination among control farms. In contrast, witnessed contact between deer and rabies reservoir species was relatively common (43% of farms reported contact with skunks, and 36% reported contact with raccoons) (Table 2).

Each of the 4 deer farmers from case farms received rabies PEP because of exposures to the rabid deer. Potential sources of exposure were common. All deer farmers reported bare skin contact with animal saliva, 50% reported being scratched by an animal, 29% reported being bitten by an animal, and 29% reported bare skin contact with animal tissue (Table 2). Case farms had significantly higher self-reported knowledge about rabies, probably because of their direct experience with the disease (Table 2). However, knowledge of rabies among control farms was low (90% of farmers reported knowledge as basic), and none of the farmers indicated that they should wash with soap and water if potentially exposed to the rabies virus (Table 2).

Conclusions

Since 1990, a total of 104 rabid deer have been reported in the United States. However, to the best of our knowledge, the cases in this report are the only reported cases in captive deer. The number of captive deer in the United States is increasing because of the growth of the deer farming industry. In Pennsylvania alone, ≈1,200 deer farms currently operate in 63 of the 67 counties (*5*). Without appropriate prevention and control efforts integrating the concepts of One Health (*6*), rabies in captive deer has the potential to threaten human and animal health.

The primary method of preventing rabies in animals is vaccination, which in turn reduces the risk for transmission to humans. Although not licensed for use in deer, rabies vaccine is expected to have a similar safety and efficacy profile in deer as it does in other ruminants. The Compendium of Animal Rabies Prevention and Control recommends that rabies vaccination be considered in livestock that

Table 1. Characteristics and practices of farms with rabid deer, Pennsylvania, USA, 2007–2010

Characteristic/practice	Case farms, n = 4	Control farms, n = 10	Total, n = 14	Odds ratio (95% CI)
Mean age of farmer, y	41.3	46.6	45.1	0.96 (0.88–1.10)
Farmer education, some high school or above, no. (%); reference = some high school or below	1 (25)	5 (50)	6 (43)	0.33 (0.03–4.40)
Mean time farming, y (range)	10.8 (5–17)	9.3 (2–17)	9.7 (2–17)	1.1 (0.83–1.37)
Mean size of farm, acres (range)	15.3 (2–45)	27.4 (3–170)	23.9 (2–170)	0.99 (0.95–1.03)
No. deer on farm, mean (range)	69.8 (13–159)	75.8 (16–230)	74.1 (13–230)	0.99 (0.98–1.02)
No. deer/acre in smallest pen, mean (range)	34.7 (10.7–90.9)	36.8 (8–89.3)	36.2 (8–90.0)	0.99 (0.96–1.04)
No. deer brought onto farm annually, mean	1.25	3.0	2.5	0.67 (0.23–1.92)
Farms reporting deer vaccination, no. (%)	4 (100)	7 (70)	11 (79)	0.78 (0.06–10.86)
Farms reporting deer vaccinated against rabies, no. (%)	4 (100)	0	4 (28)	Not calculated
Farms reporting feeding method, no (%)				
Trough	4 (100)	9 (90)	13 (93)	Not calculated
Bottle	2 (50)	9 (90)	11 (79)	0.11 (0.01–1.91)
Tube feeder	1 (25)	3 (30)	4 (29)	0.78 (0.06–10.86)
Farms reporting use of sweet feed, no. (%)	3 (75)	7 (70)	10 (71)	1.29 (0.09–17.95)
Farms reporting deer contact with rabies reservoir species, no. (%)				
Skunks	2 (50)	4 (40)	6 (43)	1.5 (0.15–15.46)
Raccoons	2 (50)	3 (30)	5 (36)	2.3 (0.22–25.25)
Foxes	0	1 (10)	1 (7)	Not calculated

Table 2. Rabies knowledge, exposures, and health-seeking behavior among deer farmers, Pennsylvania, USA, 2007–2010

Variable	No. (%) respondents			Odds ratio (95% CI)
	Case farm, n = 4	Control farm, n = 10	Total, n = 14	
Self-reported knowledge of rabies, basic; reference = advanced	1 (25)	9 (90)	10 (71)	0.04 (0.002–0.79)
Farms reporting human rabies vaccination	4 (100)	0	4 (29)	Not calculated
Farms reporting an animal bite on farm	0	4 (40)	4 (29)	Not calculated
Farms reporting an animal scratch on farm	2 (50)	5 (50)	7 (50)	1.0 (0.1–10.17)
Farms reporting bare skin contact with animal saliva	4 (100)	10 (100)	14 (100)	Not calculated
Farms reporting bare skin contact with animal tissue	2 (50)	2 (20)	4 (29)	4.0 (0.33–48.66)
Advocated action if exposed to rabies virus				
Seek medical care	3 (75)	7 (70)	10 (71)	1.29 (0.09–17.95)
Get rabies vaccination	2 (50)	4 (40)	6 (43)	1.50 (0.15–15.46)
Call county health department	1 (25)	0	1 (7)	Not calculated
Have animal tested for rabies	0	1 (10)	1 (7)	Not calculated
Wash with soap and water	0	0	0	Not calculated

have frequent contact with humans or in livestock that are particularly valuable (*6*). Captive deer would meet both criteria on the basis of the human exposures reported in the survey and their economic value (individual deer can cost hundreds to hundreds of thousands of dollars). Interstate transportation of deer is state regulated and generally prohibits importation of deer from states with endemic chronic wasting disease. However, the survey did document interstate travel of captive deer, which raises concern for the possible translocation of a raccoon rabies virus variant across oral rabies vaccination boundaries. To decrease this risk, rabies vaccination should be strongly considered in any deer transported between farms or across state borders.

Although no specific management practices were identified as major risk factors among affected deer farms, the rabies virus most likely was transmitted to the deer through contact with wildlife, as reported in the survey. However, deer-to-deer transmission could not be excluded at farms where multiple deer were affected (i.e., farms A and D). At both locations, adult does died, followed by buck fawns, suggesting that infection could have occurred during close maternal activities involving saliva transmission. However, not all of the rabid fawns belonged to the rabid does, and some of the rabid fawn's mothers remained healthy, even though doe can display maternal behavior toward multiple fawns. Regardless, measures to decrease contact between captive deer and rabies reservoir species should be implemented. Such measures might include trapping and removing such species, eliminating brush, groundhog burrows, or other potential sources of dens or cover for terrestrial carnivores; avoiding planting crops or storing food near deer pens; and using elevated, closed feeders (such as tube feeders) placed away from pen fences.

The survey also demonstrated that animal exposures, such as contact of bare skin with animal saliva, commonly occur on deer farms, and provide a potential route of transmission in the presence of any open cuts or wounds. This point is particularly important given deer farmer knowledge about rabies appears limited, especially with regard to exposures. Although 71% of respondents indicated knowing they should seek medical care if exposed to the rabies virus, none indicated they should wash with soap and water. These findings provide evidence of the danger of rabies virus transmission to humans from captive deer and the need to educate deer farmers.

Acknowledgments

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Dr Petersen is a lieutenant commander in the US Public Health Service and an Epidemic Intelligence Service Officer in the rabies program at the Centers for Disease Control and Prevention. His work focuses on the epidemiology of rabies and its application to domestic and international prevention and control efforts.

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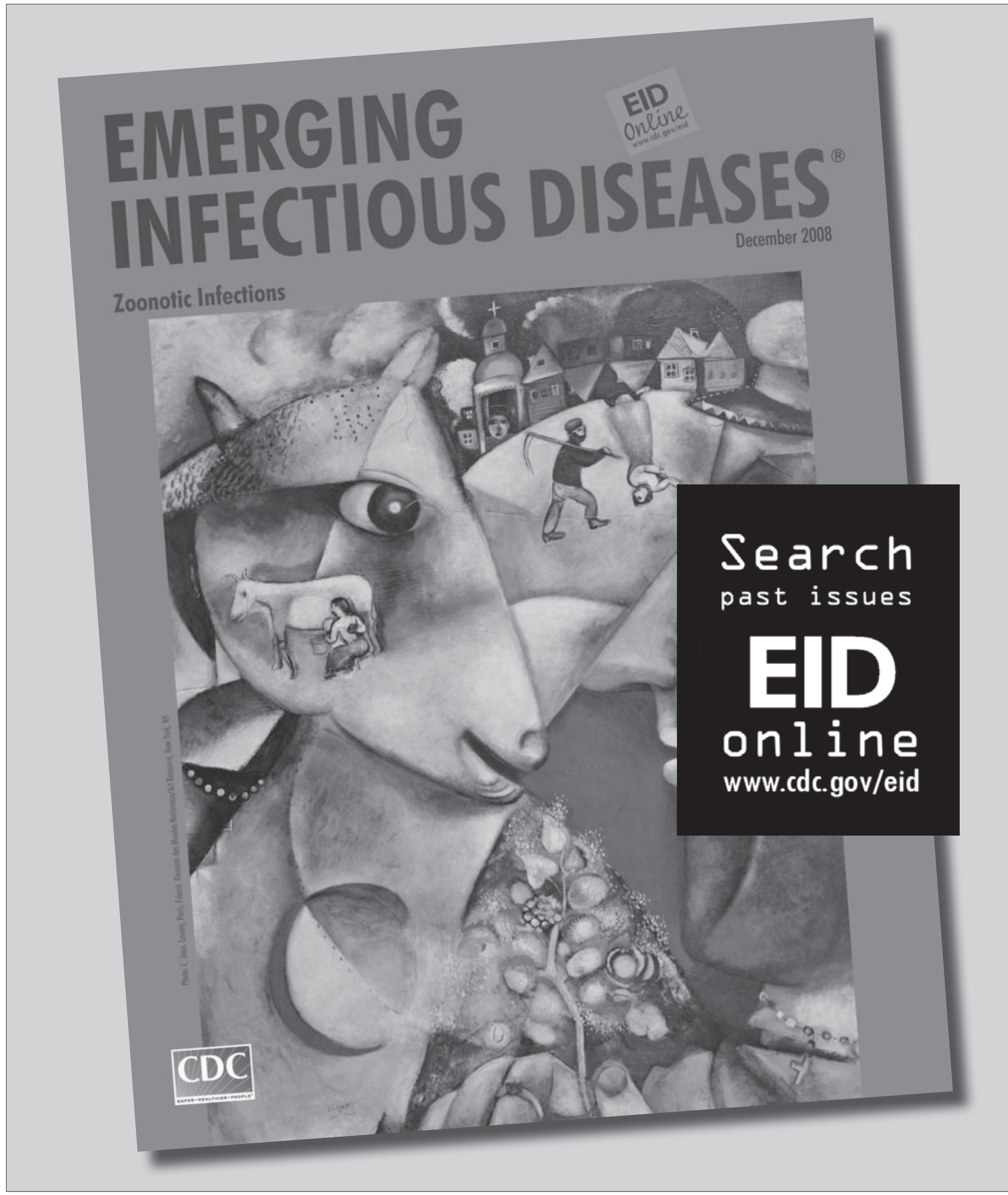

Oral Transmission of L-type Bovine Spongiform Encephalopathy in Primate Model

Nadine Mestre-Francés, Simon Nicot, Sylvie Rouland, Anne-Gaëlle Biacabe, Isabelle Quadrio, Armand Perret-Liaudet, Thierry Baron, and Jean-Michel Verdier

We report transmission of atypical L-type bovine spongiform encephalopathy to mouse lemurs after oral or intracerebral inoculation with infected bovine brain tissue. After neurologic symptoms appeared, transmissibility of the disease by both inoculation routes was confirmed by detection of disease-associated prion protein in samples of brain tissue.

Transmissible spongiform encephalopathies, also known as prion diseases, are fatal neurodegenerative disorders that affect humans and animals. An atypical form of bovine spongiform encephalopathy (BSE) was recently identified in cattle in Europe (*1,2*), North America (*3*), and Japan (*4*). This atypical BSE was designated L-type BSE (L-BSE) because Western blot analysis showed that the disease-associated protease-resistant prion protein (PrP^{res}) was of lower apparent molecular mass than in the agent of classical BSE, which is involved in the major foodborne epizooty in cattle and in variant Creutzfeldt-Jakob disease in humans (*5*).

Evidence from experimental studies in primate models (*6,7*) and transgenic mice expressing human prion protein (PrP) (*8,9*) suggests that the rare and putatively sporadic form of L-BSE (*10*) presents a higher risk than classical BSE for transmission to humans. However, a major unresolved issue is whether L-BSE can be transmitted by the oral route. To address this issue, we inoculated gray mouse lemurs (*Microcebus murinus*), a nonhuman primate model, by the oral and intracerebral (IC) routes with the agent of L-BSE.

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The Study

A total of 12 mouse lemurs of both sexes (Center for Breeding and Experimental Conditioning of Animal Models, University Montpellier 2, Montpellier, France) were maintained in animal Biosafety Level 3 facilities, according to requirements of the French ethics committee (authorization CE-LR-0810). Young and adult lemurs were fed (8 animals) or IC inoculated (4 animals) with 5 or 50 mg of L-BSE–infected brain tissue (10% homogenate in 5% glucose) (Table). The isolate for the L-BSE agent (02–2528) was derived from cattle in France (*11*). When progression of prion disease was evident, the lemurs were euthanized and their brains were isolated. Brains were processed for Western blot analysis with SHa31 monoclonal antibody against PrP for PrP^{res} detection, as described in mice (*11*); for histologic examination by using hematoxylin and eosin staining; and for disease-associated prion protein (PrPd) immunochemical detection by using the paraffin-embedded tissue blot method or immunohistochemical analysis with monoclonal antibody 3F4 against PrP.

Beginning ≈3 months before the terminal stage of the disease (19–22 months after inoculation), neurologic symptoms developed in the 4 mouse lemurs that received IC inoculations (Table). In all 4 animals, initial clinical signs and symptoms were blindness, thigmotaxic behavior, and poor appearance of the fur. Appetite and general fitness were maintained; anxiety and aggressiveness were not observed. Next, locomotion became slower, followed by incoordination and loss of balance in the last month of life. Ipsilateral circling behavior was reported, indicating unilateral degeneration of the striatum. This behavior stopped 15 days after onset, suggesting damage to the contralateral striatum. Disequilibrium, with frequent falls, became more noticeable. At the terminal stage of the disease, the animals were prostrate.

One orally inoculated lemur, which was fed 5 mg of infected brain and euthanized 27 months later, had signs and symptoms of disease similar to those in IC-inoculated animals, except for the ipsilateral circling behavior. In 2 lemurs fed 50 mg and 2 others fed 5 mg of L-BSE–infected brain, clinical signs and symptoms of prion disease developed just a few weeks before the animals were euthanized (18 and 32 months and 33 and 34 months after inoculation, respectively). Disease was characterized by progressive prostration, loss of appetite, and poor appearance of the fur, without incoordination or disequilibrium. The 3 remaining lemurs were orally

Table. Experimental transmission of cattle-derived L-BSE agent to 12 mouse lemurs, by 2 routes of inoculation*

Inoculation route	L-BSE dose, mg	Inoculated animals			
		No. inoculated (no. alive)	Age at inoculation	Survival after inoculation, mo	Positive for PrPd†
Intracerebral	5	4	1 y	19; 19.5; 22; 22	4/4
Oral	50	3 (1‡)	2 mo or 2 y	18‡; 32	2/2
Oral	5	5 (2‡)	2 mo or 2 y	27; 33; 34	2/3

*L-BSE, L-type bovine spongiform encephalopathy source; PrPd, disease-associated prion protein. Source of L-BSE, 02–2528.
†Results obtained by Western blot analysis and/or paraffin-embedded tissue-blot analysis and/or immunohistochemical analysis.
‡Animals were inoculated at 2 y of age.

inoculated at 2 years of age and were still alive and healthy 28 months after inoculation (Table).

PrP^{res} was readily detected by Western blot analysis in brain extracts (thalamus/hypothalamus region) from 8 of the 9 animals examined (Table), although at lower levels in the lemur that was euthanized earlier (i.e., 18 months after inoculation). Western blot analyses showed uniform PrP^{res} molecular profiles, irrespective of the route or dose of inoculation, with a low apparent molecular mass (≈19 kDa, similar to the PrP^{res} in the original cattle brain) (Figure 1). However, the PrP^{res} profile in mouse lemurs was characterized by a higher proportion of di- and monoglycosylated species (>95% of the total signal) than in the inoculum of the agent of bovine L-BSE (≈80%). In addition, PrP^{res} was detected by Western blot in the spleens of 3 (1 IC inoculated and 2 fed with 5 mg of cattle brain) of the 9 animals examined (Figure 1).

Histopathologic analysis showed severe spongiform changes in the brains of the 4 IC-inoculated mouse lemurs (Figure 2, panel A). The brains displayed a pattern of vacuolation characterized by intense spongiosis with many confluent vacuoles in the basal telencephalon (septum, striatum, caudate putamen nuclei), midbrain (thalamus, hypothalamus), mesencephalon (colliculi), and in some parts of the brainstem (tegmental ventral area, raphe nuclei). Lesions in the cortex and hippocampus were less severe than in the subcortical areas. Cerebellum showed occasional small-size vacuoles. Among the 5 orally inoculated animals, 2 (1 fed 5 mg, the other fed 50 mg) showed histopathologic features similar to those observed in IC-inoculated animals. In the other 3 orally inoculated animals, spongiosis was characterized by fewer vacuoles and was restricted to the striatum (Figure 2, panel B), thalamus, colliculi, and brainstem.

Distribution of PrPd in the brain was assessed by paraffin-embedded tissue blot (Figure 2, panels C and D) or immunohistochemical analysis with 3F4 antibody (Figure 2, panels E and F). Results for IC-inoculated animals showed that PrPd strongly accumulated in a dense synaptic pattern associated with nonamyloid plaques in the striatum, several thalamic nuclei (Figure 2, panel E), the external cortex of the colliculi, and the tegmental area. Other areas that were slightly less affected (e.g., neocortex and hippocampus) showed few coarse granules and synaptic deposits. The cortical molecular layer and the corpus callosum were devoid of PrPd (Figure 2, panel C). In orally inoculated animals, PrPd was strongly accumulated in the striatum and thalamus (Figure 2, panel D) but weakly accumulated in the cortex. Immunohistochemical analysis showed synaptic deposits (Figure 2, panel F), and some focal deposits were evident in animals that survived longer. No plaques were detected in orally inoculated animals.

Conclusions

We demonstrated that the agent of L-BSE can be transmitted by the oral route from cattle to mouse lemurs. As expected, orally inoculated animals survived longer than IC-inoculated animals. Orally inoculated lemurs had less severe clinical signs and symptoms, with no evidence of motor dysfunction. It was previously suggested that the agent of L-BSE might be involved in the foodborne transmission of a prion disease in mink (*11,12*), a species in which several outbreaks of transmissible mink encephalopathy had been identified, notably in the United States (*13*).

Our study clearly confirms, experimentally, the potential risk for interspecies oral transmission of the agent of L-BSE. In our model, this risk appears higher than that for the agent of classical BSE, which could only be transmitted to mouse lemurs after a first passage in macaques (*14*). We report oral transmission of the L-BSE agent in young and adult primates. Transmission by the IC route has also been

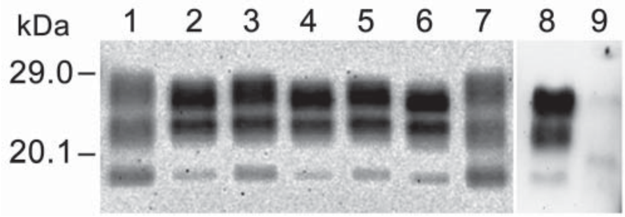

Figure 1. Western blot analysis of protease-resistant prion protein in the brain (thalamus/hypothalamus) and spleen of mouse lemurs inoculated with a cattle-derived L-type bovine spongiform encephalopathy (BSE) isolate by oral and intracerebral routes by using SHa31 monoclonal antibody against prion protein. Lanes 1, 7: cattle L-type BSE isolate (02-2528); lanes 2, 3: brain sample from intracerebral inoculation at 5 mg; lane 4: brain sample from oral inoculation at 50 mg; lanes 5, 6: brain sample from oral inoculation at 5 mg; lanes 8, 9: spleen samples from oral inoculation at 5 mg, positive and negative, respectively.

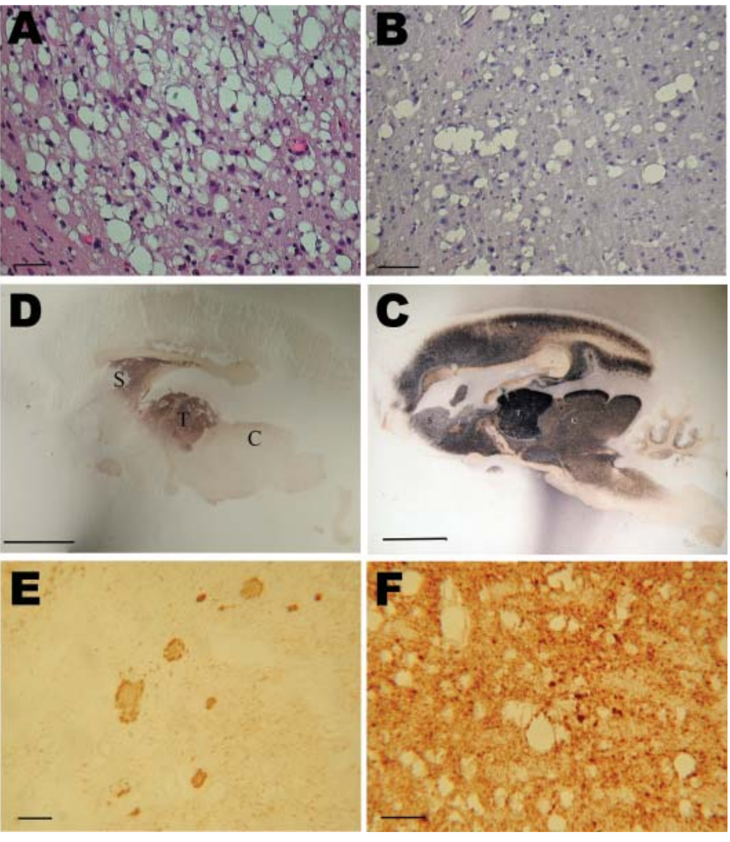

Figure 2. Histopathologic and disease-associated prion protein (PrPd) immunodetection in the brain of 2 mouse lemurs after intracerebral (5 mg) or oral (50 mg) inoculation with a cattle-derived L-type bovine spongiform encephalopathy isolate. A, B) Spongiosis in the striatum; scale bars = 30 μm. C, D) Paraffin-embedded tissue blot analysis of sagittal brain section; scale bars = 500 μm. E, F) PrPd immunodetection; scale bars = 30 μm. Analyses in C–F were performed by using the 3F4 monoclonal antibody against PrP. C, colliculus; S, striatum; T, thalamus.

reported in young macaques (*6*,*7*). A previous study of L-BSE in transgenic mice expressing human PrP suggested an absence of any transmission barrier between cattle and humans for this particular strain of the agent of BSE, in contrast to findings for the agent of classical BSE (*9*). Thus, it is imperative to maintain measures that prevent the entry of tissues from cattle possibly infected with the agent of L-BSE into the food chain.

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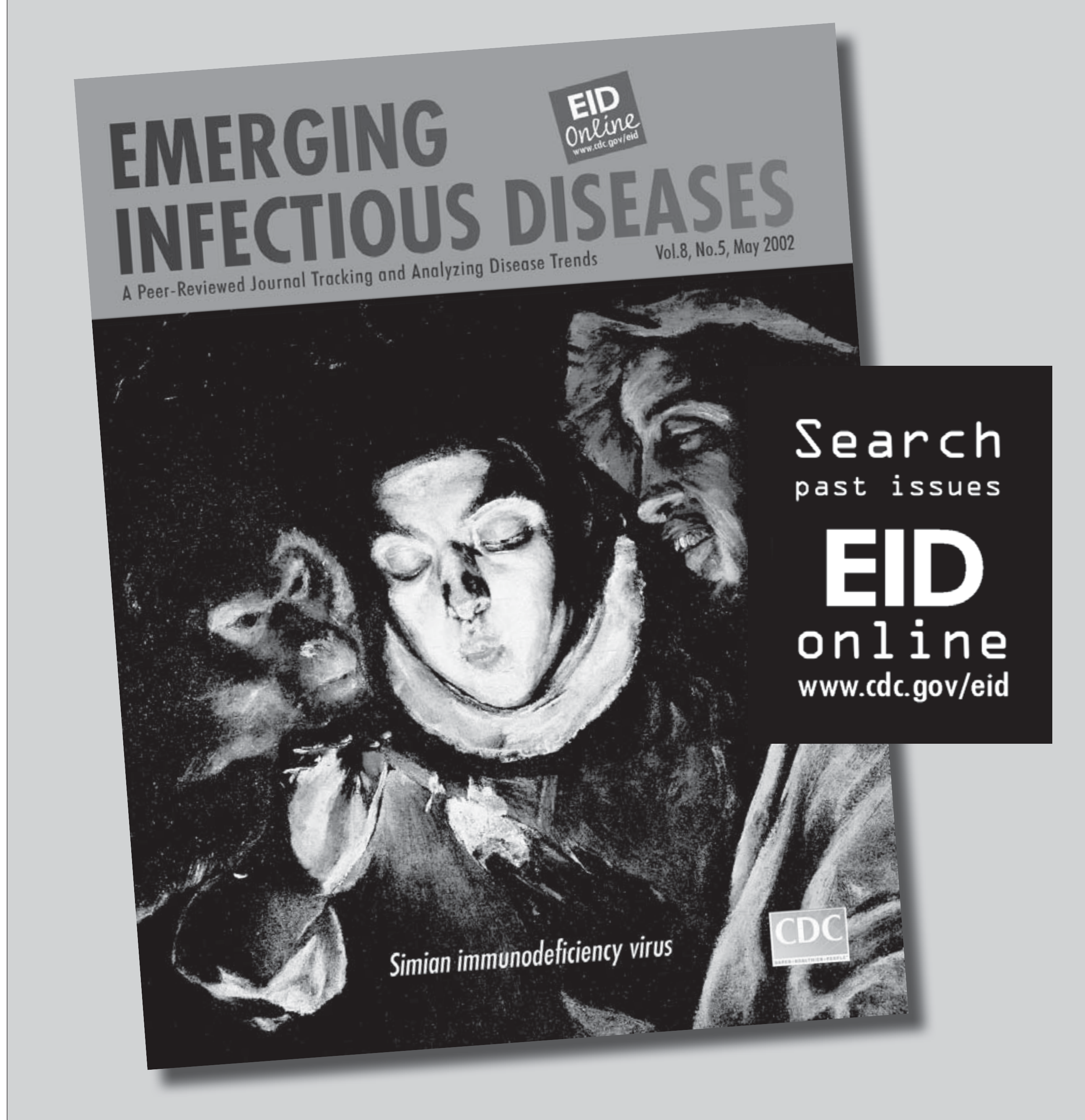

Early Detection of Pandemic (H1N1) 2009, Bangladesh

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To explore Bangladesh's ability to detect novel influenza, we examined a series of laboratory-confirmed pandemic (H1N1) 2009 cases. During June–July 2009, event-based surveillance identified 30 case-patients (57% travelers); starting July 29, sentinel sites identified 252 case-patients (1% travelers). Surveillance facilitated response weeks before the spread of pandemic (H1N1) 2009 infection to the general population.

After 2 children in North America were confirmed to have pandemic (H1N1) 2009 infections on April 17, 2009 (*1*), the virus rapidly spread throughout the world. By July 2, 2009, Southeast Asia had reported 1,866 cases (*2*). Officials worried about the effects of pandemic (H1N1) 2009 on the 147,030,000 million population (1,021 persons/km^2) of Bangladesh (*3*), where 41% of children <5 years of age are underweight (*4*). These concerns prompted Bangladesh to leverage 3 existing surveillance systems (*5*), preparedness plans, and personal protective equipment and oseltamivir stockpiles to guide the response to the pandemic.

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During April 2009, Bangladesh enhanced surveillance by implementing border screenings. Upon identification of pandemic (H1N1) 2009 in the general population, Bangladesh encouraged physicians to empirically treat patients who had acute respiratory infection with free oseltamivir if they had risk factors for complications from influenza (i.e., age <5 years or ≥65 years; diabetes; chronic heart, lung, or liver disease; asthma; neurologic, neuromuscular, hematologic, or metabolic disorders; immune suppression; cancer; obesity; pregnancy; danger signs [rapid, labored or noisy breathing, lethargy, cyanosis, inability to drink, or convulsion], or hospitalization) (*6*). We report the effects of this strategy on a case-series of laboratory-confirmed pandemic (H1N1) 2009 infection identified through enhanced surveillance.

The Study

During 2007, Bangladesh started event-based surveillance for the early detection of public health events of international concern. At 6 government and 6 private hospitals (Figure 1), physicians identified ≥2 epidemiologically linked severe acute respiratory infections, defined as subjective fever within the past 21 days and cough or sore throat (*5*), or severe pneumonia, defined as cough or difficulty breathing, chest in-drawing, stridor while calm, convulsions, inability to drink, lethargy, unconsciousness, or intractable vomiting. During April through November 2009, staff also administered ≈455,000 questionnaires to incoming land and air passengers, contacts, and referrals and collected throat and nasal swab specimens from those who reported cough, sore throat, or shortness of breath and had fever >38°C when assessed with thermal scanners.

During 2007, Bangladesh started sentinel-site surveillance for the early detection of novel influenza. During 2 days per month, physicians collected swab specimens from ambulatory case-patients at hospital clinics with influenza-like illnesses defined as sudden onset fever and cough or sore throat. Physicians also collected swab specimens from children <5 years of age hospitalized with severe pneumonia and person ≥5 years of age with severe acute respiratory infections (Figure 1).

To explore the epidemiology of seasonal influenza, community-based surveillance began in Bangladesh during 2004. Teams visited an estimated 6,600 preselected households 2×/week to identify acute respiratory infections, defined as the manifestation of 1 major sign (i.e., reported fever; rapid, labored or noisy breathing; lethargy; cyanosis; inability to drink; or convulsion) or 2 minor signs (i.e., cough, rhinorrhea, sore throat, muscle/joint pain, chills, headache, irritability, decreased activity, or vomiting). During 2008, Bangladesh also established a birth cohort of 334 children to explore the potential effects of influenza on

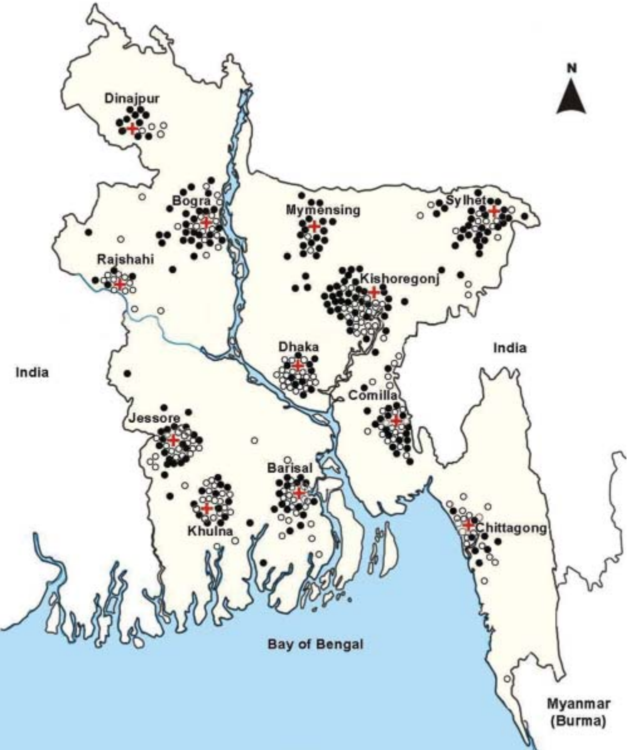

Figure 1. Location of 12 sentinel-site surveillance hospitals (red crosses) and of persons confirmed as infected with pandemic (H1N1) 2009 virus during June 2009–October 2010, Bangladesh. Open circles indicate case-patients identified during 2009; solid circles indicate case-patients identified during 2010. Testing was conducted on the basis of cases of severe pneumonia in hospitalized case-patients (<5 years of age), severe acute respiratory infection in hospitalized case-patients (≥5 years of age), influenza-like illness, and acute respiratory infection in ambulatory case-patients (all age groups) identified as part of event-based, sentinel-site, and community-based surveillance systems.

their development. Teams visited preselected households 2×/week to identify acute respiratory infections among children ≤2 years of age. At both sites, teams referred case-patients to physicians who collected nasal wash specimens and provided free care.

Laboratorians tested samples from the 3 surveillance systems for pandemic (H1N1) 2009 virus by using real-time reverse transcription PCR (*7*). Investigators shipped a convenience subset of 28 virus samples to the Centers for Disease Control and Prevention, Atlanta, for antiviral testing and strain characterization.

Investigators described the epidemiology, health-seeking, treatment, and outcome of case-patients who had laboratory-confirmed subtype H1N1 infection by using Pearson χ^2, Fisher exact, and Wilcoxon rank-sum tests when appropriate. To estimate case-fatality proportion, teams telephoned case-patients or their families ≥1 month after illness onset.

The Government of Bangladesh conducted enhanced event-based surveillance in the context of emergency response. Ethics committees approved sentinel and community-based surveillance protocols.

During June 2009–October, 2010, Bangladesh tested ≈500 passengers, 6 severe acute respiratory infection/severe pneumonia clusters, 5,000 persons identified by sentinel surveillance, and 6,000 persons identified by community-based surveillance and identified 1,371 laboratory-confirmed cases of pandemic (H1N1) 2009 infection (Table 1). During June–July 2009, most (29/30 [97%]) case-patients were identified through event-based surveillance; 17 (63%) were travelers. A rapid increase in the number of sentinel-site case-patients during July 2009 signaled the spread of pandemic (H1N1) 2009 to the general population (Figure 2).

Isolates from case-patients were antigenically related to A/California/07/2009 (H1N1) and sensitive to oseltamivir. Of the 1,271 case-patients at high risk for complications, 535 (42%) sought treatment within 72 hours of symptom onset, and 7 (3%) of 207 case-patients at sentinel hospitals received oseltamivir (Table 2). The proportion of treatment-eligible case-patients who received oseltamivir decreased from 100% to 0% from June 2009 to October 2010.

We identified 3 (2%) of 182 event-based and 25 (5%) of 527 sentinel-site decedents (p<0.001) (Table 2). Case-patients who subsequently died, sought treatment a median

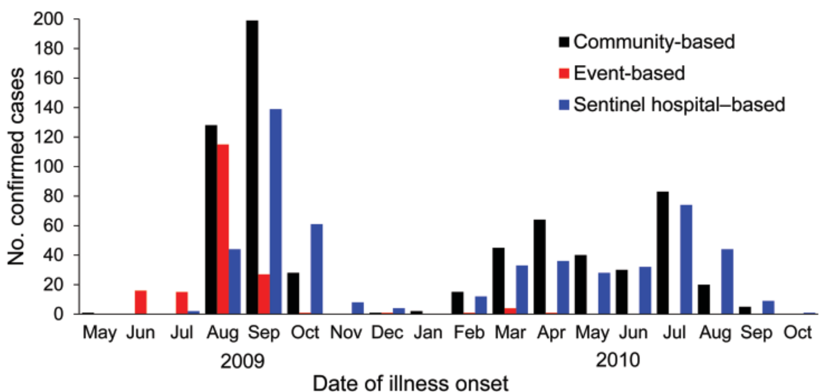

Figure 2. Date of onset of confirmed illness in case-patients with pandemic (H1N1) 2009 by surveillance platform, May 2009–October 2010, Bangladesh.

Table 1. Demographics of confirmed case-patients with pandemic (H1N1) 2009 virus by surveillance platform, Bangladesh, June 2009–October 2010

Surveillance type	Age, median (range)	No. (%) female patients	No. (%) travelers
Event-based, n = 182	24 y (4 mo–72 y)	71 (39)	51 (28)*
Sentinel-site, n = 527	20 y (1 mo–70 y)	182 (35)†	21 (4)
Kamalapur community-based, n = 621	6 y (3 mo–76 y)‡	303 (49)	0
Mirpur community-based, n = 41	16 mo (3–26 mo)	17 (41)	2 (5)

*Comparison between event-based vs. other surveillance sites, Pearson χ^2 p<0.0001.
†Comparison between sentinel-based vs. other surveillance sites, Pearson χ^2 p<0.0001.
‡Comparison between Kamalapur-based vs. other surveillance sites, rank-sum p<0.0001.

of 4 days (interquartile range 3–6 days) and received oseltamivir 12 days (interquartile range 5–14 days) after symptom onset compared with 3 days among survivors (p<0.001 and p = 0.01, respectively).

Integration of 3 influenza surveillance systems facilitated response. To delay the spread of the pandemic virus in the general population, Bangladesh used event-based surveillance to identify and treat infected travelers. When sentinel-sites signaled pandemic (H1N1) 2009 among the general population, the government distributed 3.4 million capsules of oseltamivir to hospitals, trained hospital leadership to presumptively treat case-patients with free oseltamivir, and mounted a risk-communication campaign to urge persons at risk for complications to seek care within 3 days of illness development. Officials targeted messages to avoid overwhelming Bangladesh's hospitals, where there are typically 11 hospitalized patients for every 10 beds (*8*). Meanwhile, officials continued to learn about the epidemiology of pandemic (H1N1) 2009 through population-based surveillance.

Despite government efforts, case-patients sought treatment late, and oseltamivir was underutilized. Less than half (42%) of high-risk patients sought care within 48 hours of disease onset, when oseltamivir is most efficacious. As in other studies (*9*), even severely ill persons who subsequently died were late in seeking treatment. Ill persons frequently were unfamiliar with risk communication messages and may have avoided the expense of seeking treatment. During 2009, only 34% of surveyed households recalled risk communication messages, none could identify oseltamivir (*10*), and those with a history of influenza-like illness paid an average of US $3 when seeking care (i.e., 9% of monthly household expenditure) (*11*).

While the government of Bangladesh provided initial case-patients with oseltamivir, community clinicians provided oseltamivir once pandemic (H1N1) 2009 had spread to the general population. Only a fraction of eligible case-patients then received oseltamivir. Possible explanations for the underutilization of oseltamivir include clinicians' lack of suspicion of influenza, awareness of treatment guidelines, familiarity with antiviral agents, access to oseltamivir stockpiles, or knowledge of the potential severity of pandemic virus.

Our findings are based on a small case series. Although we identified only 28 decedents, an ongoing study suggests that ≈6,000 persons died as a result of the pandemic (*12*). Nevertheless, we believe that our findings are generalizable to Bangladesh because hospitals were selected as sentinel sites to provide geographically representative data.

Table 2. Clinical description of case-patients infected with pandemic (H1N1) 2009 virus seeking treatment, by surveillance platform, Bangladesh, June 2009–October 2010

Description of case-patient	Event-based surveillance, n = 182 (%)	Sentinel-site surveillance, n = 527 (%)	Kamalapur community-based surveillance, n = 621 (%)	Mirpur community-based surveillance, n = 41 (%)
At high risk for complications from influenza illness	79 (43)	331 (63)	285 (4)	41 (100)
Preexisting conditions*	54 (30)†	182 (35)	22 (3)	0
Danger signs (i.e., difficulty breathing or shortness of breath)	50 (27)†	230 (44)	47 (8)	5 (12)
Treated with oseltamivir‡ when treatment indicated†	4/56 (84)	7/207 (3)	81/272 (30)	1/41 (2)
Median days from symptom onset to treatment with oseltamivir	2 (2–4)	5 (2–8)	4 (1–5)	5
Hospitalization	29 (16)†	259 (49)	0	0
Death	3 (2)†	25 (5)	0	0

*Preexisting conditions among event and sentinel site surveillance case-patients included asthma (71[10%]), chronic obstructive lung disease (31 [4%]), obesity (47 [7%]), immune suppression (20 [3%]), diabetes (12 [2%]), chronic heart disease (10 [1%]), neuromuscular disorders (10 [1%]), liver disease (10 [1%]), and hematologic disorders (8 [1%]), cancer (3 [0.4%]), and pregnancy (3 [0.4%]), while 2 community-based surveillance case-patients had immunosuppression (0.3%), 2 (0.3%) had asthma, and 1 (0.1%) had diabetes.
†Comparison between surveillance sites, Pearson χ^2 p<0.0001.
‡Oseltamivir 5 mg 2×/d for 5 days.

Conclusions

Bangladesh has an effective surveillance system in place for detection of emerging infectious diseases. In spite of timely surveillance, prompt risk communications and free oseltamivir, response may have been hampered by persons' delays in seeking treatment and by the underutilization of oseltamivir. Our investigation suggests the utility of diverse surveillance systems, the limitations of antiviral drug campaigns, and the importance of influenza prevention through vaccines (e.g., 15 million pandemic [H1N1] 2009 vaccine doses donated to Bangladesh during 2010) and nonpharmaceutical interventions. Such campaigns remain insufficiently used in low-income countries where vaccines are expensive, access to clean water is inadequate, and covering a cough is not customary (*13,14*).

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Human Herpesvirus 8 Seroprevalence, China

Tiejun Zhang, Xiaodan Shao, Yue Chen, Tao Zhang, Veenu Minhas, Charles Wood, and Na He

To summarize the seroprevalence of human herpesvirus 8 (HHV-8) in mainland China, we conducted a systematic review and meta-analysis based on available literature. Data show that differences in HHV-8 prevalence vary considerably among different ethnic groups and geographic regions. Blood-borne transmission could be a potential route for HHV-8 infection in China.

Human herpesvirus 8 (HHV-8) is the infectious etiologic agent associated with Kaposi sarcoma, primary effusion lymphoma, and multicentric Castleman disease. Worldwide seroprevalence of HHV-8 varies: generally low to moderate for populations in Western countries and Asia (*1–4*) but as high as 50% for the general population in sub-Saharan Africa and higher for HIV-positive populations (*5–7*). The transmission modes of HHV-8 may also differ in different geographic areas and subpopulations; sexual and nonsexual transmission have been described (*8–10*). Blood-borne transmission may exist, especially among intravenous drug users (IVDUs) and blood recipients (*11*).

The Ministry of Health of China, the United Nations Program on HIV/AIDS, and the World Health Organization estimate that ≈320,000 HIV/AIDS cases have been reported in China (*12*). However, the epidemiologic characteristics of HHV-8 infection, a severe HIV/AIDS opportunistic infection, have not been well described for China. Therefore, we conducted a systematic review and meta-analysis on the basis of available data for HHV-8 epidemiology from mainland China to have a better understanding of the prevalence, variation, and factors associated with its transmission.

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The Study

A comprehensive literature search of published studies indexed in global and databases in China during 1995–2010 was conducted. Initially, 125 reports published in English and 223 in Chinese concerning the seroprevalence in mainland China were identified. Among them, 85 articles published in England and 178 articles published in China were excluded after title and abstract screening. After reading the full text, we excluded another 33 English and 26 Chinese articles. Finally, 26 publications were included in this systematic review and have been summarized in online Technical Appendix Table 1 (wwwnc.cdc.gov/EID/pdfs/10-2070-Techapp.pdf). These studies were cross-sectional and were conducted in 8 of the 34 provinces. A substantial number (35.5%) of these studies were conducted in the Xinjiang Uygur Autonomous Region. Most samples tested were serum or plasma with few exceptions (1 whole blood, 1 peripheral blood mononuclear cells); sample sizes ranged from 37 to 4,461 (median 242, interquartile range 199–520). Overall, 18,547 participants were involved in the present analysis, and among them 15,913 were from the general population, 1,970 were immunocompromised patients, and 664 were IVDUs. Laboratory methods for all included studies were reported (19 detected HHV-8 by ELISA, 3 by PCR, and 4 by immunofluorescent assay.

The prevalence of HHV-8 pooled from reviewed studies was 11.3% (95% CI 7.2–15.5) for the general population, 22.2% (95% CI 12.7–31.8) for immunocompromised patients, and 31.2% (95% CI 27.7–34.7) for IVDUs. The prevalence among the general population was found to be the lowest in Guangdong Province and the highest in Xinjiang Province. A similar regional variation was found for immunocompromised persons. Among IVDUs, the prevalence was 34.3% (95% CI 28.3–40.3) in Zhejiang and 29.6% (95% CI 25.3–33.9) in Xinjiang Uygur Autonomous Region (online Technical Appendix Table 2; Figure).

Five studies, including 4,637 persons of Han ethnicity and 4,011 persons of ethnic minorities (2,040 Uygur, 1,169 Kazak, 200 Khalkas, 173 Hue, and 429 other) conducted in the Xinjiang Uygur Autonomous Region were analyzed for association of ethnicity with HHV-8 prevalence (online Technical Appendix Figure, panel A). The risk was significantly lower for the Han group than for other ethnic groups (odds ratio [OR] = 0.59, 95% CI 0.55–0.76). For the Han group, the pooled prevalence of HHV-8 in Xinjiang Uygur Autonomous Region was significantly higher when compared with that for other regions, 14.4% (95% CI 9.0–19.8) versus 6.4% (95% CI 4.1–8.6). Ten combined studies, with 5,716 male and 4,708 female participants, respectively, were included in meta-analysis of association between sex and HHV-8 infection (online Technical Appendix Figure, panel B).

There was no significant difference between the sexes: pooled OR 0.94 (95% CI 0.84–1.04).

Seven studies, with 863 HIV-positive patients and 3,438 negative controls, were included in the analysis. All studies yielded a significant difference in HHV-8 infection between HIV-positive and HIV-negative participants; ORs for individual studies ranged from 1.50 to 4.27, and the pooled OR was 2.97 (95% CI 2.22–3.97) (online Technical Appendix Figure, panel C). However, a significant publication bias was detected (Egger test p = 0.013; Begg test p = 0.016). A visual inspection of the funnel plot suggested that some large or small studies with negative or null results were not published (data not shown).

Few studies were designed to address the issue of possible transmission routes among the population of china. Six studies had information on possible blood transmission. Two blood transfusion studies and 4 studies of IVDUs included 837 persons who reported having been exposed to blood contact i.e., needle sharing and 1,397 who were never exposed (online Technical Appendix Figure, panel D). Substantial heterogeneity (I^2 87%, p<0.001, by test for heterogeneity) was detected among those studies; therefore, a random-effects model was used to estimate the OR. No publication bias was detected (Begg test p = 0.707; Egger test p = 0.363). OR showed a marginal association of HHV-8 prevalence with blood transfusion (OR 2.01, 95% CI 0.89–4.56) for possible blood transmission.

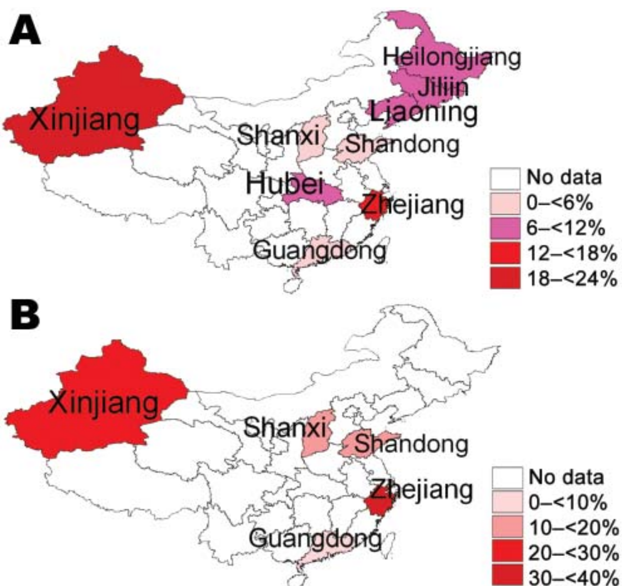

Figure. Regional distribution of pooled human herpesvirus 8 (HHV-8) prevalence in A) the general population and B) immunocompromised patients, China.

Conclusions

This systematic review indicated that HHV-8 prevalence in China varies in different regions. Pooling of data from 26 studies provided us with a large sample size, which is one of the strengths of the study. Also, we included studies that were published in the Chinese language and were not accessible to the international community. The results of this meta-analysis show that HHV-8 prevalence was higher in the Xinjiang Uygur Autonomous Region than other areas in general and among high-risk populations. Historically, Xinjiang Uygur Autonomous Region has been regarded as an area in which Kaposi sarcoma is endemic (*13*). Notably, geographic variations of HHV-8 infection within China are not well known and need to be investigated as well.

It has been well documented that HHV-8 prevalence is higher among HIV-infected persons (*14,15*). In mainland China, we found a 3-fold increase in HHV-8 infection among persons with HIV compared with HIV-noninfected persons. Given the rapid increase of HIV/AIDS cases in China, HHV-8 could become a severe public health issue in the future.

According to data from the Xinjiang Uygur Autonomous Region, minority groups were at higher risk for HHV-8 infection than the Han ethnic group. Although there was evidence for considerable heterogeneity among the studies, the association between ethnicity and HHV-8 risk showed that minorities were at higher risk for HHV-8 infection when compared with the Han ethnic group. The reasons behind this association are not well elucidated. Because all of the comparisons of HHV-8 difference between minority groups and the Han ethnic group are from the Xinjiang Uygur Autonomous Region, epidemiologic confirmation of this observation would require data from other regions, which is currently unavailable. Our analysis showed a marginally significant association between blood contact and HHV-8 infection; heterogeneity among studies was substantial. These data indicate that blood-borne transmission could occur among the Chinese population, a finding that is consistent with previous reports from other countries (*11*).

This study has some limitations. The studies included in this meta-analysis were not evenly distributed throughout China because information was not available from all the regions. Also, all of the studies might have used different methods for HHV-8 detection because of the lack of a standard assay; prevalence estimates may have been underestimated.

In summary, this meta-analysis clearly shows that the distribution of HHV-8 seroprevalence varies in China. The available information is still too limited to fully understand HHV-8 prevalence and the risk factors associated with transmission. Further studies are urgently needed to explore the epidemiology of HHV-8 infection in different subpopulations in China.

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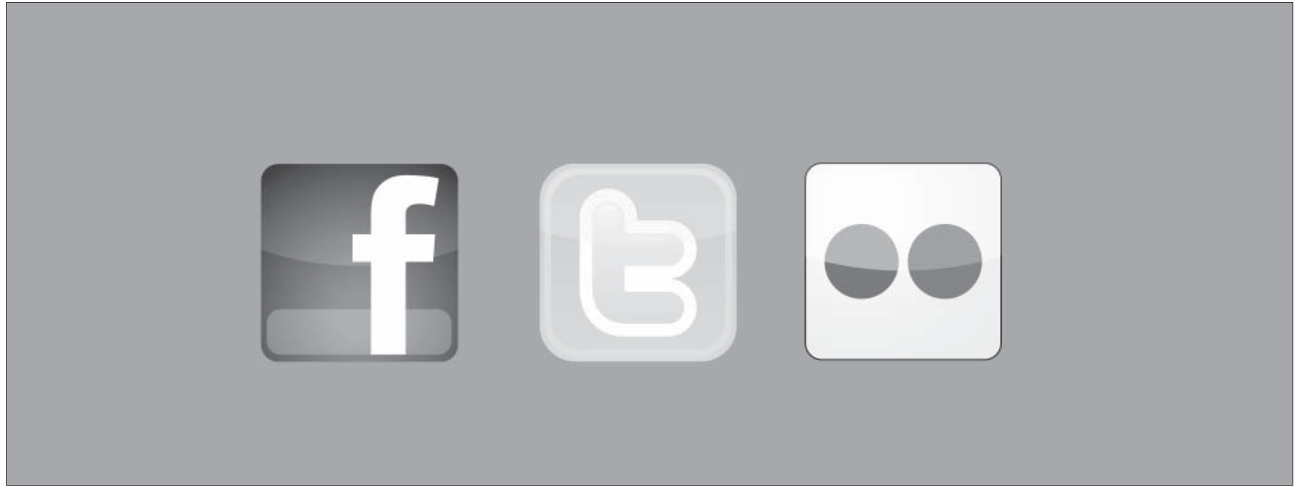

The Plague of Thebes, a Historical Epidemic in Sophocles' Oedipus Rex

Antonis A. Kousoulis, Konstantinos P. Economopoulos, Eff e Poulakou-Rebelakou, George Androutsos, and Sotirios Tsiodras

Sophocles, one of the most noted playwrights of the ancient world, wrote the tragedy Oedipus Rex in the first half of the decade 430–420 BC. A lethal plague is described in this drama. We adopted a critical approach to Oedipus Rex in analyzing the literary description of the disease, unraveling its clinical features, and defining a possible underlying cause. Our goals were to clarify whether the plague described in Oedipus Rex reflects an actual historical event; to compare it with the plague of Athens, which was described by Thucydides as occurring around the same time Sophocles wrote; and to propose a likely causative pathogen. A critical reading of Oedipus Rex and a comparison with Thucydides' history, as well as a systematic review of historical data, strongly suggests that this epidemic was an actual event, possibly caused by *Brucella abortus.*

> "Wailing on the altar stair, wives and
> grandams rend the air, long-drawn
> moans and piercing cries blent with
> prayers and litanies"—Sophocles,
> Oedipus Rex, lines 184–186

Sophocles is one of the most noted playwrights of the ancient world and, along with Aeschylus and Euripides, belongs to the trinity of the Attic tragedians who flourished during the golden century of Pericles in Athens (Figure 1). Sophocles lived between 496 and 406 BC; although he seems to have written 123 plays, only 7 have survived in a complete form (*1*). He lived his entire life in Athens and introduced many innovations in the dramatic arts (*1*).

The writing of the tragedy Oedipus the King (original Greek title Οιδίπους τύραννος, most commonly known as Oedipus Rex) is placed in the first half of the decade 430–420 BC. The play has been labeled an analytical tragedy, meaning that the crucial events which dominate the play have happened in the past (*2*,*3*).

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Oedipus Rex, apart from the undeniable literary and historic value, also presents significant medical interest because the play mentions a plague, an epidemic, which was devastating Thebes, the town of Oedipus' hegemony. Several sections, primarily in the first third of the play, refer to the aforementioned plague; the epidemic, however, is not the primary topic of the tragedy. The epidemic, in fact, is mostly a matter that serves the theatrical economy by forming a background for the evolution of the plot.

Given the potential medical interest of Oedipus Rex, we decided to adopt a critical perspective by analyzing the literary descriptions of the plague, unraveling its clinical features, defining the underlying cause, and discussing possible therapeutic options. The ultimate goals of our study were to clarify whether the plague described in Oedipus Rex could reflect an actual historical event, compare it with the plague of Athens, which was described by the historian Thucydides as occurring not long before the time that Sophocles' work appeared (*4*), and propose the most likely causative pathogen.

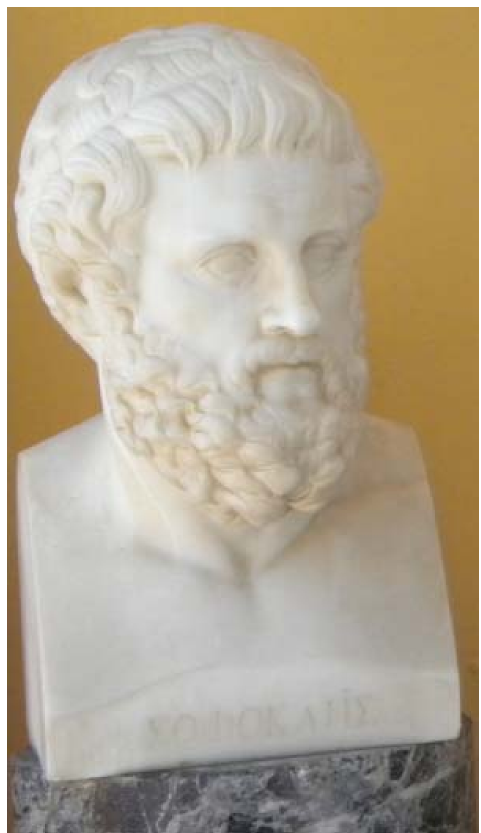

Figure 1. Bust of Sophocles in the Colonnade of the Muses in the Achilleion, Corfu, Greece, July 2011. Photo courtesy Antonis A. Kousoulis.

An Epidemic in Oedipus Rex

In the first scene of the play, Sophocles presents the basic social and historical axes around which he will unfold the plot. The devastating plague that dominates Thebes is presented to the audience through the dialogue between Oedipus and the Priest (lines 1–67) (*2*,*3*). The king has already taken some action to deal with this harm by sending his brother-in-law, Creon, to the oracle at Delphi to ask for a salvation plan (lines 68–72). The oracle announces that the plague is a result of religious pollution and that the god Apollo requests that the people of Thebes exile the previously unknown "miasma" (a word of Greek origin with a sense of moral noxious pollution) away from the town (lines 96–98) (*2*,*3*). Oedipus asks the citizens to stop praying and focus on finding the cure (lines 142–146) (*2*,*3*). In lines 167–215, the Chorus stays on stage to summarize the situation and beg for salvation (*2*,*3*).

Searching for the miasma, Oedipus summons the blind prophet Tiresias to reveal who is responsible for this evil (lines 300–313) (*2*,*3*). At the moment that Tiresias reveals to Oedipus that the king himself is the cause of the plague (lines 350–353), the epidemic becomes a secondary issue, and, as a result, there are only occasional references to the plague during the remainder of the play (lines 665–666, 685–686, 1380–1383, 1424–1428) (*2*,*3*).

Figure 2. Scene from a National Theatre of Greece production of Oedipus Rex at the Odeon of Herodus Atticus, Athens, Greece January 1995. Photo courtesy Effie Poulakou-Rebelakou.

Therefore, although the first part of the play is rife with references to the plague and its consequences, in the second part there are only sporadic referrals to the epidemic. The fate of Oedipus emerges as a truly tragic one, not so much because he caused the plague, but because of the character's own personal tragedy (Figure 2).

A Medical Critical Approach to Oedipus Rex

From the start of the drama, the plague in Thebes is a serious matter, as in line 23 where it is referred to as "weltering surge of blood" (φοινίου σάλου). In line 28 the word plague (λοιμός) appears for the first time, with the Greek word for disease (νόσος) being used in lines 150, 217, and 303 (*2*,*3*).

Sophocles describes the main characteristics of the epidemic through sporadic sentences. Early in the play it is clarified that the disease is a cattle zoonosis of high mortality rate ("a blight upon the grazing flocks and herds," line 26, with the herds being cattle,) (*2*,*3*). The lethality of this epidemic is particularly terrifying for the protagonists of the play, and the disease's severity is evinced by the first sentences of the tragedy ("reek of incense everywhere," line 4). Oedipus fears mass destruction of the city of Thebes ("with the god's good help success is sure; 'tis ruin if we fail," line 146), while the words "weltering surge of blood" (line 24), "fiery plague" (line 166), "the land is sore distressed" (line 685), and "wailing on the altar stair, wives and grandams rend the air, long-drawn moans and piercing cries blent with prayers and litanies" (lines 184–186) (*2*,*3*) all illustrate vividly the severity of the situation.

The references to the decline of land and fields could be an example of poetic exaggeration or a suggestion that the fruits or ears may participate in the transmission route of the plague ("a blight is on our harvest in the ear," line 25) (*2*,*3*). Regarding the specific clinical features of the disease, it is clear that the causative pathogen leads to miscarriages or stillbirths ("a blight on wives in travail," lines 26–27, meaning women give birth to dead babies) (*2*,*3*). The plague's effects are also pointed out by the Chorus: "earth her gracious fruits denies" and "women wail in barren throes" (lines 151, 215) (*2*,*3*).

Lines 179–181 turn out to be of high interest: "wasted thus by death on death all our city perishes; corpses spread infection round" (*2*,*3*). A word with a meaning of something that brings death is used in the original Greek (θαναταφόρα) to refer to the plague, which suggests that at the time of Sophocles his fellow Greeks were aware of the threat posed by infectious disease. The knowledge of the existence of a highly contagious and fatal disease is phrased clearly in these rhymes, strongly suggesting that Thebans were aware of the oncoming—most possibly from the adjacent city

of Athens—danger (*2–4*). This hypothesis regarding the source of the disease seems the most reasonable in medical terms, contrary to the philological approach, which declares that the epidemic derived from the gods.

In addition, the Chorus provides us with a major social aspect, as they put the blame on god of war, Ares ("Ares whose hot breath I feel, though without targe or steel he stalks, whose voice is as the battle shout," lines 190–191) (*2*,*3*). It is not quite clear why Ares is being called responsible for this plague, since there is no other such reference in the play. In fact, it is noteworthy that there is no other historic or poetic reference that links Ares to the spreading of a disease (*4*). However, Thucydides' correlation of the plague of Athens with the Peloponnesian War (431–404 BC) (*5*) gives us the opportunity to state that Sophocles connects this epidemic of Thebes with the plague of Athens and attempts to point out the disastrous effects wars always have.

Regarding the play's approach to treatment of the disease, reading through the drama we once again come across with the theocratic perceptions of ancient Greece. The citizens have become suppliants to the monuments of the gods, asking for mercy ("Why sit ye here as suppliants, in your hands branches of olive filleted with wool?," lines 2–3; "the common folk, with wreathed boughs crowd our two market-places, or before both shrines of Pallas congregate, or where Ismenus gives his oracles by fire," lines 19–21) (*2*,*3*). Consequently, a solution for the situation is requested from the oracle at Delphi (lines 68–72), while the Chorus plead for Athena, Zeus, Artemis, and Apollo to save the town from the disaster (lines 160–165) (*2*,*3*). The aforementioned aspects strongly support the notion that the disease was incurable at this time.

Possible Pathogens Responsible for the Plague in Thebes

The pathogen of the plague described in Oedipus Rex reflects the complexity of every historically emerging zoonosis. Any proposed pathogen should be a highly contagious, zoonotic disease of cattle that causes stillbirth, miscarriages, and infertility, is characterized by high mortality rates, and has the potential to have caused an epidemic in the 5th century BC. The characteristics of pathogens that might be responsible for the plague on the basis of Sophocles' descriptions in Oedipus Rex are summarized in the online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/11-0449-Techapp.pdf).

After a close inspection of the characteristics, the pathogens that include most (5 of 7) of the features described by Sophocles in Oedipus Rex are *Leishmania* spp., *Leptospira* spp., *Brucella abortus*, *Orthopoxviridae*, and *Francisella tularensis*. Among the diseases caused by these pathogens that can affect humans are the following: 1) tularemia, which is a disease mainly transmitted through rabbits; 2) smallpox, which is not a cattle zoonosis; 3) leishmaniasis, which is not a highly contagious disease; and 4) leptospirosis, which has been associated with epidemics after rainfall and flooding in relation to rodent infestation. Thus, the most probable cause of the plague in Thebes is *B. abortus*. Brucellosis is a highly contagious zoonosis caused by ingestion of unsterilized milk or meat from infected cows or close contact with their secretions. The mortality rate for untreated brucellosis is difficult to determine from the literature of the preantibiotic era (*6*); nevertheless, an 80% rate has been reported in situations of comorbidity with endocarditis (*7*). Epidemics, stillbirths, and miscarriages caused by *B. abortus* have been reported since the time of Hippocrates, which is when this disease was initially described.

However, taking into account that in modern times brucellosis in humans is a severe granulomatous disease characterized by extremely rare direct transmission from person-to-person, insidious onset in sporadic cases (mainly among veterinarians), and low mortality rates, it may be difficult for 21st century physicians and veterinarians to accept *B. abortus* as the causative agent of the plague of Thebes. Alternatively, the plague of Thebes could be a composite of ≥2 causative agents, as it has been suggested for the contemporary plague of Athens (*6*,*7*). In this case scenario, we could assume that cattle in Thebes may have been having brucellosis, leptospirosis, or listeriosis, while humans could have been affected by a different pathogen such as *Salmonella enterica* serovar Typhi (*8*,*9*). It should be noted that exploring the diseases of history requires examining the social, economic, and demographic aspects of each era because this is the only way to better understand how diseases work over centuries (*10*). Finally, we cannot reject the possibility of dealing with a *Brucella* strain that has evolved to become less deadly than a more lethal ancestor (*6*).

Thucydides' Plague of Athens and Sophocles' Epidemic

The plague that is described in Oedipus Rex could possibly be related to the plague that struck Athens in 430–429 BC (*11*), the primary source for which is the papers of historian Thucydides (where he refers to an epidemic that has been named the plague of Athens) (*5*). The following 5 points support this correlation.

Proximate Eras

The first writing of Oedipus Rex most probably took place during the time of the plague of Athens. Sophocles' epidemic seems to have enough strength to appear as a historical base on which the theatrical economy of the play is evolving (*12*). The opening of the drama, with the city of Thebes in the midst of plague has often been, historically,

taken as a reference to the plague that devastated Athens in the opening years of the Peloponnesian War and has been used to assist in the dating of this play (*13*).

Similar Descriptions by Thucydides and Sophocles

Thucydides and Sophocles use similar terms when describing attempts to deal with the epidemic. In the historical case (Athens) and the dramatic case (Thebes), the populace turned to the temples looking for a divine solution to the disaster.

Correlation with Recent Warfare

As mentioned above, in Sophocles' drama, god of war Ares gets the blame for the plague (lines 190–191). The particularity of this reference (*4*), it seems that Sophocles correlates the epidemic that strikes Thebes with the plague of Athens, which, according to Thucydides, came about as a result of the Peloponnesian War (*5*).

Similarities Regarding the Nature of the Diseases

It is difficult to compare a historical record to a poetic drama, but, keeping that in mind, both Sophocles and Thucydides refer to animal illness and death (*2*,*8*). In addition, the realistic descriptions of the historian and the nightmarish lyrical rhymes of the poet, talk about a disease with a high mortality rate (*2*,*10*). As for the clinical features, although Thucydides does not mention the pregnancy or labor pains as described in Sophocles' text, he does refer to abdominal and vulvovaginal symptoms (*5*).

Common Assumptions about the Possible Causative Pathogen

Historical medical literature has suggested many infectious diseases over time, but few have lasted as the most probable. These diseases mainly include typhoid fever, epidemic typhus, smallpox, plague, measles, and influenza, all of which could be initial candidates for the plague in Oedipus Rex and have been taken into account in this study (online Technical Appendix) (*9*,*10*).

Although the above points are of great relevance, they lack the possibility of historical verification and are mainly based on the comparative and critical assessment of Sophocles' and Thucydides' work. Historical certainty can be added by studying the alliances and the warfare involving Thebes during the era of Sophocles (e.g., Boeotian prefecture, Athenian dynasty, Spartan alliance, Persian wars) (*14*). Bearing in mind the aforementioned observations and the fact no other epidemics were reported in the eastern Mediterranean during the 5th century BC, we posit that the plague described by Sophocles in the tragedy Oedipus Rex has an actual basis in the plague of Athens described by Thucydides in his histories.

Discussion

A severe plague is described in Sophocles' drama Oedipus Rex. According to the World Health Organization, an epidemic is defined as a disease outbreak and, therefore, the occurrence of cases of disease in excess of what would normally be expected in a defined community, geographic area, or season (*15*). Thus, according to the tragedy's rhymes and with respect to literary talk, this plague should be treated as an epidemic.

It would not be irrational to trust the historical credibility of a literary text. Literature usually reflects the echo of the past. A somewhat similar example is that of archeologist Heinrich Schlieman; before Schlieman, the writings of Homer had been considered a collection of mythological poems. However, with his excavations in Troy, which used the Iliad as a guide, Schlieman provided a perfect example of how literary work can have a factual base (*16*).

Moreover, we could not overlook that Sophocles is the most realist of the Greek tragedians (*1*), and ancient tragedies were often placed into a historical frame strongly influenced by major contemporary events (*13*). Finally, although many of the features of the plot and passages have been interpreted as historical allusions, the plague seems to be recognized as the most critical element that reflects a historical event, with enough strength and clarity to be used even for the dating of the tragedy (*12*).

Conclusions

The critical reading of Oedipus Rex, its comparison with Thucydides' history, as well as the systematic review of the existing historical data, lead us to strongly suggest that this epidemic, for which the name Plague of Thebes may be used, was an actual historical fact, likely caused by *B. abortus*. With the deadly plague, which struck one of the most historic Greek cities, on the one hand and the tragic fate of a character who has become among the most recognizable in world theater on the other, Sophocles masterminded a dramatic frame and offered a lyrical, literary description of a lethal disease. As the protagonist approached his tragic catharsis, the moral order much desired by the ancient Greeks was restored with the end of the epidemic.

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etymologia

Prion

[pri′on, pre′on]

From *pro*tein + *in*fection. Nobel laureate Stanley B. Prusiner, American neurologist and biochemist, coined the word prion in 1982 to describe the noninfectious agents he proposed as the cause of scrapie. Prusiner noted that, "Because the dominant characteristics of the scrapie agent resemble those of a protein, an acronym is introduced to emphasize this feature… the term 'prion' (pronounced *pree-on*) is suggested." Prions are now recognized as etiologic agents of other transmissible spongiform encepalopathies, including bovine spongiform encephalopathy and Creutzfeld-Jakob disease.

Sources: Dorland's Illustrated Medical Dictionary. 31st ed. Philadelphia: Saunders; 2007; The Nobel Prize in Physiology or Medicine 1997: Stanley B. Prusiner [cited 2011 Oct 25]; http://nobelprize.org/nobel_prizes/medicine/laureates/1997/prusiner-autobio.html; Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science. 1982;216:136–44. doi:10.1126/science.6801762

Novel Prion Protein in BSE-affected Cattle, Switzerland

To the Editor: Bovine spongiform encephalopathy (BSE) is a feed-borne prion disease that affects mainly cattle but also other ruminants, felids, and humans (*1*). Currently, 3 types of BSE have been distinguished by Western immunoblot on the basis of the signature of the proteinase K–resistant fragment of the pathologic prion protein (PrP^{res}): the classic type of BSE (C-BSE) and 2 so-called atypical types of BSE with higher or lower molecular masses of PrP^{res} (H-BSE and L-BSE, respectively) (*2*). C-BSE is transmitted to cattle by ingestion of contaminated meat-and-bone meal, a feed supplement produced from animal carcasses and by-products. H-BSE and L-BSE have been identified by active disease surveillance, and incidence in aged cattle is low; but little is known about their epidemiology, pathobiology, and zoonotic potential (*3*). We describe 2 recent cases of BSE in aged cattle in Switzerland in which a PrP^{res} phenotype distinct from those of C-, L- and H-BSE was unexpectedly displayed.

In April 2011, an 8-year-old cow (cow 1) died of accidental injury, with no apparent precedent clinical signs, on a farm in the canton of St. Gallen, Switzerland. In the context of active surveillance for BSE, the medulla oblongata was tested and found to be BSE positive by using the PrioStrip test (Prionics AG, Schlieren, Switzerland), a lateral-flow immunochromatographic assay for detection of PrP^{res}. One month later, another cow (cow 2), 15 years of age, in the canton of Berne, Switzerland, was slaughtered because of a hind limb fracture. Information on this animal's health status before death was unavailable. Statutory testing of the medulla oblongata gave a BSE-positive result by using the Prionics Check Western, a rapid Western blot technique (*4*). Medulla oblongata samples from the 2 animals were forwarded to the National Reference Laboratory for confirmatory testing.

In accordance with the guidelines of the World Organisation for Animal Health (*5*), BSE was confirmed for each animal by positive test results in independent, approved screening tests, of which 1 must be a Western blot (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/18/1/11-1225-Techapp.pdf). Because the tissues were severely autolyzed, target structures for the diagnosis of BSE could not be identified, and histopathologic and immunohistochemical results were inconclusive.

The Prionics Western blot detected a similar 3-band PrP^{res} glycoprofile with molecular masses of roughly 16, 20, and 25 kDa for each animal, lower than equivalent PrP protein bands detected in animals with C-BSE (Figure). Sequencing of the open reading frame of the *PRNP* gene of cow 2 (which was unsuccessful for cow 1) indicated that the encoded protein was identical to the common bovine PrP amino acid sequence (as translated from GenBank accession no. AJ298878) and therefore was not likely to account for the differences observed by Western blot testing.

We next investigated which region of the prion protein was present in these abberant PrP^{res} fragments by probing with a panel of antibodies in the Western blot that bind to different regions of the prion protein (online Technical Appendix). PrP^{res} in cows 1 and 2 was readily detected by antibodies Sha31, 94B4, and JB10. By contrast, antibody 9A2, which maps to the PrP^{res} N terminus, bound only to PrP^{res} in samples from animals with C-, L- and H- BSE but not in samples from cows 1 and 2.

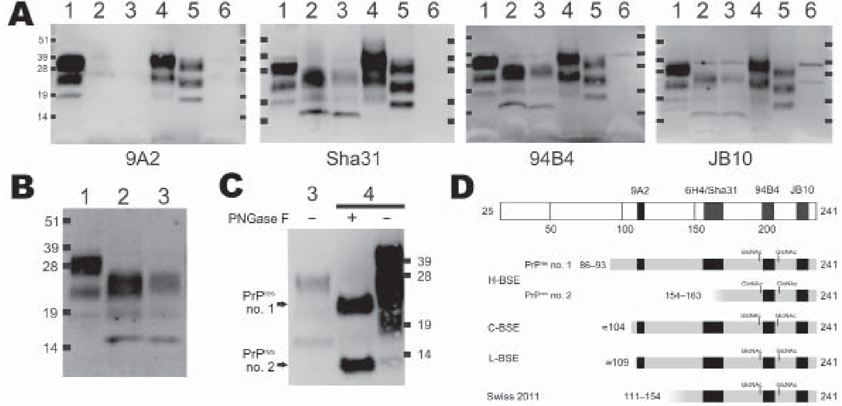

Figure. Molecular typing of the pathologic prion protein from 2 cows with bovine spongiform encepalopathy (BSE), Switzerland. A) Epitope mapping using antibodies 9A2, Sha31, 94B4, and JB10. B) Confirmatory Western blotting using antibody 6H4. C) Comparison PrP^{res} in cow 2 and H-BSE with (+) and without (–) deglycosylation with PNGaseF (antibody 94B4, sodium sulfate–polyacrylamide gel electrophoresis (PAGE) with NuPAGE MES instead of NuPAGE MOPS running buffer [Invitrogen,Carlsbad, CA, USA]). PrP^{res} 1 and 2 in H-BSE samples are indicated. Molecular mass standards are shown in kiloDaltons. D) The illustration at the top represents the full-length, mature bovine prion protein and the binding sites of the antibodies used for epitope mapping (black boxes). N-terminal and C-terminal residues are indicated by numbers. PrP^{res} fragments are partially mono- and di-glycosylated, which results in the characteristic 3-band patterns in the Western immunoblot. Sites of N-linked glycosylation are shown at positions 192 and 208 (-GlcNAc). C-BSE, classic BSE; cow 1, an 8-year-old BSE-positive cow; cow 2, a 15-year-old BSE-positive cow; PrP^{res}, proteinase K–resistant fragment of the prion protein; H-BSE, atypical BSE with higher molecular mass of PrP^{res}; L-BSE, atypical BSE with lower molecular mass of PrP^{res}. Lane 1, C-BSE; lane 2, cow 1; lane 3, cow 2; lane 4, H-BSE; lane 5, L-BSE; lane 6, negative. A description of the methods is provided online (www.cdc.gov/EID/article/18/1/11-1225-F1.htm).

The molecular masses of the PrP^{res} moieties from the 2 cows were also clearly distinct from those from controls with L- and H-BSE (Figure). For samples from animals with H-BSE, enzymatic deglycosylation demonstrated PrP^{res} subtypes, 1 and 2, the latter being a C-terminal PrP^{res} fragment of ≈12–14 kDa (*6*). To investigate whether the novel PrP^{res} type corresponds to PrP^{res} subtype 2, we compared samples from cow 2 with those from the H-BSE control by Western blot. The PrP^{res} type from the 2 cows reported here and PrP^{res} subtype 2 from the H-BSE control were indeed distinct (Figure).

We report a novel PrP^{res} signature in 2 cows with BSE diagnoses determined according to established criteria. Combining Western blot analysis with an epitope mapping strategy, we ascertained that these animals displayed an N terminally truncated PrP^{res} different from currently classified BSE prions (Figure). The interpretation of these findings remains difficult because neuropathologic and systematic clinical data for the 2 cases are not available. Moreover, the tissue samples were autolyzed, and the question of whether this affected the PrP^{res} molecular signature is of concern. Nonetheless, our findings raise the possibility that these cattle were affected by a prion disease not previously encountered and distinct from the known types of BSE. To confirm this possibility and to assess a potential effect on disease control and public health, in vivo transmission studies using transgenic mouse models and cattle are ongoing. Until results of these studies are available, molecular diagnostic techniques should be used so that such cases are not missed.

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Hantavirus in Bat, Sierra Leone

To the Editor: Hantaviruses (family *Bunyaviridae*) are transmitted from rodent reservoirs to humans. These viruses cause life-threatening human diseases: hantavirus cardiopulmonary syndrome in the Americas and hemorrhagic fever with renal syndrome in Asia and Europe (*1*). Since 2006, indigenous hantaviruses were reported also from Africa. Sangassou virus was found in an African wood mouse (*Hylomyscus simus*) in Guinea (*2*). Discovery of newer African hantaviruses, Tanganya virus and recently Azagny virus, was even more surprising because they were found in shrews (*3,4*).

The detection of hantaviruses in small mammals other than rodents, such as shrews and also moles (*4*), increasingly raises questions regarding the real hantavirus host range. Bats (order Chiroptera) are already known to harbor a broad variety of emerging pathogens, including other bunyaviruses (*5*). Their ability to fly

and social life history enable efficient pathogen maintenance, evolution, and spread. Therefore, we conducted a study on hantaviruses in bats from Africa.

A total of 525 tissue samples from 417 bats representing 28 genera were tested for the presence of hantavirus RNA. Samples originated from different regions in western and central Africa and were collected during 2009 and early 2011. Total RNA was extracted from tissue samples and reverse transcribed. cDNA was screened by PCR specific for sequences of the large genomic segment across the genus *Hantavirus* (*2*).

One sample yielded a product of the expected size and was subjected to cloning and sequencing. The positive sample (MGB/1209) was obtained from 1 of 18 investigated slit-faced bats (family Nycteridae). The animal was trapped at the Magboi River within Gola National Park, Sierra Leone (7°50.194′N, 10°38.626′W), and the identification as *Nycteris hispida* has been verified with the voucher specimen (RCJF529). Histologic examination of organs of the animal showed no obvious pathologic findings.

The obtained 414-nt sequence covers a genomic region, which was found to correspond to nt position 2,918–3,332 in the large segment open reading frame of prototypic Hantaan virus. Bioinformatic analysis on the amino acid level showed highest degrees of identity to shrew- and mole-associated hantaviruses (Thottapalayam virus 73.0%, Altai virus 69.7%, Nova and Imjin virus 69.3%). On the basis of tree topology of a maximum-likelihood phylogenetic tree, the sequence does not cluster with rodent-associated hantaviruses but groups with those found in shrews and moles (Figure).

Considering that bats are more closely related to shrews and moles than to rodents (*6*), a certain genetic similarity of a putative bat-borne hantavirus with shrew- and mole-associated hantaviruses seems reasonable. Notably, shrew-associated Thottapalayam virus (India) and Imjin virus (South Korea) seem to be closer relatives, and African Tanganya virus (Guinea) and Azagny virus (Côte d'Ivoire) are more distantly related. Additional sequence data is needed for more conclusive phylogenetic analyses.

Because the new amino acid sequence is at least 22% divergent from those of other hantaviruses, we conclude that the bat was infected with a newly found hantavirus. We propose the putative name Magboi virus (MGBV) for the new virus because it was detected in an animal captured at the Magboi River in Sierra Leone. The MGBV nucleotide sequence is novel and has not been known or handled before in our laboratory. Before this study, hantavirus nucleic acid was found in lung and kidney tissues of bats from

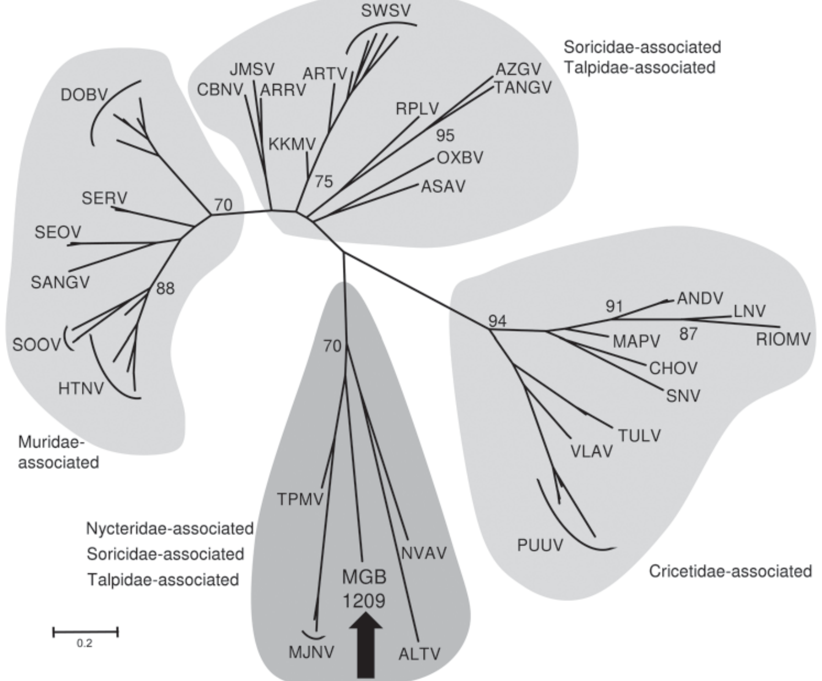

Figure. Maximum-likelihood phylogenetic tree of MGB/1209 virus based on partial large segment sequence (414 nt) and showing the phylogenetic placement of the novel sequence from *Nycteris* spp. bat compared with hantaviruses associated (i) with shrews and moles: Altai virus (ALTV), Artybash virus (ARTV), Asama virus (ASAV), Ash River virus (ARRV), Azagny virus (AZGV), Camp Ripley virus (RPLV), Cao Bang virus (CBNV), Imjin virus (MJNV), Jemez Springs virus (JMSV), Kenkeme virus (KKMV), Nova virus (NVAV), Oxbow virus (OXBV), Seewis virus (SWSV), Tanganya virus (TGNV), Thottapalayam virus (TPMV), and (ii) with rodents: Andes virus (ANDV), Choclo virus (CHOV), Dobrava-Belgrade virus (DOBV), Hantaan virus (HTNV), Laguna Negra virus (LNV), Maporal virus (MAPV), Puumala virus (PUUV), Rio Mamore virus (RIOMV), Sangassou virus (SANGV), Seoul virus (SEOV), Serang virus (SERV), Sin Nombre virus (SNV), Soochong virus (SOOV), Tula virus (TULV), Vladivostok virus (VLAV). The list of the accession numbers used in the analysis is available from the authors upon request. The tree was computed by using MEGA5 (http://www.megasoftware.net). The Tamura 3-parameter model with gamma-distributed rate heterogeneity and a proportion of invariant sites (T92 +G + I) was selected as the best fit evolutionary model according to the Baeysian information criterion calculated with MEGA5. The values at the tree branches are the bootstrap support values calculated from 500 replicates. Scale bar indicates an evolutionary distance of 0.2 substitutions per position in the sequence. The gray areas indicate association of hantaviruses with reservoir host families. The MGB/1209 partial sequence of the large genomic segment was deposited in GenBank under accession no. JN037851.

the genera *Eptesicus* and *Rhinolophus* in South Korea. However, nucleotide sequencing showed the presence of prototypical Hantaan virus indicating a spillover infection or laboratory contamination (*7*).

Further screening is necessary to confirm *N. hispida* as a natural reservoir host of the virus. Although the presented bat-associated sequence is obviously distinct from other hantaviruses, which suggests association with a novel natural host, a spillover infection from another, yet unrecognized host cannot be ruled out. However, detection of the virus exclusively in 1 organ (lung but not in liver, kidney, and spleen; data not shown) suggests a persistent infection that is typically observed in natural hosts of hantaviruses (*8*).

To date, only a few reports exist on cases of hemorrhagic fever with renal syndrome in Africa (*9,10*). However, underreporting must be assumed because the symptoms resemble those of many other febrile infections. Moreover, in cases of infections by non–rodent-associated hantaviruses, cross-reactivity with routinely used rodent-borne virus antigens should be limited and may hamper human serodiagnostics (*1*). The results suggest that bats, which are hosts of many emerging pathogens (*5*), may act as natural reservoirs for hantavirus. The effect of this virus on public health remains to be determined.

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Outbreak of Porcine Epidemic Diarrhea in Suckling Piglets, China

To the Editor: Beginning in October 2010, porcine epidemic diarrhea (PED), caused by a coronaviral infection affecting pigs, emerged in China in an outbreak characterized by high mortality rates among suckling piglets. The outbreak overwhelmed >10 provinces in southern China, and >1,000,000 piglets died. This outbreak was distinguished by ≈100% illness among piglets after birth (predominantly within 7 days and

sometimes within only a few hours) and death rates of 80%–100% (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/pdfs/11-1259-Techapp.pdf). Few sows or boars showed any clinical signs during the outbreak, which is not consistent with a recent report from Thailand (*1*). In that outbreak during late 2007, pigs of all ages were affected, exhibiting different degrees of diarrhea and no appetite. We characterized the genetic variation of the PED virus (PEDV) that caused a large-scale outbreak in China during 2010–2011 and compared it with viruses in other outbreaks. We also report a possible novel transmission pathway for PEDV.

A total of 177 samples (intestine, stool, and maternal milk) were collected from pigs from different farms who had diarrhea; 100% of farms had ≥1 porcine sample positive for PEDV. A total of 125/177 porcine samples were confirmed as positive for PEDV by reverse transcription PCR using primers as described (*2*). PEDV was detected in 105 (82.0%) of 128 fecal samples and 20 (40.8%) of 49 sow milk samples. Piglets infected with PEDV showed mild hemorrhage, undigested curdled milk in the stomach, and thin-walled intestines with severe mucosal atrophy and foamy fluid (data not shown).

The spike (S) gene of the family *Coronaviridae* has a high degree of variation and can induce neutralizing antibody (*3*). Reverse transcription PCR products of the 651-bp partial S gene of PEDV and the deduced amino acid sequences were aligned by using ClustalW (www.genome.jp/tools/clustalw), and a neighbor-joining tree with 1,000 bootstraps was constructed. Sequences of the S genes from this outbreak were 99.1%–100.0% homologous and had 88.7%–98.9% nt identity with all reference strains (online Technical Appendix Table 2), 98.5%–98.9% with Thailand strains, and 94.5%–95.1% with vaccine strain CV777. The partial S gene deduced amino acid sequences were compared and also showed a high degree of homology (98.0%–100.0%); they had 85.3%–98.7% identity with all reference strains listed in online Technical Appendix Table 2, 98.0%–98.7% with Thailand strains, and 93.3%–94.7% with vaccine strain CV777 (data not shown).

Phylogenetic analysis indicated that the PEDV in the China outbreak was different from foreign and other domestic strains on the basis of the reported partial S gene sequences. All new strains were clustered in the same branch, close to the cluster of Thailand strains, and far from the cluster of vaccine strain CV777 (Figure).

In the China outbreak, PEDV caused severe diarrheal disease in piglets; heavy economic losses in many provinces resulted, despite use of commercial vaccines (inactivated transmissible gastroenteritis [TGEV H] and porcine epidemic diarrhea [CV777]). To determine why the vaccines showed poor efficacy, we investigated evolution of the virus. Comparison of amino acid sequences from isolates from the outbreak and from the CV777 vaccine strain showed 9 amino acid mutations of fragments containing major hydrophilic regions: 16 (L→H), 18 (S→G), 22 (V→I), 44 (T→S), 89 (G→S), 100 (A→E), 107 (L→F), 130 (I→V) and 160 (I→F) (online Technical Appendix Figure, panel A). Three of these 9 mutations were at positions 16, 18, and 22 in the isolates from China; they influenced the hydrophobicity of the S protein as compared with that for CV777 (online Technical Appendix Figure, panel B).

Phylogenic analysis showed that strain CV777 did not cluster with current common strains and showed

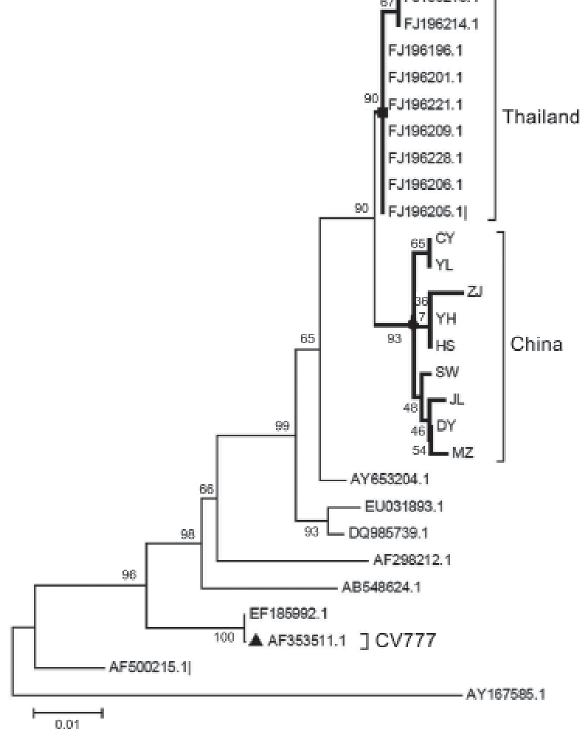

Figure. Phylogenetic tree constructed by using the neighbor-joining method based on the 9 porcine epidemic diarrhea virus (PEDV) sequences identified in a study of porcine epidemic diarrhea in China. Partially amplified spike genes of the PEDV isolates plus 18 PEDV sequences downloaded from GenBank were compared. Sequences included in each cluster are listed in online Technical Appendix Table 3 (wwwnc.cdc.gov/EID/pdfs/11-1259-Techapp.pdf). Strains from Thailand and China and the CV777 vaccine strain are indicated. Scale bar indicates nucleotide substitutions per site.

considerable genetic distance from them. Isolates in the outbreak in China had only a minor nucleotide sequence variation from the Thailand isolates, indicating that the virus has a high genetic relatedness to the Southeast Asia strain. However, previous studies showed that isolates from Europe, South Korea, and China were serologically identical to the prototype CV777 strain (*1*,*4*).

To our knowledge, fecal–oral transmission is probably the main or only route of PEDV transmission (*5*–*7*). In our study, if a fecal sample from a sick piglet was found to be positive for PEDV, we also collected and studied milk from its mother. These results showed that PEDV was present in sow milk (online Technical Appendix Table 3), but the detection rate was lower for these samples (40.8%) than for the fecal samples (82.0%).

On the basis of these results, we hypothesize that sow milk could represent a possible (and potentially major) route for the vertical transmission of PEDV from sow to suckling piglet. This hypothesis could be indirectly verified by our field observation that piglet death rates decreased as a result of fostering (data not shown). Our findings show that PEDV was identified not only in fecal samples from sick piglets, as expected, but also in the milk of the sow, which suggests vertical transmission of the virus.

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Bartonella quintana Transmission from Mite to Family with High Socioeconomic Status

To the Editor: Urban trench fever caused by *Bartonella quintana* has been reported in persons who abuse alcohol and in homeless persons in large cities worldwide. Symptoms vary from asymptomatic intermittent bacteremia to serious complications (*1*). *Pediculus humanus* lice, the known vector of the infection, are not always identified, which raises the possibility that other vectors might also be involved (*2*). We report on an outbreak of *B. quintana* infection among a young family of high socioeconomic status and their visiting relatives.

The family resides in a regional city (population 104,000) in northern Czech Republic in an old, renovated apartment located on the top floor, just under the roof. In the summer of 2007, hundreds of ectoparasitic mites migrated from a hole in the roof and settled on the inner side of a permanently open window before infesting family members. Two weeks later (day 1 of symptom onset), a papular rash and pruritic vesicular lesions were noted by the parents on the body and legs of their 2 children, a 1-year-old girl and a 3-year-old boy. On day 3, the girl's body temperature rose to 38.0°C, and the boy's temperature rose to 39.5°C. The rash resolved in ≈10 days in both children. Vesicular lesions on the girl's buccal mucosal membrane resolved in 5 days. Excoriated areas resulting from spontaneous rupture of lesions or scratching were still visible on day 14.

On day 4, a fever (temperature, 38.5°C) and intense tibialgia, which persisted for 5 days, developed in the 33-year-old father of the infected children. On day 5, a vesicular rash, which resolved in 10 days, developed in the 33-year-old mother. The children's

grandfather and both grandmothers also showed symptoms of infection within ≈14 days after having spent ≥1 days or nights in the infected family's household (Table). In addition, the regional epidemiologist who was involved in the investigation showed development of a severe infection 16 days after exposure to implicated mites that escaped from a collection tube (Table). Recurrent fevers of decreasing intensity, followed by remissions at 1-week intervals, were observed in all patients for up to 3 months.

Seven mites, which were collected by the father on day 6 after symptom onset, were identified as engorged and nonengorged members of the genus *Dermanyssus*. After treatment with ethanol, the mites were investigated by culture and DNA analysis. DNA fragments specific for *Bartonella* spp. (i.e., a 185-bp [*3*] and a 397-bp [*4*,*5*] fragment of the 16S rRNA gene) were amplified; the sequence of the 397-bp fragment was 100% similar to the *htrA* sequence of the *B. quintana* strain Toulouse (Table). Results were negative for PCRs with primers for 16S rDNA of *Anaplasma phagocytophilum* (*6*) and primers for *ospA* of *Borrelia burgdorferi* (*7*). Only *Staphylococcus cohnii* subsp. *urealyticus*, as part of human or animal commensal flora, was detected on blood agar plates that were cultured for 30 days in a microaerophilic atmosphere.

Patient samples were analyzed by using the specific 16S rRNA primers; the *Bartonella*-specific amplicon was found only in a sample that was collected on day 4 from the father. Amplification of the *htrA* gene fragment of identical size and with identical sequences also confirmed the presence of DNA specific for *B. quintana* in the father's sample. Hemocultures were not performed

Table. Patient and microbiologic data from a study of *Bartonella quintana* transmission from mites to a family with high socioeconomic status, Czech Republic, 2007*

Day after symptom onset†	Date of specimen collection	Specimen type‡	Case-patient	Main symptoms	Specimen testing		Incubation period, d
					IgG titer§	PCR¶	
1	NA	NA	Daughter, son	Papular rash, pruritic lesions	NA	NA	14
3	2007 Jul 5	Serum	Son	Rash, vesicles, fever (temperature 39°C)	Neg	Neg/ND	14
		Serum	Daughter	Rash, vesicles, fever (temperature 39.5°C)	Neg	Neg/ND	14
4	2007 Jul 6	Serum	Father	Recurrent fever (temperature 38.5°C), tibialgia, headache	256	Pos/pos	15
5	2007 Jul 7	Serum	Mother	Vesicles, tibialgia	512	Neg/ND	16
6	2007 Jul 11	Mites	NA	NA	NA	Pos/pos	NA
28	2007 Aug 2	Serum	Epidemiologist	Malaise, arthralgia, headache	256	Neg/ND	16
35	2007 Aug 9	Serum	Grandfather	Malaise, arthralgia, rash, headache	Neg	Neg/ND	14
		Serum	Grandmother 1	Fatigue, malaise	256	Neg/ND	14
		Serum	Grandmother 2	Fatigue, malaise	64	Neg/ND	14
41	2007 Aug 15	Serum	Son	Recurrent fever	256	Neg/ND	14
		Serum	Daughter	Recurrent fever	64	Neg/ND	14
		Serum	Father	Malaise and intense headache	256	Neg/ND	15
		Serum	Mother	Malaise and intense headache	512	Neg/ND	16
		Serum	Grandfather	Recurrent fatigue and malaise	Neg	Neg/ND	14
		Serum	Grandmother 1	Recurrent fatigue and malaise	256	Neg/ND	14
68	2007 Sep 11	Mites	NA	NA	NA	Pos/pos	NA
74	2007 Aug 17	Serum	Epidemiologist	Recurrent fever; fatigue and intense headache	512	Neg/ND	16
163	2007 Dec 13	Serum, B, H	Epidemiologist	Poor concentration, headache	256	Neg/ND	16
197	2008 Jan 17	Serum, B, H	Son	None	Neg	Neg/ND	14
		Serum, B, H	Daughter	None	Neg	Neg/ND	14
		Serum, B, H	Father	Poor concentration, headache	128	Neg/ND	15
		Serum, B, H	Mother	None	128	Neg/ND	16
		Serum, B, H	Grandmother 1	None	Neg	Neg/ND	14

*NA, not applicable; neg, negative; ND, not done; pos, positive; B, blood with anticoagulant EDTA; H, hemoculture. During August 9–19, 2007, children and adult case-patients received oral clarithromycin and oral doxycycline, respectively. On August 9 and 19, 2007, the apartment building in which the case-patients lived was treated with insecticide.
†Days after symptom onset do not correlate with incubation period in last column.
‡Specimens were analyzed as follows: serum by serologic testing, EDTA blood by PCR, hemoculture by culture. Patient serum samples were negative for *Anaplasma phagocytophilum* (by immunofluorescence assay [IFA], IgM, and IgG); *Borrelia bugdorferi* (by ELISA and Western blot, IgM, and IgG); *Coxiella burnetii*, *Rickettsia connorii,* and *R. prowazekii* (IFA, total immunoglobulin).
§Determined by IFA.
¶Detected by 16S rRNA and by *htrA* amplification.

at symptom onset, but results for patient serum samples cultured under the same conditions as the homogenized parasites remained negative. Significant titers of IgG against *B. quintana* and *B. henselae* or IgG seroconversion in paired serum samples were observed for all patients except the grandfather (Table).

Oral clarithromycin and doxycycline were administered to the children and adults, respectively, for 10 days. The apartment was repeatedly treated with insecticide, and the hole in the roof was repaired, leading to eradication of the mites. The few dead and dry mites that were available for additional parasitologic analysis were mounted in Swan mounting medium (information about the medium is available from the authors), but no characteristics allowing differentiation between species of the genus *Dermanyssus* were recognized during examination by light microscopy. Failed attempts were made to trap pigeons that had lived on the roof of the apartment or in the same city; however, samples from trapped synanthropic pigeons from the north (n = 20) and central (n = 33) part of the country were negative for *Bartonella* spp. by the culture and amplification methods described above. Recurrent fever reported by adult patients resolved in 3 months, and all patients made a full clinical recovery. Laboratory findings for the patients were followed for 6 months after symptom onset (Table).

The fact that the suspected vector was a hematophagous mite (*Dermanyssus* sp.), a parasite of synanthropic pigeons and a suspected vector of other bacterial pathogens (*8*,*9*), and that the 16S rRNA *Bartonella* spp. gene was detected in mites (*Steatonyssus* sp. from the superfamily *Dermanyssoidea*) (*10*) remains a challenge for additional study. Pigeons probably played the role of accidental host in this outbreak, but the source of the infection remains unclear.

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Urban Transmission of Human African Trypanosomiasis, Gabon

To the Editor: We describe a confirmed case of human African trypanosomiasis (HAT) in an expatriate returning to France from Gabon after a probable tsetse fly bite in the urban setting of Libreville. This case indicates a possible urban transmission of HAT in Gabon and stresses the need for entomologic studies in Libreville.

HAT is endemic to sub-Saharan Africa. *Trypanosoma brucei rhodesiense* (eastern Africa) and *T.b. gambiense* (western Africa) parasites are transmitted to humans by tsetse flies of the *Glossina morsitans* group (*T.b. rhodesiense*) and of the *G. palpalis* group (*T.b. gambiense*), which are found only in Africa. *T.b. gambiense* represents >90% of all reported cases

of HAT worldwide. HAT has always been a travel-associated disease. It is a rare cause of fever, cutaneous lesions, and neurologic signs in travelers returning from disease-endemic areas and involves *T.b. rhodesiense* in 70% of the cases, resulting mostly from an exposure during safari in game parks (*1,2*).

A 58-year-old previously healthy Portuguese man who worked in Gabon for 13 years for a French company was admitted to the tropical and infectious diseases ward because of a 2-month history of intermittent fever, fatigue, and a 10-kg weight loss. The patient recalled a painful unidentified insect bite on his right thigh 2 months before in his garden in Libreville (Lalala quarter). A 8-cm, indurated, erythematous, and painful plaque (chancre) progressively developed (Figure) in the following weeks after the assumed insect bite. When admitted to the hospital, the patient had a temperature of 39°C, anorexia, insomnia, pruritus of the left arm, and paresthesia of the hands and feet. Two additional large annular erythematous macules, centrally pale (trypanids), were found on his back (Figure). A subclavicular 0.5-cm lymph node was observed. There was no hepatosplenomegaly.

His laboratory results showed moderate anemia (hemoglobin 11.8 g/dL) and thrombopenia (134,000 platelets/mm^3) and elevated levels of C-reactive protein (30.6 mg/L) and gammaglobulins (23.9 g/L). A thick-blood smear showed no malaria parasites but a few trypomastigotes of *Trypanosoma* spp*.* PCR of blood identified *T.b. gambiense.* A cerebrospinal fluid sample showed moderate elevation of total proteins (0.43 g/L) and albumin (291 mg/L), 11 leukocytes, and no IgM elevation. Direct examination and PCR showed no trypanosome in the cerebrospinal fluid. Specific antibodies were found in the blood by indirect immunofluorescence (titer 200). Biopsies of 2 skin lesions (thigh, back) showed a lymphoplasmocytic vasculitis consistent with cutaneous locations of HAT; no parasite was observed in situ. The patient was treated successfully with a 7-day course of pentamidine. The case was reported to World Health Organization Control of Neglected Tropical Diseases Department.

A total of 328 HAT cases were reported to the World Health Organization in Gabon during 2000–2009; most infections were acquired in the mangrove swamp Atlantic coast focus in Noya (Estuaire Province) and some in the focus of Bendje (Ogooué-Maritime Province) (*3*). Four of 6 cases of *T.b. gambiense* imported to Europe during 2005–2009 were in expatriates with a travel history to Gabon (*1*). In the 4 case-patients infected in Gabon, an exposure in rural forest areas was assessed (*4–6*; D. Malvy, pers. comm.). In the fifth case reported here, the tsetse bite likely occurred in the urban setting of Libreville.

The patient did not report occupational exposure to tsetse bites outside Libreville during the previous year. He occasionally went in Pointe Denis during weekends but did not remember having been bitten by a tsetse fly. Although the patient did not identify the insect in his garden, the chronology of his clinical history and the presence of a typical chancre at the place of the insect bite that occurred before symptoms provide strong arguments in favor of this hypothesis. The bite occurred during the morning hours, in the patient's home garden in the Lalala area of Libreville (0.357568N, 9.475365E) near the Ogombié River. This area is located 125 km and 75 km from the Bendje and Noya HAT foci, respectively.

Two studies provided evidence for urban transmission of HAT in Kinshasa (Democratic Republic of Congo) and in Bonon (Côte d'Ivoire) (*7,8*). Concurrently, some tsetse species, such as *G. palpalis*, adapt to high human densities and are found in the largest urban centers of western Africa (*9*). Entomologic studies in Libreville should prompt further investigation into a possible urban transmission of HAT in Gabon, as we suspect in the case reported.

Figure. Thigh chancre (1) and back trypanids (2 and 3) in a patient with human African trypanosomiasis infection, Gabon. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/1/11-1384-F1.htm).

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Rickettsia felis Infections, New Zealand

To the Editor: Members of the genus *Rickettsia* have garnered much attention worldwide in recent years with the emergence of newly recognized rickettsioses. In New Zealand, only *Rickettsia typhi* and *R. felis,* belonging to the typhus and spotted fever groups, respectively, have so far been found (*1*). *R. typhi*, primarily transmitted by the oriental rat flea (*Xenopsylla cheopis*), has a worldwide distribution and causes murine typhus in humans (*2*). At the end of 2009, a total of 47 cases of murine typhus had been recorded in New Zealand. In contrast, although the cat flea (*Ctenocephalides felis*) can carry *R. felis* in New Zealand (*3*), no human infections have been reported. However, because *R. felis* shares a similar clinical profile to murine typhus, infection can be mistaken for a suspected case of *R. typhi* (*4*).

Clinical suspicion of rickettsial infection is widely confirmed by serologic tests with the indirect immunofluorescence assay (IFA) being the standard test. However, antibodies against *R. felis* in human sera are known to cross-react with *R. typhi* in IFA (*5*). Western blot (WB) and cross-adsorption assays, in combination with IFA, can differentiate between several rickettsioses (*5*,*6*). We report on the trial in New Zealand of WB and cross-adsorption assays for differentiating retrospectively between past *R. typhi* and *R. felis* infections and evidence of *R. felis* infection in persons living in the country.

Serum samples were obtained from 24 volunteers from the Institute of Environmental Science and Research Limited, Porirua, New Zealand. Samples were tested using *R. typhi* IFA slides (Australian Rickettsial Reference Laboratory [ARRL], Geelong, Victoria, Australia). After incubation (37°C for 30 min), slides were washed 3 times, incubated with fluorescein-conjugated antihuman IgG, IgM, and IgA (ARRL), and washed again before examination. All samples were then tested by using an IgG IFA kit (Focus Diagnostics, Cypress, CA, USA) against typhus group (TG) *R. typhi* and spotted fever group (SFG) *R. rickettsii*.

TG-positive and SFG-negative serum samples may represent *R. typhi* infections, and SFG-positive and TG-negative serum samples may represent *R. felis* infections. Because *R. typhi* can cross-react with SFG rickettsiae (*7*), and *R. felis* with *R. typhi* (*5*), results that are TG positive and SFG positive may be caused by either rickettsiae. Positive reactivity may also represent overseas-acquired rickettsioses. Thus, WB and cross-adsorption assays using *R. typhi* (Wilmington) and *R. felis* (URRWXCal2) antigens (Unité des Rickettsies, Marseilles, France) were used to confirm any *R. typhi* or *R. felis* infections (*6*).

Antigens (2 mg/mL) were solubilized (100°C for 10 min) in 2× Laemmli buffer (*6*) and subjected to electrophoresis (20 µg/well; 20 mA, 2.5 h) through polyacrylamide gels (12.5% resolving; 4% stacking) (BioRad, Hercules, CA, USA). Resolved antigens were electroblotted (100 V for 1 h) onto 0.45-µm polyvinylidene difluoride membranes, which were blocked by using 5% milk–Tris-buffered saline with 0.1% Tween 20. Each antigen lane was divided into 2 strips before incubation (room temperature for 1 h) with serum (diluted 1:200). After three 10-min washes with Tris-buffered saline with 0.1% Tween 20, strips were incubated (room temperature, 1 h) with horseradish peroxidase–conjugated antihuman IgG (1:150,000; SouthernBiotech, Birmingham, AL, USA) and washed again. Enhanced chemiluminescent detection of bound horseradish peroxidase (ECL Plus; GE Healthcare, Buckinghamshire,

Table. Serologic data and risk factors of volunteers that showed positive reactivity in rickettsial IFA, New Zealand*

Volunteer no.	IFA serologic titers: ARRL kit†, *Rickettsia typhi*	IFA serologic titers: Focus kit‡, *R. typhi*	IFA serologic titers: Focus kit‡, *R. rickettsii*	WB and cross-adsorption results	Risk factors in the past 4 years: Flea or animal contact	Risk factors in the past 4 years: Traveled overseas
4	Neg	64	64	Indeterminate§	Rat, cat, dog	Yes
5	Neg	Neg	64	Indeterminate	Cat, dog	Yes
8	Neg	Neg	128	Neg¶	Flea, rat, cat, dog	Yes
9	Neg	Neg	64	Indeterminate	Flea, cat, dog	Yes
16	Neg	Neg	128	Indeterminate	Rat, cat, dog	Yes
17	Neg	Neg	128	*R. felis*	Rat, cat, dog	Yes
18	128	64	64	Neg	Cat, cow, hen	Yes
19	128	Neg	64	Indeterminate	Cat, dog	Yes
21	Neg	64	256	*R. felis*	Flea, cat, dog, possum	Yes
22	Neg	Neg	64	Neg	Cat, dog	Yes
23	256	Neg	Neg	Neg	Rat, cat, dog	No
25	Neg	Neg	128	Indeterminate	None	Yes

*IFA, indirect immunofluorescence assay; ARRL, Australian Rickettsial Reference Laboratory; WB, Western blot; neg, negative.
†The cutoff titer for seropositivity was 128 as recommended by the manufacturer.
‡According to kit instructions, endpoint titers ≥64 and <256 indicate either past infection or early response to a recent infection, and ≥256 are considered presumptive evidence of recent or current infection.
§Serum samples that still reacted with *R. typhi* and *R. felis* after cross-adsorption were classified as indeterminate responses.
¶Serum samples that showed no specific reactions to *R. typhi* and *R. felis* in the WB assay were classified as negative for *R. typhi* and *R. felis*..

UK) enabled identification of reactive band sizes with Precision Plus standards (BioRad). Cross-adsorption was carried out by incubating serum diluted 1:30 in boiled antigen (37°C for 5.5 h, then 4°C overnight) before centrifugation (10,000 × *g* for 10 min) (*6*). Supernatants were applied to WB strips and results compared with *R. typhi* and *R. felis* antisera.

Of the 24 serum samples, 3 (12.5%) were positive on the ARRL slides, and 11 (45.8%) showed IgG reactivity on Focus slides (Table). Of these 11 serum samples, 8 (33.3%) were SFG positive and TG negative, and 3 (12.5%) were SFG and TG positive. Of the 12 serum samples that showed some IFA reactivity, after cross-adsorption, none had specific reactivity against *R. typhi*, and 2 were confirmed as *R. felis*. Both volunteers recorded risk factors associated with *R. felis* infection. Six serum samples were indeterminate. Detectable antibodies remained after both cross-adsorptions, which may be caused by infection by *R. felis* and *R. typhi*, or other rickettsiae or cross-reactive pathogens (*5*,*8*).

Although IgG titers decline over time, detectable levels can remain for 4 years and thus exposure to *R. felis* may have occurred any time during this period (*9*). Because both *R. felis*–infected persons had traveled overseas within the past 4 years and *R. felis* has a wide distribution (*4*), overseas exposure is possible. *R. felis* is known to be prevalent in *C. felis* fleas, including in New Zealand (*3*,*4*). This prevalence and the high rate of cat and dog ownership have public health implications and support the recognition of *R. felis* as an emerging global health threat (*4*). Infection from *R. felis* in addition to *R. typhi* should be considered in the differential diagnosis of fever, headache, myalgia, and rash.

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Identifying Risk Factors for Shiga Toxin–producing *Escherichia coli* by Payment Information

To the Editor: During May and June 2011, a large outbreak of hemolytic uremic syndrome (HUS) and diarrhea caused by Shiga toxin–producing *Escherichia coli* (STEC) occurred, centered on northern Germany (*1*,*2*). Early on, salads and raw vegetables were suspected to be food vehicles (*3*). Also in May, the staff department of a local company informed the Health Protection Authority in Frankfurt in southwestern Germany about the rapidly increasing number of patients with bloody diarrhea and HUS among employees at 2 company office sites. Both sites were served by cafeterias run by the same caterer. Main dishes were prepared in the cafeterias' kitchens and differed between the 2 sites. However, in both cafeterias various fresh foods from a salad bar and fruits, desserts, and daily asparagus dishes originated from the caterer's main kitchen. The salad bar included 30 items. Suspecting that this outbreak was linked to the one in northern Germany, we conducted an outbreak investigation to confirm the epidemiologic link to focus epidemiologic and traceback investigations.

A face-to-face survey among hospitalized employees and by email among all other employees was conducted, which included personal details, symptoms, and information about general food eaten at the cafeterias. We defined outbreak cases as infections in employees of the company at 1 of the 2 sites who by May 23, 2011, were either hospitalized with bloody diarrhea or HUS or who self-reported onset of bloody diarrhea from May 8 through May 23. A total of 320 persons responded to the survey, and 285 (89%) of 320 of the responders stated they used the cafeterias; 60 employees fulfilled our case definition. Case-patients' median age was 33 years (range 22–60 years), and 36 (60%) of 60 were female. Thirty case-patients were hospitalized; HUS developed in 18 (30%) (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/1/11-1044-FA1.htm). Disease onsets occurred over 9 days. Beginning and magnitude of the outbreak were not different between cafeteria locations. Bacteriologic diagnostics for 11 patients yielded results that are compatible with the outbreak strain (*4*).

We used billing data from the cafeterias' obligatory cashless payment system to ascertain risk factors for disease. A nested case–control study design was chosen, limited to a fraction of the cohort to obtain rapid risk estimates. Exposures included were purchases of any fruit, salad bar item, dessert, or asparagus dish in either cafeteria from May 2 through May 13. On the basis of customer identification numbers, the caterer provided billing information for persons with early cases (n = 23). Controls were randomly chosen persons from the caterer's database whose disease status was checked against the survey information (n = 30) and who did not report symptoms of diarrhea (nonbloody), vomiting, or nausea during the same period. Univariable logistic regression was performed.

In univariable analysis, salad bar purchases were highly associated with illness (odds ratio 5.19; 95% CI 1.28–21.03), and desserts, fruit, and asparagus dishes were not (Table). Three (9%) of the case-patients remained unexposed to salad bar items according to the payment system data. The analysis of main courses

Table. Univariable analysis of risk factors for bloody diarrhea among users of 2 cafeterias in Frankfurt, Germany, 2011

Risk factor	No. case-patients exposed/ total no. (%)	No. controls exposed/ total no. (%)	Univariable analysis*: Odds ratio (95% CI)	p value
Salad bar	20/23 (87)	16/30 (53)	5.83 (1.42–23.88)	0.014
Dessert	16/23 (70)	18/30 (60)	1.52 (0.48–4.81)	0.473
Fruits	5/23 (22)	10/30 (33)	0.53 (0.15–1.81)	0.312
Asparagus dish	7/23 (30)	11/30 (37)	0.76 (0.24–2.41)	0.635
Female sex	16/23 (70)	15/30 (50)	2.28 (0.73–7.15)	0.155
Age, y				
<30	12/23 (52)	6/30 (20)	2.80 (0.62–12.66)	0.181
30–<40	5/23 (22)	7/30 (23)	Reference	Reference
40–<50	4/23 (17)	13/30 (43)	0.43 (0.09–2.14)	0.303
≥50	2/23 (9)	4/30 (13)	0.70 (0.09–5.43)	0.733

*Estimates in a multivariable model remained virtually unchanged.

purchased in 1 cafeteria revealed that no such meal had been consumed by >5 (22%) of 23 case-patients. Beginning May 23, the cafeterias were closed for 1 week, and salad sales were suspended for a longer period. There were no additional cases.

These results and the identification of the same rare serotype of O104:H4 renders this a satellite outbreak to the larger outbreak in northern Germany, which is the largest outbreak in terms of HUS ever described worldwide. Sprouts are believed to be the food vehicle (*5*). Sprouts available in the Frankfurt cafeteria salad bars were traced back to a producer of fenugreek sprouts, which appear to be the common source of primary cases in the entire outbreak (*5*). Sprout consumption could not be studied directly in Frankfurt because of the intense media attention on the sprout hypothesis once it had been announced. Also, it was thought that too much time had passed to successfully recall actually selected salad bar items consumed a few weeks previous.

Cafeteria billing information allowed for a rapid investigation while avoiding exposure misclassification attributable to ill-remembered food purchases (*6*). Using data sources independent of individual memory is quite useful. In previous studies, similar tools were successfully applied for the detection of outbreak vehicles. Credit card information was used during an investigation on STEC in beef sausages in Denmark (*7*), supermarket purchase records for STEC in Iceland (*8*), and grocery store loyalty card records for cyclosporiasis in Canada (*9*). Shopper card information was used in the United States in an outbreak of *Salmonella enterica* serovar Montevideo (*10*). However, billing information also could have introduced exposure misclassification, e.g., purchased food that was left uneaten or brought for colleagues. Analysis on ingredient level is often not possible. This study emphasizes the need for recall-independent investigation methods. In settings where such methods are available, they should be exploited early and relevant data saved from routine deletion.

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Brill-Zinsser Disease in Moroccan Man, France, 2011

To the Editor: Epidemic typhus is caused by *Rickettsia prowazekii* and transmitted by human body lice. For centuries, it has been associated with overcrowding, cold weather, and poor hygiene. Brill-Zinsser disease is a recurrent form of epidemic typhus that is unrelated to louse infestation and develops sporadically years after the primary illness. Clinical features are similar to, but milder than, those of epidemic typhus (*1*). We report a case of Brill-Zinsser disease in a patient who was born in Morocco and had no history of epidemic typhus.

A 69-year-old man living in France sought care from his general practitioner on March 7, 2011, after 2 days of high-grade fever (40°C) associated with headache, myalgia, fatigue, and mild cough. Amoxicillin was prescribed for a putative diagnosis of acute respiratory infection.

He was admitted to hospital on March 9 for persistent fever. Physical examination results were unremarkable. Blood test results were as follows: C-reactive protein 111 mg/L (reference 0–8 mg/L); procalcitonin 0.49 ng/mL (reference 0.1–0.4 ng/mL), lymphocyte count 0.7×10^3 cells/μL (reference $1–4 \times 10^3$ cells/μL), platelet count 92×10^3 cells/μL (reference $150–450 \times 10^3$ cells/μL), and lactate dehydrogenase 376 U/L (reference 94–246 U/L). Chest radiograph results were normal. Results of 5 blood cultures and a urine culture were negative. Stupor developed on March 11. Cerebrospinal fluid test results were normal. Because the patient lived near a goat farm, Q fever and tularemia were considered plausible hypotheses, and oral doxycycline was introduced on March 13. The patient became afebrile on March 15, and he was discharged from the hospital and remained well.

On the basis of serologic results, the following diagnoses could be ruled out: viral infections (HIV, cytomegalovirus, Epstein-Barr virus); tularemia; Q fever; leptospirosis; salmonellosis; and *Legionella*, *Mycoplasma*, and *Chlamydia* spp. infections. Acute-phase and convalescent-phase serum samples were positive for typhus-group rickettsiae by the microimmunofluorescence assay at the World Health Organization Collaborative Center for Rickettsioses and Other Arthropod-Borne Bacterial Diseases (Marseille, France). A microimmunofluorescence assay showed titers of 100 for IgM and 6,400 for IgG. Western blot analyses and cross-adsorption studies strongly suggested *R. prowazekii* as the cause of the man's illness. Quantitative PCR result on DNA extracted from the acute-phase serum was negative (*2*).

The patient had been raised in Morocco. At 19 years of age, he emigrated to France, where he lived in a urban area. He subsequently traveled every 3 years to Morocco for 1-month summer holidays, always in urban areas. He had most recently traveled to Morocco in 2008. He denied any history of hospitalization for a severe febrile illness and any exposure to louse bites. In the weeks before disease onset, he had not taken any new drug. He had no immunoglobulin deficiency.

On the basis of serologic analysis with Western blot, we confirmed *R. prowazekii* infection in a patient with no recent travel and no contact with lice or flying squirrels. *R. prowazekii* infection may occur rarely in France; it was found in Marseille in 2002 in an asymptomatic homeless person (*3*). In contrast, the patient in our report was living in a hygienic environment, and an autochthonous infection is therefore highly unlikely.

Epidemic typhus was endemic to North Africa until the 1970s (*4*). Subsequently, this region was thought to be free from epidemic typhus, but 2 cases have been reported since 1999 in Algeria, where 1 case of Brill-Zinsser disease was observed in a man who had had epidemic typhus in 1960 during the Algerian civil war (*5*–*7*). Few published data exist about the seroprevalence of *R. prowazekii* infections in North Africa (*4*). In Tunisia, no epidemic typhus was found in 2005 among 47 febrile patients (*8*). However, a seroepidemiologic survey performed in blood donors and hospitalized patients in the Aures, Algeria, found a prevalence of 2% (*4*). This finding suggests that *R. prowazekii* infection might have occurred in this population more often than suspected. No recent published data are available from Morocco.

Since 1970, reports of only 8 cases of Brill-Zinsser disease have been published (*9*,*10*). In all cases, known risk factors were present (overcrowding, poor hygiene, or contact with flying squirrels). Brill and Zinsser described that stress or waning immunity could reactivate *R. prowazekii* infection (*2*). Corticosteroids can trigger recurrence of *R. prowazekii* in mice (*2*), but no such observations were made in humans. In the case presented here, we found no stress factor, no immunosuppression, and no medical history of epidemic typhus.

Brill-Zinsser disease can develop >40 years after acute infection. The mechanism of *R. prowazekii* latency has not been established. A recently explored reservoir for silent forms of *R. prowazekii* infection is adipose tissue because it contains endothelial cells, which are the target cells for *R. prowazekii* infection, and because of its wide distribution throughout the body (*2*). Brill-Zinsser disease should be considered as a possible diagnosis for acute fever in any patient who has lived in an area where epidemic typhus is endemic.

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Temperate Climate Niche for *Cryptococcus gattii* in Northern Europe

To the Editor: *Cryptococcus gattii* was considered to be geographically restricted to countries with tropical and subtropical climates until 1999, when an outbreak of cryptococcosis in humans and animals occurred in the temperate climate of Vancouver Island, British Columbia, Canada (*1*). Montagna et al. reported the first environmental *C. gattii* in Europe from the Mediterranean region of Italy; these authors isolated it from 11 (4.3%) of 255 samples of plant detritus of *Eucalyptus camaldulensis* trees collected from the residential locality of an autochthonous case of cryptococcal meningitis caused by *C. gattii* in Apulia (*2*). These observations were recently substantiated by the isolation of *C. gattii* from plant debris of trees belonging to *Ceratonia siliqua* (carob), *Pinus halepensis* (stone pine), and *E. camaldulensis* in Spain (*3*). We report environmental isolation of the primary pathogenic fungus *C. gattii* from a forest in Berg en Dal, the Netherlands, which extends its geographic distribution to the temperate climate of northern Europe.

We investigated 112 decayed wood samples collected from inside trunk hollows of 52 living trees belonging to 5 families during April–May 2011 in Nijmegen, the Netherlands. The trees sampled were chestnut (*Castanea sativa*, n = 24), Douglas fir (*Pseudotsuga menziesii*, n = 17), oak (*Quercus macranthera*, n = 6), walnut (*Juglans regia*, n = 3), and mulberry (*Morus alba*, n = 2). The main criterion in selecting a tree for sampling was advanced age and presence of large trunk hollows variably sheltered from sunlight. The sampled sites had no bird nests and were apparently free from avian excreta. The decayed wood samples were collected with an in-house swabbing technique by using simplified Staib niger seed agar as described (*4*). The plates were incubated at 30°C and periodically observed up to 7 days for isolation of *C. gattii* and *C. neoformans*. Suspected colonies of *Cryptococcus* spp. were purified by dilution plating and identified by their morphologic and biochemical profiles, including development of blue color on L-canavanine-glycine bromothymol blue medium.

Identity of the isolates was confirmed by sequencing the internal trascribed spacer and D1/D2 regions, and they were genotyped by using amplified fragment-length polymorphism (AFLP) fingerprinting and multilocus sequence typing (MLST). The MLST loci *CAP10*, *CAP59*, *GPD1*, IGS, *LAC1*, *MPD1*, *PLB1*, *SOD1*, *TEF1*α, and *URA5* of the environmental *C. gattii* isolates were amplified and sequenced, and data were compared with MLST data from a large *C. gattii* population study (*5*) and with a recently published set of clinical, animal, and environmental *C. gattii* isolates from Mediterranean Europe and the Netherlands (Figure) (*3,6,7*). In addition, the mating type was determined with PCR by using mating type–specific primers for the *STE12*a and α alleles (*8*).

Four strains of *C. neoformans* species complex were isolated from the 112 decayed wood samples examined from 52 trees. One strain that originated from an oak tree (*Q. macranthera*), was identified as *C. neoformans* var. *grubii*. The remaining 3 strains, all originating from different hollows in a Douglas fir tree, were identified as *C. gattii* genotype AFLP4/VGI and mating type α. The strains were deposited at the CBS-KNAW (Centraalbureau voor Schimmelcultures/Royal Netherlands Academy of Arts and Sciences) Fungal Biodiversity Centre (accession nos. CBS12349, CBS12355, and 12356), and the sequences were deposited in GenBank (accession nos. JN982044–JN982073).

MLST analysis showed that the *C. gattii* isolates in our study are more closely related to the clinical isolate from the Netherlands (*6*) and to the clinical and environmental *C. gattii* isolates (AFLP4/VGI) reported from the Netherlands and other countries in Europe than to isolates from outside Europe (*3,7,8*). The autochthonous *C. gattii* AFLP4/VGI isolate, CBS2502 (earlier identified as *C. neoformans*) isolate from the Netherlands was recovered postmortem in 1957 from the lungs of a pregnant woman with cryptococcosis (*6*). This patient came from a low socioeconomic strata, was unlikely to have traveled outside the Netherlands, and probably acquired the infection indigenously from an environmental source (*6*).

Furthermore, genotype AFLP4/VGI appears to be the genotype of *C. gattii* prevalent in Europe (*3,7,8*). Outside Europe, *C. gattii* has been reported from Douglas fir trees in Vancouver Island, British Columbia, Canada; however, those isolates represented another molecular type, i.e., AFLP6/VGII (*9*). Genotype AFLP 4/VGI *C. gattii* isolates have been implicated in human infections in that region, but to our knowledge, no environmental isolates have been found until now.

Our detection of *C. gattii* in the environment and its previous isolation from a clinical case in the Netherlands suggests that this pathogen is endemic to the temperate climate of northern Europe. This suggestion agrees with the concept emerging from a decade of investigations in Canada and the Pacific Northwest that the geographic distribution of *C. gattii* extends to the temperate region, albeit with another AFLP genotype (*1*,*9*,*10*). Further environmental studies are likely to show a wider spectrum of host trees and higher environmental prevalence of *C. gattii* in this continent than what appears in the literature.

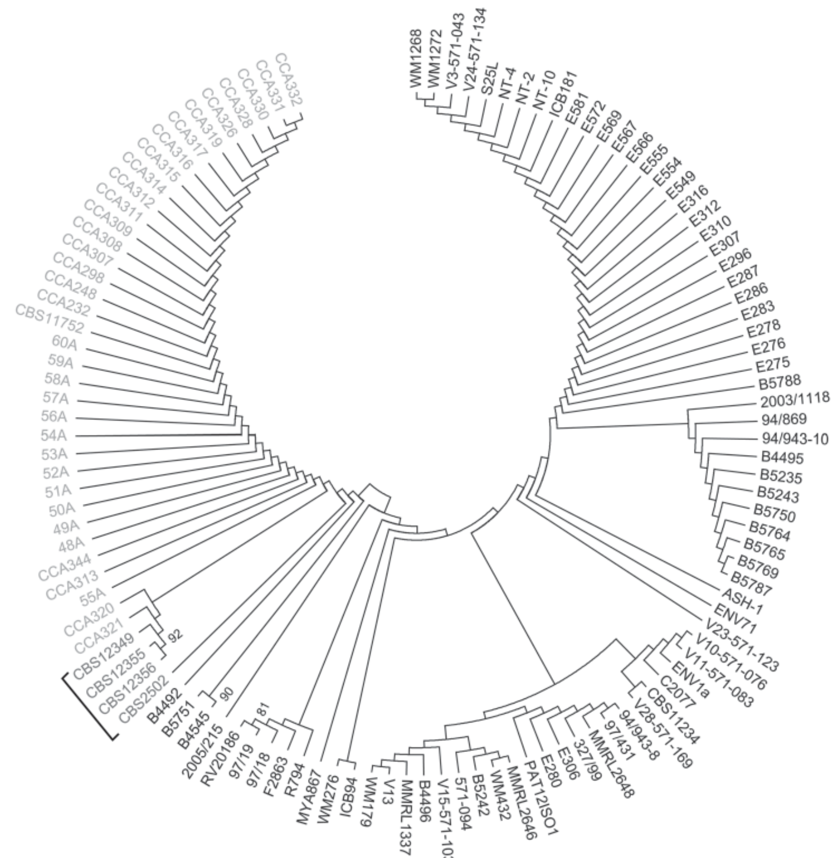

Figure. Unrooted bootstrap maximum-likelihood phylogenetic multilocus sequence typing analysis of *Cryptococcus gattii* genotype AFLP4/VGI isolates based on 7 unlinked nuclear loci (*5*). Bracket indicates the 3 *C. gattii* isolates from the Netherlands cultured from Douglas fir (*Pseudotsuga menziesii*) (CBS12349, CBS12355, CBS12356) and 1 clinical isolate from 1957 from the Netherlands (CBS2502) (*6*). Isolates in gray to the left of the bracket are the previously observed European Mediterranean cluster, with clinical, animal and environmental isolates (*3*). All other *C. gattii* AFLP4/VGI isolates originate mainly from Australia, Africa, and South America, as described (*5*). The isolates from the Netherlands are closely related to isolates that originated from the Mediterranean region. Numbers next to branches show bootstrap support (≥80). A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/1/11-1190-F1.htm).

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Pulmonary Infection Caused by *Mycobacterium conceptionense*

To the Editor: *Mycobacterium conceptionense* was first identified in 2006 from a patient with posttraumatic osteitis (*1*). Since then, 3 more isolates have been recovered from a subcutaneous abscess (*2*), a wound after breast surgery (3), and an abscess after a fat injection (*4*). During November 2009 through April 2010, *M. conceptionense* was isolated from sputum from 4 patients in 2 tertiary hospitals in South Korea.

Patient 1, a 69-year-old woman, was admitted to Seoul National University Bundang Hospital in 2005 with fever and pleuritic chest pain. She had a long history of recurrent fever and cough. Computed tomography (CT) showed multifocal nodular lung lesions with lymphadenopathy. After 7 days of treatment with cefuroxime and azithromycin, the patient's fever subsided and radiographic lesions disappeared. She was discharged with negative culture results. After discharge, she had recurrent episodes of fever, and CT showed waxing and waning pulmonary lesions. Nontuberculous mycobacteria (NTM) species were isolated from some sputum cultures: *M. smegmatis* in 2006; *M. avium* in 2007; and *M. intracellulare* in 2008 and 2009. In February and April 2010, her respiratory symptoms and chest CT findings indicated more severe disease, and *M. conceptionense* grew in sputum cultures. After treatment with clarithromycin, rifampin, and ethambutol for 2 months, the patient's symptoms improved and sputum culture results were negative.

Patient 2, a 70-year-old man with Parkinson disease, was referred to Seoul National University Bundang Hospital in November 2009 for a small nodular lung lesion detected by CT during a medical checkup. He exhibited no pulmonary symptoms. Routine laboratory test results were within normal limits. *M. conceptionense* was isolated from sputum. Clarithromycin was prescribed for 10 days, and the patient remains asymptomatic.

Patient 3, a 70-year-old man with tongue cancer, was admitted to Seoul National University Hospital in March 2010 with exacerbated dyspnea. In November 2009, CT had indicated new nodular lung lesions and chemotherapy had been started. Chest CT in 2010 showed increased size and extent of nodular infiltration, which suggested pulmonary infection rather than cancer metastasis. From 2 sputum samples, 2 isolates of *M. conceptionense* were identified. In addition, *Streptococcus pneumoniae* grew in blood and sputum cultures. Despite treatment with broad-spectrum antimicrobial drugs, the patient died of respiratory failure.

Patient 4, a 53-year-old man, sought care at Seoul National University Hospital in 2008 for chest discomfort. Other than having diabetes mellitus, he had been healthy. Chest

CT showed multiple lung nodules. Sputum culture grew *M. tuberculosis*. The patient received isoniazid, rifampin, ethambutol, and levofloxacin for 6 months, during which time sputum cultures were negative. In April 2010, follow-up sputum culture grew *M. conceptionense*. The patient was asymptomatic and followed up without treatment.

Cultures for each patient were conducted at the respective hospitals, where sputum specimens were placed on solid media (Ogawa; Shinyang, Seoul, South Korea) and in liquid media (MGIT 960; Becton Dickinson, Sparks, MD, USA) after decontamination with NaOH. For all 6 specimens, acid-fast bacilli grew 4–7 days after incubation in liquid media.

Molecular identification was conducted at Seoul National University Bundang Hospital, where PCR restriction fragment length polymorphism and multiplex real-time PCR and melting curve analyses were performed as described (*5*,*6*). Each method produced identical results for all but did not support specific identification. PCR restriction fragment length polymorphism profiles and melting peaks for the isolates from patients 1–4 were similar to those of *M. septicum* and *M. fortuitum*. Sequence analyses of the 652-bp fragment of *tuf* and the 527-bp and 1,571-bp fragments of 16S rDNA genes were performed (*7*,*8*). The *tuf* sequences of isolates from patients 1, 3, and 4 showed 100% identity with the *M. conceptionense* type strain, 98.2% homology (11-bp difference) with *M. porcinum*, and 98.1% homology with *M. fortuitum*. The *tuf* sequence of the isolate from patient 2 differed by 2 bp from the others. The 16S rDNA sequence of the isolate from patient 1 showed 100% homology with sequences of *M. conceptionense* and *M. senegalense* and 99.9% (2-bp difference) homology with *M. farcinogenes*. Broth microdilution susceptibility tests for isolates from patients 1, 2, and

Table. Summary of cases of *Mycobacterium conceptionense* infection*

Patient no.	Age, y/sex	Underlying illness	Clinical presentation	*M. conceptionense* source	Sequencing results†	Treatment	Outcome	Ref
1	69/F	Chronic lung disease	Chronic cough and recurrent fever; multifocal lung lesion and lymphadenopathy seen on chest CT image	Sputum (2×)	*tuf,* 16S rDNA (1,441 and 458 bp; 100% match)	CLA, RIF, EMB	Improved after 2 mo treatment	This article
2	70/M	Parkinson disease	Asymptomatic; small nodule seen on chest CT image	Sputum (1×)	*tuf*	CLA	Asymptomatic	This article
3	70/M	Tongue cancer	Respiratory failure, *Streptococcus pneumoniae* septicemia; lung lesion on chest CT image	Sputum (2×)	*tuf,* 16S rDNA (400 and 460 bp; 100% match)	CLA, LVX, IPM, AMK, VAN	Died	This article
4	53/M	Lung tuberculosis	Asymptomatic after completion of antituberculosis treatment	Sputum (1×)	*tuf,* 16S rDNA (459 bp; 100% match)	Observation	Asymptomatic	This article
5‡	31/F	Posttraumatic osteitis	Wound liquid outflow 3 mo after treatment for open fracture	Wound liquid, bone tissue biopsy, excised skin tissue	16S rDNA, *soda*, *hsp65*, *recA*, *rpoB*	AMC	Not reported	(*1*)
6‡	43/F	Subcutaneous abscess without trauma	Painful swelling and erythematous ankle; abscess detected by MRI	Abscess aspirate	16S rDNA (1,464 bp)	COT, CLA, DOX, LIN	Improved after 5 mo treatment	(*2*)
7‡	58/F	Breast implant infection	Fever and wound discharge	Wound discharge, surgical drainage	*rpoB*	CIP, AZY, DOX	Unremarkable results at 2-mo follow-up after 18 mo treatment	(*3*)
8	50/F	Face surgery with fat grafting	Erythematous nodules and purulent discharge	Wound discharge	16S rDNA, *rpoB*	AMK, LVX, CFX, CLA, SXT	Recovered after 1 mo treatment	(*4*)

*Ref, reference; CT, computed tomography; CLA, clarithromycin; RIF, rifampin; EMB, ethambutol; LVX, levofloxacin; IPM, imipenem; AMK, amikacin; VAN, vancomycin; AMC, amoxicillin/clavulanic acid; MRI, magnetic resonance imaging COT, cotrimoxazole; DOX, doxycycline; LIN, linezolid; CIP, ciprofloxacin; AZY, azithromycin; CFX, cefoxitin; SXT, sulfamethoxazole/trimethoprim.
†Sequences of the isolates were compared with the *tuf* gene and 16S rDNA gene sequence of the type strain CIP 108544^T (GenBank accession nos. EU191943.1 and AY859684.1, respectively).
‡Data modified from Thibeaut et al. (*3*).

4 showed susceptibility to amikacin, ciprofloxacin, clarithromycin, and doxycycline but resistance to cefoxitin, sulfamethoxazole, rifampin (MIC >16 µg/mL) and intermediate-resistance to imipenem (MIC 8–16 µg/mL).

According to the American Thoracic Society diagnostic criteria for NTM lung disease (*9*), patient 1 fulfilled all criteria and patient 3 fulfilled the radiographic and microbiological criteria. These findings suggest that *M. conceptionense* can cause lung disease. For the other patients, colonization with *M. conceptionense* is a more plausible explanation (Table).

These 4 recent cases of *M. conceptionense* infection are in accordance with the increasing prevalence of NTM (*10*). Increasing prevalence might be the result of technical advances in NTM identification, including use of liquid media and sequencing, or the result of a local outbreak or contamination event. We consider contamination to be an unlikely cause because specimens were completely separated from each other during collection and testing. Isolates from different patients yielded distinct randomly amplified polymorphic DNA patterns. In conclusion, *M. conceptionense* is not a rare NTM species in South Korea and can cause pulmonary disease.

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Mycobacterium riyadhense Pulmonary Infection, France and Bahrain

To the Editor: *Mycobacterium riyadhense* is a newly described mycobacterial species that is potentially pathogenic for humans. Extrapulmonary infection with this nontuberculous mycobacterium (NTM) has been reported (*1*).We report 2 cases of pulmonary infection with this NTM.

The first case of infection was in a 39-year-old woman who was admitted to Toulon Military Hospital, Toulon, France, in December 2005 with suspected pulmonary tuberculosis. For 1 month, the patient had a persistent cough, fever, asthenia, and weight loss. Findings on chest radiographs were suggestive of tuberculosis, with cavitation in the right upper lobe, and the tuberculin skin test reaction was positive. Sputum specimens collected on 3 consecutive days were negative for acid-fast bacilli (AFB), but broth cultures (BacT/ALERT 3D system; bioMérieux, Marcy l'Etoile, France) yielded mycobacterial growth.

We used 4 multiplex line-probe assays to identify the mycobacteria: GenoType MTBC (Hain Lifescience, Nehren, Germany) identified the organisms as members of the *M. tuberculosis* complex (MTBC; with a nonspecific reaction, banding pattern 1, 2, 3); GenoType Mycobacterium

CM (Common Mycobacteria) (Hain Lifescience) kit and GenoType Mycobacterium AS (Additional Species) (Hain Lifescience) kit identified the strains as members of the MTBC and as unspecified *Mycobacterium* species, respectively; and INNO-LiPA MYCOBACTERIA v2 (Innogenetics, Ghent, Belgium) yielded a *Mycobacterium*-positive reaction by genus probe but no species-specific result.

Following the criteria of the American Thoracic Society, we considered the isolates as the pathogens responsible for the patient's respiratory disease (*2*). The patient was treated with a combination of isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA). EMB and PZA were continued for 2 months; INH and RIF were continued for 10 months (Table), at which time the patient was considered cured.

The second case of infection was in a 43-year-old man who was admitted to Awali Hospital, Awali, Bahrain, in November 2006. The patient reported malaise, insomnia, cough, weight loss, and anorexia. Radiographs showed features suggestive of tuberculosis (left upper lobe consolidation with focal cavitation). Sputum specimens collected on 3 consecutive days were positive for AFB and mycobacterial growth. To identify the pathogen(s), we used the same 4 mutiplex line-probe assays as used for case-patient 1, and results were similar. The identified strain was considered to be the pathogen responsible for the respiratory disease (*2*).

The patient was treated with a combination of clarithromycin (CLR) and ciprofloxacin (CIP) for 12 months; however, he had a clinical and microbiological (i.e., positive for AFB and culture results with the same NTM) relapse during this treatment. In November 2007, 3 sputum specimens from the patient were positive for AFB, and cultures yielded a mycobacterial strain identical to that identified by the assays. The patient was treated with antituberculous drugs (INH, RIF, EMB, PZA, plus CLR and CIP) for 6 months, and then INH, RIF, CLR, CIP were continued for 2 additional months (Table), after which the patient showed clinical improvement.

In the 2 cases, molecular identification of the isolates as *M. riyadhense* was achieved by using partial *hsp65* and *rpoB* gene sequencing, which was based on the high level of sequence identities with the type strain of *M. riyadhense* and a distance score of 3.5 and 4.6, respectively, to the next species, "*M. simulans*" (Table). Broth microdilution panels (SLOMYCO Sensititer; Trek Diagnosis Systems, Cleveland, OH,

Table. Clinical characteristics, drug susceptibility testing, and outcome for 3 case-patients with *Mycobacterium riyadhense* infection, Saudi Arabia, France, and Bahrain*

Patient age, y/sex	Clinical situation	Molecular-based identification of *M. riyadhense*: Gene	% Sequence similarity with type strain	GenBank accession nos.	Drug susceptibility pattern, drug (MIC, µg/mL) interpretation	Antimicrobial drug therapy	Treatment duration, outcome
19/M†	Bone infection in left maxillary sinus	16S rRNA, *rpoB*, *hsp65*	Type strain	EU27464, FJ786256, EU921671	AMK (10.0) R; CYC (20.0) S; CIP (2.0) S; CLF (<0.5) S; CLR (<2.0) S; EMB (5.0) S; INH (1.0) I; PAS (>1.0) R; PRO (<1.0) S; RFB (0.2) S; RIF (0.2) S; STR (5.0) S‡	INH, RFP, EMB; then INH, RFP	9 mo, cured
39/F§	Pulmonary infection	16S rRNA¶, *rpoB*, *hsp65*	99.8, 99.8, 100	JF896094, JF896096, JF896098	AMK (≤1.0) S; CIP (1.0) S; CLR (0.12) S; DOX (16.0) R; EMB (≤0.5) S; ETH (0.3) S; INH (0.5) S; LZD (≤1.0) S; MOX (≤0.12) S; RFB (≤0.25) S; RIF (≤0.12) S; STR (1.0) S; TMP/SMX (≤0.12/2.38) NA#	INH, RFP, EMB, PZA; then INH, RFP	1 y, cured
43/M**	Pulmonary infection	16S rRNA¶, *rpoB*, *hsp65*	99.8, 99.7, 99.1	JF896095, JF896097, JF896099	AMK (≤1.0) S; CIP (0.12) S; CLR (0.12) S; EMB (≤0.5) S; ETH (0.3) S; DOX (4.0) R; INH (0.25) S; LZD (≤1.0) S; MOX (≤0.12) S; RFB (≤0.25) S; RIF (≤0.12) S; STR (≤0.5) S; TMP/SMX (≤2.0/38.0) NA#	CLR, CIP; then INH, RFP, EMB, PZA, CLR, CIP; then INH, RFP, CLR, CIP	1 y, relapse; 8 mo, cured

*AMK, amikacin; R, resistant; CYC, cycloserine; S, susceptible; CIP, ciprofloxacin; CLF, clofazimine; CLR, clarithromycin; EMB, ethambutol; INH, isoniazid; I, intermediate; PAS, para-aminosalicylate sodium; PRO, prothionamide; RFB, rifabutin; RIF, rifampin; STR, streptomycin ; RFP, rifapentine; DOX, doxycycline; ETH, ethionamide; LZD, linezolid; MOX, moxifloxacin; TMP/SMX, trimethoprim/sulfamethoxazole; NA, not available; PZA, pyrazinamide.
†Patient in Saudi Arabia; reported by van Ingen et al. (*1*).
‡Drug susceptibility testing was performed by using the agar dilution method.
§Patient in France.
¶Low 16S rRNA gene polymorphism between several mycobacterial species.
#Drug susceptibility testing was performed by using broth microdilution panels (SLOMYCO Sensititer; Trek Diagnosis Systems, Cleveland< OH, USA) and interpreted according to standards of the National Committee for Clinical Laboratory Standards (*3*).
**Patient in Bahrain.

USA) were used to determine drug susceptibility (Table) (*3*).

Commercial probes are frequently used for rapid identification of mycobacterial species (*4*); however, *M. riyadhense* and other recently proposed NTMs (e.g., *M. kumamotonense* and "*M. simulans*") cross-react with MTBC DNA probes and may be missed by line-probe assays (*5*,*6*). With the emergence of new NTM species, commercial probes could fail to discriminate between species, leaving clinical isolates either unidentified or misidentified. Because of its ease of use, accuracy, and discriminatory power, multilocus sequence analysis may soon become the standard for routine NTM species identification.

We have shown evidence for the pathogenic role of *M. riyadhense* in pulmonary diseases, a pathogen that has previously been reported to have extrapulmonary pathogenicity (*1*). Clinical and radiologic signs and symptoms of pulmonary infection caused by *M. riyadhense*, including cough, weight loss, fever, and cavitating lung lesions, were similar to those in typical cases caused by MTBC strains. van Ingen et al. (*7*) suggested that the region of difference 1 (RD1) virulence locus identified in MTBC members may also play a crucial role in virulence of some NTM species. These authors found RD1 genes in NTMs that were causing human disease, including *M. kansasii*, *M. szulgai*, *M. marinum*, and the type strain of *M. riyadhense* (*7*).

We confirmed the presence of RD1 *esat-6* and *cfp-10* genes in the *M. riyadhense* isolates reported here (GenBank accession nos. JF896090–JF896093). Because *M. riyadhense* is an emerging pathogen with, to our knowledge, only 1 previously reported extrapulmonary case of infection (*1*), the optimal treatment for infected patients is unknown. Our results and drug susceptibility testing indicate that antituberculous drugs, including INH, RMP, and EMB, are effective against *M. riyadhense* infection (Table), but the combination of CLR plus CIP was not effective in 1 case-patient reported here, despite in vitro susceptibility to both drugs.

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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.

Crimean-Congo Hemorrhagic Fever Virus in Ticks, Southwestern Europe, 2010

To the Editor: Crimean-Congo hemorrhagic fever virus (CCHFV; family *Bunyaviridae*, genus *Nairovirus*) causes outbreaks of severe hemorrhagic fever in humans, with case-fatality rates ≤30% (*1*,*2*). The disease was initially recognized by Russian scientists in the 1940s (*3*), and the virus was first isolated in the Democratic Republic of Congo some years later (*4*). CCHFV is reported throughout broad regions of Africa, Europe, the Middle East, and Asia. Reports linking transmission of the virus with an infected vector have involved ticks of the genus *Hyalomma* (*5*). It appears that maintenance of active foci of CCHFV in the field is dependent on *Hyalomma* spp., even within periods of silent activity. Several vertebrates are involved in the natural transmission cycle (*6*). Transmission of CCHFV to humans occurs through tick bites, direct contact with blood or tissues of infected animals, person-to-person spread, or by nosocomial infection (*1*).

In southeastern Europe, the Balkans are the known western limit for CCHFV (*7*). This finding is of special interest because *Hyalomma marginatum*, the main tick vector in the western Paleartic (an ecozone that includes temperate and cold areas of Eurasia and North Africa and several archipelagos and islands in the Atlantic and Pacific Oceans), is common throughout the Mediterranean Basin (*7*), where clinical cases of the disease or the virus have not been reported. Unsupported claims of the effects of climate on virus distribution have been reported but never empirically demonstrated (*8*).

We report the detection of CCHFV in ticks collected in southwestern Europe. A total of 117 semi-engorged adult *H. lusitanicum* ticks were collected from 28 adult red deer (*Cervus elaphus*) in November 2010, at a site (39.63°N, 7.33°W) in Cáceres, Spain. Live ticks were transported to the special pathogens laboratory at Hospital San Pedro–CIBIR in Logroño (northern Spain), classified, and frozen at −80°C. For RNA extraction, specimens were washed in 70% ethanol and then in Milli-Q water (Milli-Q Advantage water system; Millipore Ibérica, S.A., Madrid, Spain) that had been autoclaved. Each tick was cut lengthwise; half was used for additional processing and the remainder was stored. Before use, each half was crushed in sterile conditions. RNA was individually extracted by using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and frozen at −80°C.

The RNA was distributed in 12 pools and retrotranscribed by using the Omniscript RT kit (QIAGEN) according to the manufacturer's instructions and then frozen at −20°C. Nested PCRs were performed by using specific primers for the small segment of CCHFV as described (*9*). Negative controls (with template DNA but without primers and with primers and containing water instead of template DNA) were included in all assays.

For the second round of PCRs, 2 of 12 pools showed amplicons of the expected size (211 bp). Only 1 amplicon could be sequenced. MEGA5 (www.megasoftware.net) was used to compare the sequence with representative small segment sequences of CCHFV available in GenBank (Figure). (Aligned sequences are available from the authors.) Pools of cDNA were submitted to the Spanish National Center of Microbiology (Madrid), where results were confirmed. The CCHFV sequence we report showed 98% genetic similarity (204/209 bp) with sequences recorded for CCHFV in Mauritania and Senegal, on the western coast of Africa.

This finding suggests the circulation of CCHFV in southwestern Europe. The close affinity of the strain from Spain with strains circulating in western Africa and the lack of similarity with isolates from eastern Europe suggest the introduction of this virus from nearby countries of northern Africa. Migratory movements of birds could explain the presence of the virus in southwestern Europe because birds are common hosts of immature *H. marginatum*, which was reportedly introduced into Europe through annual migratory flights along the western coast of Africa (*10*). Because of the lack of genetic similarities among virus strains, trade movements of domestic or wild ungulates from eastern Europe do not support our finding.

We cannot state whether this virus was circulating previously or if other strains are present in the area because CCHFV detection in the western Mediterranean region has not been previously addressed. *H. lusitanicum* ticks exist as relatively isolated populations in a narrow strip from Sicily to Portugal. The Mediterranean rabbit and ungulates, the main hosts for immature and adult *H. lusitanicum* ticks, respectively, are residents of the collection area; however, the movement of these animals through trade has not occurred for several years. Thus, *H. lusitanicum* ticks could not serve as a spreading vector in the western Mediterranean region. The CCHFV strain from southwestern Europe has been found in ticks restricted to hosts that cannot spread long distances. Therefore, although it would be unlikely, given the strain's similarity with CCHFV isolates from Senegal and Mauritania, we should not exclude the possibility of an ancient existence for this strain. Additional data collected in the Mediterranean Basin are necessary to establish the actual range of CCHFV.

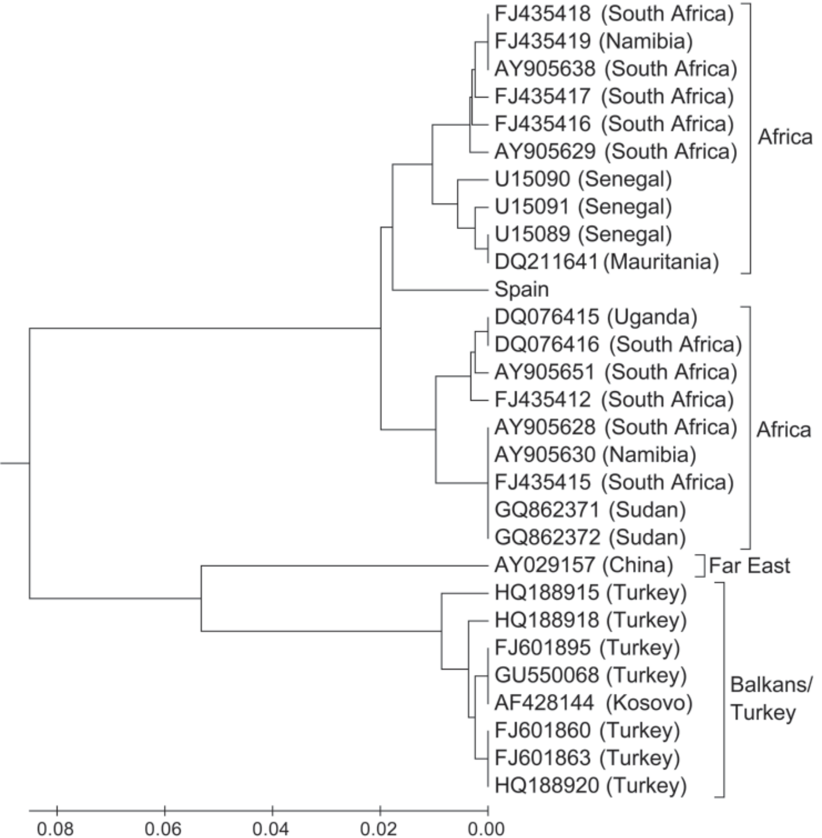

Figure. Evolutionary relationships of Crimean-Congo hemorrhagic fever virus strains from Spain and other representative sites. Evolutionary history was inferred by using the unweighted pair group method with arithmetic mean. The optimal tree is shown (sum of branch length, 0.36861921). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed by using the maximum composite likelihood method and are in the units of the no. of base substitutions per site. Analysis involved 29-nt sequences. The first, second, third, and noncoding codon positions were included. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted by using MEGA5 (www.megasoftware.net).

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Crimean-Congo Hemorrhagic Fever Virus in Ticks from Imported Livestock, Egypt

To the Editor: Crimean Congo hemorrhagic fever, a tick-borne illness caused by Crimean Congo hemorrhagic fever virus (CCHFV), is endemic to Africa, the Balkans, the Middle East, and parts of Asia. The hard ticks (Ixodidae), especially those of the genus *Hyalomma*, serve as reservoirs and vectors for CCHFV, and a variety of animals, such as cattle, sheep, and camels, are considered amplifying hosts for the virus. Although CCHFV may cause little or no disease in zoonotic hosts, the virus can cause severe disease in humans who may be exposed by tick bites or by contact with blood or tissues from infected patients or animals (*1*). Surveillance for CCHFV in animal and vector populations provides an opportunity to monitor a disease of potentially severe impact.

In North Africa and the Middle East, trade in live animals, meat, and meat products poses noticeable risk to human and animal health (*2*,*3*) and can serve as a mobile pool of diseases with potentially large economic and health effects. Animals often originate in distant areas of a country or its neighbors, where they may be exposed to zoonotic pathogens not endemic to their final location, and may collect vectors that carry additional pathogens.

As part of a broader study examining occupational risk of exposure to vector-borne and zoonotic pathogens in a high-risk abattoir worker population, we collected ectoparasites from freshly-slaughtered livestock and examined them for CCHFV. This study and was conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the Guide for the Care and Use of Laboratory Animals (*4*).

Sample collection took place over a 2-week period in July 2009 at the Muneeb abattoir in the Giza Governorate of Egypt. Ectoparasites were removed from 43 freshly slaughtered animals by using blunt forceps and were placed in glass vials. In total, 342 ectoparasites were collected: 70 (20.5%) from 14 cattle, 52 (15.2%) from 17 buffalo, 6 (1.8%) from 2 sheep, and 214 (62.6%) from 10 camels. Cattle, buffalo, and sheep originated in Egypt; camels were imported from Sudan and Somalia.

Ectoparasites were transported to US Naval Medical Research Unit No. 3 in Cairo, Egypt, for taxonomic identification and pathogen detection. Ninety-seven percent (334) of the ectoparasites were ticks from the family Ixodidae. The genus *Hyalomma* accounted for 254 (76.0%) of these

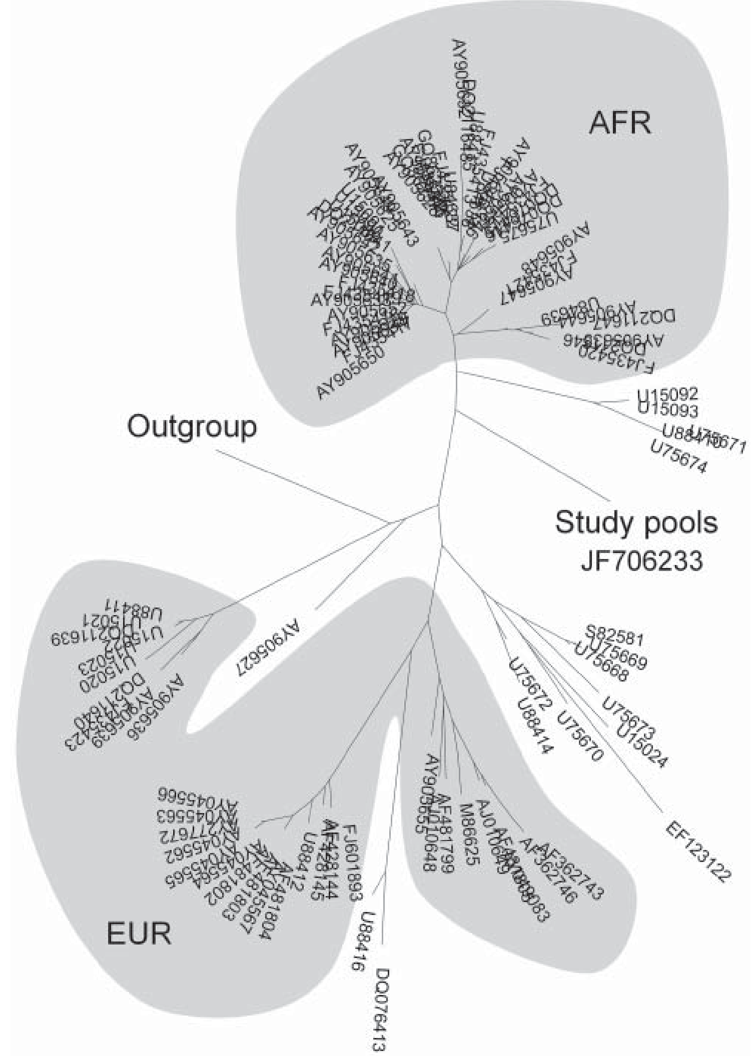

Figure. Phylogenetic relationship of 110 Crimean-Congo hemorrhagic fever virus sequences obtained from this study (6 tick pools), the positive control, and 99 published isolates from GenBank. Sequences were aligned by using ClustalX (www.clustal.org), and a phylogenetic tree was constructed by using MEGA4 (neighbor-joining, Kimura 2-parameter, complete deletion of missing data, tree condensed when bootstrap calculated branch support was <0.5) (www.megasoftware.net). The 2 shaded areas indicate clustering of African isolates (AFR) and predominantly European isolates (EUR); unshaded isolates originate primarily from the Middle East.

ticks, including nearly all of those collected from sheep (100%) and camels (99.5%) but only 60.4% and 9.1% of those collected from buffalo and cattle, respectively. The remaining ticks of the family Ixodidae belonged to genus *Boophilus*.

After identification, ticks were grouped into pools by species, sex, and animal source. RNA was then extracted by using the QIAamp Viral RNA Kit (QIAGEN, Valencia, CA, USA). CCHFV small fragment RNA was detected with SuperScript III Platinum SYBR Green One-Step qRT-PCR (Invitrogen, Carlsbad, CA, USA) and published primers as described (*5*).

Of 138 pools tested (258 ticks of the genera *Hyalomma* and *Boophilus*), 6 pools were positive for CCHFV. These 6 pools contained ticks collected from 1 camel imported from Somalia and 4 from Sudan. One positive pool comprised female *H. excavatum* (Koch, 1844), and 5 comprised female *H. dromedarii* (Koch, 1844), both proven vectors of CCHFV.

Sequence analysis showed that each CCHFV-positive pool contained an identical yet previously unrecorded 229-bp RNA fragment (GenBank accession no. JF706233; Figure). This fragment had 92% identity with many published CCHFV isolates (BLAST analysis [www.ncbi.nlm.nih.gov/BLAST], GenBank nonredundant database); however, most base changes were synonymous substitutions. Translated protein queries (tblastx) identified an amino acid (serine at codon 26) in this new variant that differed from all but 1 (AJ010648.1) of the 100 most similar published sequences. The functional consequence of a S26N substitution is unknown but may be minimal because of the similar physicochemical properties of asparagine and serine.

Despite the low number of camels sampled in this study, 5 of the 10 camels were found to harbor ticks carrying RNA from an undocumented variant of CCHF. Although none of the domestic animals harbored infected ticks, it is not possible to conclude if these data reflect importation of CCHFV or infection acquired within Egypt because details about conditions under which animals were kept before slaughter are unavailable. Previous serologic studies in Egypt have shown antibodies to CCHFV were prevalent among imported camels at a quarantine station in Aswan governorate (*6*) and among domestic cattle, buffalo, sheep, and goats in Sharkia Governorate (*7*). We plan to further investigate the presence of CCHFV within camel and ectoparasite populations in Egypt by expanding protocol activities to a camel market in the same area as the abattoir.

Acknowledgments

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Cutaneous Leishmaniasis Acquired in Jura, France

To the Editor: Cutaneous leishmaniasis is well established in the Mediterranean basin. However, the disease is spreading and new foci have been reported (*1–3*). Because of climate change, it is feasible that vector-borne diseases such as cutaneous leishmaniasis may spread northward into Europe (*4*). We report a patient who acquired cutaneous leishmaniasis while on holiday in Jura, France.

A previously healthy 49-year-old white man from the Netherlands traveled to France in August 2007. During August 2–17, he stayed at a camp site in Clairvaux-les-Lacs in a forested area near a lake. He made regular trips by foot in the surrounding area. Three months later, he noticed a swelling on his nose. In February 2008, he consulted a dermatologist who treated him twice with cryotherapy under a diagnosis of actinic keratosis, after which the lesion nearly disappeared.

Three months later, the patient again consulted the dermatologist when the lesion recurred. A biopsy result from the lesion was interpreted as an acute ulcerative inflammation without further specification. Treatment was continued with imiquimod 5% cream followed by erythromycin 2% cream with clobetasol 0.05% ointment. Because of a lack of improvement, this treatment regimen was alternated with tacrolimus 0.1% ointment until May 2008.

In November 2008, he consulted another dermatologist, who obtained a biopsy specimen in which a large number of intracellular microorganisms compatible with leishmaniasis were observed in histiocytes. The patient was then referred to the Department of Dermatology at the Academic Medical Centre in Amsterdam. On examination, we found a plaque with a crusting surface and an erythematous border on the bridge of the nose (Figure, panel A). Regional lymph nodes were not palpable.

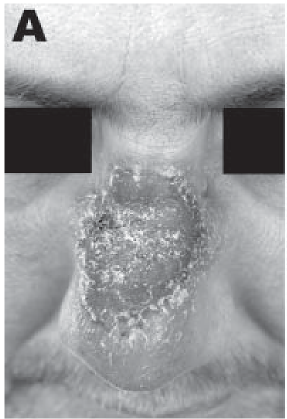
Revised histopathologic examination of the biopsy specimen showed a dermal inflammatory infiltrate of histiocytes containing numerous intracellular *Leishmania* amastigotes and epithelioid cells, lymphocytes, and few plasma cells (Figure, panel B). A direct smear from the biopsy specimen was positive for Leishman-Donovan bodies. Culture on Novy-MacNeal-Nicolle medium was positive for *Leishmania* spp. A PCR result for *Leishmania* performed on a biopsy specimen from the lesion was positive; sequence analysis showed DNA of *Leishmania donovani/infantum* complex.

Treatment was initiated with oral itraconazole (100 mg, 2×/d) for 6 weeks without improvement and was then continued with miltefosine (50 mg 3×/d) for 28 days. Other than nausea, the patient did not experience side effects. Regular monitoring of liver function showed values within normal limits. The lesion healed completely.

There are several reports of leishmaniasis acquired in Europe in locations north of the Mediterranean basin. Naucke et al. (*5*) reported 11 cases of endemically acquired leishmaniasis (human, canine, feline, and equine infections) in Germany since 1991. In 1992, a child with visceral leishmaniasis was described who had spent weekends and holidays near Calais, France (*6*).

We assume that our patient acquired cutaneous leishmaniasis in mainland Europe at 46°N. He had not visited leishmaniasis-endemic areas before this holiday in the French Jura.

Cutaneous leishmaniasis in France is found mainly in the region Pyrénées-Orientales, with 2 sandflies, *Phlebotomus ariasi* and *Phlebotomus perniciosus*, as vectors (*7*). One of the causative factors for the northward emergence of leishmaniasis in Europe is the spread of visceral and cutaneous leishmaniasis from disease-endemic areas in the Mediterranean to neighboring temperate areas with vectors without disease (*8*). A northward spread of leishmaniasis with an extension of the geographic range of *Ph. perniciosus* and *Ph. neglectusus* sandflies has been found in Italy (*9*), and northward



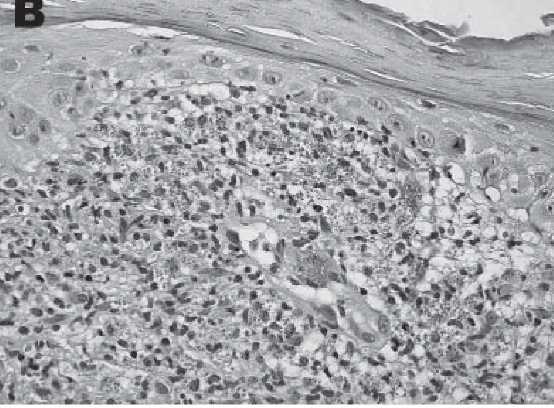


Figure. A) Crustosus plaque on the nose of the patient. B) The epidermis shows parakeratosis, atrophy, and a single apoptotic keratinocyte. An inflammatory infiltrate is present in the papillary dermis, mainly composed of (epithelioid) histiocytes, admixed with lymphocytes and few plasma cells. B) Most macrophages in the infiltrate are parasitized by numerous *Leishmania* spp. amastigotes (hematoxylin and eosin stain, original magnification ×400). A color version of this figure is available online (www.cdc.gov/EID/article/18/01/11-0408-F1.htm).

spread of the proven sandfly vector *Ph. (Laroussius) perniciosus* and the competent sandfly vector *Ph. (Transphlebotomus) mascittii* into Germany (*5*). It has been hypothesized that sandflies have always been sporadically present in central Europe, but that climate change will lead to extended distribution (*10*).

It is tempting to assume that climate change resulted in cutaneous leishmaniasis at 46°N in France. In any event, our case and those reported by others should make clinicians aware of the possibility of cutaneous leishmaniasis outside the well-known disease-endemic areas.

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Visceral Leishmaniasis during Italian Renaissance, 1522–1562

To the Editor: Leishmaniasis, an infectious disease caused by parasites of the genus *Leishmania*, is transmitted to humans through the bite of a female sandfly. The 3 forms of leishmaniasis are visceral (VL) and cutaneous (CL), which are typical of the Old World, and mucocutaneous leishmaniasis, which occurs primarily in Central and South America. VL (also called kala-azar) is caused by species of the *L. donovani* complex (including *L. infantum*), and CL is mainly caused by *L. major* or *L. tropica* (*1*). In Italy, VL and CL are caused by *L. infantum*. The origin and spread of leishmaniasis are a matter of debate. Widespread in antiquity, visceral leishmaniasis has been identified only in mummies from ancient Egypt and upper Nubia (*2*). Similarly, only 4 cases of mucocutaneous leishmaniasis have been identified in skulls from northern Chile (*3*).

We describe the identification of *L. infantum* infection in Eleonora from Toledo (1522–1562), wife of Cosimo I de' Medici and member of one of the major political Italian families during the Renaissance. The positive identification of *Leishmania* infection was achieved in bone samples by 2 independent approaches. First, a molecular ancient DNA (aDNA) analysis identified a specific 123-bp fragment of a conserved region of the minicircle molecule of the parasite´s kinetoplastid mitochondrial DNA (*4*,*5*) which on direct sequencing showed a *Leishmania*-specific sequence compatible with *L. infantum* (Figure; online Appendix Figure, wwwnc.cdc.gov/EID/article/18/1/10-2001-FA1.htm). This PCR result was independently replicated in 2 laboratories and additionally supported by the second approach, a protein assay showing a concomitant positive reaction by detecting IgG against *L. infantum* by Western blot sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Direct sequencing of the *Leishmania* aDNA identified a strain with high homology to *L. infantum*. Accordingly, we obtained a 98% concordance rate between our sequence and that of *L. infantum* (expect rate 6e-47, identity rate 113/118) (online Appendix Figure). The rates for other *Leishmania* species indicated that concordance for those species was less probable.

For the protein assay, fractionated proteins from a lysate of late-log-phase promastigotes of *L. infantum* ZMON-1 (World Health Organization

code MHOM/TN/1980/IPT-1) were electroblotted onto nitrocellulose membrane, and antibody detection was conducted on a Bio-Rad (Hercules, CA, USA) Multiscreen apparatus (*6*). Antibodies against *L. infantum* selectively reacted in a supernatant of protein extract from Eleonora, thereby confirming the immunologic identification of the protozoal infection. The response of IgG against *L. infantum* whole-parasite antigens revealed specific recognition of 8 polypeptides ranging from 14–16 kD to 184 kD. This pattern of bands is consistent with a symptomatic form of VL as shown by the 14 to 16–kD bands.

Although it was initially proposed that the antigenicity of ancient proteins may be altered by diagenesis, further investigations have shown that ancient immunoglobulins can persist across geologic times (*7*). Potential pitfalls in protein-based detection of ancient pathogens have been addressed by incorporating proper controls during the analysis. False-positive data, which can result either from contamination of ancient material by modern materials or from lack of microbe specificity of the test (*7*), have been ruled out by the parallel testing of several blanks (buffer without ancient material) and by testing, in parallel, samples of ancient bone tissue harvested from persons who died of known diseases other than leishmaniasis (e.g., plague). All negative controls used in aDNA and protein research and all blanks yielded negative results. To avoid contamination, we used no positive controls.

The disease history of Eleonora from Toledo is as follows. Her clinical history was dominated by a large number of pregnancies. When 18–32 years of age, she gave birth to as many as 11 infants. On the basis of additional clinical reports of court doctors, it was assumed that pulmonary tuberculosis developed when she was 29 years of age (*8*). In the last years of her life, Eleonora from Toledo had various severe ailments. Irregular bouts of fever, wasting and constant vomiting, stomach pain, weight loss, anemia, and hemorrhage were recorded. Autopsy revealed that her most damaged organs were the lungs and that the lung lesions were consistent with a chronic pulmonary infection. Hepatomagaly and splenomegaly were also recorded (*9*). Although these signs and symptoms could have come from the tuberculosis infection, they are also consistent with those in patients with symptomatic VL, i.e., progressive fever, weight loss, splenomegaly, hepatomegaly, hypergammaglobulinemia, and pancytopenia. Complications include immunosuppression, secondary bacterial infections, hemorrhage, and anemia (*10*). All these observations lend support to the notion that Eleonora from Toledo was not immunocompetent. In addition to a supposed tuberculosis co-infection, VL infection may have been a key event leading to her death at age 40.

Our molecular and serologic identification of *Leishmania* infection in a historically prominent person from southern Europe has major relevance. This information might be useful for monitoring the infection and its pathogen throughout history and might provide data on the host–pathogen interaction over different periods.

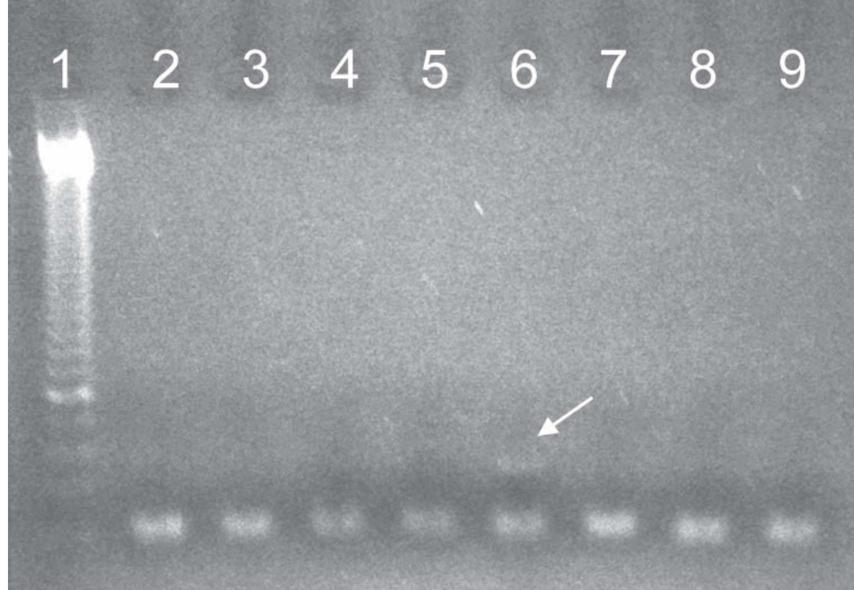

Figure. PCR amplification of a 123-bp fragment of kinetoplastid mitochondrial DNA of *Leishmania* spp. from Eleonora from Toledo (lane 6, arrow). Lane 1, molecular mass standard; lanes 2–5, ancient controls; lanes 7–9, blank controls.

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Plague Epidemic in the Kingdom of Naples, 1656–1658

To the Editor: In 1656, an epidemic of plague occurred in the Kingdom of Naples, Italy. Earlier the disease had spread from Algiers to Spain; in June 1647, it appeared in Valencia, and in the spring of 1648, it appeared in Aragon and several other Spanish areas of Valencia, Andalusia, and Catalonia. In 1652, plague had spread to Sardinia and then to the cities and territories of Naples, Rome, and Genoa. Within the Kingdom of Naples, plague first reached the town of Naples in the spring of 1656. Despite measures restricting population movement, by the summer of 1656, the disease had reached several provinces in southern Italy (*1,2*).

Historical records indicate that the epidemic in Barletta, in southern Italy, developed after the arrival of a ship from Naples. On May 26, 1656, the ship Sant' Andrea arrived from Naples at the port of Barletta. However, after sanitary inspection, the ship was prevented from landing and obliged to depart, but this measure was not sufficient to prevent the disease from entering the port. The Barletta epidemic peaked in October, after which the number of cases diminished; and on June 22, 1657, Barletta was declared free of plague. Of this city's original population of 20,000, the disease killed 7,000–12,000 persons. It is hypothesized that throughout the Kingdom, the plague killed ≈1,250,000 persons (*1,2*).

Since the 14th century, noble families of Barletta had been buried in tombs in underground tunnels of Sant' Andrea church. During restoration of the church in 2009, more underground tunnels containing many skeletons were discovered. It has been hypothesized that the church had also been used as a cemetery during the plague epidemic. During an inspection of the skeletons, 5 skulls of young persons were identified and collected. For a negative control, the skull of a person buried in a tomb before the epidemic was also collected.

The skulls were radiographed to identify unerupted teeth (Figure), which were then aseptically extracted. After classification, each tooth was cut along a sagittal line to uncover the dental pulp, which was then hydrated in sterile phosphate-buffered saline (pH 7.2) for 48 h at 37°C. The DNA was extracted by using DNAeasy Blood and Tissue Kits (QIAGEN, Hilden, Germany) and by modifying the first step, which was conducted overnight at 56°C with 600 μL of ATL buffer (QIAGEN) and 50 μL of proteinase K. To verify the presence of inhibiting substance, the control DNA extracts were screened by using a PCR for human mitochondrial DNA (*3*).

To investigate the cause of the deaths, we adopted a PCR suicide method and searched for *Yersinia pestis*. We amplified the *pla* gene for *Y. pestis* by using Sybr green PCR in real time with a modification of a previous protocol (*4*) coupled with conventional PCR according to Drancourt et al. (*5*). Conventional PCRs were adopted for *Bacillus anthracis* by targeting the *pag* and *capC* genes (*6*) and for *Salmonella enterica* serovar Typhi by targeting the *narG* gene (*7*). To prevent cross-contamination, we conducted all PCRs with a negative control and in the absence of positive controls. Melting curve analysis and agarose

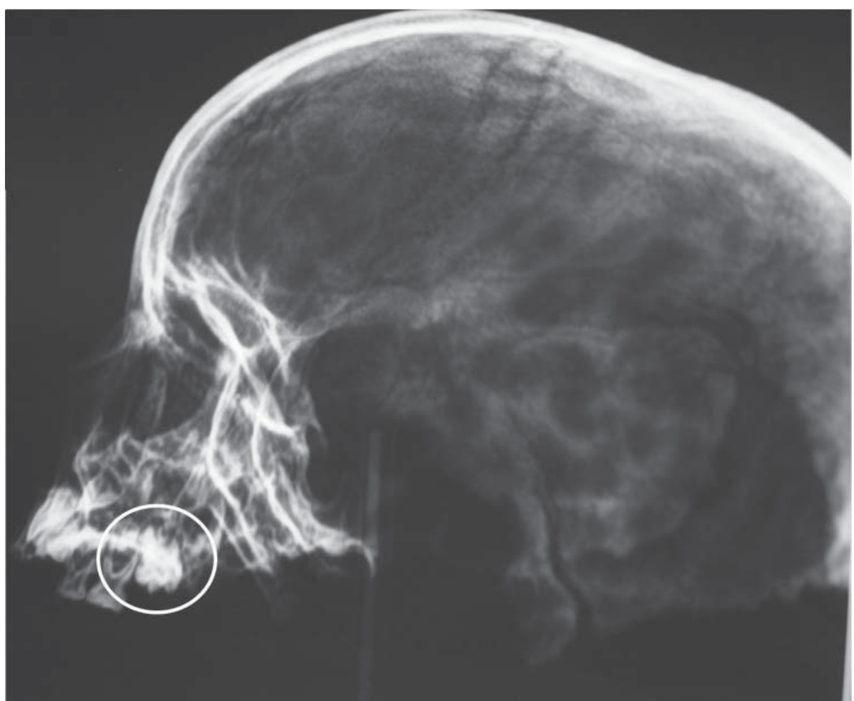

Figure. Radiograph of skull found under Sant' Andrea church in Barletta, Italy, in 2009, showing unerupted teeth (circled) that were later extracted aseptically.

gel electrophoresis of PCR products indicated suspected positive samples. All amplicons relative to conventional and suicide PCR were submitted for sequencing analysis confirmation. The negative DNA was reanalyzed to confirm the results.

From the 26 dental pulp samples analyzed from the 5 skulls of young persons, 7 samples were positive for the *pla* gene of *Y. pestis* by the Sybr green real-time PCR, and 2 of these were positive for this gene by conventional PCR. All were negative for *B. anthracis* and *S. enterica* ser. Typhi. GenBank BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) results of the 2 sequenced amplicons found a 100% match with the reference sequences (GenBank accession no. AL109969.1); query coverage was 100%. The sequences obtained were deposited in the GenBank sequence database under accession nos. JN208020–1.

In conclusion, the confirmed finding of DNA of *Y. pestis* in 2 skeletons and suspected finding in the remaining 3 suggests that these persons died of plague during the 1656–1658 epidemic in southern Italy. Although it has not been universally agreed upon, several studies have confirmed that the agent of 16th to 18th century "plague" epidemics in Europe were caused by *Y. pestis*. Different methods have documented *Y. pestis* as the agent in 10 Black Death burial sites scattered over 5 countries (*8*). In northern Italy, the presence of *Y. pestis* has been confirmed in Venice (14th–17th centuries) (*8*), Genoa (Bastione dell'Acquasola) (14th century) (*9*), and Parma (16th–17th centuries) (*10*). This study confirms that the plague that infected the Kingdom of Naples, which spanned almost all of southern Italy, was also caused by *Y. pestis*.

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Leprosy, Still Present in La Réunion

To the Editor: During recent decades, a considerable and consistent decrease in worldwide incidence of leprosy has been observed, mainly because of the recommendation to introduce multidrug therapy in 1981 (*1*) and the implementation of free therapy in 1994 (*2*) by the World Health Organization (WHO). The prevalence rate of the disease has been reduced globally by >90% since 1985 (*3*). Since 2000, WHO has recommended the implementation of a leprosy surveillance system in leprosy-endemic countries with indicators for screening, treatment, and monitoring of patients (*4*). From these indicators, WHO establishes an annual official report on the global status of the disease. According to official reports received from 141 countries, the global registered prevalence of leprosy was 211,903 cases in 2010 (*5*).

La Réunion is a French overseas department located in the Indian Ocean 700 km east of Madagascar. La Réunion's health care system is similar to that of continental France. Although new cases of leprosy have been punctually reported by health professionals during the past few years, which suggests that the disease is still present, the situation in La Réunion is poorly documented because of the lack of a specific surveillance system. Thus, the goal of eliminating leprosy as a public health problem (i.e., prevalence <1/10,000) (*6*) cannot be assessed because the goal requires a good knowledge of the epidemiologic status of the disease. Furthermore, the risk of leprosy recrudescence linked to a relapse of patients with autochthonous cases or patients with leprosy migrating from neighboring leprosy-endemic countries, such as Madagascar, Comoros, and Mayotte (*5*), is present. In 2009, a total of 1,572 new cases of leprosy were detected in Madagascar, 319 in Comoros, and 37 in Mayotte (*5*,*7*). If one considers the geographic proximity and the many tourist exchanges between La Réunion and those neighboring islands, the risk of importation, although low, is constant.

In that context, Cire Indian Ocean (the Regional Office of French Institute for Public Health Surveillance), in collaboration with health professionals involved in diagnosis and treatment of the disease, has implemented a specific surveillance system for leprosy in La Réunion. The objectives are to guide potential preventive measures by determining incidence of leprosy, following the disease's evolution, and characterizing the patients affected.

The surveillance system was based on the notification of every case by health professionals likely to diagnose and treat subjects according to the WHO case definition (*8*), i.e., clinicians, private or hospital dermatologists, and infectious disease specialists. The notification was realized through a standardized questionnaire that included sociodemographic, clinical, and microbiological data. Concurrently, the pathology laboratories were consulted to detect any nondeclared cases and to improve the completeness of data.

This surveillance was retrospective for 2005–2010, then prospective for 2011. In total, 17 patients responding to the case definition of leprosy and given a diagnosis during 2005–2010 were reported for an average population of 804,025 inhabitants in La Réunion (data from the National Institute of Statistics and Economics Studies). The mean annual incidence during this period was 3.4 cases/10^6 inhabitants. The male:female sex ratio was 2.2, and the median age was 54 years (range 8–77 years). More than half the patients were born in La Réunion (n = 9); the other patients' birthplaces were Comoros Islands (n = 4), Mayotte (n = 3), and Madagascar (n = 1). Among the patients born in La Réunion, 6 had never left the island, 3 had traveled but had always resided in La Réunion, and 6 patents resided in the same area of a city in the southwestern part of the island.

An active search for other cases in this area was performed by contacting all the health professionals likely to diagnose leprosies; 1 clinician reported a suspected case among his patients. That patient is currently being screened. Of the patients overall, 15 were screened by skin biopsy or smear from the ear.

According to the microbiological classification, 14 patients had a multibacillary form (positive smear) and 1 patient had a paucibacillary form (negative smear). Clinical signs suggested multibacillary leprosy (>5 patches or lesions on the patient's skin) for 15 patients and paucibacillary leprosy (1–5 patches or lesions on the skin) for 2 patients. The median time between diagnosis and treatment was 6 days (range 0–20 days). Four patients had a severe disability with a grade 2. Overall, 15 patients had lepromatous leprosy and 2 had tuberculoid leprosy.

Although elimination of leprosy was achieved in La Réunion, the implementation of a leprosy surveillance system enabled us to highlight an autochthonous circulation of *Mycobacterium leprae*, leading to a cluster of cases recently diagnosed in the southwestern part of the island. During the investigation of this cluster, it was noticed that most of the doctors were unaware of the existence of leprosy in La Réunion or of the disease's clinical signs. Incidence of leprosy could therefore be largely underestimated because of this lack of knowledge, and actions to raise awareness among health care professionals will be established to improve the detection and rapid treatment of patients.

We thank the physicians and biologists for their participation in the surveillance of leprosy in La Réunion.

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Vaccinia Virus Zoonotic Infection, São Paulo State, Brazil

To the Editor: Since 1999, vaccinia virus (VACV) has been isolated frequently from dairy cattle and humans (*1–3*). During bovine vaccinia outbreaks, VACV can be transmitted to farmers and those who milk cows; it frequently causes lesions on the hands and forearms. Bovine vaccinia causes economic losses and affects public health services in Brazil (*1–4*). One of the first VACV viruses isolated during Brazilian bovine vaccinia outbreaks was Araçatuba virus (ARAV), which was collected in São Paulo State, and since that time, other VACVs have been isolated in this state (*2*,*5*,*6*).

The circulation of VACV in São Paulo forests was described in the 1960s and 1970s, although such isolates seem to be phylogenetically distinct from ARAV and other VACVs that currently circulate in Brazil (*7*). Although VACV in several Brazilian states has been reported (*1–3*), the intrastate spread of VACV concerns veterinary and medical authorities and presents a challenge to the sanitary barriers and prophylactic measures implemented to date. We report 2 zoonotic bovine vaccinia outbreaks in the midwestern region of São Paulo State, Brazil.

The Institutional Ethics and Animal Welfare Commission from the Faculdade de Midicina Veterinária e Zootecnia–Universidade Estadual Paulista Júlio de Mesquita Filho/Campus de Botucatu approved this study. In 2009 and 2010, exanthematic outbreaks were reported in rural areas of Itatinga (23°6′7″S, 48°36′57″W) and Torre de Pedra (23°14′38″S, 48°11′42″W) counties, respectively. Between the 2 outbreaks, lesions were observed on the teats and udders of 10 lactating cows. The lesions appeared

as macules, evolved into vesicles, pustules, and ulcers and healed after 2–3 weeks. Lesions developed on the hands and arms of the milkers after occupational contact with sick animals. The milkers also described headache, lymphadenopathy, and fever.

Specimens from 7 scabs and 1 vesicle were collected for virus identification by laboratory assays. After DNA extraction (InvitekDNA, Berlin, Germany), the samples were subjected to a specific orthopoxvirus PCR for the amplification of the A56R gene of vaccinia virus (*8*). A fragment of ≈950 bp was amplified from 5 exanthematic lesions. Two milk samples collected from sick cows were also positive for A56R. Parapoxvirus DNA was not detected in any collected sample (*9*). Material from the bovine and human exanthematic lesions induced characteristic poxvirus cytopathic effects in baby hamster kidney cells. In addition, 13 of the 18 collected bovine serum specimens were positive for orthopoxvirus according to a plaque reduction neutralization test and an ELISA (*4*). Human serum specimens were negative for orthopoxvirus by the plaque reduction neutralization test but positive by IgM ELISA, indicating the occurrence of an acute infection process (*4*).

A56R-PCR amplicons from 2 exanthematic lesions and 2 milk samples were sequenced in both orientations by using the Mega-BACE-sequencer (GE Healthcare, Little Chalfont, UK). Optimal alignment of our samples and other orthopoxvirus A56R gene sequences with ClustalW (www.ncbi.nlm.nih.gov/pmc/articles/PMC308517) by using MEGA3.1 (www.megasoftware.net) showed that a signature deletion was present in the sequences of several Brazilian VACV isolates (*1–3*). Three of the 4 sequenced amplicons exhibited 100% identity: the milk samples and a lesion collected from a same county. VACV samples from Itatinga and Torre de Pedra showed high identity with ARAV (*2*) and other Brazilian VACVs, including the Cantagalo (*1*) and Mariana viruses (*10*). A phylogenetic tree based on the A56R gene was constructed with the neighbor-joining method, 1,000 bootstrap replicates, and the Tamura 3-parameter model (MEGA3.1) (Figure). VACVs from Itatinga and Torre de Pedra clustered with several VACVs isolated during bovine vaccinia outbreaks. The A56R sequences obtained in this study were deposited in GenBank (accession no. It1446645).

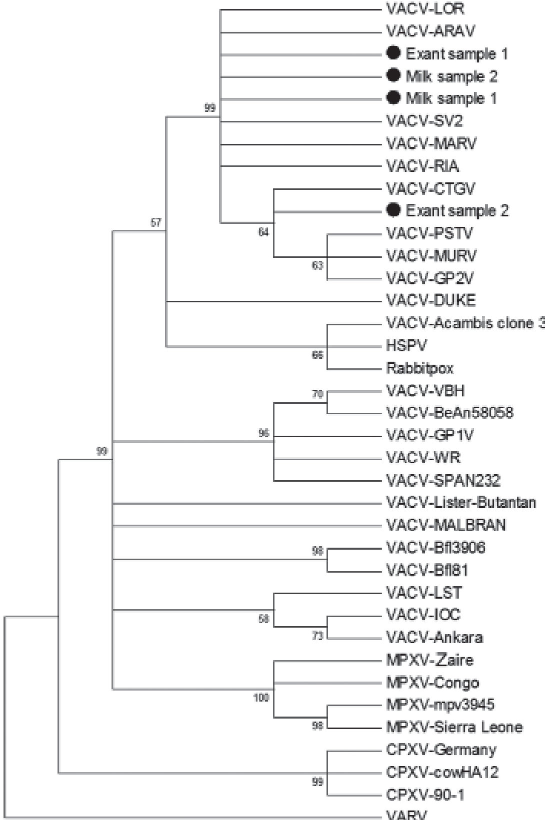

Figure. Consensus bootstrap phylogenetic tree based on the nucleotide sequences of the A56R-hemagglutinin gene of vaccinia virus. The tree was constructed with hemagglutinin sequences by using the neighbor-joining method with 1,000 bootstrap replicates and the Tamura 3-parameter model in MEGA3.1 software (www.megasoftware.net). Bootstrap values >50% are shown. Nucleotide sequences were obtained from GenBank. Black dots indicate the vaccinia virus (VACV) analyzed in this study. HSPV, horsepoxvirus; VARV, variola virus; CPXV, cowpoxvirus; MPXV, monkeypoxvirus.

We describe a new zoonotic outbreak of bovine vaccinia in São Paulo State, Brazil. Our molecular data suggest that this outbreak was caused by a VACV that is genetically related to viruses isolated in previous years, including ARAV, which was isolated in 1999 (*2*). The emergence and reemergence of this virus in previously bovine vaccinia–free microregions of São Paulo State suggest that VACV could have adapted to a specific microbiome and that the virus may be circulating not only in cattle and humans but also in some wild reservoir (*10*). Although genetic and ecologic studies of Brazilian VACVs have advanced in the past several years, little has been achieved in terms of bovine vaccinia prevention and control. Therefore, bovine vaccinia surveillance and public communication are critical in areas where VACV circulates.

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Mumps Vaccine Effectiveness Against Orchitis

To the Editor: Yung et al. reported in the April 2011 issue of Emerging Infectious Diseases on the epidemiologic characteristics of the nationwide mumps outbreak in England and Wales in 2004−2005 (*1*). The associated effect of disease was considerable, with >43,000 reported cases and >2,600 hospitalizations. Compared with the prevaccine era, the average age of infection was higher, with infection occurring mostly in older teenagers and young adults (*2*). Older age at infection is associated with a higher risk of certain complications, particularly orchitis (*3*). Yung et al. reported that among cases of mumps, previous mumps measles rubella (MMR) vaccination offered considerable protection against orchitis, meningitis, and hospitalization (*1*).

In the Netherlands, mumps vaccination, using a 2-dose schedule with the MMR vaccine against measles, mumps, and rubella, was introduced in 1987, including catch-up vaccination of 3 birth cohorts (1983–1985). From birth cohort 1985 onwards, the coverage of the first and second dose of MMR has been consistently >92% (*4*). This coverage led to immediate control of mumps, with mumps related hospitalization dropping from 390 cases in 1987 to 11 in 1990 (*5*).

However, a major reemergence of mumps in the Netherlands occurred during August 2007–May 2009, when a large genotype D mumps outbreak affected mainly unvaccinated persons with a religious objection to vaccination (*6*). Subsequently, a genotype G outbreak of mumps started at the end of 2009, affecting mainly vaccinated adolescents. The outbreak started among university students in different cities, with a sudden increase in transmission after a large party for students in early 2009 (*7*).

The Dutch Centre for Infectious Disease Control advised Municipal Health Services in January 2011 to recommend MMR vaccination for university students who were unvaccinated or who had received only 1 dose of vaccine in the past. This policy was further implemented in the new academic year that began in August 2011. Information regarding the effectiveness of previous MMR vaccination against mumps complications is needed to support this policy and to predict the effect on mumps-related disease.

To study this policy, we analyzed mumps notifications in the Netherlands during December 1, 2009–June 14, 2011. Notifications include information about vaccination

Table. Mumps complications by MMR vaccination status, the Netherlands, December 1, 2009–June 14, 2011*

Complication	MMR doses received	No. mumps cases	No. (%) cases with complications	OR	aOR†	p value	Adjusted VE,‡ % (95% CI)
Orchitis§	0	86	20 (23)	Ref	Ref	Ref	Ref
	1	48	5 (10)	0.38	0.34	0.05	66 (1 to 88)
	2	338	31 (9)	0.32	0.26	<0.01	74 (49 to 87)
Other complications¶	0	117	1 (1)	Ref	Ref	Ref	Ref
	1	85	1 (1)	1.38	0.88	0.93	12 (–14 to 95)
	2	571	6 (1)	1.23	0.75	0.80	25 (–5 to 91)
Hospitalization	0	130	4 (3)	Ref	Ref	Ref	Ref
	1	83	2 (2)	0.80	0.70	0.69	30 (–312 to 88)
	2	535	6 (1)	0.40	0.43	0.25	57 (–84 to 90)

*Only those for whom complication and vaccination status were known are included; therefore, totals may differ. MMR, mumps, measles, rubella; OR, odds ratio; aOR, adjusted odds ratio; VE, vaccine effectiveness; ref, reference categories.
†OR and VE adjusted for age group (<18, 18−25, >25 y) and sex, except for orchitis, where the OR and VE were adjusted only for age group.
‡VE = 1 – OR where the OR is an approximation of the relative risk.
§Only male patients ≥12 years of age are included.
¶Includes the following reported complications: pancreatitis (n = 2), meningitis (3), thyroiditis (1), bronchitis (1), high fever and shortness of breath (1).

status and complications (e.g., orchitis, meningitis, encephalitis, pancreatitis). Vaccination status was confirmed by checking the national vaccination register, the general practitioner or patients' vaccination booklets. Vaccine effectiveness against complications and hospitalizations was estimated by using logistic regression, adjusting for age group and sex.

In the study period, 958 cases were reported, and 16 case-patients were hospitalized (1.9% of case-patients with a known hospitalization status; n = 842). The median age of case-patients was 22 years (range 1–86 years), and 58.7% were male. We had information on the vaccination status of 905 case-patients (94.5%). For this group, 68% of these vaccination statuses were confirmed. Of the 905 case-patients, 16% were unvaccinated, and 10% and 68% had received 1 and 2 doses, respectively; 6% were vaccinated at least once, but number of doses was unknown. Of case-patients with information on the occurrence of complications (95.7%, n = 917), 73 (8.0%) reported ≥1 complication. Orchitis was by far the most frequently reported complication (66 case-patients, 11.8% of men). Other complications included pancreatitis (2, 0.2%), meningitis (3, 0.3%), and thyroiditis (1, 0.1%).

Previous vaccination with 1 or 2 doses reduced the risk for mumps orchitis among male mumps case-patients ≥12 years of age by ≈70% (Table). This finding is consistent with that reported by Yung et al. (*1*). Because of a lower number of cases, we could not reliably estimate the effect of vaccination in preventing hospitalization and other complications. The estimated proportion of case-patients hospitalized derived from the enhanced mumps surveillance by Yung et al. is remarkably similar to our estimate (3% and 2%, respectively). It is likely that we underestimated the overall effect of disease associated with this outbreak. Notification is known to be incomplete and complications developing after the date of notification are not included. However, because the reporting of complications is unlikely to be associated with vaccination status, we believe our estimates of the vaccine's protective effects among cases of mumps are unbiased.

Whereas objection to vaccinate was the predominant cause for the 2007–2009 mumps outbreak in the Netherlands, the current outbreak seems to be caused by secondary vaccine failure. Potential causes of this failure include waning of vaccine induced immunity, a relative mismatch between vaccine and outbreak strain, and intense social contact in the affected group (*8*). Our observations that orchitis was the most frequently reported complication, and that previous MMR vaccination considerably reduced the risk of orchitis among cases of mumps, are important to justify recommending mumps vaccination to unvaccinated persons.

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Genetic Characterization of Peste des Petits Ruminants Virus, Sierra Leone

To the Editor: Peste des petits ruminants (PPR) is a highly infectious disease of small ruminants, characterized by high rates of illness and death and caused by a single-stranded RNA virus (peste des petits ruminants virus [PPRV]). PPRV can be divided into 4 genetically distinct lineages based on the nucleocapsid (*N*) gene (*1*). The lineages correlate well with geographic distribution of the virus, with lineages I and II mainly restricted to western and central Africa, lineage III to eastern Africa and the Arabian peninsula, and lineage IV to Southeast Asia, the Middle East, and more recently northern Africa (*2*).

PPRV is endemic to most of western Africa, and considered a major constraint on the livestock industry. In Sierra Leone, a country bordered by Guinea, Liberia, and the Atlantic Ocean, and having high goat and sheep populations, PPRV is believed to be the cause of outbreaks of respiratory disease with high death rates. Inadequate veterinary infrastructure and diagnostic capacity, exacerbated by the civil war in 1991–2002, however, has prevented confirmation. In this study, we confirmed presence of PPRV in Sierra Leone, which led to the official report of PPR to the World Organisation for Animal Health (Paris, France).

The study was conducted in April 2009 as part of a training mission organized at Teko Central Veterinary Laboratory, Makeni, Sierra Leone, by the World Organisation for Animal Health Collaborating Centre for Biotechnology–based Diagnosis of Infectious Diseases in Veterinary Medicine (www.sva.se/oie-cc) in collaboration with the Food and Agriculture Organization–Emergency Center for Transboundary Animal Diseases, Bamako, Mali. During the training, blood and serum samples were collected from goats (n = 9) and sheep (n = 1) from 2 smallholders with suspected outbreaks of PPR in the area around Makeni in central Sierra Leone. In addition, serum from 5 goats with respiratory disease was sampled at a livestock market in Kabala 100 km north of Makeni.

Serologic testing was performed at Teko. All serum samples (n = 15) were tested for PPRV antibodies by using a commercial ELISA (BDSL, Ayrshire, UK; *3*); 12 (80%) of the samples were positive for PPRV.

Blood samples were collected on Nobuto filter strips (Advantec MFS Inc., Tokyo, Japan) and transported to the BioSafety Level 3 laboratory at the National Veterinary Institute, Uppsala, Sweden, for nucleic acid detection (*4,5*). RNA was eluted from the blood impregnated filter strips and screened for PPRV by using real-time RT-PCR specific for the *N* gene (*6*). Viral RNA was detected in 13 (87%) of the samples, with most of the positive samples indicating high viral load (cycle threshold <20).

For determination of the genetic lineage of detected viruses, RNA from all samples was subjected to PCR amplification of a 351-bp segment of the *N* gene by using the NP3/NP4 primer pair (*7*), but with a modified protocol using the One-Step RT-PCR kit (QIAGEN, Hilden, Germany) (*5*). Amplified PCR products were separated by electrophoresis, gel extracted, purified, and processed for sequencing by using ABI PRISM BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

N gene sequences were obtained from 10 (67%) of the samples, and showed 83%–100% nt identity level compared with sequences available in GenBank using the BLASTn tool (www.ncbi.nlm.nih.gov/blast) and 93%–100% identity between each other. Phylogenetic analysis was performed with 4 representative sequences (GenBank accession nos. JN602079–JN602082) from this study by using neighbor-joining and the Kimura 2-parameter model in MEGA5 (CEMI, Tempe, AZ, USA), including *N* gene sequences representing all 4 lineages.

The PPR viruses from Sierra Leone clustered in lineage II with viruses from Mali, Nigeria, and Ghana, and could further be distinguished into 2 clusters (Figure). One virus from Kabala clustered closely with viruses from Mali (Mali 99/1), whereas all others showed 100% identity with a virus from Nigeria (Nig/75/1), in many countries used as vaccine virus strain. In Sierra Leone at the time, however, PPR vaccination was not being performed, suggesting that obtained sequences originated from circulating field viruses related to Nig/75/1 rather than being vaccine derived. This suggestion was strongly supported by the clinical presentation typical of PPR. Surprisingly, no

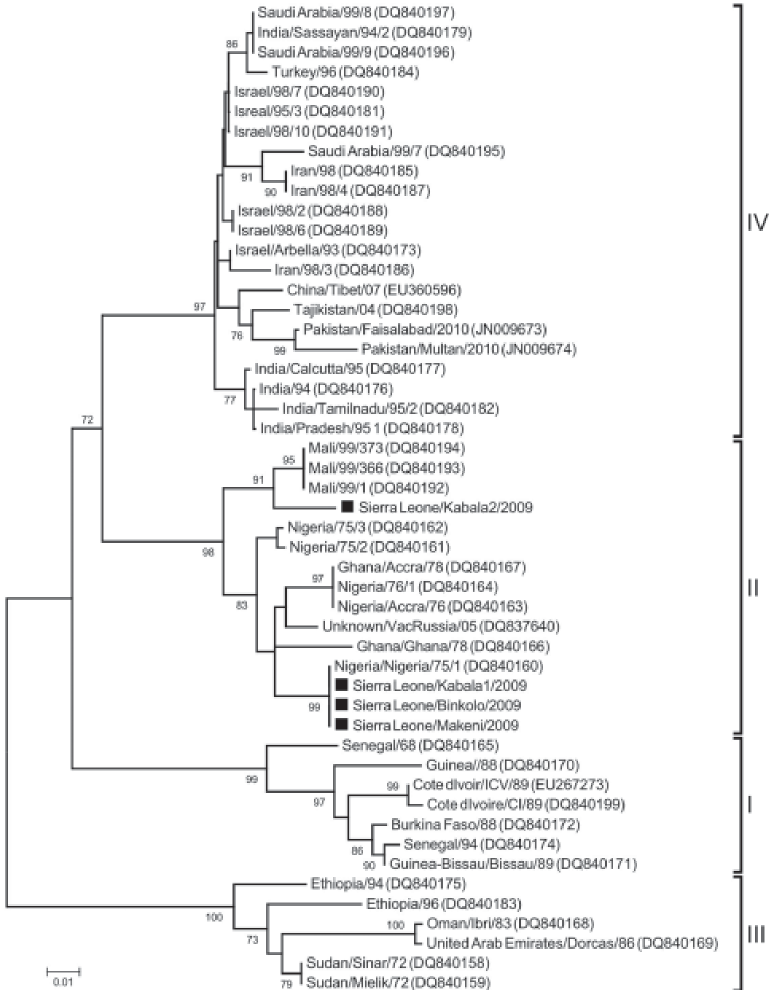

Figure. Majority rule consensus tree of peste des petits ruminants viruses (PPRV) based on the variable region of the *N* gene (255 bp), constructed using the neighbor-joining method and the Kimura-2-parameter model in MEGA5 (www.megasoftware.net). Numbers indicate the bootstrap values (2,000 replicates); only values >70% are shown. Horizontal distances are proportional to sequence distances. The figure indicates a clear division of the 4 lineages of PPRV and the sequences obtained in this study clustered in lineage II and are marked with black squares. Scale bar indicates nucleotide substitutions per site.

relationship was found with PPRV strains so far described from Guinea, the immediate neighboring country and closest livestock trading partner, or those from Senegal, Guinea-Bissau, Côte d'Ivoire, and Burkina Faso, which constitute lineage I.

In conclusion, we confirm the presence of PPRV in Sierra Leone, and provide genetic characterization of detected viruses, knowledge that is fundamental for control, prevention, and in the long run, eradication of the disease. The detection of 2 different sublineages at the livestock market in Kabala shows how markets can serve as mixing vessels, and also gives evidence of at least 2 separate introductions of PPRV into the country, underlining the transboundary nature of the disease, particularly in regions with uncontrolled livestock movements. Since this study, an official vaccination program based on Nigeria/75/1 has been launched.

Acknowledgments

We thank Hermann Unger for providing the PPRV cELISA.

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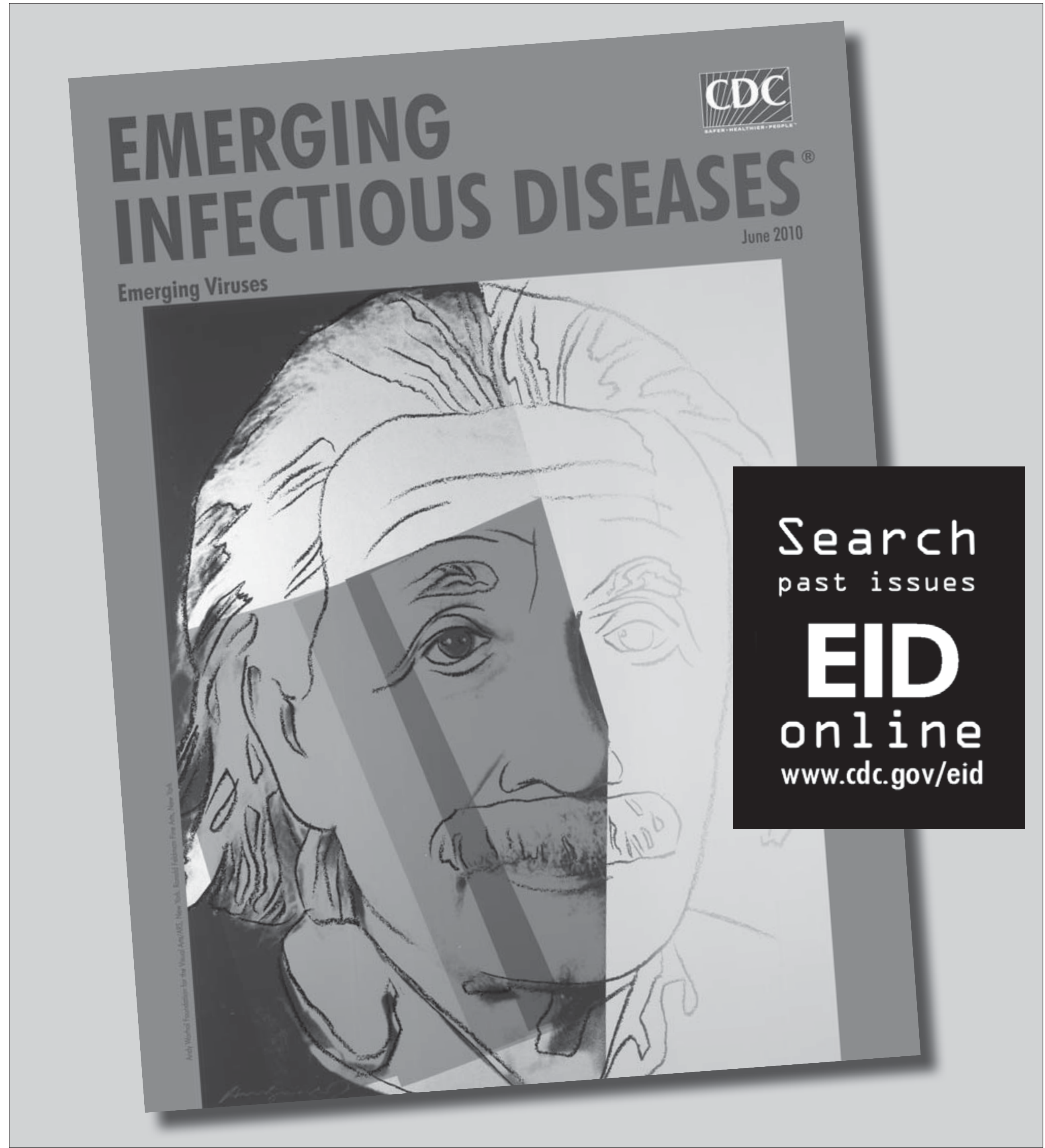

Dioskourides of Samos, *Musici Ambulanti*, Mosaic (c. 2200 years ago) (41 cm × 43 cm). Museo Archeologico Nazionale, Piazza Museo Nazionale, Napoli, Italy

Tough Art and Microbial Drama

Polyxeni Potter

Glass, ceramic, marble or limestone, shells, pebbles, enamel, ivory, mother-of-pearl, gold, painted and semiprecious stones—in the most refined form especially cut into hard cubes known as *tesserae*—were set at different angles and depths and arranged into tactile patterns depicting mythologic subjects, everyday or theatrical scenes, and many other themes to adorn architectural structures. Even though they were sometimes found outside buildings, mosaics were primarily used on interior surfaces. As the practice became more common, *tesserae* of colored glass were expressly produced to provide intense blue, red, and green hues, "Like very rubies from gold patinas gleaming," as Dante Alighieri would write in the Divine Comedy when he viewed the ancient mosaics in Ravenna, Italy.

Surface decoration made of small particles set in a foundation to form a unified whole likely began as assembled colored stones as far back as the third millennium BCE. Organizing these stones into patterns, or mosaic making, became an art form and flourished in antiquity, and because of its structural strength and durability, survived on the floors and walls of ancient palaces, cathedrals, and affluent homes inspiring new technical and artistic greatness throughout history and continuing to thrive as monumental decoration in the 19th and 20th centuries.

As art form, the mosaic offers pure color expression through the setting (*opus*) of individual fragments into clay, plaster, or mortar. The laborious process relies on materials that, unlike brushstrokes, are inherently inflexible. Because the final image is meant to be viewed from a distance, color gradation, an optical illusion, is achieved in the eyes of the viewer. The *tesserae* vary in size and shape—small stones for faces and hands, larger ones for backgrounds—and allow for precision in laying the mosaics and for special visual effects. They are tilted and spaced irregularly to create glitter and refract natural light. They capture, reflect, absorb, and splinter the light which, multiplied as with prisms, breaks into an infinite number of chromatic units. Or, as an unknown poet put it also in reference to the Ravenna mosaics, "Either light was born or imprisoned here. It reigns supreme."

Musici Ambulanti, on this month's cover, found in the ruins of a structure known as the Villa of Cicero near the city of Pompeii, is one of the finest examples of ancient mosaic making. Signed by Dioskourides of Samos, known to us only from mosaics bearing his name, this work has been linked to images found more than 3 centuries later in the House of Menander in Mytilene. These mosaics contain scenes from the plays of Menander, most famous of the writers of Attic New Comedy in the second half of the 4th century BCE. He was known for portraying ordinary people and their lives. The Dioskourides panel roughly resembles a mosaic scene in Mytilene that likely originated from an illustrated manuscript or a painting of Menander's play *Theophoroumene* (The Girl Possessed).

Only fragments of the play remain, so the story line is sketchy. The heroine was possessed by Kybele, mother of

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the gods and mistress of nature, whose cult was associated with ecstatic states and dances performed to drums or cymbals. Illustrations of the play have become a visual record of Greek drama as well as of painting and music in the Hellenistic period.

In the Dioskourides panel, three actors wearing masks are performing as musicians. The figures are individualized through their actions and postures. A man on the right plays the tympanon. Another man with cymbals, in the center, is turned toward the tympanon player. And a woman with a *hetaera* (courtesan) mask, playing the aulos (a reed instrument) is accompanied by an assistant, an unmasked boy on the left. The actors' bodies cast shadows on the wall. The scene brims with hilarity and easy, flowing movement.

The image is architectural, with columns supporting a coffered roof. Pillars on the side extend to the top. Central figure representations are contained in marble frames. The background is simple, mainly neutral bands against which the figures stand out and a street with a house door behind them. The mosaic is made with minute glass *tesserae* (none larger than 0.25 cm, in many parts of the figures smaller than 1 mm square) in *opus vermiculatum* (worm-like), a technique in which one or more rows of *tesserae* curve around the figures, emphasizing them and foreground elements in a halo effect. As in many of the best mosaics of the period, the mortar is tinted to match the *tesserae*. In this case, the mortar was painted over with a very fine brush after the *tesserae* were set. The colors in the tunics of the men and the aulos player vary in tone to produce shimmering light.

The term "mosaic," derived from the Greek *Mouseios* (of the muses), has come to mean anything made in mosaic style and can apply to literary and other compositions outside art. So filled with possibilities for metaphorical interpretation, the term has been widely used, most famously in regards to multiculturalism. An ancient form of art become a common modern metaphor, the mosaic has shown remarkable resilience quite apart from its concrete solidity of structure.

"Painting in stone," as mosaic making has sometimes been called, is painstaking and technically demanding, particularly in its inspired form, which combines seamlessly and in complete balance color, light, and rhythm. Yet it has persisted through the ages, its tiny ingredients refined and enriched but otherwise essentially the same. The lyrical scene *Musici Ambulanti* with its ancient humor and contemporary perspective opens up the topic for metaphorical discussion. Here is a medium that owes its perseverance to its inflexibility.

In this issue, along with current reports on influenza, prion disease, and multidrug-resistant tuberculosis, we offer some historical reports, reminders that tough art is not all that survives the ravages of time, persisting through the ages. Pathogenic agents do too. This is why we still speculate on the plague of Thebes and the possibility, among many others examined, that brucellosis was the culprit. Brucellosis, a highly transmissible zoonosis, remains endemic to the Mediterranean basin. This is too why we read with interest about a dengue fever epidemic in Athens viewed through a daily newspaper in 1927–1931. Dengue fever, also an ancient scourge of global proportions, still causes outbreaks in tropical areas around the globe and sometimes even in Florida.

Unlike mosaics, which owe their resilience to the inflexibility of their medium, microbes often owe their persistence to extraordinary flexibility brought about by genetic plasticity and a short life cycle. When their genes divide, the offspring are unlike their parents in ways that may make them survive or cause disease more effectively. They adapt quickly and remain with us throughout the ages. Like the acting musicians in Dioskourides' mosaic, they play and they dance to their own rhythm, compelling, often masked, and impervious to the march of time.

Acknowledgment

Many thanks to David Swerdlow for his invaluable assistance in obtaining permission to use *Musici Ambulanti* on the cover of this issue.

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Article Title

Intestinal Toxemia Botulism in 3 Adults, Ontario, Canada, 2006–2008

CME Questions

1. You are seeing a 52-year-old woman with abdominal pain as well as neurologic complaints, and you consider whether she might have adult intestinal toxemia botulism. Which of the following statements regarding adult intestinal toxemia botulism is most accurate?

A. It produces an ascending rigid paralysis
B. It occurs after direct ingestion of neurotoxin but not spores
C. There have been fewer than 20 cases reported over the last 30 years
D. The primary infecting organism is *Clostridium butyricum*

2. In working through the differential diagnosis for this patient, what should you consider as an important risk factor for adult intestinal toxemia botulism?

A. Regular use of proton pump inhibitors
B. Recent infection with *Salmonella* species
C. The presence of Crohn disease
D. Chronic laxative use

3. Which of the following statements regarding the clinical presentation of patients with adult intestinal toxemia botulism in the current case series is most accurate?

A. All patients presented with ocular symptoms and signs
B. All patients presented with fasciculations
C. The most common source of botulism was undercooked meat
D. The most common source of botulism was fresh vegetables

4. Which of the following statements regarding the workup and prognosis of cases of adult intestinal toxemia botulism in the current case series is most accurate?

A. All patients had positive stool testing for more than 30 days
B. Nerve conduction studies were not helpful in the workup of any case
C. No patient required mechanical ventilation
D. All patients died as a result of complications of adult intestinal toxemia botulism

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Accelerating Control of Pertussis in England and Wales

CME Questions

1. Which of the following is part of the pertussis vaccine schedule during childhood in England and Wales?

A. The vaccine schedule begins in the neonatal period
B. The third vaccine dose is given at age 6 months
C. A total of 6 injections are necessary during childhood
D. A pertussis booster is required in the preschool period

2. Which age group had the highest increase in laboratory-confirmed cases and notifications of pertussis between 1998 and 2009 in the current study?

A. Less than 3 months
B. 6–11 months
C. 5–9 years
D. 9 years and older

3. What was the most common contact source of pertussis in the current study?

A. Home
B. The workplace
C. School
D. Health care facilities

4. Which of the following statements regarding pertussis vaccine effectiveness in the current study is most accurate?

A. Vaccine efficacy declined rapidly between 6–11 months and 12–39 months of age
B. Age-specific vaccine efficacy was reduced among infants compared with older children
C. The estimated vaccine efficacy for 4 doses was 95%
D. The vaccine effectiveness of the first dose was approximately 30%

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Pathogenic Responses among Young Adults during the 1918 Influenza Pandemic

Unsuspected Dengue Fever and Acute Febrile Illness in Young Adults, Southern Sri Lanka

Pathogenesis of Avian Bornavirus in Experimentally Infected Cockatiels

Effect of Surveillance Method on Lyme Disease Reports, Connecticut, 1996–2007

Human Bocavirus Infection in Children

Declining Guillain-Barré Syndrome after Campylobacteriosis Control, New Zealand, 1988–2010

Characterization of Nipah Virus from Outbreaks in Bangladesh, 2008–2010

Invasive Pneumococcal Disease and Pandemic (H1N1) 2009, Denver, Colorado

Diphtheria in the Postepidemic Period, Europe, 2000–2009

Invasive Pneumococcal Pneumonia with Influenza and Respiratory Syncytial Virus Infection

Survey of Infections Transmissible between Baboons and Humans, Cape Town, South Africa

Shuni Virus as Cause of Neurologic Disease in Horses

Extended Outbreak in Pediatric Hospitals Caused by *Cryptosporidium hominis*, China

Pandemic (H1N1) 2009 Virus Infection in Captive Cheetah

Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus Infections, United States, 2010–2011

Multiorgan Dysfunction Caused by Acute *Trypanosoma brucei rhodesiense* Infection Acquired on Safari, Zambia

Diarrhea Associated with *Plesiomonas shigelloides* Infection, Northwestern Ecuador

Phylogeography of *Francisella tularensis* subsp. *holarctica*, Europe

Complete list of articles in the February issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

February 7–24, 2012
16th Public Health Summer School
University of Otago
Wellington, New Zealand
http://www.uow.otago.ac.nz/summerschool.html

March 5–8, 2012
19th Conference on Retroviruses and Opportunistic Infections (CROI 2012)
Washington State Convention Center
Seattle, WA, USA
http://www.retroconference.org

March 11–14, 2012
ICEID 2012
Atlanta, GA, USA

May 9–13, 2012
8th International Congress on Autoimmunity 2012
Granada, Spain
http://www2.kenes.com/autoimmunity/pages/home.aspx?gclid=CMWIldrLvawCFVAntAodgzJ9pA

June 13–16, 2012
15th International Congress on Infectious Diseases (ICID)
Bangkok, Thailand
http://www.isid.org/15th_icid

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Emerging Infectious Diseases 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 as separate files, in the native format when possible (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpg or .tif files. Other file formats may be acceptable; contact fue7@cdc.gov for guidance. Figures should not be embedded in the manuscript file. Use color only as needed. Use Arial font for figure lettering. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced to print size. Large figures may be made available online only. Place figure keys within the figure; figure legends should be provided at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only and should contain 500–1,000 words. They should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.