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On the Cover

Caspar David Friedrich (1774–1840)
The Polar Sea (1824) Oil on canvas
(97.8 cm × 128.3 cm)

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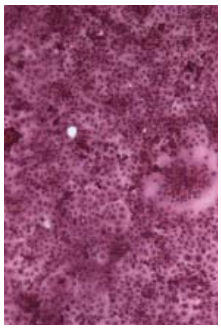

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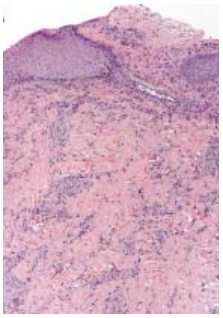

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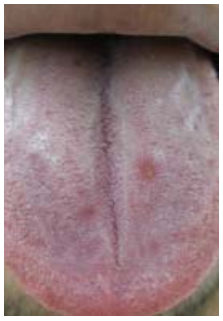

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Zoonoses in the Bedroom

Bruno B. Chomel and Ben Sun

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Learning Objectives

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

- Describe transmission of plague by pets living in very close contact to their owners
- Describe *Pasteurella* spp. infections transmitted from pets to their owners
- Describe parasitic zoonoses associated with pet ownership

Editor

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In most industrialized countries, pets are becoming an integral part of households, sharing human lifestyles, bedrooms, and beds. The estimated percentage of pet owners who allow dogs and cats on their beds is 14%–62%. However, public health risks, including increased emergence of zoonoses, may be associated with such practices.

As modern society is becoming more urbanized, the presence in our households of traditional pets, or even exotic creatures, is increasing in popularity. Pets have become an integral part of the family and are often considered to be extended family (*1*). Having pets brings many benefits, such as psychological support, friendship, and even good health practices (exercising or reducing stress) (*1*). However, in many countries, pets have become substitutes for childbearing and child care, sometimes leading to excessive pet care. For example, one of the most recent trends in pet care in Asia is hair dyeing. A recent news story claims, "Dyeing pets is popular in many developed countries like Japan and Korea, but China is quickly catching on" (www.wibw.com/home/headlines/101783553.html [cited 2010 Nov 29]). Not only are pets present in our daily environment, but they have also conquered our bedrooms. Sharing our resting hours with our pets may be a source of psychological comfort, but because pets can bring a wide range of zoonotic pathogens into our environment, sharing is also associated with risks.

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Sleeping with "Man's Best Friends"

In the United States, >60% of households have pets (*2*); pet ownership increased from 56% in 1988 to 62% in 2008 (www.americanpetproducts.org/press_industrytrends.asp [cited 2010 Jun 23]). Among dog owners, 53% consider their dog to be a member of the family. A surprising 56% of dog owners sleep with their dog next to them; ≈50% of

dogs sleep on the bed. Among dogs that sleep with their owners, 62% are small dogs, 41% are medium sized, and 32% are large (http://pets.webmd.com/features/pets-in-your-bed [cited 2010 Jun 23]). In a 2005 survey about dog ownership conducted by the American Kennel Club, 21% of dog owners interviewed said that they slept with their dog regularly; women were more likely than men to allow the practice (25% to 16%) (www.akc.org/pdfs/press_center/press_releases/2006/ValentineSurvey.pdf [cited 2010 Nov 29]). Another 16% said that their dogs snuck into their beds at least once in a while (Sacramento Bee, April 9, 2006, L1–L2). Among cats, 62% slept with their adult owners and another 13% slept with children.

In the United Kingdom, an estimated 6.5 million dogs live in ≈25% of households (*3*). In a survey of 260 dog-owning households in a community in Cheshire, 19% of the dogs were sleeping on the bedroom floor and 14% on their owner's bed (*3*). A survey conducted in 1995 with regard to cats >12 years of age throughout the United Kingdom found that among 1,236 of these older cats, 45% were sleeping regularly on the owner's bed (www.fabcats.org/behaviour/ understanding/oldcats.html [cited 2010 Jun 23]).

In the Netherlands, the pet population is ≈2 million dogs and 3 million cats (*1*). The percentage of households with pets increased from 50% in 1999 to 55% in 2005. A recent study indicated that among 159 households with pets, 50% of pet owners interviewed allowed the pet to lick their face; 60% of pets visited the bedroom; 45% of dogs and 62% of cats were allowed on the bed; and 18% and 30% of the dogs and cats, respectively, were allowed to sleep with the owner in bed (*1*).

In France, the estimated pet population is ≈8.1 million dogs in 25% of households and ≈9 million cats in 26% of households. The number of dogs increased from ≈4 million in the late 1950s to its current 8.1 million (www.naturanimal.com/chiens/pratique/chiffres-chiens.php [cited 2010 Nov 29]). Le Monde in 2002 reported that ≈45% of cat owners and ≈30% of dog owners slept with their pet (www.lemonde.fr/cgi-bin/ACHATS/acheter.cgi?offre=ARCHIVES&type_item=ART_ARCH_30J&objet_id=785025 [cited 2010 Jun 23]).

Although such trends (Table 1) should be considered with some caution because they were obtained from media sources and may not accurately reflect the true prevalence of this behavior, the zoonotic disease risks associated with such behavior should be evaluated on the basis of the scientific literature. We therefore searched PubMed for any peer-reviewed publication that clearly documented human exposure to zoonotic diseases by sleeping with, sharing a bed with, kissing, or being licked by pets.

Bacterial, Parasitic, and Viral Zoonoses

Plague

During a 1974 outbreak of plague in New Mexico, USA, 7 cases of bubonic plague were investigated. One patient noticed flea bites the morning after he allowed his flea-infested cat to share his bed (*4*). Similarly, in a series of 23 cases of plague related to cat exposure, a 9-year-old boy from Arizona had handled and slept with a sick cat (*5*). Another case, which occurred in 1983 in New Mexico, was likely acquired after indoor/outdoor cats slept with the patient (*6*). More recently, a 2008 matched case–control study (*7*) surveyed 9 plague survivors, 12 household members of these survivors, and 30 age- and neighborhood-matched controls about household and individual exposures. Four (44%) survivors and 3 (10%) controls (matched odds ratio 5.7, 95% confidence interval [CI] 1.0–31.6) reported sleeping in the same bed with a pet dog, which remained significantly associated with infection in a multivariate logistic regression model ($p = 0.046$). Such behavior is of concern because dogs may facilitate transfer of infected fleas into the home and, unlike cats, rarely show clinical signs of infection that could serve as a warning.

Chagas Disease

A study in northwest Argentina showed that dogs and cats infected with the Chagas disease agent, *Trypanosoma cruzi*, increased risk for domestic transmission of *T. cruzi* to the Chagas disease vector, *Triatoma infestans* bugs (*8*). Infection rates were significantly higher when infected dogs shared sleeping areas with humans than when they did not (relative risk 1.79; 95% CI 1.1–2.91).

Cat-Scratch Disease

Cat-scratch disease is mainly transmitted to humans when they are scratched by a cat that harbors *Bartonella*

Table 1. Estimated pet dog and cat populations in developed countries and estimated percentage of these pets sleeping on/in owner's bed , 1974–2010

	Dogs		Cats	
Country	Estimated population, millions	% Sleeping on/ in owner's bed	Estimated population, millions	% Sleeping on/ in owner's bed
United States	60	21–33	75	60
United Kingdom	8	14	8	45
France	8	30	9	45
The Netherlands	2	45	3	62

henselae–infected fleas and flea feces (*9*). However, a few documented cases have been associated with sleeping or being licked by a household pet. For example, a systemic case of cat-scratch disease with hepatic, splenic, and renal involvement caused by *B. henselae* was diagnosed by immunofluorescence assay, PCR, computed tomography, and histologic examination. The patient was a 9-year-old aboriginal girl from Taiwan, who had been sleeping with a cat at night (*10*). In addition, *B. hensalae* infection was suspected and confirmed by serologic testing of a 50-year-old man from Japan, who had left cervical lymphadenopathy and owned a dog that often licked his face (*11*). In a study of risk factors associated with cat-scratch disease in Connecticut, USA, case-patients were more likely than matched controls to have been scratched or bitten by a kitten, licked on the face by a kitten, slept with a kitten, or combed a kitten (*12*).

Pasteurella spp. and *Capnocytophaga canimorsus* Infections

Several reports describe human infections by *Pasteurella* spp. that were acquired after close contact with pets, including sharing a bed, being licked by, or kissing the pets. In 1985, a case of meningitis caused by *P. multocida* in a 60-year-old housewife living in the United Kingdom was reported (*13*). She admitted to regularly kissing the family dog. *P. multocida* isolates from buccal and nasal swabs of the dog were identical to isolates from the woman. Two cases of meningitis in newborn children (≤1 month of age) have been reported; 1 was associated with a pet cat stealing a baby's pacifier and using it as a toy, and the other was associated with a pet dog that often licked the baby's face (*14*). Of 38 reported cases of *P. multocida* meningitis in infants, 27 (87%) of 31 infants that had been exposed to animals had been exposed directly or indirectly to the animals' oropharyngeal secretions through licking or sniffing (*14*). A case of *P. multocida* infection of a hip replacement site occurred in a 69-year-old man (*15*). This man indicated that the dog had shared his bed before and after his operations, sleeping under the covers on the side of the affected leg, as it had done every night for the past 10 years (*15*).

Being licked by pets is a common source of human infection with *P. multocida* (*16*–*19*), but in a case described by Wade et al. (*16*), transmission to an infant occurred from another person. After the 2 family dogs had licked the hands of the infant's 2-year-old brother, the older boy allowed the infant to suck on his little finger. Heym et al. (*18*) describe a case in France in which a total knee arthroplasty site became infected with *P. multocida* after the patient's dog licked a small wound on the third toe of the leg that had been operated on. In another case, *P. multocida* was cultured from a wound abscess that developed in a 48-year-old obese woman 6 weeks after hysterectomy and panniculectomy for endometrial cancer (*20*); her cat had licked the wound. In France, meningitis caused by *P. multocida* developed in a 67-year-old patient with chronic, purulent otorrhea of the right ear. His dog frequently licked the patient's right ear (*21*), and cultures from the dog's saliva also grew *P. multocida*. The isolates had identical biochemical patterns, and pulsed-field gel electrophoresis (PFGE) confirmed genotypic similarities. After digestion of genomic DNA with the infrequently cleaving restriction endonuclease *Sma*I, banding-pattern analysis showed clonal similarity between the isolates from the patient and the dog. In Japan, paranasal sinusitis caused by *P. multocida* was diagnosed for a 39-year-old woman with rhinorrhea and headache (*22*). The patient's cat awakened her every morning by licking her. *P. multocida* isolates from the woman's nasal discharge and the cat's saliva were similar with respect to biochemical properties, serotype, and drug susceptibility.

Kissing pets can also transmit zoonoses. A study in Japan of 24 pet owners (11 cats and 3 dogs) found no *Pasteurella* spp. in the oral cavity of the 19 owners who had not kissed their cat, but isolated *P. stomatis* from the oral cavity of 1 of 2 owners who had kissed their cat and in 2 of 3 dog owners who had kissed their dog (*23*). Also in Japan, meningitis caused by *P. multocida* developed in a 44-year-old woman who admitted that she was regularly kissing the dog's face and feeding it by transferring food mouth to mouth (*24*). As suggested by Kawashima et al. (*24*), "recent increase in pet ownership is likely to increase human exposure to *P. multocida*." These authors identified at least 2 other cases of *P. multocida* meningitis between 2000 and 2010; these cases developed after the patients kissed a pet dog and a pet rabbit.

Capnocytophaga canimorsus infections in humans have been associated with being licked by or sleeping with a dog or cat. In Finland from 1988 through 1994, several cases of *C. canimorsus* septicemia were identified; 2 cases were associated with sleeping with and/or being licked by a pet (*25*). For an 81-year-old woman with cellulitis of the right leg and an ulcer between the fourth and fifth toe, *C. canimorsus* was isolated from a blood culture. This patient indicated that she slept with her cat in her bed and that the cat licked her feet and toes. A 60-year-old patient with chronic eczema died of septic shock and renal failure and disseminated intravascular coagulation caused by *C. canimorsus* (*25*). The ulcerous chronic eczema of his legs was the most probable port of entry for the organism because his dog used to lick his legs. In Kansas, USA, a splenectomized 44-year-old man died after infection with *C. canimorsus* (*26*). The man had lived in a trailer and collected scrap metal to sell; he had several cuts and scratches on his forearms and hands. His recently acquired German shepherd puppy reportedly licked the open abrasions on

the man's hands, but no bite was reported. In Australia, septicemia and multiorgan failure developed in a 48-year-old woman after her fox terrier puppy licked a minor burn wound on the top of her left foot (*27*).

Staphylococcus intermedius Infections

Staphylococcus intermedius is a common commensal bacterium in dogs and cats and has rarely been identified as causing human infection (*28*). However, in Japan, *S. intermedius* developed in the mastoid cavity of a 51-year-old woman after mastoidectomy for chronic otitis media with cholesteatoma (*28*). Her dog had licked her ears, and bacterial strains from the dog's saliva and the patient's otorrhea were confirmed by PFGE to be identical. Similarly, a 28-year-old woman with a history of endoscopic pituitary adenoma resection reported 3 weeks of foul-smelling nasal discharge (*29*). Nasal endoscopy indentified a purulent sinus infection caused by methicillin-resistant *S. intermedius*. Cultures from the patient's pet bulldog also grew *S. intermedius* strains that were confirmed by PFGE to be identical to those of the patient. The patient reported having had close physical contact with her dog, including frequent licking of her face, and that the dog had recent bouts of pyoderma requiring treatment with antimicrobial drugs.

Methicillin-Resistant *Stapylococcus aureus* Infections

A 48-year-old man with diabetes and his wife had recurrent methicillin-resistant *Staphylococcus aureus* (MRSA) infections (*30*). Culture of nares samples from the family dog grew mupirocin-resistant MRSA that had a PFGE chromosomal pattern identical to the MRSA isolated from the patient's nares and his wife's wound. The couple reported that the dog routinely slept in their bed and frequently licked their faces. Further recurrence of MRSA infection and nasal colonization in the couple was prevented only after successful eradication of MRSA from the dog's nares.

Rabies

In many developing countries, being licked by dogs that are rabid or suspected to be rabid is considered to pose a major risk. A survey of rabies exposure among 296 Norwegian missionaries and foreign aid workers traveling abroad showed that of 48 persons for whom postexposure vaccination was recommended, two thirds had only cared for or been licked by the suspected rabid animal (*31*). Rabies remains a problem in Southeast Asia, where many backpackers visit each year. In the early 1990s, foreign travelers (74% of whom were European), who had been in Thailand for an average of 17 days, were surveyed about potential rabies exposure during their visits. Among 1,882 travelers, 1.3% had been bitten and 8.9% had been licked by dogs (*32*). During May–June 2008, another survey of 870 foreign backpackers (median age 25.5 years) in Bangkok, Thailand, found that 3.56% had been licked by a dog (*33*).

Parasitic Infections

In the United States, the most common parasitic zoonoses associated with dogs are caused by hookworms (*Ancylostoma* spp.) and roundworms (*Toxocara canis*) (*2*). In the Netherlands, prevalent parasitic zoonoses are caused by *Toxocara* spp., *Giardia* spp., *Cryptosporidium* spp., and *Toxoplasma* spp. (*1*). Among the ways that toxocariasis can be transmitted to humans, contact with embryonated eggs on a dog's hair coat was recently proposed (*34*). Similarly, a recent study in the Netherlands identified *Toxocara* spp. eggs on the fur of 18 dogs (12.2%) and 2 cats (3.4%) and in the feces of 4 dogs and 1 cat (*1*). That same study found *Giardia* spp. in the feces of 14 dogs and 3 cats and *Cryptosporidium* spp. in feces of 8 dogs and 1 cat (*1*). A case of *Cheyletiella blakei* infection was reported in a 76-year-old woman with pruritic eruption of vesicles and bullous lesions on her trunk and arms (*35*). *Cheyletiella* spp. dermatitis was suspected because of the appearance and distribution of the elementary lesions and because before the eruption, the patient had acquired a cat that sometimes slept in her bed. The diagnosis was confirmed by a veterinary examination and isolation of *C. blakei* from the cat's skin. The patient's condition resolved after the cat was treated with ivermectin, the household was disinfected with permethrin, and the patient was treated with benzyl benzoate.

Other Dangers

Another major health hazard can be created by keeping dominant and possessive dogs in a bedroom where young infants are sleeping. An analysis of risk factors associated with nonplay dog bites in Kingston, Jamaica, found that a dog sleeping in a family member's bedroom was a risk factor for biting (relative risk 2.54, 95% CI 1.4–4.54) (*36*). In a review of fatal dog attacks in the United States during 1989–1994, Sacks et al. (*37*) reported that among 109 dog bite–related deaths, 57% were of children <10 years old and 11 were of a sleeping infant.

Recommendations

Zoonotic infections acquired by sleeping with a pet are uncommon. However, severe cases of *C. canimorsus* infection or plague in humans have been documented. More zoonotic agents that are transmitted by kissing a pet or being licked by a pet have been identified, especially zoonotic pathogens that are commensal in the oral cavity of carnivores, such as *Pasteurella* spp. and *C. canimorsus*. Because young children are often at higher risk than adults for exposure to zoonotic pathogens, especially

Table 2. Zoonoses acquired from close contact with pet, 1974–2010*

Zoonosis	Type of pet contact (reference)		
	Sleeping with	Kissing	Being licked by
Plague	Dogs (*7*), Cats (*4–6*)	–	–
Chagas disease	Dogs and cats (*8*)	–	–
Cat-scratch disease	Cats, kittens (*10,12*); dog (*11*)	–	Kittens (*12*)
Pasteurellosis	Dog (*15*)	Dog (*13*); dogs and cats (*23,24*); rabbit (*24*)	Dogs (*16,18,21*); cats (*14,17,19,20,22*); dogs and cats (*14*)
Capnocytophaga canimorsus septicemia	Cat (*25*)	–	Dog (*25–27*); cat (*25*)
Staphylococcosis	–	–	Dogs (*28,29*)
MRSA infection	Dog (*30*)	–	–
Rabies	–	–	Dogs (*31–33*)
Toxocariasis	Dogs and cats (*1*)	Dogs and cats (*1*)	Dogs and cats (*1*)
Giardiasis	Dogs and cats (*1*)	Dogs and cats (*1*)	Dogs and cats (*1*)
Cryptosporidiosis	Dogs and cats (*1*)	Dogs and cats (*1*)	Dogs and cats (*1*)
Cheyletiellosis	Dog (*35*)	–	–
Pet bites	Dogs (*36,37*)	–	–

*MRSA, methicillin-resistant *Staphylococcus aureus*; –, none reported.

when animals are displayed in public settings, the National Association of State Public Health Veterinarians issued specific recommendations (*38*). However, the concerns associated with sharing a bed with pets, being licked by pets, or kissing pets were not addressed in these recommendations. Similarly, although the risk for introduction of zoonotic agents by pets in hospitals or nursing homes has been evaluated (*39*) and recommendations made (*40*), the recommendations do not specifically address the risk for transmission through being licked by, kissing, or even sleeping with a pet.

Our review suggests that persons, especially young children or immunocompromised persons, should be discouraged from sharing their bed with their pets or regularly kissing their pets. Any area licked by a pet, especially for children or immunocompromised persons or an open wound, should be immediately washed with soap and water. Pets should be kept free of ectoparasites (especially fleas), routinely dewormed, and regularly examined by a veterinarian. Preventive measures such as anthelmintic drug intervention for puppies within the first few weeks after birth or, even better, for bitches during the last few weeks of pregnancy, could help prevent most cases of human toxocariasis. Similarly, evaluation of patients with recurrent MRSA colonization or infection or *Pasteurella* spp. infection with no obvious source should prompt queries about any regular contact with pet dogs, particularly in household settings.

Conclusion

Although uncommon with healthy pets, the risk for transmission of zoonotic agents by close contact between pets and their owners through bed sharing, kissing or licking is real and has even been documented for life-threatening infections such as plague (Table 2). Carriage of ectoparasites or internal parasites is certainly of major concern when it comes to this type of behavior. To reduce such risks, pet owners should seek regular veterinary care for their pets.

Dr Chomel is a professor of zoonoses at the School of Veterinary Medicine, University of California, Davis, with an interest in the epidemiology of zoonotic diseases, especially new and emerging zoonoses.

Dr Sun is the state public health veterinarian for California and is involved with several national committees concerning zoonotic diseases.

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Hepatitis E Virus and Neurologic Disorders

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Learning Objectives

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

- Describe the overall spectrum of neurological manifestations of hepatitis E virus (HEV) infection, based on a case series
- Describe diagnosis of HEV infection in patients who present with neurological symptoms and liver function test abnormalities
- Describe peripheral nerve involvement associated with HEV infection.

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Information about the spectrum of disease caused by hepatitis E virus (HEV) genotype 3 is emerging. During 2004–2009, at 2 hospitals in the United Kingdom and France, among 126 patients with locally acquired acute and chronic HEV genotype 3 infection, neurologic complications developed in 7 (5.5%): inflammatory polyradiculopathy (n = 3), Guillain-Barré syndrome (n = 1), bilateral brachial neuritis (n = 1), encephalitis (n = 1), and ataxia/proximal myopathy (n = 1). Three cases occurred in nonimmunocompromised patients with acute HEV infection, and 4 were in immunocompromised patients with chronic HEV infection. HEV RNA was detected in cerebrospinal fluid of all 4 patients with chronic HEV infection but not in that of 2 patients with acute HEV infection. Neurologic outcomes were complete resolution (n = 3), improvement with residual neurologic deficit (n = 3), and no improvement (n = 1). Neurologic disorders are an emerging extrahepatic manifestation of HEV infection.

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Hepatitis E virus (HEV) infection is a well-known cause of acute hepatitis in developing countries (*1*). However, autochthonous (locally acquired) HEV infection is also emerging in industrialized countries (*1*), where it is caused by HEV genotype 3 and thought to be a zoonosis transmitted by pigs (*2*). Within the past few years, HEV has been responsible for chronic hepatitis, which can rapidly evolve to cirrhosis, in immunocompromised patients (*3*–*8*). However, little data regarding HEV-related extrahepatic manifestations have been published, although an association between neurologic manifestations (e.g., Guillain-Barré syndrome, neuralgic amyotrophy, acute transverse myelitis) and acute HEV infection has been suggested (*9*–*13*).

Previously, the association between neurologic signs and symptoms and HEV infection has been based on detection of anti-HEV immunoglobulin (Ig) M in serum. However, Rianthavorn et al. reported a case of HEV genotype 3–induced neuralgic amyotrophy in which HEV RNA was detected in the serum of patients with neurologic signs and symptoms (*14*), and we recently detected HEV RNA in the cerebrospinal fluid (CSF) of a kidney-transplant recipient with chronic HEV infection and neurologic signs and symptoms (*15*). We describe 7 cases of HEV-associated neurologic disorders in patients from the Royal Cornwall Hospital, Truro, Cornwall, UK, and Toulouse University Hospital, Toulouse, southwestern France.

In Cornwall, among 55 patients with locally acquired hepatitis E, neurologic signs and symptoms developed among 3 (5.5%). From January 2004 through April 2009, in the organ-transplant unit of Toulouse University Hospital, among 50 solid-organ–transplant patients with HEV, neurologic signs and symptoms developed among 3 (6%). In addition, from January 2005 through December 2009, in the Department of Hepatology of Toulouse University Hospital, among 21 patients with acute HEV infection, neurologic signs and symptoms developed in 1 (4.76%). We describe these 7 cases of HEV-induced neurologic disorders, which occurred in 3 nonimmunocompromised patients with acute HEV infection, in 2 kidney transplant recipients and 1 kidney–pancreas transplant recipient with chronic HEV infection, and in 1 HIV-positive patient with chronic HEV infection (Tables 1, 2).

Methods

The diagnosis of HEV infection was based on the presence of HEV RNA in serum. Serologic analysis showed negative results for hepatitis A, B, and C viruses for all 7 patients and negative HIV results for all but 1 (patient 7). Organ-transplant recipients had negative results for HBV DNA, HCV RNA, and cytomegalovirus (CMV) DNA. Epstein-Barr virus (EBV) DNA was found in the blood of 2 patients (patients 4 and 5).

For the patients from Toulouse, anti-HEV status was determined by using Adaltis EIAgen HEV IgG and IgM kits (Ingen, Chilly Mazarin, France). For patients from the United Kingdom, HEV serology kits from Wantai (Beijing, People's Republic of China) or Genelabs (Singapore) were used. Serum HEV RNA was detected by real-time PCR with amplification within the open reading frame 2 region (*3*,*5*,*16*). Detected strains were sequenced and compared with reference HEV strains (GenBank) as reported (*5*,*17*).

The Patients

Patient 1

A 42-year-old man from Cornwall sought care for severe low-back pain, which progressed to paresthesia in the legs, then the arms, and then weakness with normal sphincter control. The man had not traveled outside the United Kingdom and had had no contact with pigs. Physical examination found weakness of his entire upper limbs and proximal legs. Pinprick sensation was impaired in areas on the right side innervated from C2–4 and distally but asymmetrically in his legs; additionally, S2–5 were involved on the right. Reflexes were diminished or absent in all 4 limbs.

CSF analysis showed high protein levels with lymphocytic pleocytosis (protein 1.27 g/L [reference 0.15–0.45

Table 1. Summary of 7 cases of HEV–associated neurologic disorders, Cornwall, UK, and Toulouse, France*

Patient no.	Relevant medical status	HEV infection phase†	HEV genotype	Serum				Cerebrospinal fluid		
				HEV IgG/IgM	HEV RNA	ALT, IU/L†	Bilirubin, µmol/L	HEV RNA	Protein level, g/L	Leukocytes, cells/mm^3
1	Not immunocompromised	Acute	3e	+/+	+	623	14	–	1.27	145
2	Not immunocompromised	Acute	3e	+/+	+	1160	70	ND	–	–
3	Not immunocompromised	Acute	3f	+/+	+	384	35	–	2	14
4	Kidney–pancreas transplant recipient	Chronic	3f	+/+	+	171	19	+	0.71	1
5	Kidney transplant recipient	Chronic	3f	–/+	+	110	12	+	0.8	8
6	Kidney transplant recipient	Chronic	3f	+/+	+	105	12	+	0.76	7
7‡	HIV positive	Chronic	3a	+/+	+	150	9	+	0.47	1

*HEV, hepatitis E virus; Ig, immunoglobulin; ALT, alanine aminotransferase; +, positive; –, negative; ND, not done.
†At time of examination for neurologic symptoms.
‡This patient had positive IgG and IgM Wantai assay results throughout but had negative HEV IgG and IgM results for 3 separate Genelabs assays during 2007–2008.

Table 2. Clinical presentation for 7 patients with HEV–associated neurologic disorders, Cornwall, UK, and Toulouse, France*

Patient no.	Neurologic signs and symptoms	Therapy	Outcome
1	Acute inflammatory polyradiculoneuropathy	–	Complete resolution
2	Bilateral brachial neuritis	–	Resolution with residual weakness
3	Guillain-Barré syndrome	IV Ig	Resolution at HEV clearance
4	Ataxia, severe proximal weakness of lower limbs, urine retention, and cognitive dysfunction	IS modification	Resolution with residual motor deficit
5	Encephalitis	IS cessation, foscavir, IV Ig	Complete resolution
6	Peripheral demyelinating polyradiculoneuropathy	IS modification, IV Ig	No improvement
7	Painful sensory peripheral neuropathy	Peg-IFN/ribavirin	Complete resolution

*HEV, hepatitis E virus; –, no specific therapy; IV Ig, intravenous immunoglobulins; IS, immunosuppressant, Peg-IFN, pegylated interferon.

g/L], glucose 3.5 mmol/L, and leukocytes 145×10^9 cells/L [90% lymphocytes]). Magnetic resonance image (MRI) of the pelvis and lumbar spine showed no abnormalities. Nerve-conduction studies showed distal sensory and motor activity to be within normal limits for all limbs; however, substantial tibial F-wave responses after ankle stimulation were noted, with relative prolongation on the right (right 58.50 milliseconds [ms], left 47.00 ms [reference 52.3 ±4.3 ms, interleg latency difference <5.7 ms]).

Liver function tests showed serum bilirubin within reference range but elevated alanine aminotransferase (ALT) (623 IU/L [reference 3–35 IU/L]). Serologic testing was negative for *Borrelia burgdorferi* and *Treponema pallidum*. Anti-HEV IgM and IgG were detected in the serum, as was HEV RNA, confirming a diagnosis of acute HEV. Molecular characterization showed that the serum HEV was genotype 3e (GenBank accession no. FN869556). CSF was negative for HEV RNA, CMV DNA, EBV DNA, and varicella zoster virus (VZV) DNA.

The best explanation for the clinical and laboratory findings was acute inflammatory polyradiculoneuropathy. The patient was given no specific treatment; neurologic signs and symptoms resolved fully in 3 months, and laboratory parameters returned to reference range within 6 months.

Patient 2

A 38-year-old man from Cornwall, with type 1 diabetes had a 5-day history of diarrhea, followed by pain, paresthesia, and weakness in his upper arms. He had neither recently traveled outside the United Kingdom nor had contact with pigs. Sensation to pinprick over the C5–6 dermatomes was bilaterally reduced as was strength during elbow flexion, shoulder abduction, and external rotation (worse on the right). Electrophysiologic studies confirmed a diagnosis of bilateral brachial neuritis with denervation of the supraspinatus, infraspinatus, and triceps muscles, which was more severe on the right. CSF was not examined.

Liver function tests showed elevated total serum bilirubin (70 μmol/L [reference 3–17 μmol/L]) and ALT (1,160 IU/L [reference 3–35 IU/L]). Serum was positive for anti-HEV IgG, anti-HEV IgM, and HEV RNA. Molecular characterization showed that the HEV isolated from the serum was genotype 3e (GenBank accession no. FN869555).

The patient was given no specific treatment. Within 6 weeks, liver enzyme levels returned to reference range and HEV RNA became undetectable. Signs and symptoms of brachial neuritis and other neurologic deficits gradually improved over the next 18 months, but residual weakness in his upper right arm remained.

Patient 3

A 60-year-old woman from Toulouse, France, with type 1 diabetes, had a 1-week history of severe asthenia, jaundice, and progressive weakness in her legs. She had no history of recent travel outside France or contact with animals. She was bedridden with lower limb weakness and complete loss of deep-tendon reflexes but no paresthesia. She had no fever and no biological markers of inflammation, i.e., C-reactive protein was <1 mg/L. CSF protein was 2g/L, glucose 6.2 mmol/L, and leukocyte count 14×10^9 cells/L. Liver function tests showed elevated total serum bilirubin (35 μmol/L [reference 2–21 μmol/L]) and elevated ALT (384 IU/L [reference 5–45 IU/L]). C-reactive protein and creatinine phosphokinase levels were within normal limits. Anti-HEV IgM and IgG were detected in the serum. HEV RNA was also detected in serum and fecal samples, confirming a diagnosis of acute HEV. Molecular characterization showed that the serum HEV was genotype 3f (GenBank accession no. EU 221001.1). CSF was negative for HEV RNA.

The patient's clinical and laboratory findings are best explained by acute inflammatory demyelinating polyneuropathy (Guillain-Barré syndrome) associated with HEV infection. She was given intravenous immunoglobulin at 0.4 g/kg 1×/d for 5 days. Neurologic signs and symptoms improved rapidly, and liver enzyme levels progressively returned to reference limits within 4 weeks. HEV RNA became undetectable 1 month after initial examination. Her neurologic condition gradually improved over the next 18 months, but residual weakness in her lower limbs remained.

Patient 4

In a 60-year-old man, acute autochthonous HEV (genotype 3f; GenBank accession no. EU221003) infection developed 27 months after a kidney–pancreas transplant. Acute polyradiculoneuropathy with moderate ataxia and severe proximal weakness of his lower limbs developed 30 months after HEV infection, occurring concomitantly with severe cognitive impairment and intermittent frontal dysfunction. CSF protein was 0.71 g/L, glucose 2.9 mmol/L, and leukocyte count 1×10^9 cells/L. MRI of the cerebrum showed an old lenticular infarction and no acute changes. MRI of the spine showed no abnormalities.

Immunosuppressive therapy for transplantation was a combination of tacrolimus (trough level 6 ng/mL), mycophenolate mofetil, and low-dose prednisolone (5 mg/d). Liver function test results showed total bilirubin within normal limits (19 μmol/L [reference 2–21 μmol/L]) but elevated ALT (171 IU/L [reference 5–45 IU/L]). Liver biopsy sample showed features of chronic active hepatitis; Metavir score was A2F3. CD4 count was 219×10^9 cells/L Serum HEV RNA concentration was 1,572 copies/mL. CSF was negative for anti-HEV IgG but positive for anti-HEV IgM. HEV RNA was detected in CSF obtained at the time of admission. CSF contained no detectable CMV DNA, EBV DNA, Herpes simplex viruses 1 and 2 DNA, VZV DNA, JC virus DNA, cryptococcal antigen, *Toxoplasma gondii* DNA, or *Candida* spp.

Because the patient was aphasic, confused, and drowsy, tacrolimus was replaced by low-dose sirolimus. After 10 days, neurologic signs and symptoms improved. However, 10 months later, despite rehabilitation and physiotherapy, motor deficit in the lower limbs remained and he was still unable to walk. Four months after conversion from tacrolimus to sirolimus, HEV RNA became undetectable in the serum and remains so as of September 2010. The patient declined follow-up lumbar puncture.

Patient 5

In a 35-year-old man, acute autochthonous HEV (genotype 3f; GenBank accession no. EU220999) infection developed 48 months after kidney transplantation. Three years later, drowsiness and fever (38°C) developed, and neurologic assessment revealed signs and signs and symptoms of encephalitis characterized by confusion and drowsiness without focal signs. CSF protein was 0.8 g/L, glucose 2.5 mmol/L, and leukocyte count 8×10^9cells/L. Initial computed tomographic scan of the brain showed no abnormalities. However, a few hours later, his level consciousness deteriorated and he required mechanical ventilation. Cerebral MRI, performed 24 hours later, showed features of encephalitis with diffuse white matter signal abnormalities in the supratentorial and infratentorial regions.

Immunosuppressive therapy was a combination of tacrolimus (trough level 3 ng/mL), mycophenolate mofetil, and low-dose prednisolone (5 mg/d). Liver function tests showed total bilirubin level within reference range (12 μmol/L [reference 2–21 μmol/L]) and an elevated ALT level of 110 IU/L (reference 5–45 IU/L). A liver biopsy sample showed features of chronic active hepatitis; Metavir score was A2F2. CD4 count was 149×10^9 cells/L Serum EBV DNA concentration remained unchanged from 6 months earlier, at 4.24 $\log_{10}$ copies/mL. Serum was positive for anti-HEV IgM but negative for anti-HEV IgG. Serum HEV RNA concentration was 2,154,000 copies/mL. CSF was negative for anti-HEV IgG and IgM. HEV RNA and EBV DNA were detected in CSF obtained at the time of admission. CMV DNA, Herpes simplex 1 and 2 DNA, VZV DNA, JC virus DNA, cryptococcal antigen, *Toxoplasma gondii* DNA, and *Candida* spp. were absent in the CSF.

Immunosuppressive therapy was stopped, and Foscavir (6 g/d) and intravenous immunoglobulins (total dose 2 g/kg) were added to the broad spectrum antimicrobial drugs given since admission. MRI showed improvement by day 10, and the patient was extubated. Two months later, despite the absence of neurologic signs and symptoms, CSF protein was 1 g/L, glucose 4.9 mmol/L and leukocyte count 16×10^9 cells/L (96% lymphocytes). HEV RNA and EBV DNA were still detected in the serum and CSF. One year later, HEV spontaneously cleared from serum, but the patient declined a third lumbar puncture.

Patient 6

In a 44-year-old man from Toulouse, France, acute autochthonous HEV (genotype 3f; GenBank accession no. FJ665423) infection developed 50 months after a kidney transplant (*15*). After 33 months of chronic HEV infection, the patient experienced progressive bilateral muscular weakness, difficulty walking, and palmar and plantar dysesthesia without fever. Neurologic examination revealed peripheral nerve involvement (with proximal muscular weakness that affected all limbs) and central nervous system involvement (bilateral pyramidal signs). Electrophysiologic studies showed signs of peripheral demyelinating polyradiculoneuropathy. MRI of the cerebrum showed no abnormalities. CSF protein was 0.76 g/L, glucose 3.9 mmol/L, and leukocyte count 7×10^9 cells /L.

Immunosuppressive therapy consisted of tacrolimus (trough level 8 ng/mL), mycophenolate mofetil (1 g/d), and low-dose prednisolone (2.5 mg/d). Liver function tests showed total bilirubin level within normal limits (12 μmol/L [reference 2–21]) and elevated ALT level (105 IU/L [reference 5–45 IU/L]). Liver biopsy sample showed cirrhosis; Metavir score was A2F4. Serum CD4-positive count was 167×10^9 cells/L. Serum, but not CSF, contained anti-HEV

IgG and IgM. Serum HEV RNA was 260,000 copies/mL, and HEV RNA was detected in CSF. No signs of infection were detected in the serum and CSF, except for EBV DNA, which had remained detectable in the blood since transplantation and at an unchanged concentration of 4.4 $\log_{10}$ copies/mL.

After 3 months, because the patient had severe ataxia and loss of sphincter control, neuromuscular biopsy was performed and showed nonspecific signs of neurogenic muscular atrophy but no signs of vasculitis in either muscule or nerve specimens. Consequently, the tacrolimus dosage was markedly reduced to target a trough level of 2.5 ng/mL, and intravenous immunoglobulins were administrated (0.4 g/kg/d for 5 days, total dose 2 g/kg). However, no substantial improvement was observed. After another month, decompensated cirrhosis developed and the patient died of bleeding esophageal varices.

Patient 7

A 48-year-old man from Cornwall was examined for persistently abnormal liver function that was complicating HIV disease. HIV-1 infection had been diagnosed in 2001 when the patient lived in Cambodia; he was subsequently treated for miliary tuberculosis in 2003.

When back in the United Kingdom, before receiving any antiretroviral medications, the patient had mildly elevated ALT (51 IU/L, reference 3–35 IU/L); other liver enzymes were within reference range. CD4 count was 30 $\times 10^9$ cells/L, and HIV-1 viral load was 8.3×10^4 copies/mL. Accordingly, in January 2007, the patient was given tenofovir/emtricitabine and lopinavir/ritonavir. In February 2007, the regimen was changed to abacavir/lamivudine and efavirenz; after this time, because the patient had difficulty tolerating these drugs, the regimen was again changed to abacavir/lamivudine and lopinavir/ritoanvirfor, which led to serum HIV RNA clearance in June 2007. In March 2007, ALT had risen to 114 IU/L, but there was no serologic evidence of syphilis or acute hepatitis A, B, or C. From July 2007 through July 2009, ALT remained elevated (118–195 IU/mL). In July 2007, HEV IgM and IgG were detected by enzyme immunoassay. HEV infection was confirmed by detection of HEV RNA (genotype 3a; GenBank accession no. FN869554) in serum. Testing of stored plasma samples for HEV RNA showed that the patient had been viremic since July 2007 and had remained so for 30 months, confirming chronic HEV infection. Liver biopsy sample showed cirrhosis; Metavir score was A3F4. The time HEV infection was acquired and its geographic origin remain uncertain.

In 2005, soon after completing antituberculous chemotherapy, the patient experienced progressive and painful sensory peripheral neuropathy with decreased pinprick sensation and proprioception and weakness in the distal lower limbs. At the time, these neurologic signs and symptoms were thought to have resulted from either HIV-associated neuropathy or previous isoniazid-containing antituberculous chemotherapy. In May 2009, CSF contained 0.47 g protein/L, 3.2mmol glucose/L, 1×10^9 leukocytes/L and HEV RNA.

In July 2009, because of chronic HEV liver infection, the patient was given pegylated interferon-α-2a and ribavirin. During the course of this treatment, the neurologic signs and symptoms improved, and by the time the virus cleared, they were virtually gone. One month after completion of therapy and symptom resolution, CSF levels of protein, glucose, erythrocytes, and leukocytes were within reference range; however, HEV RNA was still detected. An exact estimate of HEV viral load was not performed, but the semiquantitative technique used showed substantial reduction of HEV (barely detectable) in a follow-up CSF sample.

Discussion

Data about neurologic sequelae of HEV infection are scarce and come mainly from the Indian subcontinent. These data probably refer to HEV genotype 1 infection because this is the predominant genotype in this area.

In industrialized countries, autochthonous HEV infection has been described for a large number of persons who have not traveled to areas where HEV has traditionally been considered endemic (*1*). Hepatitis E for these persons is thought to be a porcine zoonosis and is generally caused by HEV genotype 3 (and genotype 4 in the People's Republic of China and Japan). The clinical features of hepatitis E in persons in industrialized countries are quite distinct from those in developing countries: HEV occurs most often in middle-aged and elderly men, and associated mortality rate is 5%–10% (*1*). Information about the spectrum and magnitude of disease caused by HEV genotype 3 is still emerging. For example, in recent years chronic HEV infection (with rapid development of cirrhosis) in immunocompromised persons has been demonstrated (*3*–*8*).

For the 7 cases of HEV genotype 3 infection with associated neurologic disorders reported here, the spectrum of neurologic injury associated with HEV infection was quite wide and was found in patients with acute and chronic HEV infection. However, these neurologic signs and symptoms can be divided in 2 clinical pictures. The first and dominant clinical picture is peripheral nerve involvement, which was observed for 5 of the 7 patients. These 5 patients had acute or chronic polyradiculoneuropathy. In these cases, proximal peripheral nerve involvement was similar to that associated with immune or other infectious diseases. In addition to this dominant clinical picture, 1 patient had central and peripheral manifestations, and 1 patient had encephalitis. Only 1 of the 2 patients had fever, and meningitis with lymphocytic CFS was mild or absent in that patient.

For several reasons, we think that the association between HEV genotype 3 infection and the neurologic signs and symptoms in the 7 patients reported here is causal. First, similar neurologic illnesses have been described in 2 clinically and geographically distinct populations. Second, for all patients, the diagnosis of HEV was confirmed by molecular techniques, which excludes the possibility of cross-reacting antibodies causing a spurious association between HEV infection and neurologic illness. Third, HEV RNA was detected in the CSF of some patients. Finally, there was a temporal association between clearance of HEV viremia and resolution of the neurologic signs and symptoms.

The mechanisms of neurologic damage in our patients are unknown. Many viruses (including hepatotropic viruses) trigger neurologic signs and symptoms, especially Guillain-Barré syndrome (*18*). Such infections may elicit an immune response that cross-reacts with axolemmal or Schwann cell antigens and thereby damages peripheral nerves (*18*). Among the 7 cases reported here, HEV RNA was detected in the CSF of 4 patients with chronic HEV infection and neurologic signs and symptoms, suggesting that local viral replication is occurring in the central nervous system, which may cause direct neuronal damage. Additional evidence for viral replication in the central nervous system is the discovery that different HEV quasispecies coexisted in the serum and CSF of a patient with chronic HEV infection (patient 6) (*15*). Neurologic signs and symptoms may result from infection with, or emergence of, neurotropic HEV variants (*15*).

On the basis of our observations, we are unable to estimate how frequently HEV genotype 3 infections cause neurologic damage. In the series of (mainly) acute cases in the United Kingdom, neurologic signs and symptoms were present in ≈5% of patients; in the series of chronic HEV infection in Toulouse, incidence was ≈6%. The true rate of neurologic sequalae associated with HEV 3 infection may be higher because autochthonous genotype 3 infections in industrialized countries are not widely recognized by many clinicians (including neurologists). This may be partly because of the understated clinical presentation of HEV infection. Only 1 patient reported here was icteric at the time of initial examination, and liver function test results of the immunocompromised patients were only modestly elevated. HEV-induced neurologic disorders occurred with 3 subtypes of HEV (i.e., HEV 3a, HEV 3e, and HEV 3f). These data indicate that neurologic injury induced by HEV genotype 3 is not subtype specific; because subtypes a, e, and f are found throughout Europe and North America, the geographic range of disease may well be extensive.

In conclusion, neurologic signs and symptoms are an emerging extrahepatic manifestation of HEV genotype 3 infection. We recommend that clinicians strongly consider the possibility of HEV infection in patients with neurologic disorders, especially those with peripheral nerve involvement and liver abnormalities indicated by blood tests. The diagnosis may be suggested by HEV serology but should be confirmed by molecular documentation of HEV RNA in the serum, CSF, or both.

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Human Infections with Non-O157 Shiga Toxin–producing *Escherichia coli*, Switzerland, 2000–2009

Ursula Käppeli, Herbert Hächler, Nicole Giezendanner, Lothar Beutin, and Roger Stephan

We characterized 97 non-O157 Shiga toxin (stx)–producing *Escherichia coli* strains isolated from human patients during 2000–2009 from the national reference laboratory in Switzerland. These strains belonged to 40 O:H serotypes; 4 serotypes (O26:H11/H$^-$, O103:H2, O121:H19, and O145:H28/H$^-$) accounted for 46.4% of the strains. Nonbloody diarrhea was reported by 23.2% of the patients, bloody diarrhea by 56.8%. Hemolytic uremic syndrome developed in 40.0% of patients; serotype O26:H11/H$^-$ was most often associated with this syndrome. Forty-five (46.4%) strains carried *stx*2 genes only, 36 strains (37.1%) carried *stx*1, and 16 (16.5%) strains carried *stx*1 and *stx*2. Genes encoding enterohemolysin and intimin were detected in 75.3% and 70.1% of the strains, respectively. Resistance to ≥1 antimicrobial agent was present in 25 isolates. High genetic diversity within strains indicates that non-O157 stx–producing *E. coli* infections in Switzerland most often occurred as single cases.

Shiga toxin (stx)–producing *Escherichia coli* (STEC) is among the most common causes of food-borne diseases (*1*). This organism is responsible for several human gastrointestinal illnesses, including nonbloody or bloody diarrhea. Especially in children, these diseases may be affected by neurologic and renal complications, including hemolytic uremic syndrome (HUS). Most outbreaks and sporadic cases of bloody diarrhea and HUS have been attributed to strains of STEC serotype O157:H7. However, in Europe and recently in the United States, the role of non-O157 STEC strains (e.g., O26:H11/H$^-$, O91:H21/H$^-$, O103:H2, O111:H$^-$, O113:H21, O121:H19, O128:H2/H$^-$, and O145:H28/H$^-$) as causes of HUS, bloody diarrhea, and other gastrointestinal illnesses is being increasingly recognized (*1*).

The common feature and main virulence factor of STEC is production of stx1 or stx2 proteins. Human virulent STEC strains often may also contain other virulence factors such as intimin (*eae*), a protein essential for the intimate attachment and the formation of attaching and effacing lesions on gastrointestinal epithelial cells, and *E. coli* hemolysin (*ehxA*) (*2*).

Little data are available for clinical non-O157 STEC infections in humans, including those in Switzerland, a country with a small but disproportionately high population of travelers. Therefore, we characterized all non-O157 STEC strains collected by the Swiss National Centre for Enteropathogenic Bacteria (Zurich, Switzerland) during 2000–2009, characterized these strains according to clinical and anamnestic data, and compared these results with data from other countries in Europe and the United States.

Materials and Methods

Strains

A total of 97 non-O157 STEC strains obtained from the Swiss National Centre for Enteropathogenic Bacteria were characterized. Strains were isolated during 2000–2009 from fecal samples of human patients with a reasonable clinical suspicion of infection with STEC. Samples sent to the Centre from hospitals or private practitioners are representative for Switzerland and the period screened.

Serotyping

STEC isolates were serotyped by using O (O1–O186)–specific and H (H1–H56)–specific rabbit antiserum produced at the Federal Institute for Risk Assessment (Ber-

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lin, Germany). Nonmotile strains were investigated with respect to their flagellar genotypes by using PCR and *Hha*I digestion of PCR products as described (*3*).

Strain Characterization

Fermentation of sorbitol was tested by using sorbitol MacConkey agar (Oxoid Ltd., Basingstoke, UK). PCRs targeting the *stx*1 and *stx*2 (*4*,*5*) *eae* (*6*), and *ehxA* (*7*) genes were performed as described.

Genotyping

Pulsed-field gel electrophoresis (PFGE) was performed according to the PulseNet protocol (Centers for Disease Control and Prevention, Atlanta, GA, USA) (*8*) and by using restriction enzyme *Xba*I and the CHEF-DR III system (Bio-Rad, Hercules, CA, USA). Pulse times were ramped from 5 to 50 sec for 19 h at an angle of 120°. *Salmonella enterica* serovar Braenderup strain H9812 (BAA 664; American Type Culture Collection, Manassas, VA, USA) was used as a reference. GelCompar II software (Applied Maths NV, Sint-Martens-Latem, Belgium) was used for pattern comparison. PFGE patterns were considered clonally related if they had a similarity coefficient >80% (Dice similarity index and unweighted pair-group with arithmetic mean method).

Antimicrobial Drug Susceptibility Testing

Strains were tested for antimicrobial drug resistance by the disk-diffusion method according to protocols of the Clinical and Laboratory Standards Institute (*9*). The panel of antimicrobial drug disks (Becton Dickinson, Sparks Glencoe, MD, USA) used contained ampicillin, amoxicillin/clavulanic acid, ceftazidime, cefalothin, ciprofloxacin, cefpodoxime, cefotaxime, cefuroxime, cefepime, cefoxitin, gentamicin, and tetracycline. *E. coli* strain 25922 (American Type Culture Collection) was used as a quality control.

Results

Strains

The 97 strains were isolated from 95 patients. Two STEC strains (968–03I and 968–03II; 2244–08I and 2244–08II) were isolated from 2 patients (online Appendix Table, www.cdc.gov/EID/content/17/2/178-appT.htm). During the period of screening, an increasing number of strains per year were registered. This increase could have been caused by the official reporting system for STEC, which was initiated in Switzerland in 1999; or by a country-wide reporting program for HUS, which was initiated in Switzerland in 2004 and may have increased physician awareness for HUS.

Anamnestic Data

Anamnestic data for the 95 patients are shown in Figure 1. HUS developed in 38 patients (40%); 18 (47.4%) were male, 20 (52.6%) were female, and median age was <2 years (range <1–81 years). Bloody diarrhea was noted for 54 (56.8%) patients, nonbloody diarrhea for 22 (23.2%), and anemia for 16 (16.8%). For 7 patients, no anamnestic data were available. Ten (10.5%) patients were ≥60 years of age and 63 (66.3%) were ≤10 years of age. For some patients, there was epidemiologic evidence of contact with animals, traveling, or eating animal-derived food.

No data were available for use of antimicrobial drugs. However, it is generally assumed in Switzerland that no antimicrobial drugs should be given to patients with acute diarrhea if laboratory test results are not available.

Characterization of STEC Serotypes and Virulence Genes

The strains belonged to 40 O:H serotype; 4 serotypes (O26:H11/H$^-$; O103:H2, O121:H19, and O145:H28/H$^-$) accounted for 46.4% of the strains (online Appendix Table). No significant differences were found between most prevalent serogroups and symptoms observed.

When grown on sorbitol MacConkey agar, 80 (82.5%) strains fermented sorbitol and 17 (17.5%) did not. Forty-five (46.4%) strains had *stx*2 genes, 36 strains (37.1%) had *stx*1, and 16 (16.5%) strains had *stx*1 and *stx*2. Genes for enterohemolysin and intimin were detected in 75.3%, and 70.1% of the strains, respectively.

PFGE Genotyping

PFGE conducted for strains of the most prevalent serogroups (O26, O103, O121, and O145) showed that the patterns of O26 strains were heterogeneous (similarity coefficient range 49%–94%) except for 5 strains (Figure 2). Patterns of O145 strains were also heterogeneous (similarity coefficient range 56%–95%) except for 2 strains (Figure 3). Patterns of O103 and O121 strains were heterogeneous (similarity coefficients ranges 86%–97% and 62%–91%, respectively) (Figures 4, 5). For O26 and O145 strains with the same PFGE patterns, no obvious epidemiologic link was observed between patients and different regions of Switzerland. Moreover, no information was available for about risk factors for these patients.

Antimicrobial Drug Susceptibility Testing

All non-O157 STEC strains were susceptible to 5 antimicrobial drugs (ceftazidime, ciprofloxacin, cefotaxime, cefepime, and cefoxitin). Results are summarized in the online Appendix Table. Among 97 strains, 13 (13.4%) were resistant to ampicillin, 3 (3.1%) to amoxicillin/clavulanic acid, 12 (12.4%) to cefalothin, 1 (1%) to cefpodoxime, 1 (1%) to cefuroxime, 2 (2.1%) to gentamicin,

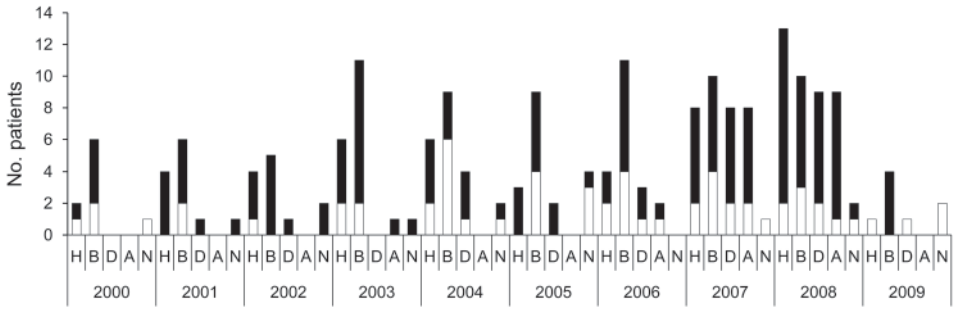

Figure 1. Anamnestic data for 97 non-O157 Shiga toxin–producing *Escherichia coli* (STEC) (black bar sections) and 44 O157 STEC (white bar sections) strains isolated from human patients, Switzerland, 2000–2009. H, hemolytic uremic syndrome; B, bloody diarrhea; D, nonbloody diarrhea; A, anemia; N, no anamnestic data available.

and 21 (21.6%) to tetracycline. The most frequent combination was resistance to ampicillin, cefalothin, and tetracycline, which was detected in 8 isolates, once each with additional resistance to amoxicillin/clavulanic acid or gentamicin. One strain, 1972–08, appeared to hyperexpress a broad-spectrum β-lactamase (resistance to ampicillin, amoxicillin/clavulanic acid, cefalothin, cefuroxime, and cefpodoxime).

Discussion

Over the 10-year study period (2000–2009), HUS caused by infection with non-O157 STEC was detected in 38 (40%) of the 95 patients investigated. This frequency of HUS cases in our study was higher than that in other studies (*10*,*11*). In contrast to other studies, in which all diarrhea samples were screened for STEC, the set of non-O157 strains in our study was isolated from patients with a reasonable clinical suspicion of infection with STEC. This fact could be the reason for a higher proportion of HUS and bloody diarrhea cases in our study group. Fourteen (36.8%) HUS cases were caused by STEC O26:H11; the remaining cases were caused by other serotypes. Of the patients with HUS, 20 (52.6%) were female and 18 (47.4%) were male. Furthermore, 30 (78.9%) were ≤5 years of age and only 4 (10.5%) were ≥60 years of age.

In a study in Minnesota, USA, over a 7-year period (2000–2006) in which 108 non-O157 STEC isolates were obtained, HUS did not develop in any of the patients; 57% were female (*10*). In Germany, Austria, and Australia, O111 strains were most frequently associated with HUS (*11*). In our study, 32 (84.2%) of the HUS isolates had *stx*2 as the only STEC gene (75%) or in combination with *stx*1 (25%), and 6 (15.8%) isolates had only *stx*1. This frequency of *stx2* in HUS isolates is similar to that in other countries, such as the United States (*12*).

Among the 30 detected STEC serogroups, O26 was most common (28 strains), followed by O145 (10 strains), O103, and O121 (6 strains each). These frequently found serogroups and others (O20, O113, O128, O146, O148, and O174) identified in our strain collection have also been found in sheep and cattle in Switzerland (*13*,*14*).

Our finding that O26 isolates (28.9%) were the most common non-O157 STEC serogroup found in Switzerland is similar to findings reported from Belgium, Germany, Japan, Spain, and the United States (*15*–*19*). Similar to other countries (*16*), in Switzerland, STEC O26:H11/H$^-$ also caused several HUS cases.

Of the remaining major O groups, O145 (6 isolates of O145:[H28] and 4 isolates of O145:H25/H$^-$) was the second most common non-O157 serogroup isolated in this study (10.3% of all isolates). Eight O145 isolates had positive results for *stx*2, *eae*, and *ehxA*; 1 isolate that did not ferment sorbitol had positive results for *stx*1, *eae*, and *ehxA*; and 1 isolate that had positive results for *stx*1 and *stx*2 also had positive results for *eae* and *ehxA*. These strain characteristics are similar to those of isolates from Finland

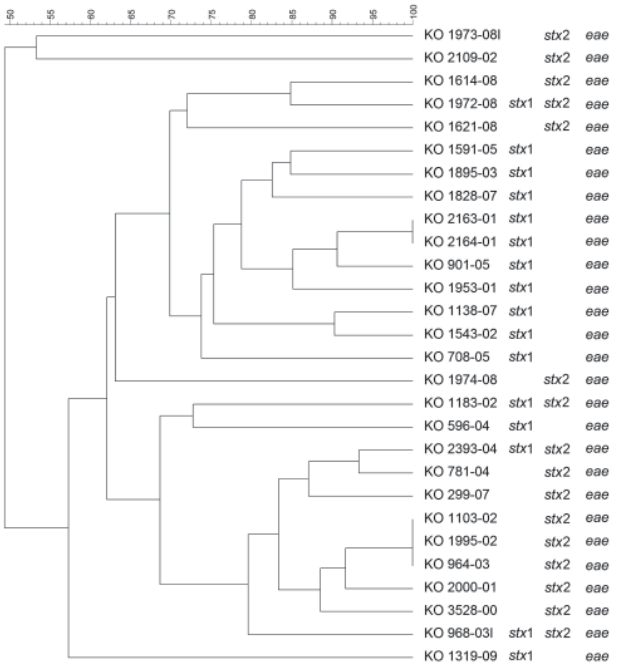

Figure 2. Dendrogram of Shiga toxin–producing *Escherichia coli* O26 strains isolated from human patients, Switzerland, 2000–2009. *stx*, Shiga toxin gene; *eae*, intimin gene. Scale bar indicates degree of similarity (%).

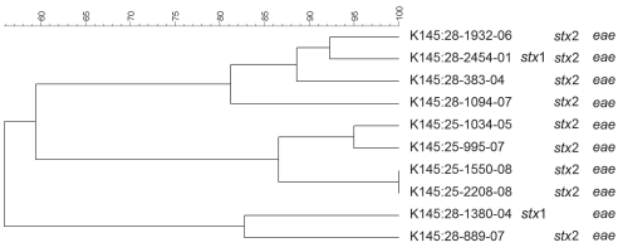

Figure 3. Dendrogram of Shiga toxin–producing *Escherichia coli* O145 strains isolated from human patients, Switzerland, 2000–2009. *stx*, Shiga toxin gene; *eae*, intimin gene. Scale bar indicates degree of similarity (%).

and Germany (*20*,*21*). Four of the O145 strains were associated with bloody diarrhea, and 5 were associated with HUS, which is similar to symptom distribution associated with STEC O145 reported by Karch et al. (*18*).

The third most common non-O157 STEC serotypes isolated were O103:H2/H$^-$ (6 strains) and O121:H19 (6 strains). In contrast to findings from Germany (*19*), none of the O103 strains from Switzerland were associated with HUS.

Among the STEC O121:H19 group, all but 1 of the strains were motile, fermented sorbitol, and were *stx*2, *eae*, and *ehxA* positive; the 1 exception was *ehxA* negative. Four patients had bloody diarrhea and HUS developed in 2 patients. These findings confirm the results of Johnson et al. (*11*), which showed that strains of serogroup O26, O103, O121, and O145 are more likely to be associated with cases of HUS.

Although STEC O111 is one of the most common serogroups in countries such as Germany, Austria, and Australia and is often associated with HUS (*11*,*22*), we found only 2 O111:H$^-$ strains that had the H8 genotype. One of these strains had *stx*1, *eae*, *ehxA*, and the other strain had *stx*1, *stx*2, *eae* and *ehxA*. The patients from whom these 2 strains were isolated sought treatment for bloody diarrhea and HUS, respectively.

According to FoodNet 2009 (*23*), the most common non-O157 STEC serogroups in the United States are O26 (28.9%), O103 (20.0%), and O111 (14.9%). The frequency of O26 (28.9% of all isolates) in the United States is the same as that in Switzerland. However, the frequencies of STEC O103 and O111 in the United States are higher than those in Switzerland (STEC O103, 6.2%; STEC O111, 2.1%).

In Germany, STEC O91 (H14/H21) is currently the fourth most common STEC serogroup isolated (*24*). However, we have detected only 1 O91:H10, *stx*2-positive, *eae*- and *ehxA*-negative, sorbitol-fermenting strain in Switzerland during the past decade. This strain was isolated from a 60-year-old woman in whom HUS developed. STEC O91 isolates that express flagellar antigen H10 have been detected in different countries, albeit at low frequencies (*25*–*27*). O91 is usually *eae* negative and the most common serogroup isolated from adult patients (*25*). Another *eae*-negative serogroup is O117, which is often associated with travelers, mainly to Asia, Africa, and Cuba (*28*). We identified 3 (3.1%) O117:H7 strains that were *stx*1 positive and *stx*2, *eae*, and *ehxA* negative. This virulence pattern was identical to that for 20 STEC strains reported by Olesen et al. (*28*). One of our strains was associated with a patient who traveled to India, a country that has been reported as the origin of O117 infections (*29*). However, no information was available regarding travel for the other 2 patients in our study from whom the O117:H7 *stx*1-positive, *stx*2-, *eae*-, and *ehxA*-negative strains were isolated. Two of these patients had bloody diarrhea, but no clinical data were available for the third patient.

To detect genetic similarities and epidemiologic relationships among STEC strains, we performed PFGE on representatives of serogroups that occurred at high frequencies. O26 PFGE patterns were heterogeneous except for 2 strain sets, which had 2 and 3 strains without any obvious epidemiologic relationship to each other. Set 1 contained 2 *stx*1-positive, *eae*-positive strains isolated in 2001 (KO 2163–01 and KO 2164–01) from an 80-year-old woman and a 2-year-old-boy, respectively, each of whom had bloody diarrhea. Set 2 contained 3 *stx2*-positive, *eae*-positive strains isolated in 2002 and 2003 (KO 1103–02, KO 1995–02, and KO 964–03), from 3 female patients (1, 2, and 41 years of age), all of whom had HUS and bloody diarrhea. However, no information for risk factors, such as contact with animals, traveling, or eating animal derived-food, was available for these 5 patients.

PFGE patterns for O145 strains were also heterogeneous, except for 2 similar strains without any epidemiologic relationships to each other that were isolated in 2008 (K 145:25–1550–08 and K 145:25–2208–08; both *stx*2 and *eae* positive, 1 from a 2-year-old girl who was treated for HUS, and 1 from a 1-year-old boy who had bloody diarrhea). However, no information for risk factors, such as contact with animals, traveling, or eating animal-derived food, was available for these 2 patients. PFGE patterns of O103 and O121 strains were the most heterogeneous; none of the strains had the same patterns.

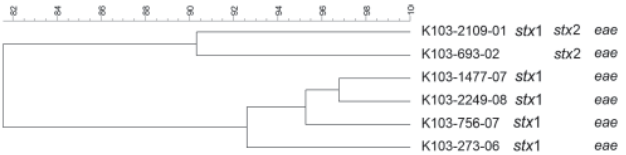

Figure 4. Dendrogram of Shiga toxin–producing *Escherichia coli* O103 strains isolated from human patients, Switzerland, 2000–2009. *stx*, Shiga toxin gene; *eae*, intimin gene. Scale bar indicates degree of similarity (%).

The *stx* gene distribution among all 97 non-O157 STEC isolates showed that 45 (46.4%) strains had only *stx*2, 36 (37.1%) had only *stx*1, and 16 (16.5%) had *stx*1 and *stx*2. Studies from Spain (*17*) and Finland (*20*) showed a similar distribution of *stx* genes among non-O157 STEC isolates. These findings are in contrast to those from the United States (Minnesota), in which non-O157 STEC strains generally showed a higher frequency of isolates that had *stx*1 (*10*).

The presence of the *eae* gene has been reported to have some predictive value for STEC seropathotypes that are associated with epidemic disease and consequently associated with severe disease such as bloody diarrhea and HUS (*12*,*30*,*31*). Therefore, we performed statistical analysis with multinomial regression and binary logistic regression for our data.

Strains associated with HUS, compared to those associated with bloody diarrhea, were more likely to harbor *stx*2 and *eae*, but the presence of only 1 of these virulence factors was not significantly associated. In our study, of 29 patients who provided *eae*-negative isolates, 17 had bloody diarrhea, 4 had bloody diarrhea and HUS, 4 had HUS, 1 had nonbloody diarrhea, and 3 had no clinical data available. Strains from the 8 patients with HUS comprised a variety of serogroups (O20, O82, O91, O148, O153, O181, and ONT); only 5 had the *stx*2 gene. In our study, 21.1% of isolates from HUS patients were *eae* negative. The 3 *eae*-negative, *stx*2-negative strains had only *stx*1 or *stx*1 and *ehxA*. Strains with such a pattern of virulence factors are notable because they are less likely to cause HUS than strains harboring *stx*2. However, these findings are consistent with epidemiologic data from other countries (*19*,*27*), which indicate that certain *eae*-negative STEC strains cause hemorrhagic diseases in humans. A report from the United States (Minnesota) indicated that non-O157 isolates that had only *stx*1 can cause severe illness (bloody diarrhea or HUS) (*32*).

Resistance to ≥1 of the 12 antimicrobial drugs tested was identified in 25 (25.8%) non-O157 STEC strains. This finding is consistent with results for a study in Spain, in which 238 (41%) of 581 non-O157 STEC strains were resistant to ≥1 of 26 antimicrobial drugs (*33*). In our study, 13 (13.4%) of 97 strains were resistant to ampicillin, 3 (3.1%) to amoxicillin/clavulanic acid, 12 (12.4%) to cefalothin, 1 (1%) to cefpodoxine, 1 (1%) to cefuroxime, 2 (2.1%) to gentamicin, and 21 (21.6%) to tetracycline. In STEC strains, the most frequent drug-resistance combination was resistance to ampicillin, cefalothin, and tetracycline, which was detected in 8 isolates, once with resistance to amoxicillin/clavulanic acid and once with resistance to gentamicin. Comparably, Schroeder et al. (*34*) tested 137 non-O157 *E. coli* human isolates (including 37 STEC strains) from the United States, Saudi Arabia, Argentina, Canada, Mexico, Zambia, and Singapore and reported STEC drug-resistance frequencies of 14% for ampicillin, 5% for amoxicillin/clavulanic acid, 11% for cefalothin, and 32% for tetracycline.

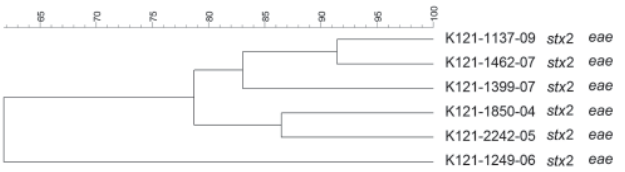

Figure 5. Dendrogram of Shiga toxin–producing *Escherichia coli* O121 strains isolated from human patients, Switzerland, 2000–2009 in Switzerland. *stx*, Shiga toxin gene; *eae*, intimin gene. Scale bar indicates degree of similarity (%).

In conclusion, high genetic diversity within strains indicates that non-O157 Shiga toxin–producing *E. coli* infections in Switzerland most often occurred as single cases. Because little data are available for clinical non-O157 STEC infections in humans, our results may provide useful information for analysis of these strains.

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Ms Käppeli is a doctoral candidate at the Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zurich. Her main research interest is characteristics of non-O157 Shiga toxin–producing *E. coli*.

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Severe Cases of Pandemic (H1N1) 2009 in Children, Germany

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In a hospital-based observational study in Germany, we investigated children admitted to pediatric intensive care units and deaths caused by confirmed pandemic (H1N1) 2009 to identify risk factors and outcomes in critically ill children. Ninety-three children were eligible for our study, including 9 with hospital-acquired infections. Seventy-five percent had underlying chronic medical conditions; neurodevelopmental disorders were most prevalent (57%). The proportion of patients having ≥1 risk factor increased with age in years (odds ratio 1.21, p = 0.007). Of 15 deaths, 11 occurred in a pediatric intensive care unit (case-fatality rate 12%, 95% confidence interval 6%–21%). Only 9% of the children had been vaccinated against pandemic (H1N1) 2009; all survived. Our results stress the role of underlying risk factors, especially neurodevelopmental disorders, and the need for improving preventive measures to reduce severe disease and adverse outcomes of pandemic (H1N1) 2009 in children.

The novel strain of influenza known as pandemic (H1N1) 2009 virus that originated in Mexico and the United States resulted in the first pandemic of the 21st century. Cases were observed in 214 countries, and 18,097 laboratory-confirmed deaths caused by this virus have been reported (*1*). In Germany, where the first cases were confirmed on April 29, 2009, the number of reported cases was 226,158 (including 255 deaths) as of May 18, 2010 (*2*).

Children were particularly affected by pandemic (H1N1) 2009. This finding is evident in the age distribution of patients, which is skewed toward younger age groups, and in high hospitalization rates for children identified in many settings worldwide (*3*–*5*). Severity has been mostly assessed in terms of admission to intensive care units (ICUs) and case-fatality rates. In a cohort study in Australia and New Zealand, the highest age-specific incidence rate for ICU admission was for children <1 year of age (*6*). Observational studies in ICU settings in the early pandemic phase in Mexico (*7*) and Canada (*8*) highlighted high rates of adolescents among critically ill patients.

Studies conducted in pediatric ICU (PICU) settings originate predominantly from the Americas (*9*–*11*). In Europe, Lister et al. summarized experiences from 4 ICUs in the United Kingdom and identified 13 critically ill children with pandemic (H1N1) 2009 during June–July 2009 (*12*). These studies and national surveillance systems contributed to a better understanding of determinants and risk factors for severe disease in children. However, information from countries in Europe about severe cases of pandemic influenza (H1N1) 2009 in children who are particularly vulnerable is still limited (*12*,*13*). To obtain information on risk factors, course of disease, and outcome of critically ill children with pandemic (H1N1) 2009, we prospectively performed a nationwide observational study covering the fall wave of pandemic (H1N1) 2009 in Germany.

Methods

Study Design

We investigated cases of critically ill children with confirmed pandemic (H1N1) 2009 in pediatric hospitals in Germany. The 375 study sites participating in the established nationwide active surveillance network Survey Center for Rare Pediatric Diseases in Germany (ESPED) comprise all pediatric hospitals in Germany.

The study included pediatric cases of pandemic (H1N1) 2009 reported during August 2009–April 30, 2010. Cases were defined as illness in patients <15 years of age who had a laboratory-confirmed infection with pandemic (H1N1) 2009 virus (determined by PCR, virus isolation, or antigen detection) and were admitted to a PICU or died.

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Data Collection

A structured questionnaire, adapted from an earlier study on seasonal influenza by Liese et al. (*14*), was distributed to the hospitals that reported cases, completed by the treating physician, and collected by the ESPED study center. Monthly reporting was requested even if no cases were identified. Up to 3 reminders were sent if questionnaires were not returned. To take into account the reporting delay, we included reports received by the study center until the end of April 2010. Of 211 distributed questionnaires requested by 132 hospitals, 176 (83%) were returned to the study center (Figure 1). After excluding 2 patients with cases who had been notified twice and 81 questionnaires from persons who did not fulfill the case definition, there remained 93 (53%) eligible questionnaires from 55 hospitals. Data were double entered into an electronic database by using EpiData software (EpiData Association, Odense, Denmark). Individual datasets were inspected for missing information, plausibility, and data entry errors. The contact persons of the participating hospitals were notified up to 2 times when data were incomplete in the questionnaire form.

The structured questionnaire included patient information; data on the hospital stay; clinical signs and symptoms of illness; clinical and laboratory diagnosis; specific treatments; underlying chronic medical conditions (chronic respiratory diseases, cardiac diseases, immunodeficiency and neurodevelopmental disorders, including developmental delay, cerebral palsy, epilepsy, and other cognitive disorders); status of influenza vaccination; and complications of the disease. Answer categories were predetermined, but other diagnoses and concurrent conditions could additionally be specified by the respondents as free text.

Statistical Analysis

Descriptive statistics comprised the calculation of median and interquartile ranges (IQRs) for continuous variables and absolute numbers and proportions (together with 95% binomial exact confidence intervals [CIs] where appropriate) for categorical variables. For the calculation of inpatient periods, patients were excluded if they had acquired pandemic (H1N1) 2009 while hospitalized. Comparative analyses were performed on the basis of the Wilcoxon rank-sum test for continuous variables and Fisher exact test for categorical variables only for patients admitted to a PICU. Odds ratios (ORs) and 95% CIs were calculated. Logistic regression was performed for continuous independent variables. All reported p values were 2-sided, and $p<0.05$ was considered significant. Statistical analyses were performed by using Stata 11.0 (StataCorp LP, College Station, TX, USA).

Data Protection and Ethical Clearance

Adherence to national data protection laws was approved by the Federal Commissioner for Data Protection and Freedom of Information of Germany. Ethical approval was granted by the Ethics Committee, Charité, Universitätsmedizin, Berlin, Germany.

Results

Characteristics of Study Population

During the study period, we included 93 critically ill children with confirmed pandemic (H1N1) 2009. Their dates of disease onset were September 21, 2009–February 23, 2010; a peak in November 2009 included 58% of the cases (Figure 2). Sixty percent of the patients were boys. The age distribution is shown in Figure 3. Median age was 4.5 years (IQR 1.3–9.3 years), 19 (20%) were <1 year of age, and 16 (17%) children were <6 months of age. Seventy-eight patients survived and 15 died. Among those who died, 4 patients were not admitted to a PICU (Figure 1). Nine patients, of whom 1 died, had acquired pandemic (H1N1) 2009 while hospitalized. The PICU cohort comprised 89 patients with a case-fatality rate of 12% (95% CI 6%–21%). The 89 reported patients correspond to an incidence rate for severe PICU-admitted cases of 27.8 cases/million children in infants <1 year of age and 8.0 cases/million children in children <15 years of age (all children of the same age group). No difference

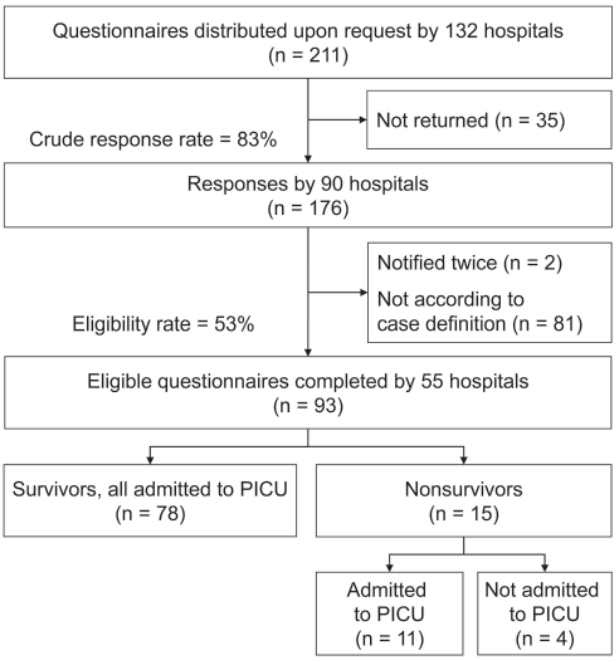

Figure 1. Overview of study participation and participant groups among children with severe pandemic (H1N1) 2009, Germany, 2009–2010. PICU, pediatric intensive care unit.

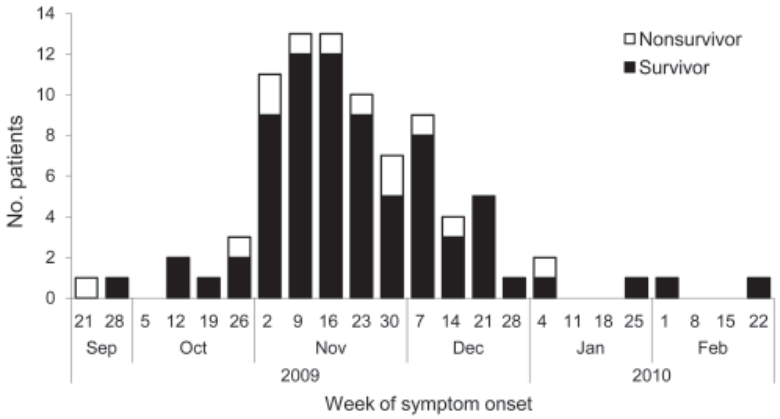

Figure 2. Date of symptom onset for 86 children with severe pandemic (H1N1) 2009, Germany, September 21, 2009–February 22, 2010. Only children with available information are included.

was found in the age distribution between survivors and those who died.

Underlying Chronic Medical Conditions and Vaccination Status

Seventy-five percent (67/89 with available information) of the patients had ≥1 underlying chronic medical condition known as a risk factor for seasonal influenza. The age distribution by presence or absence of ≥1 underlying chronic medical condition is shown in Figure 4. The proportion of patients having ≥1 risk factor increased with age in years (OR 1.2, 95% CI 1.1–1.4; p = 0.007). Neurodevelopmental disorders were most frequently reported (57% of the cases), followed by chronic respiratory diseases (38%), immunodeficiency (16%), and cardiac diseases (13%) (Table 1). Neurodevelopmental disorders were associated with a chronic respiratory disease in 60% (25/42) of the cases and were present in 79% (11/14) of those who died. Among the 53 children ≥6 months of age for whom information was available, 5 patients (9%) had been vaccinated against pandemic (H1N1) 2009; all of them survived.

Clinical Manifestations

Pneumonia was the most frequent clinical diagnosis. It was documented in 70 (75%) of 93 patients and was the only diagnosis for 37% of them. The second most frequent diagnosis was acute respiratory distress syndrome (ARDS) in 22 (24%) of 93 patients. This diagnosis was only reported for patients admitted to a PICU and was associated with death (OR 7.4, 95% CI, 1.6–37.8; p = 0.004). Six patients had a diagnosis of encephalopathy and 2 had a diagnosis of myocarditis (Table 2).

Hospital Course and Treatment

The median duration from symptom onset to hospital admission was 2 days (IQR 1–5 days), and the median duration from hospitalization to PICU admission was 0 days (IQR 0–1 days) for all patients admitted to the PICU. Both of these periods were not different between surviving patients and those who died in the PICU. Among those who died who were treated in the PICU, the median time from symptom onset to death was 8 days (IQR 3–12 days), and the median length of stay in the PICU was 2 days (IQR 0–8 days) (Table 3).

Among patients admitted to the PICU, oseltamivir was administered to 61% (51/84) of the patients; there was no difference in its use between survivors and those who died (Table 4). Median time from symptom onset to antiviral treatment for both groups was 4 days (IQR 1–7 days for survivors and 2–8 days for those who died) (Table 3). Other treatments in the PICU included catecholamines (28/81 PICU patients, 35%) and mechanical ventilation (56/86, 65%). Both of these treatments were administered more often to those who died (p = 0.007 and p = 0.007, respectively).

Discussion

During the peak phase of the pandemic, active surveillance in pediatric hospitals identified 93 severe cases of pandemic (H1N1) 2009 in children with available information on prior medical history, course of disease, and outcome. When we compared absolute numbers, deaths of children caused by pandemic (H1N1) 2009 were reported 23× more frequently in our study than in a prospective study on seasonal influenza (*14*). In this study, which used

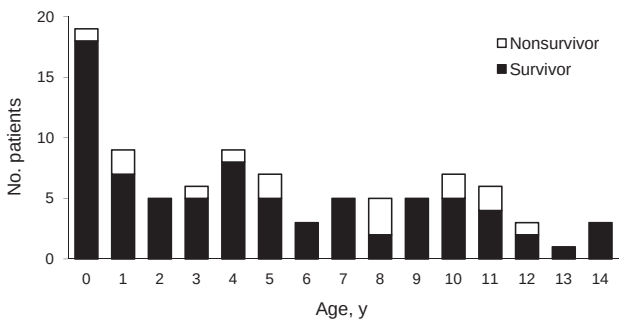

Figure 3. Age distribution of 93 children with severe pandemic (H1N1) 2009, Germany, 2009–2010.

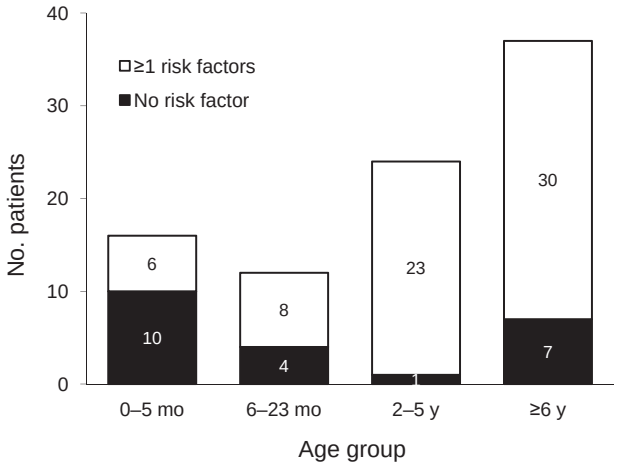

Figure 4. Age group distribution of 89 children with severe pandemic (H1N1) 2009, by number of underlying chronic medical conditions (risk factors), Germany, 2009–2010. Only children with available information are listed. Risk factors are chronic respiratory diseases, cardiac diseases, immunodeficiency, and neurodevelopmental disorders.

an analogous case definition and the same hospital network, the deaths of only 2 patients were reported for 3 influenza seasons (2005–06, 2006–07, and 2007–08 seasons) in Germany. Similarly, in the United States, more deaths in children caused by pandemic (H1N1) 2009 were reported than for each of the 3 previous influenza seasons (*15*).

The higher number of reported deaths caused by pandemic (H1N1) 2009 might be partially explained by the high level of suspicion among physicians during the pandemic, which resulted in more frequent testing and diagnosis of influenza. This hypothesis is supported by a prospective study for seasonal influenza in the United States, in which only 43% of children admitted to a PICU with laboratory-confirmed influenza were independently given a diagnosis of influenza by the treating physician (*16*).

Our study indicated a PICU case-fatality rate of 12%, which is consistent with results from a study in Canada, which reported a case-fatality rate of 7% among 57 case-patients admitted to a PICU (*11*). However, case-fatality rates for children with pandemic (H1N1) 2009 vary considerably across study sites, as shown in 2 other studies in PICU settings. In a cohort of 147 children in Argentina, a case fatality rate of 39% was reported (*9*), which was similar to a case-fatality rate of 38% in a cohort of 13 patients in the United Kingdom (*12*). Both studies reported a higher case-fatality rate than for seasonal influenza. Differences in health care organization, including PICU admission criteria, age structure of the cohorts, and selection of study sites may partly explain the different findings.

The incidence rate for severe cases in PICU-admitted patients <15 years of age was 8.0 cases/million children, which was 5× times as high as the cumulative incidence over the 3 previous influenza seasons in the same population group reported by Liese et al. (1.7 cases/million children in the same age group) (*14*). This finding is consistent with studies from Australia and New Zealand, which showed the highest age-specific incidence in this age group (*6*).

Children <1 year of age represented 20% of our cohort, and thus a higher proportion than in the cohort investigated in the Netherlands (15%) (*13*). Special awareness is clearly needed for diagnosing influenza in infants because of the variable clinical manifestations in this age group. This awareness might be particularly relevant in low-resource settings that have limited virologic diagnostic capacities.

In our study, ARDS and pneumonia were the most frequent diagnoses among those who died. ARDS was the only diagnosis strongly associated with a fatal outcome (PICU case-fatality rate 32%). In Argentina, 80% of the children in a PICU had ARDS, and this condition was also associated with death (*9*). The frequency of other complications, which included 6 cases of encephalopathy and 2 cases of myocarditis did not differ between survivors and those who died.

Nine of 93 children in our study had acquired pandemic (H1N1) 2009 while hospitalized. The risk for nosocomial transmission of pandemic (H1N1) 2009 has also

Table 1. Underlying chronic medical conditions and vaccination status for children with severe pandemic (H1N1) 2009, Germany, 2009–2010*

Characteristic	Total	Nonsurvivors not in PICU	Admitted to PICU				
			Survivors	Nonsurvivors	Subtotal	p value	OR (95% CI)
Underlying chronic medical conditions							
Any	67/89 (75)	4/4 (100)	56/76 (74)	7/9 (78)	63/85 (74)	1	1.3 (0.2–13.3)
Neurodevelopmental disorders	51/89 (57)	4/4 (100)	40/75 (53)	7/10 (70)	47/85 (55)	0.501	2.0 (0.4–13.1)
Respiratory disease	31/82 (38)	2/3 (67)	25/70 (36)	4/9 (44)	29/79 (37)	0.718	1.4 (0.3–7.3)
Immunodeficiency	13/80 (16)	1/4 (25)	12/67 (18)	0/9 (0)	12/76 (16)	0.339	0.0 (0.0–2.1)
Cardiac disease	11/84 (13)	0/4 (0)	9/70 (13)	2/10 (20)	11/80 (14)	0.621	1.7 (0.2–10.6)
Vaccination status in patients ≥6 mo of age							
Any influenza	9/56 (16)	0/4 (0)	9/48 (19)	0/4 (0)	9/52 (17)	1	0.0 (0.0–4.7)
Pandemic (H1N1) 2009	5/53 (9)	0/4 (0)	5/45 (11)	0/4 (0)	5/49 (10)	1	0.0 (0.0–9.2)

*Values are no. positive/no. with available information (%) except as indicated. PICU, pediatric intensive care unit; OR, odds ratio; CI, confidence interval. ORs were calculated only among patients admitted to the PICU.

Table 2. Clinical diagnosis for children with severe pandemic (H1N1) 2009, Germany, 2009–2010*

Clinical diagnosis	Total†	Nonsurvivors not in PICU†	Admitted to PICU				
			Survivors†	Nonsurvivors†	Subtotal†	p value	OR (95% CI)
Pneumonia	70/93 (75)	4/4 (100)	59/78 (76)	7/11 (64)	66/89 (74)	0.465	0.6 (0.1–2.9)
ARDS	22/93 (24)	0/4 (0)	15/78 (19)	7/11 (64)	22/89 (25)	0.004	7.4 (1.6–37.8)
Secondary pneumonia	15/93 (16)	0/4 (0)	13/78 (17)	2/11 (18)	15/89 (17)	1	1.1 (0.1–6.3)
Bronchitis/bronchiolitis	12/93 (13)	1/4 (25)	10/78 (13)	1/11 (9)	11/89 (12)	1	0.7 (0.0–5.8)
Encephalopathy	6/93 (6)	0/4 (0)	5/78 (6)	1/11 (9)	6/89 (7)	0.558	1.5 (0.0–15.1)
Sepsis	6/93 (6)	1/4 (25)	5/78 (6)	0/11 (0)	5/89 (6)	1	0.0 (0.0–5.6)
Status asthmaticus	2/93 (2)	0/4 (0)	2/78 (3)	0/11 (0)	2/89 (2)	1	0.0 (0.0–14.5)
Febrile seizure	2/93 (2)	0/4 (0)	2/78 (3)	0/11 (0)	2/89 (2)	1	0.0 (0.0–14.5)
Myocarditis	2/93 (2)	0/4 (0)	1/78 (1)	1/11 (9)	2/89 (2)	0.233	7.7 (0.1–611.9)
Other diagnosis‡	26/93 (28)	0/4 (0)	21/78 (27)	5/11 (45)	26/89 (29)	0.287	2.3 (0.5–9.9)

*Values are no. positive/no. with available information (%) except as indicated. PICU, pediatric intensive care unit; OR, odds ratio; CI, confidence interval; ARDS, acute respiratory distress syndrome. ORs were calculated only among the cases admitted to the PICU.
†Children may have had >1 diagnosis.
‡Acute exacerbation of a chronic disease or new diagnosis.

been documented in other studies (*17*,*18*). In both of these reports, pandemic (H1N1) 2009 was likely transmitted by health care workers. Additionally, children with underlying chronic medical conditions might have a higher risk for being hospitalized and therefore are particularly exposed to the risk for nosocomial infection. As reported for seasonal influenza (*19*,*20*), this result stresses the need for appropriate preventive strategies in hospital settings, such as early use of diagnostic tests and vaccination of health care workers who are involved in the care of patients with risk factors for severe disease.

We observed that patients who died had a median time in the hospital of 3 days, including 2 days in a PICU. Death occurred despite maximum intensive care therapy, as demonstrated by the higher rate of catecholamine treatment and mechanical ventilation among those who died. This observed rapid course of fatal disease despite intensive care, which was also reported in the United Kingdom (*12*), underlines the need for prevention.

The proportion of patients having ≥1 underlying chronic medical condition was high (75% overall) and increased with age. Our findings are consistent with those from a case series of 235 hospitalized children with pandemic (H1N1) 2009 in Canada (median age 4.8 years, range 0–16 years). A total of 60% of the patients in this study had ≥1 underlying chronic medical conditions (33% were children <2 years of age and 72% were older children) (*21*). Neurodevelopmental disorders were reported for more than half of the children and in more than three fourths of those who died. These results are consistent with the results from other PICU-setting studies in which neurodevelopmental disorders were the first or second most prevalent risk factor (*9*–*12*). According to the surveillance system for pediatric deaths associated with pandemic (H1N1) 2009 in the United States, 92% of the children with high-risk medical conditions had neurodevelopmental disorders (*22*).

In our study, only 5 children had been vaccinated against pandemic (H1N1) 2009. Their vaccination dates were not given, and it remains unclear whether the interval was sufficient to acquire immune protection. A considerable proportion of the patients with investigated cases could not benefit from immunization because the pandemic (H1N1) 2009 vaccine was not publicly available in Germany until after November 2, 2009, and 17% of all children in this study were <6 months of age. In Germany, neurodevelopmental disorders had not been explicitly included in the

Table 3. Clinical course for children with severe pandemic (H1N1) 2009, Germany, 2009–2010*

Characteristic	Total	Nonsurvivors not in PICU	Admitted to PICU			
			Survivors	Nonsurvivors	Subtotal	p value
Time course of illness, d						
Symptom to hospital admission	77, 2 (1–5)	3, 1 (1–2)	65, 2 (1–5)	9, 1 (1–3)	74, 2 (1–5)	0.700
Hospitalization to PICU admission	74, 0 (0–1)	NA	65, 0 (0–1)	9, 1 (0–3)	74, 0 (0–1)	0.236
PICU length of stay	80, 8 (3–17)	NA	69, 9 (3–18)	11, 2 (0–8)	80, 8 (3–17)	NC
Hospital length of stay	83, 14 (5–23)	3, 5 (3–12)	69, 16 (7–25)	11, 3 (2–12)	80, 14.5 (5.5–23.5)	NC
Symptom onset to outcome†	85, 16 (8–26)	4, 5.5 (5–9.5)	72, 18.5 (10.5–29.5)	9, 8 (3–12)	81, 17 (8–27)	NC
Time to treatment, d						
Symptom onset to oseltamivir treatment	45, 4 (1–7)	1, 4‡	39, 4 (1–7)	5, 4 (2–8)	44, 4 (1–7)	0.551

*Values given are total no. with available information, median (IQR), except as indicated. PICU, pediatric intensive care unit; IQR, interquartile range; NA, not applicable (not admitted to PICU); NC, not compared because of different outcomes (release for survivors and death for nonsurvivors).
†Including patients with hospital-acquired pandemic (H1N1) 2009 infection.
‡Only 1 observation.

Table 4. Treatment administered to children with severe pandemic (H1N1) 2009, Germany, 2009–2010*

Treatment	Total	Nonsurvivors not in PICU	Admitted to PICU				
			Survivors	Nonsurvivors	Subtotal	p value	OR (95% CI)
Oseltamivir	53/88 (60)	2/4 (50)	44/74 (59)	7/10 (70)	51/84 (61)	0.733	1.6 (0.3–10.2)
Antimicrobial drug	80/91 (88)	4/4 (100)	67/77 (87)	9/10 (90)	76/87 (87)	1	1.3 (0.2–64.7)
Catecholamine	28/85 (33)	0/4 (0)	20/70 (29)	8/11 (73)	28/81 (35)	0.007	6.7 (1.4–41.8)
Mechanical ventilation	56/90 (62)	0/4 (0)	45/75 (60)	11/11 (100)	56/86 (65)	0.007	NA (1.8–NA)

*Values are no. positive/no with available information (%) except as indicated. PICU, pediatric intensive care unit; OR, odds ratio; CI, confidence interval, NA, not applicable.

chronic medical conditions in the vaccination recommendations for seasonal influenza (*23*) and were only specified in recommendations for pandemic (H1N1) 2009 vaccine (*24*). In contrast, in the United States, neurodevelopmental disorders had already been recognized as a risk factor for seasonal influenza in 2005 (*25*).

Recent reports on adults and children with pandemic (H1N1) 2009 suggested that oseltamivir therapy benefitted patients with severe cases. Early treatment within 2 days after symptom onset was statistically associated with a lower risk for ICU admission and death in hospitalized pandemic (H1N1) 2009 patients (n = 272; median age 21 years) than with later treatment (*26*). In our study, the median time to oseltamivir treatment was 4 days and did not differ between survivors and those who died. Therefore, our study might not have been able to detect the benefit of this treatment. Nevertheless, this finding should be viewed with caution because our study was not designed to evaluate the effectiveness of oseltamivir for treatment of children with pandemic (H1N1) 2009. However, 1 ICU-setting study (n = 58; median age 44 years) suggested a benefit for patients who were treated with oseltamivir >48 hours after illness onset (*7*).

The representativeness of our study was assessed by comparing our data with those from the national databases. First, the timeline of our cases was compared with the Praxis Index, which derives from the syndromic surveillance system of the national working group on influenza and accounts for all notifications of influenza-like illness cases in Germany. The Praxis Index curve and the epidemiologic curve of patients investigated in our study show similar shapes. Second, of the 15 identified deaths in our study, 14 could be matched with the 29 deaths in children <15 years of age reported in the National Surveillance System. This difference might be explained by the fact that only children admitted to pediatric hospitals were captured in our study. Because our study was a nationwide study, the 93 cases originated from 55 hospitals in 14 of the 16 Federal States of Germany.

Our study has several limitations. These limitations include potential underreporting, although this might have been minimized by increased awareness during the influenza pandemic in Germany. In addition, patients with influenza could not be included when the questionnaires were not returned despite written reminders. Another limitation might be that not all children are hospitalized in pediatric hospitals. However, patients with severe cases requiring intensive care would likely have been transferred to a PICU and thus should have been captured in our study. This suggestion is supported by the fact that 11 patients had been transferred from other hospitals. An additional limitation might be that knowledge of clinical features of patients was only based on information provided in the questionnaires. Furthermore, ascertainment of underlying chronic medical conditions was not standardized and may differ from 1 physician to another. Because the survey instrument captured temporal information in days, the time from symptom onset to initiation of treatment could not be calculated in hours. Finally, even with an unexpected high number of reported severe cases, the total number of deaths in PICUs was too small to perform a multivariable analysis for factors associated with death.

This study identified a considerable number of severe cases of pandemic (H1N1) 2009 among children in Germany, confirming observations in the Americas. Our results stress the role of underlying risk factors, especially neurodevelopmental disorders, in children with severe cases of pandemic (H1N1) 2009. The results also indicate that measures that would prevent severe disease and adverse outcomes in children, including vaccination and other preventive measures, as well as early diagnosis and prompt treatment of this infection, are not used to their full extent despite availability of maximum care resources.

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Risk Factors for *Cryptococcus gattii* Infection, British Columbia, Canada

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To determine whether particular environmental, medical, or behavioral risk factors existed among *Cryptcoccus gattii*–infected persons compared with the general population, we conducted a sex-matched case–control study on a subset of case-patients in British Columbia (1999–2001). Exposures and underlying medical conditions among all case-patients (1999–2007) were also compared with results of provincial population–based surveys and studies. In case–control analyses, oral steroids (matched odds ratio [MOR] 8.11, 95% confidence interval [CI] 1.74–37.80), pneumonia (MOR 2.71, 95% CI 1.05–6.98), and other lung conditions (MOR 3.21, 95% CI 1.08–9.52) were associated with infection. In population comparisons, case-patients were more likely to be ≥50 years of age (p<0.001), current smokers (p<0.001), infected with HIV (p<0.001), or have a history of invasive cancer (p<0.001). Although *C. gattii* is commonly believed to infect persons with apparently healthy immune systems, several immunosuppressive and pulmonary conditions seem to be risk factors.

Cryptococcus gattii emerged on Vancouver Island, British Columbia (BC), Canada, in 1999, resulting in one of the highest incidences of this infection worldwide (*1*,*2*). The natural reservoir of *C. gattii* seems to be soil and plant debris, and it has been associated with numerous tree species (*3*,*4*). When inhaled, this encapsulated basidiomycetous yeast may infect humans as well as diverse animal species (*5*). Infected humans may be asymptomatic but usually exhibit pulmonary infection characterized by cough, shortness of breath, and single or multiple pulmonary nodules visible on radiographs of the lung. Body sites such as brain, skin, and bone are affected less commonly (*6*,*7*). Approximately 18% of patients in British Columbia have disseminated disease, including meningitis and brain cryptococcomas (*1*). The case-fatality rate among BC residents during 1999–2007 was 8.7% (*1*).

C. gattii is believed to infect persons with uncompromised immune systems (*8*,*9*), unlike *C. neoformans,* a relatively common opportunistic pathogen in HIV-infected patients and other immunocompromised patients. Although true differences in species-specific pathogenicity may exist, *C. gattii* may also commonly infect persons with undetected immune deficiencies. Early studies have identified several risk factors for *Cryptococcus* spp. infection; however, these do not distinguish between species. In experimental studies, steroids have been shown to decrease host resistance to infection (*10*). Case-series investigations have linked corticosteroid use with an increased risk of dying from cryptococcal meningitis (*11*) and with an increased risk for disseminated disease (*12*). A nationwide survey of 163 HIV-negative cryptococcosis patients in France (1985–1993) showed that having malignancies (32%), undergoing organ transplantation (19%), and receiving corticosteroid therapy (33%) were the main predisposing factors (*13*). Other frequently identified predisposing conditions for cryptococcosis include sarcoidosis, hyper-immunoglobulin (Ig) M and hyper–IgE syndromes, and CD4+ T-cell lymphopenia in those who are HIV negative (*14*). Case reports have also suggested that cirrhosis is a risk factor for cryptococcal peritonitis (*15*).

Cryptococcal infections are rare in children, no matter their HIV status (*16*). Historical studies of cryptococcosis patients have shown that higher proportions of male

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patients had the disease, both before and after the HIV epidemic (*14*). The risk for disseminated infection among HIV-positive persons with cryptococcal infection was almost 4× higher for those who smoked at the time of diagnosis than for those who did not (*17*).

Many studies that examined predisposing factors for *Cryptococcus* infection in humans have done so in selected populations (e.g., HIV-positive patients), and assessment has been frequently limited to medical rather than behavioral or environmental exposures. Often laboratory testing was not undertaken to distinguish patients with *C. gattii* infection from those with *C. neoformans* infection. In newer studies, which have obtained subtyping information, typically, the number of *C. gattii* isolates was insufficient to determine risk factors. Risk factors suggested in the medical literature have arisen from case reports and case series that described the proportion of patients with particular underlying conditions. Although some investigations have compared risk factors between patients with *C. gattii* and those with *C. neoformans* infections (*8*,*9*), we could find no examples in which case-patients were compared with healthy controls to determine risks for disease acquisition.

Ecologically, epidemiologically, and clinically, *C. gattii* is sufficiently different from its fungal relative *C. neoformans* (including *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*) to warrant its own species designation (*18*). It therefore seems reasonable to assume that the risk factors for disease acquisition may not be the same. We undertook this current investigation to determine whether, compared with the general population, particular medical, behavioral, or environmental risk factors existed among case-patients with *C. gattii* infection in British Columbia, Canada.

Methods

Risk factors for *C. gattii* infection were evaluated in 2 ways. First, a case–control study was conducted on a subset of case-patients (1999–2001) to examine which medical and environmental exposures increased the odds of infection. Second, risk behaviors and underlying medical conditions of all case-patients (1999–2007) were compared with those of the general BC population by using existing data from population-based surveys and studies.

Case Ascertainment

Patients who became study participants included those with culture-confirmed *C. gattii* infection (for which genotyping had been done) as well as those with laboratory evidence of cryptococcal infection (determined from antigen detection and histopathologic or microscopic examination) who were also HIV negative and had been exposed to a local *C. gattii*–endemic area in the year before disease onset (*1*,*2)*. HIV status and geographic exposure were included as part of the case definition for patients from whom culture results were unavailable because hospitalization rates had increased sharply in this population group in 1999, signaling the onset of the *C. gattii* outbreak in British Columbia (*2*). Information on case-patients was obtained from the BC Cryptococcus Database, which contains laboratory and interview data from patients with a diagnosis since 2001 when infection with *Cryptococcus* species became reportable. All available isolates are routinely sent to the British Columbia Centre for Disease Control Public Health Microbiology and Reference Laboratory for culturing, serotyping, and molecular characterization. Cases diagnosed before 2001 were identified either through reporting by laboratory physicians or through a review of the Provincial Electronic Hospital Separation Database for the years 1995–2001 for records containing the International Classification of Diseases, 9th Revision (ICD-9), code 117.5 (cryptococcosis) without ICD-9 codes V08 and 042.X (HIV/AIDS). Stored clinical isolates were also typed retrospectively (*2*). Specimens were identified as *C. gattii* as previously described (*19*–*21*).

Case–Control Study

Thirty-eight BC residents whose *C. gattii* infections were diagnosed from January 1999 through December 2001 were eligible for inclusion in the case–control study. Two controls per case-patient were identified through the general practitioners of the infected persons. Controls were matched with case-patients by sex since this was considered a likely confounder for many behavioral risk factors. Controls were required to have had a chest radiograph showing absence of active pulmonary disease after their matched case-patient's date of diagnosis.

Case-patients and controls were interviewed twice by using structured, in-depth questionnaires. The first interview was conducted face to face at the participants' homes. Information collected included demographic variables; medical history (e.g., has a doctor ever diagnosed you with X?), smoking status; clinical symptoms; occupation; travel history; recreational activities; gardening and landscaping activities; construction activities; and exposure to botanical gardens, zoos, aquariums, agriculture, animals, compost, bark mulch, various tree species, and wooded areas. Case-patients were asked to consider activities in the 3 months before onset of symptoms. Controls were interviewed about the exposure period corresponding to their matched case-patient. Current smokers were those who self-identified as smokers of cigarettes or cigars at the time of interview. Respondents who had ever smoked were persons who had smoked cigarettes or cigars on a regular basis at some point in their lives.

Frequency analysis was performed by using SPSS version 10.0 (SPSS Inc, Chicago, IL, USA). Matched

odds ratios and 95% confidence intervals were calculated by using S-Plus version 6.0 (MathSoft Inc, Seattle, WA, USA). Stratified analyses were conducted to explore potential confounding relationships. Logistic regression was not possible because of the collinearity of the data and small sample size.

Population Comparison

Information on a smaller number of risk factors (than obtained from the questionnaire) was also routinely collected from case-patients, their physicians, or both, during standardized public health interviews of all BC case-patients from 1999 through 2007. Data were collected from symptomatic and asymptomatic case-patients. Past medical conditions were included; steroid use was evaluated over the 3-month period before onset of infection for case-patients whose conditions were diagnosed during 1999–2001 and over a 12-month period for later identified case-patients. The proportion of *C. gattii*–infected case-patients was calculated for the following age groups: 0–19 20–39, 40–49, 50–59, 60–69, 70–79, and ≥80 years. Proportions were also tabulated of case-patients who were male, were current smokers at the time of interview, were infected with HIV, had chronic obstructive pulmonary disease (COPD) or asthma, had taken systemic corticosteroids, or had a history of invasive cancer. Invasive cancer included all cancers reported, including melanoma (but no other skin cancers). Prevalence proportions were calculated among case-patients for whom data on a particular risk factor were available. Prevalence proportions for *C. gattii* case-patients were then compared with prevalence proportions for the general BC population obtained from existing data sources. These included provincial statistics on age and sex distribution (*22*), BC smoking prevalence estimates from a national community health survey (*23*), and type-specific cancer prevalence from the BC Cancer Agency (*24*). Provincial estimates of HIV prevalence, derived by previously published methods (*25*), were provided by BC Centre for Disease Control (M. Gilbert, pers. comm.). We used the χ^2 test for small dependent populations in Microsoft Excel (Microsoft Corp., Redmond, WA, USA) to compare prevalence of risk factors among *C. gattii* case-patients with the overall BC population.

Results

Case–Control Study

During 1999–2001, 38 case-patients met the case definition criteria for inclusion in the case–control study. Nineteen cases were diagnosed by culture, the remaining 19 by histopathologic examination. Isolates from 18 case-patients with culture-confirmed infection were *C. gattii*, serotype B; 1 isolate could not be retrieved for subtyping. None of the 38 patients was HIV positive. The mean age at diagnosis was 59.7 years (range 20–82 years; SD 13.49); 22 were male. Thirty-six case-patients were Caucasian, and 2 were of Asian descent, which is consistent with provincial ethnicity statistics (data not shown). Thirty were retired or unemployed at the time of their illness. Ten case-patients had cryptococcal meningitis when they sought treatment; the remainder exhibited respiratory infection. The most common self-reported symptoms were cough (21 patients), shortness of breath (20 patients), night sweats (20 patients), and fever (20 patients).

Thirty matched sets of case-patients and controls were interviewed (8 case-patients could not be matched). No significant difference in age was found (p = 0.24). Case-patients were more likely than controls to report having ever received a physician's diagnosis of pneumonia (matched odds ratio [MOR] 2.71, 95% confidence interval [CI] 1.05–6.98) or other lung conditions (MOR 3.21, 95% CI 1.08–9.52) (Table 1). Case-patients were also more likely than controls to have taken systemic corticosteroids (MOR 8.11, 95% CI 1.74–37.80), including prednisone (12 case-patients) and methylprednisolone (1 case-patient), during the

Table 1. ORs for risk factors for *Cryptococcus gattii* infection for 30 matched case-patient and general population sets, British Columbia, Canada, 1999–2007*

Risk factors	MOR (95% CI)
Medical	
Lung conditions†	**3.21 (1.08–9.52)**
Pneumonia	**2.71 (1.05–6.98)**
Asthma	0.45 (0.12–1.66)
Diabetes	0.65 (0.17–2.50)
Anemia	2.64 (0.74–9.44)
Arthritis	0.97 (0.37–2.49)
Liver disease	4.00 (0.36–44.10)
Cancer	2.03 (0.63–6.81)
Other fungal infections	1.69 (0.23–12.20)
Tuberculosis	3.24 (0.29–36.60)
Oral steroid use‡	**8.11 (1.74–37.80)**
Current smoker	1.00 (0.34–2.93)
Ever smoked	1.18 (0.44–3.20)
Environmental‡§	
Living with 1 mile of woods	1.70 (0.17–2.02)
Outdoor building or repairing house	4.00 (1.00–16.00)
Cutting/chopping wood	**0.17 (0.04–0.76)**
Pruning	**0.28 (0.09–0.88)**
Cleaning up branches	**0.29 (0.10–0.84)**
Digging earth	0.93 (0.38–2.30)
Camping	1.23 (0.23–2.91)
Gardening	1.15 (0.47–2.79)

*MOR, matched odds ratio; CI, confidence interval. **Boldface** indicates significant risk factors.
†Includes emphysema, chronic bronchitis, chronic obstructive pulmonary disease, sarcoidosis.
‡In 3 mo before symptom onset.
§Other environmental risk factors not significant at α = 0.05: animal or crop farm within 1 mile of residence; construction or landscaping activities; cleaning of buildings, eaves, troughs, or bird feeders; contact with individual tree species; visits to botanical gardens; boating; use of compost materials and bark mulch.

3 months before their illness. No single indication for steroid therapy predominated among case-patients. Although oral steroid use had the strongest association with *C. gattii* infection, stratified analysis did not identify confounding effects on other variables. Neither smoking nor a history of cancer was associated with illness. Chopping wood, pruning trees, and cleaning up branches were significantly less commonly reported among case-patients than among controls, whereas 1 activity, conducting outdoor repairs to a house or building, was more common among case-patients.

Population Comparison

Among 218 *C. gattii* case-patients reported in British Columbia from 1999–2007, 124 (56.9%) had a culture-confirmed infection. The mean age was 58.7 years. Of case-patients with risk factor information available, 65 (41.9%) of those ≥12 years of age smoked at the time of diagnosis, 6 (3.7%) had HIV infection, and 38 (24.7%) had a history of invasive cancer. The most common forms of cancer reported were leukemia (n = 7), lymphoma (n = 6), and lung (n = 6). Seventy (38.0%) case-patients who provided information were considered to be immunocompromised (i.e., had an HIV infection, an organ transplant, a history of invasive cancer, and/or used systemic corticosteroids in the year before diagnosis).

Those with *C. gattii* infection were more likely than the general population to be ≥50 years of age (p<0.001), be current smokers (p<0.001), be infected with HIV (p<0.001), or have a history of invasive cancer (p<0.001) (Table 2). *C. gattii*–infected persons were more likely than the general population to belong to age groups ≥50 years (i.e., 50–59 years, 60–69 years 70–79 years, and ≥80 years). The incidence of *C. gattii* infection was highest in those 70–79 years of age (2.5/100,000 population). Male sex was not associated with infection (p = 0.198) (Table 2). Those with *C. gattii* infection were not more likely to have a history of COPD or asthma than were the general population. Although 30 (27.0%) of 111 patients with available information had used systemic steroids in the 12 months before diagnosis, no valid population controls were found for comparison.

Discussion

This controlled study identifies demographic, medical, and behavioral risk factors for *C. gattii* acquisition. Notably, ≈40% of *C. gattii*–infected patients were immunocompromised. This represents a departure from the prevalent view that *C. gattii* infects otherwise healthy persons. However, the proportion of immunocompromised patients remains lower than estimates of immunocompromise for patients infected by *C. neoformans*, many of whom have severe immunologic disorders (*26*).

In contrast to previously reported findings of an association between cryptococcal species infection and male sex, even when adjusting for HIV status (*14*), *C. gattii* patients in British Columbia were not statistically more likely to be male. It has been hypothesized that sex differences may be the result of differential environmental exposure during occupational or leisure activities. In British Columbia, sex differences are unlikely, given the widespread detection of the fungus in the environment, including urban and semi-urban settings. Previous studies that have demonstrated sex differences were not species specific and likely included a disproportionate number of persons with *C. neoformans* infection; thus, gender may not influence acquisition of *C. gattii* specifically.

C. gattii patients in British Columbia were more likely than the provincial population to be ≥50 years of age. The relative absence of pediatric case-patients (4 patients in 9 years) confirms reports in other jurisdictions (*16*). This finding may have resulted from differential exposure, as suggested by serosurveys in Australia (*16*). In a New York study, however, most children had acquired antibodies to *C. neoformans* by the age of 5 years (*27*). Alternately, advancing age may be a risk factor simply because older persons are more likely to have medical risk factors for *C. gattii* infection or have had their immune function decline with age. In the case–control analysis, no significant difference

Table 2. Comparison of risk factor prevalence among *Cryptococcus gattii*–infected patients and the general population, British Columbia, Canada, 1999–2007

Risk factor	Prevalence, % (95% confidence interval)		p value
	C. gattii–infected case-patients	General population	
Age ≥50 y	72.4 (64.9–79.8)	31.3	<0.001
Male sex	55.8 (46.4–65.1)	49.6	0.198
Current smoker	41.9 (35.3–48.6)	17.8 (16.9–18.6)*	<0.001
All invasive cancers	24.7 (21.7–27.7)	3.6	<0.001
Leukemia/lymphoma only	8.4 (7.6–9.2)	0.3	<0.001
Lung only	3.9 (3.3–4.5)	0.1	<0.001
HIV infection	3.7 (2.9–4.4)	0.2	<0.001
Chronic obstructive pulmonary disease	4.1 (–0.5 to 9.6)	8.0†	0.090
Asthma	3.4 (–1.1 to 7.9)	7.8	0.054

*From original data source.
†Estimated based on range provided.

in age was detected between case-patients and controls; therefore, this relationship could not be studied. The lack of age association between case-patients and controls may have been because of bias toward the inclusion of older controls because they were selected on the basis of a recent chest radiograph showing no abnormalities—a procedure more likely to be routinely performed in older patients.

In the case–control study, *C. gattii*–infected case-patients were significantly more likely than controls to have received systemic corticosteroid therapy. Increased susceptibility to cryptococcal infection after corticosteroid therapy has previously been described (*28*). Mouse experiments by Levine et al. in the 1950s demonstrated that mice, which usually were able to localize an induced subcutaneous cryptococcocal infection, could not do so after an intraperitoneal injection of 2.5 mg of cortisone acetate (*10*). Among US patients with culture-confirmed cryptococcal meningitis, persons who had received ≥20 mg of prednisone or who had Hodgkin lymphoma or other lymphoreticular malignancies were at significantly higher risk of dying than those without these conditions (*11*). In another case series of *Cryptococcus*-infected patients, disseminated disease developed in 59% of those treated with corticosteroids (*12*). More recently, corticosteroid use has been identified as a risk factor for disseminated disease in HIV-negative patients with pulmonary cryptococosis (*29*). Because oral steroids are sometimes used to treat cryptococcal infections, these findings present a medical challenge.

In the case–control study, a history of pneumonia or other lung conditions (including emphysema, chronic bronchitis, COPD, sarcoidosis) was associated with cryptococcosis. Pneumonia alone may not be an independently associated risk factor because it is associated with many lung conditions. Certain respiratory conditions, including bronchial obstruction, predispose the patient to recurrent bacterial pneumonia (*30*,*31*), and bronchopulmonary disorders may predispose a person to pulmonary cryptococcosis (*32*) because airway damage leads to a decreased barrier to infection. *C. gattii*–infected case-patients were not more likely than population controls to have experienced COPD, when that factor was examined alone. Notably, asthma was not identified as a risk factor by either the case–control or population-level analyses, although this condition, along with COPD, has been suggested as a risk factor (*31*).

Invasive cancers of all types were significantly more common in *C. gattii*–infected patients than in the general population. This finding held true for subgroups of patients with lung cancer and leukemia/lymphoma. Lymphoproliferative malignancies have traditionally been considered risk factors for cryptococcal infection and disseminated disease (*14*), and this appears to remain true for *C. gattii*–infected populations specifically. Although invasive cancers likely lead to an increased risk for infection, *C. gattii* infection may have been an incidental finding in patients with lung cancer, with the detection of the former facilitated through imaging and invasive procedures used for diagnosing the latter. In the case–control study, MORs also suggested that odds of infection were elevated for those with a history of invasive cancers; however, this difference was not significant.

During 1999–2007, six cases of culture-confirmed *C. gattii* infections were documented in HIV-positive residents of British Columbia. Although such cases are recognized (*33*,*34*), HIV infection has generally been associated with *C. neoformans* infection. This study highlights that HIV infection, although rare in *C. gattii* patients, is significantly more likely to be found in this patient group than in the general population. In fact, the strength of this relationship may have been underestimated: since HIV-positive persons with cryptococcal infection of undetermined species were excluded from the case definition, some *C. gattii* infections among HIV-positive patients may have been missed. Although the specific reason is unknown, HIV status may be associated with *C. gattii* infection because of differences in strain pathogenicity and host immune response.

Three outdoor activities—chopping wood, pruning, and cleaning up branches—were protective against infection. The reason for this is not clear because these activities would potentially have exposed the person to *C. gattii* in trees. Ongoing low-level exposure possibly results in immunity, preventing symptomatic infection if the person is exposed to a higher dose. More likely, these more vigorous activities are undertaken by healthy persons and reflect a general state of wellness rather than a protective effect of these specific behaviors. In contrast, outdoor building repairs represented a risk factor for infection. This may have been due to the disturbance of rotting wood colonized with *C. gattii*. Environmental experiments in British Columbia have demonstrated substantially increased airborne concentrations of *C. gattii* when colonized trees are felled or chipped (*35*).

Population comparisons suggest that significantly more *C. gattii*–infected case-patients are current smokers. Because smoking compromises the protective barriers in airways, it has been linked to increased risk for many lung infections (*36*). A previous study of AIDS patients with *C. neoformans* infection, which identified smoking as a risk factor for disseminated disease, suggested that fungus may be inhaled along with smoke particulates and deposited in small airways (*17*). Air-sampling studies of *C. gattii* in British Columbia have found that the airborne propagule is sufficiently small to be deposited in the upper lung (i.e., from 3.3 μm to >7 μm (*37*).

This study has several limitations. First, to ensure that asymptomatic *C. gattii*–infected patients were not included

as controls, we required controls to have had chest radiographs showing no abnormalities. A selection bias may have been introduced, which could have reduced the likelihood of showing significant differences among variables, such as smoking, that might be associated with having a radiograph taken, leading to more conservative estimates or an inability to detect true differences in exposures between case-patients and controls. Similarly, the small sample size of the case–control study may have limited its power to detect significant exposures. Therefore, when possible, population comparisons were also performed to validate case–control findings.

In addition, risk factors for both analyses were collected by self-report, which may have been inaccurate. Also, the case–control analysis examined risk factor information in the 3 months before disease onset. Subsequent research has shown that the incubation period for this disease is typically longer (*38*). This long incubation period may have affected the study's ability to accurately detect behavioral and environmental risk factors; medical risk factors were unaffected because these were reported as "ever diagnosed with." Despite a case definition specifically designed to exclude case-patients infected with *C. neoformans*, some misclassification may have occurred, given that only 50% of infections in case-patients included in the case–control study and 57% of infections in case-patients included in population comparisons could be confirmed by laboratory typing.

Conclusion

Although most cases of *C. gattii* infection do occur in otherwise healthy persons, our findings suggest that infection is associated with immunosuppressive states induced by oral corticosteroid use and invasive cancers as well as with weakened pulmonary function resulting from previous lung infections and smoking (Table 3). Despite HIV infection being rare among *C. gattii*–infected case-patients, it also occurred more frequently in infected persons than in the general population. Recent evidence indicates that *C. gattii* is now spreading in the Pacific Northwest of the United States (*19*,*39*). Physician awareness of risk factors should assist with diagnosis of this serious but treatable infection in areas where the disease is emerging.

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Table 3. Risk factors for *Cryptococcus gattii* infection as evaluated by case–control study and population-based comparison, British Columbia, 1999–2007*

	Case–control study†		Population comparison‡	
Risk factor	Evaluated	Association with disease	Evaluated	Association with disease
Age	Yes	None	Yes	Positive
Sex	No		Yes	None
Oral steroid use	Yes	Positive	No	
Invasive cancer	Yes	None	Yes	Positive
Pneumonia	Yes	Positive	No	
Other lung problems§	Yes	Positive	No	
COPD	No		Yes	None
Asthma	Yes	None	Yes	None
Current smoker	Yes	None	Yes	Positive
HIV infection	No		Yes	Positive
Environmental exposures	Yes	Negative¶	No	

*COPD, chronic obstructive pulmonary disease.
†n = 30 matched pairs.
‡n = 218 case-patients with *C. gattii* infection.
§Includes emphysema, chronic bronchitis, COPD, sarcoidosis.
¶Negative associations were found for cutting/chopping wood, pruning, and cleaning up branches.

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Possible Increased Pathogenicity of Pandemic (H1N1) 2009 Influenza Virus upon Reassortment

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Since emergence of the pandemic (H1N1) 2009 virus in April 2009, three influenza A viruses—seasonal (H3N2), seasonal (H1N1), and pandemic (H1N1) 2009—have circulated in humans. Genetic reassortment between these viruses could result in enhanced pathogenicity. We compared 4 reassortant viruses with favorable in vitro replication properties with the wild-type pandemic (H1N1) 2009 virus with respect to replication kinetics in vitro and pathogenicity and transmission in ferrets. Pandemic (H1N1) 2009 viruses containing basic polymerase 2 alone or in combination with acidic polymerase of seasonal (H1N1) virus were attenuated in ferrets. In contrast, pandemic (H1N1) 2009 with neuraminidase of seasonal (H3N2) virus resulted in increased virus replication and more severe pulmonary lesions. The data show that pandemic (H1N1) 2009 virus has the potential to reassort with seasonal influenza viruses, which may result in increased pathogenicity while it maintains the capacity of transmission through aerosols or respiratory droplets.

The influenza virus A (H1N1) that caused the first influenza pandemic of the 21st century, pandemic (H1N1) 2009, continues to be detected worldwide (*1*,*2*). The pandemic overall has been relatively mild; disease has ranged from subclinical infections to sporadic cases of severe pneumonia and acute respiratory distress syndrome (*3*–*8*). The virus responsible is a unique reassortant virus containing neuraminidase (NA) and matrix genes from the Eurasian swine influenza virus lineage, and the other 6 gene segments are derived from the North American triple reassortant swine influenza virus lineage (*9*). From the pandemic's start, there have been concerns the virus may mutate or reassort with contemporary influenza viruses and give rise to more pathogenic viruses.

Cocirculation of multiple strains of influenza virus A in humans provides an opportunity for viral genetic reassortment (mixing of genes from ≥2 viruses) (*10*). Genetic reassortment of pandemic (H1N1) 2009 virus with seasonal influenza A (H3N2) or seasonal influenza A (H1N1) viruses might thus represent a route to enhanced pathogenicity. No reassortment events between pandemic (H1N1) 2009 and seasonal viruses have been reported in humans. However, a triple-reassortant swine influenza virus A (H1N1), distinct from pandemic (H1N1) 2009 virus and containing the hemagglutinin (HA) and NA genes of seasonal influenza virus A (H1N1), was described recently (*11*). Dual infections by seasonal influenza A (H1N1) and seasonal influenza A (H3N2) viruses have been reported (*12*), as well as mixed infections of pandemic (H1N1) 2009 and seasonal influenza A (H3N2) viruses (*13*,*14*), highlighting the potential for reassortment of currently circulating influenza viruses. Subtype H1N2 reassortant influenza viruses that contain the HA of seasonal influenza A (H1N1) and the NA of seasonal influenza A (H3N2) viruses have been isolated from humans during previous influenza seasons, confirming that such HA/NA combinations can emerge in humans (*15*,*16*).

To investigate the potential for reassortment between seasonal influenza A and pandemic (H1N1) 2009 viruses, we used an in vitro selection method using reverse genetics and serial passaging under limited dilution conditions. Pathogenicity and transmission of these viruses were tested

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by using a ferret model. We report here the identification of 4 reassortants with different gene constellations.

Materials and Methods

Cells and Viruses

MDCK cells were cultured in Eagle minimum essential medium as described (*17*). Influenza virus A/Netherlands/602/2009 was isolated from the first patient with pandemic (H1N1) 2009 virus infection in the Netherlands (*18*). Influenza virus A/Netherlands/213/2003 (seasonal influenza A [H3N2]) and influenza virus A/Netherlands/26/2007 (seasonal influenza A [H1N1]) were isolated from patients during epidemics in the Netherlands. After these viruses were passaged in MDCK cells 2×, all 8 gene segments were amplified by reverse transcription–PCR, cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000 (*19*,*20*), and subsequently used to generate recombinant virus by reverse genetics as described elsewhere (*19*).

Generation of the Reassortant Viruses

Mixtures of reassortant viruses were generated in 293T cells by using reverse genetics, by co-transfecting 8 plasmids that encode the pandemic (H1N1) 2009 virus genome together with 7 plasmids encoding the seasonal influenza A (H3N2) or seasonal influenza A (H1N1) virus genome. We omitted HA of the seasonal viruses to ensure that only reassortants containing the pandemic (H1N1) 2009 virus HA could arise, against which a large proportion of the human population is still immunologically naïve (*21*). The 293T-cell supernatants were passaged in quadruplicate under limiting dilution conditions by using 10-fold serial dilutions in MDCK cells 3× to enable selective outgrowth of viruses with high in vitro replication rates. After 3 passages, the genome composition of these viruses was determined by sequencing with conserved primers targeting noncoding regions of each gene segment. Reverse genetics was also used to produce specific reassortant viruses (pandemic [H1N1] 2009–seasonal influenza A [H1N1] basic polymerase [PB] 2, pandemic [H1N1] 2009–seasonal influenza A [H1N1] PB2 acidic polymerase [PA], pandemic [H1N1] 2009–seasonal influenza A [H3N2] NA, and pandemic [H1N1] 2009–seasonal influenza A [H3N2] NAPB1) by transfection of 293T cells and subsequent virus propagation in MDCK cells.

In Vitro Characterization of Viruses

Multicycle replication curves were generated by injecting MDCK cells at a multiplicity of infection of 0.01 50% tissue culture infective dose ($TCID_{50}$) per cell in 2-fold (*17*). Virus titers from samples of inoculated MDCK cells, as well as nasal and throat swabs or homogenized tissue samples from inoculated ferrets, were determined by endpoint titration in MDCK cells, as described (*22*).

Ferret Experiments

All animal studies were approved by an independent animal ethics committee. Experiments were performed under animal BioSafety Level 3+ conditions. The ferret model to test pathogenicity and transmission of pandemic (H1N1) 2009 virus was described previously (*17*,*18*). To study pathogenicity, 5 groups of 6 influenza virus–seronegative female ferrets (*Mustella putorius furo*) were inoculated intranasally with 10^6 $TCID_{50}$ of wild-type pandemic (H1N1) 2009 virus, or the reassortant viruses pandemic (H1N1) 2009–seasonal influenza A (H1N1) PB2, pandemic (H1N1) 2009–seasonal influenza A (H1N1) PB2PA, pandemic (H1N1) 2009–seasonal influenza A (H3N2) NA, and pandemic (H1N1) 2009–seasonal influenza A (H3N2) NAPB1, divided between both nostrils (2 × 250 μL). In the transmission experiment, 4 female ferrets for wild-type pandemic (H1N1) 2009 virus and 2 ferrets for each reassortant virus were individually housed in transmission cages and inoculated intranasally with 10^6 $TCID_{50}$ of virus divided between both nostrils (2 × 250 μL). Animal daily weights were used as an indicator of disease.

Immunohistochemistry and Histopathology

Immunohistochemical testing and pathologic examination were performed by using lungs of inoculated ferrets. For each virus, 3 ferrets were euthanized at 3 and 7 days postinoculation (dpi) by exsanguination. Necropsies and tissue sampling were performed according to standard protocol. After fixation in 10% neutral-buffered formalin and embedding in paraffin, samples were sectioned at 4 μm and stained with an immunohistochemical method by using a mouse monoclonal antibody against the nucleoprotein of influenza virus A (*23*). Influenza virus antigen expression in lung sections was scored for bronchial surface epithelium, bronchial submucosal gland epithelium, bronchiolar epithelium, alveolar type I pneumocytes, and alveolar type II pneumocytes. Scoring was categorized as 0, no positive cells; 1, few positive cells; 2, moderate number of positive cells; and 3, many positive cells. Serial lung sections were stained with hematoxylin and eosin for detection and description of pathologic changes. Samples were scored for influenza virus–associated inflammation in bronchi (bronchitis), bronchial submucosal glands (bronchoadenitis), bronchioles (bronchiolitis), and alveoli (alveolitis). Scoring of severity of inflammation was 0, no inflammation; 1, mild inflammation; 2, moderate inflammation; and 3, marked inflammation. Researchers who examined the sections had no knowledge of the identity of the ferrets.

Results

In Vitro Selection of Reassortants

The proportion of gene segments 1–8 (except HA) analyzed was ≈60%, 60%, 65%, 90%, 95%, 100%, and 100%, respectively. Minor virus variants were not detected. No point mutations were observed in the proportions of the genome analyzed. Upon pandemic (H1N1) 2009–seasonal influenza A (H1N1) transfection and passaging, 3 reassortants contained the PB2 gene of seasonal influenza virus A (H1N1), 2 of which had also incorporated the seasonal (H1N1) PA gene. All 4 pandemic (H1N1) 2009–seasonal influenza A (H3N2) virus reassortants had the NA gene of seasonal influenza A (H3N2), and 3 of 4 reassortants also incorporated the seasonal influenza A (H3N2) PB1 gene (Table 1).

In Vitro Characterization of Pandemic (H1N1) 2009–Seasonal Influenza A (H1N1) and Pandemic (H1N1) 2009–Seasonal Influenza A (H3N2) Reassortants

The replication kinetics of pandemic (H1N1) 2009–seasonal influenza A (H1N1) PB2 and pandemic (H1N1) 2009–seasonal influenza A (H1N1) PB2PA were similar to those of wild-type pandemic (H1N1) 2009 virus, and the pandemic (H1N1) 2009–seasonal influenza A (H3N2) NA and pandemic (H1N1) 2009–seasonal influenza A (H3N2) NAPB1 reassortant viruses displayed slightly higher virus titers at 24 and/or 48 h after inoculation, with a maximum difference in virus titer of 1.0 $\log_{10}$ $TCID_{50}$ (Figure 1). The fact that each reassortant virus replicated at least at the same rate as the wild-type pandemic (H1N1) 2009 virus agrees with results from the in vitro selection experiment described above.

Pathogenicity of the Reassortant Viruses in Ferrets

The mean maximum weight loss was 7% for animals inoculated with the pandemic (H1N1) 2009 virus. Animals inoculated with pandemic (H1N1) 2009–seasonal influenza A (H1N1) PB2PA, pandemic (H1N1) 2009–seasonal influenza A (H1N1) PB2, pandemic (H1N1) 2009–seasonal influenza A (H3N2) NAPB1 and pandemic (H1N1) 2009–seasonal influenza A (H3N2) NA had a maximum weight loss of 4%, 2%, 2%, and 6%, respectively (data not shown).

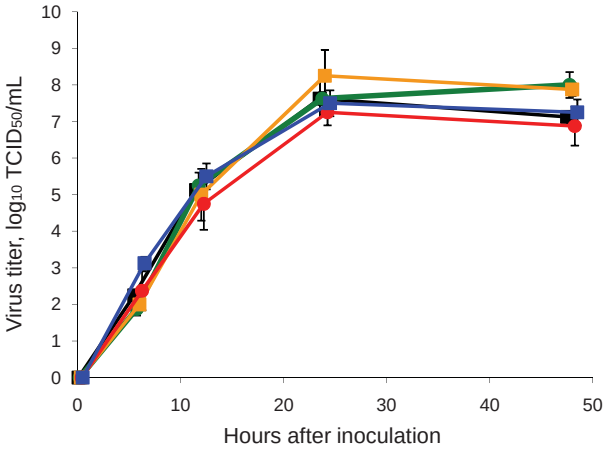

Figure 1. Replication of wild-type and reassortant pandemic (H1N1) 2009 viruses in MDCK cells. MDCK cells were injected in duplicate with 0.01 50% tissue culture infective dose ($TCID_{50}$) per cell of each virus: black, wild-type pandemic (H1N1) 2009; red, reassortant pandemic (H1N1) 2009–seasonal influenza (H1N1) basic polymerase (PB) 2 acidic polymerase; blue, reassortant pandemic (H1N1) 2009–seasonal influenza (H1N1) PB2; green, reassortant pandemic (H1N1) 2009–seasonal influenza (H3N2) PB1 neuraminidase (NA); orange, reassortant pandemic (H1N1)–seasonal influenza (H3N2) NA. Supernatant samples were harvested 6, 12, 24 and 48 h after injection. Supernatant samples were titrated in MDCK cells. Geometric mean titers and standard deviation were calculated from 2 independent experiments.

Nose and throat swabs were collected daily, and virus titers were determined. Infectious virus shedding continued until 6–7 days dpi from noses (Figure 2, panels A and C) and throats (Figure 2, panels B and D) of most inoculated animals. Total virus shedding from the nose, as calculated from the area under the curve for ferrets in the experiment for 7 days (n = 3), was significantly lower in animals inoculated with the pandemic (H1N1) 2009–seasonal influenza A (H1N1) PB2 reassortant virus ($p = 0.003$ by *t* test) and significantly higher in the animals inoculated with the pan-

Table 1. Predominant virus genome composition upon in vitro selection of mixtures of pandemic (H1N1) 2009–seasonal (H1N1) and pandemic (H1N1)–seasonal (H3N2) influenza virus reassortants*

Replicates	PB2	PB1	PA	HA	NP	NA	M	NS
Pandemic (H1N1) 2009–seasonal (H1N1) 1	sH1	pH1	sH1	pH1	pH1	pH1	pH1	pH1
Pandemic (H1N1) 2009–seasonal (H1N1) 2	sH1	pH1	sH1	pH1	pH1	pH1	pH1	pH1
Pandemic (H1N1) 2009–seasonal (H1N1) 3	sH1	pH1	pH1	pH1	pH1	pH1	pH1	pH1
Pandemic (H1N1) 2009–seasonal (H1N1) 4	pH1	pH1	pH1	pH1	pH1	pH1	pH1	pH1
Pandemic (H1N1) 2009–seasonal (H3N2) 1	pH1	sH3	pH1	pH1	pH1	sH3	pH1	pH1
Pandemic (H1N1) 2009–seasonal (H3N2) 2	pH1	pH1	pH1	pH1	pH1	sH3	pH1	pH1
Pandemic (H1N1) 2009–seasonal (H3N2) 3	pH1	sH3	pH1	pH1	pH1	sH3	pH1	pH1
Pandemic (H1N1) 2009–seasonal (H3N2) 4	pH1	sH3	pH1	pH1	pH1	sH3	pH1	pH1

*PB, basic polymerase; PA, acidic polymerase; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix; NS, nonstructural; sH1, seasonal influenza A virus (H1N1); pH1, pandemic (H1N1) 2009 virus; sH3, seasonal influenza A (H3N2) virus.

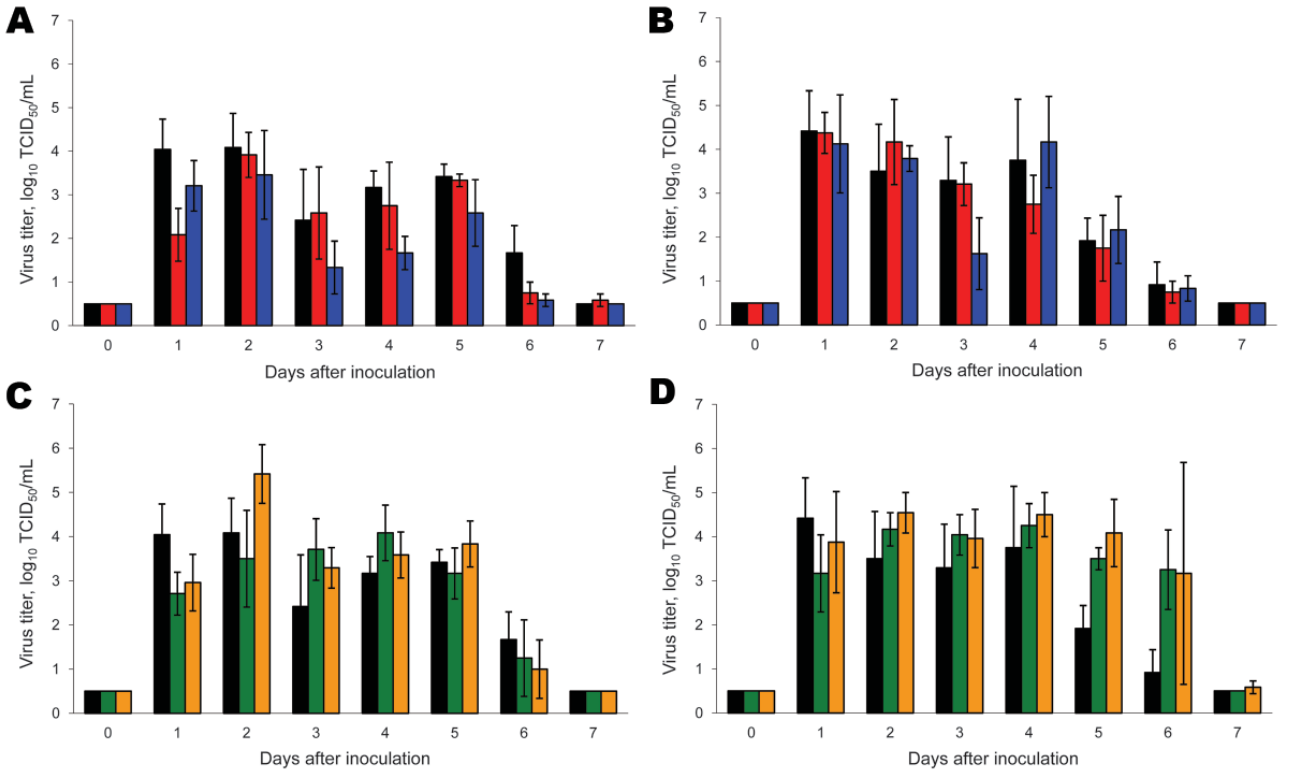

Figure 2. Virus shedding from the nose and throat of ferrets inoculated with wild-type and reassortant pandemic (H1N1) 2009 viruses. Virus shedding from nose (A, C) and throat (B, D) is shown for pandemic (H1N1) 2009–seasonal influenza (H1N1) (A, B) and pandemic (H1N1) 2009–seasonal influenza (H3N2) (C, D) reassortant viruses. Black, wild-type pandemic (H1N1) 2009; red, pandemic (H1N1) 2009–seasonal influenza (H1N1) basic polymerase (PB) 2 acidic polymerase; blue, pandemic (H1N1) 2009–seasonal influenza (H1N1) PB2; green, pandemic (H1N1) 2009–seasonal influenza (H3N2) PB1 neuraminidase (NA); orange, pandemic (H1N1)–seasonal influenza (H3N2) NA. Geometric mean titers are shown; error bars indicate SD. The lower limit of detection is 0.5 $\log_{10}$ 50% tissue culture infective dose/mL ($TCID_{50}$/mL). After day 3, only 3 animals remained in each group.

demic (H1N1) 2009–seasonal influenza virus A (H3N2) NA ($p = 0.023$ by *t* test), than in animals inoculated with wild-type pandemic (H1N1) 2009 virus. Total virus shedding from the throat, as calculated from the area under the curve for ferrets in the experiment for 7 days, was not significantly different between the groups of ferrets.

At 3 and 7 dpi, 3 ferrets from each group were euthanized, and samples from nasal turbinates, trachea, and lungs were collected for virologic examination. At 7 dpi, virus was undetectable or detected at only very low levels in these samples from all groups of ferrets. At 3 dpi, no virus or relatively low virus titers were detected in the lungs and trachea respectively, of ferrets inoculated with pandemic (H1N1) 2009–seasonal influenza A (H1N1) reassortant viruses (Figure 3, panels A and B), and titers in the nasal turbinates were similar to those for wild-type pandemic (H1N1) 2009 virus (Figure 3, panel C). These data indicate that both pandemic (H1N1) 2009–seasonal influenza A (H1N1) viruses were attenuated with respect to replication in the lower respiratory tract of ferrets.

At 3 dpi, virus was detected in the lungs, trachea, and nasal turbinates of ferrets inoculated with pandemic (H1N1) 2009–seasonal influenza A (H3N2) NAPB1 and pandemic (H1N1) 2009–seasonal influenza A (H3N2) NA viruses at approximately the same levels as upon inoculation with wild-type pandemic (H1N1) 2009 virus (Figure 3, panels D–F). Virus titers detected in the lungs and trachea of animals inoculated with pandemic (H1N1)–seasonal influenza virus A (H3N2) NA at 3 dpi were 1.0 $\log_{10}$ $TCID_{50}$ higher than those in animals inoculated with wild-type pandemic (H1N1) 2009 virus (not statistically significant). These data indicate that both pandemic (H1N1) 2009–seasonal influenza A (H3N2) viruses tested were not attenuated in ferrets. If anything, shedding of pandemic (H1N1)–seasonal influenza A (H3N2) NA virus from the nose (Figure 2, panel C), lungs (Figure 3, panel D), and trachea (Figure 3, panel E) was higher than shedding of wild-type pandemic (H1N1) 2009 virus.

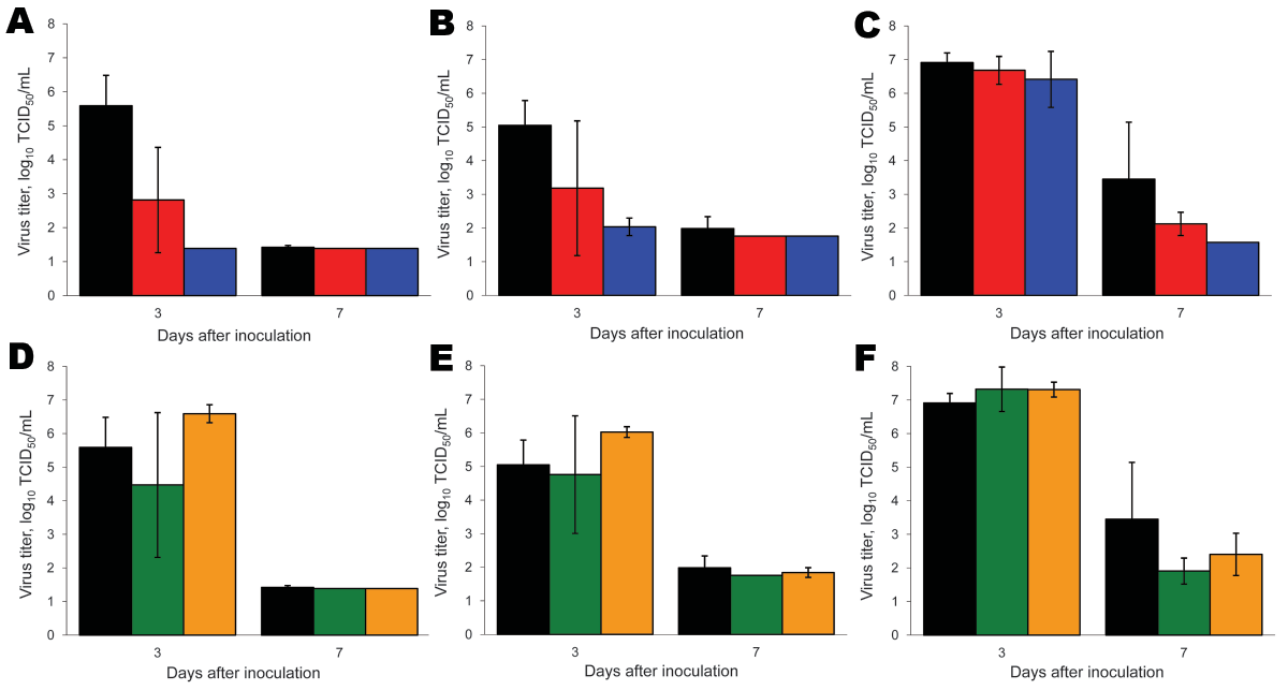

Figure 3. Virus detection in respiratory tissues of ferrets inoculated with wild-type and reassortant pandemic (H1N1) 2009 viruses. Virus detection in lungs (A, D), trachea (B, E), and nasal turbinates (C, F) is shown for pandemic (H1N1) 2009–seasonal influenza (H1N1) (A–C) and pandemic (H1N1) 2009–seasonal influenza (H3N2) (D–F) reassortant viruses. Black, wild-type pandemic (H1N1) 2009; red, pandemic (H1N1) 2009–seasonal influenza (H1N1) basic polymerase (PB) 2 acidic polymerase; blue, pandemic (H1N1) 2009–seasonal influenza (H1N1) PB2; green, pandemic (H1N1) 2009–seasonal influenza (H3N2) PB1 neuraminidase (NA); orange, pandemic (H1N1)–seasonal influenza (H3N2) NA. Three ferrets of each group were euthanized at 3 and 7 days postinoculation. Geometric mean titers are shown; error bars indicate SD. The lower limit of detection is 0.5 $\log_{10}$ 50% tissue culture infective dose/mL ($TCID_{50}$/mL).

Pathologic Changes in the Respiratory Tract of Ferrets Inoculated with Pandemic (H1N1) 2009 and Reassortant Viruses

At 7 dpi, virus antigen expression was undetectable in lung tissue of any of the euthanized ferrets, and lesions were absent or resolving. At 3 dpi, neither viral antigen expression nor lesions were detected in lungs of ferrets inoculated with pandemic (H1N1) 2009–seasonal influenza A (H1N1) PB2. Only 1 of 3 ferrets inoculated with pandemic (H1N1) 2009 had scant virus antigen expression and mild associated lesions in bronchial submucosal glands and bronchioles at 3 dpi (Table 2, Figure 4). In contrast, all 3 ferrets inoculated with pandemic (H1N1) 2009–seasonal influenza A (H3N2) NA had moderate to abundant virus antigen expression in bronchial submucosal glands, bronchioles, or both, associated with moderate to marked inflammation (Table 2, Figure 4). Virus antigen expression and associated lesions in the lungs of ferrets inoculated with pandemic (H1N1) 2009–seasonal influenza A (H3N2) PB1NA were intermediate between those of wild-type pandemic (H1N1) 2009 and pandemic (H1N1)–seasonal influenza A (H3N2) NA (Table 2).

Cell types in which virus antigen expression was detected were ciliated epithelial cells of bronchi, epithelial cells of bronchial submucosal glands, ciliated and nonciliated cells of bronchioles, and both squamous and cuboidal epithelial cells (interpreted as type I and type II pneumocytes, respectively) of alveoli (Figure 4). Virus antigen expression was also seen in desquamated epithelial cells and cell debris in lumina of above tissues.

Lesions associated with virus antigen expression can be categorized as acute, focal or multifocal, necrotizing bronchitis, bronchoadenitis, bronchiolitis, and alveolitis. These lesions were characterized by degeneration and necrosis of epithelial cells, infiltration of the affected tissues and their lumina by many neutrophils and few eosinophils, and exudation of edema fluid and fibrin into tissue lumina.

Transmission of Reassortant Viruses in Ferrets

Transmission of pandemic (H1N1) 2009 and reassortant influenza viruses through aerosol or respiratory droplets was tested in the ferret model. Ferrets in groups of 4 for pandemic (H1N1) 2009 virus and 2 for the reassortant viruses were inoculated intranasally with 10^6 $TCID_{50}$ of virus. At 1 dpi, an uninfected ferret was placed in a cage adjacent to each inoculated ferret. All viruses were transmitted from the inoculated to the uninfected ferrets in 4/4 ferrets for pandemic (H1N1) 2009 virus and 2/2 ferrets for

Table 2. Virus antigen expression and severity of lesion in different tissues of the lung of 3 ferrets inoculated with pandemic (H1N1) 2009 or reassortant pandemic (H1N1) 2009 viruses*

	Cumulative score per tissue							
	Bronchial surface epithelium		Bronchial submucosal epithelium		Bronchiolar epithelium		Alveolar epithelium	
Virus	IHC	H&E	IHC	H&E	IHC	H&E	IHC	H&E
Pandemic (H1N1) 2009	0, 0, 0†	0, 0, 0	0, 0, 1	0, 0, 1	0, 0, 1	0, 0, 1	0, 0, 0	0, 0, 0
Pandemic (H1N1) 2009–seasonal influenza A (H1N1) PB2PA	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
Pandemic (H1N1) 2009–seasonal influenza A (H1N1) PB2	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
Pandemic (H1N1) 2009–seasonal influenza A (H3N2) PB1NA	1, 1, 0	0, 1, 0	2, 1, 0	1, 1, 0	2, 1, 0	1, 1, 0	1, 1, 0	1, 1, 1
Pandemic (H1N1) 2009–seasonal influenza A (H3N2) NA	1, 1, 1	0, 1, 1	1, 0, 3	1, 0, 3	2, 2, 3	2, 2, 3	1, 0, 1	1, 1, 1

*IHC, immunohistochemistry to detected virus antigen expression; H&E, hematoxylin and eosin staining to analyze severity of inflammation; PB, basic polymerase; PA, acidic polymerase; NA, neuraminidase.
†Individual scores (as indicated in the Methods section) for 3 ferrets are listed.

each of the reassortant viruses. The first day of virus detection in the previously uninfected animals was 2 days post exposure, similar for all viruses tested.

Discussion

We used an in vitro selection method to identify reassortant viruses between pandemic (H1N1) 2009 virus and seasonal influenza A (H1N1) and influenza A (H3N2) viruses of interest for testing in a ferret model. Studying the effects of reassortment on changes in influenza virus phenotype is cumbersome because the number of reassortants that can be generated between 2 viruses is high; $2^8 = 256$ different viruses. After 3 passages, a limited number of specific virus populations were selected in vitro. Minor virus variants representing <20% of the virus population would remain undetected in our approach of PCR amplification and direct determination of the consensus sequence of the amplicons. However, upon repeating the procedure 4 times for both reassortment combinations, the seasonal influenza virus genes that were selected in the pandemic (H1N1) 2009 virus backbone were more or less consistent, with NA of seasonal influenza virus A (H3N2) being selected in 4/4 attempts, PB1 of seasonal influenza A (H3N2) and PB2 of seasonal influenza virus A (H1N1) in 3/4 attempts, and PA of seasonal influenza virus A (H1N1) in 2/4 attempts. Replication in MDCK cells may not be the best selection criterion for the identification of reassortants of interest to human health. Nevertheless, we chose this in vitro selection method because previous work has shown that pandemic (H1N1) 2009 outcompetes seasonal influenza A (H1N1) and seasonal influenza A (H3N2) viruses rapidly, reducing the opportunity for reassortment (*24*). This growth advantage over seasonal viruses was in agreement with the fact that selected viruses mostly contained pandemic (H1N1) 2009 genes. The use of reverse genetics enables production of all gene segments at approximately similar copy numbers on transfection, whereas after double infection with 2 viruses, in vitro or in ovo viruses may differ in replication capacity, resulting in a bias of reassortants produced.

Notably, the polymerase gene segments of seasonal influenza A (H1N1) and seasonal influenza A (H3N2) viruses frequently substituted for the polymerase genes of the pandemic (H1N1) 2009 virus in vitro. In minigenome assays, the polymerase complex activity of the wild-type pandemic (H1N1) 2009 virus was relatively low, and replacement of various polymerase genes of the pandemic (H1N1) 2009 virus increased this activity. However, polymerase complexes with the highest activity in minigenome assays were not necessarily the ones detected in the reassortant viruses (data not shown). This apparent discrepancy is probably a result of the different parameters under investigation in the 2 assays, in particular, the production of mRNA vs. all viral RNAs.

Virus titers for pandemic (H1N1) 2009–seasonal influenza A (H1N1) PB2PA and pandemic (H1N1) 2009–seasonal influenza A (H1N1) PB2 in the lungs and trachea of ferrets were lower than titers in ferrets inoculated with wild-type pandemic (H1N1) 2009 virus, suggesting that both pandemic (H1N1) 2009–seasonal influenza A (H1N1) reassortant viruses were attenuated in ferrets, at least for replication in the lower respiratory tract. The reassortants between pandemic (H1N1) 2009 and seasonal influenza A (H3N2) viruses replicated at slightly higher rates than wild-type pandemic (H1N1) 2009 virus in vitro. Moreover, virus shedding of pandemic (H1N1) 2009–seasonal influenza virus A (H3N2) NA from the nose, lungs, and trachea of inoculated ferrets was slightly higher than wild-type pandemic (H1N1) 2009 virus. Although the differences in replication and shedding were small and not statistically significant because of the small numbers of animals in each group, the pandemic (H1N1) 2009–seasonal influenza A (H3N2) viruses were not attenuated in ferrets.

Inoculation of the reassortant pandemic (H1N1)–seasonal influenza A (H3N2) NA (either with or without the

PB1 of seasonal influenza virus A [H3N2]) resulted in higher expression of virus antigen and more severe lesions at all levels of the lower respiratory tract compared with inoculation of wild-type pandemic (H1N1) 2009 virus (Table 2; Figure 4). In a previous study, the wild-type pandemic (H1N1) 2009 virus was detected more abundantly in the lower airways of ferrets than in the present study (*18*). We attribute this difference to the use of a virus isolate rather than a virus generated by reverse genetics and to a different batch of ferrets in the previous study. In the present study, all viruses were produced with reverse genetics and can thus be compared directly. Moreover, the reassortant pandemic (H1N1) 2009 virus with the NA of the seasonal influenza virus A (H3N2) was more pathogenic than both sources of pandemic (H1N1) 2009 virus, either the wild-type isolate or the virus derived by reverse genetics. Increased severity of lesions may be related to higher virus replication in the lung, to stronger host immune responses, or both (*25*).

We conclude that the pandemic (H1N1) 2009 virus has the potential to reassort with seasonal influenza virus A (H1N1) and influenza virus A (H3N2) and that such reassortment events could result in viruses with increased pathogenicity in ferrets. Although increased pathogenicity in ferrets cannot be extrapolated directly to increased pathogenicity in humans, ferrets are susceptible to natural infection and respiratory disease and lung pathology develop in a manner similar to the that in humans infected with seasonal, avian, or pandemic influenza viruses. Thus, the ferret model is generally thought to be a good animal model for influenza in humans (*26*,*27*). Patterns of influenza virus attachment to cells of the respiratory tract are also similar

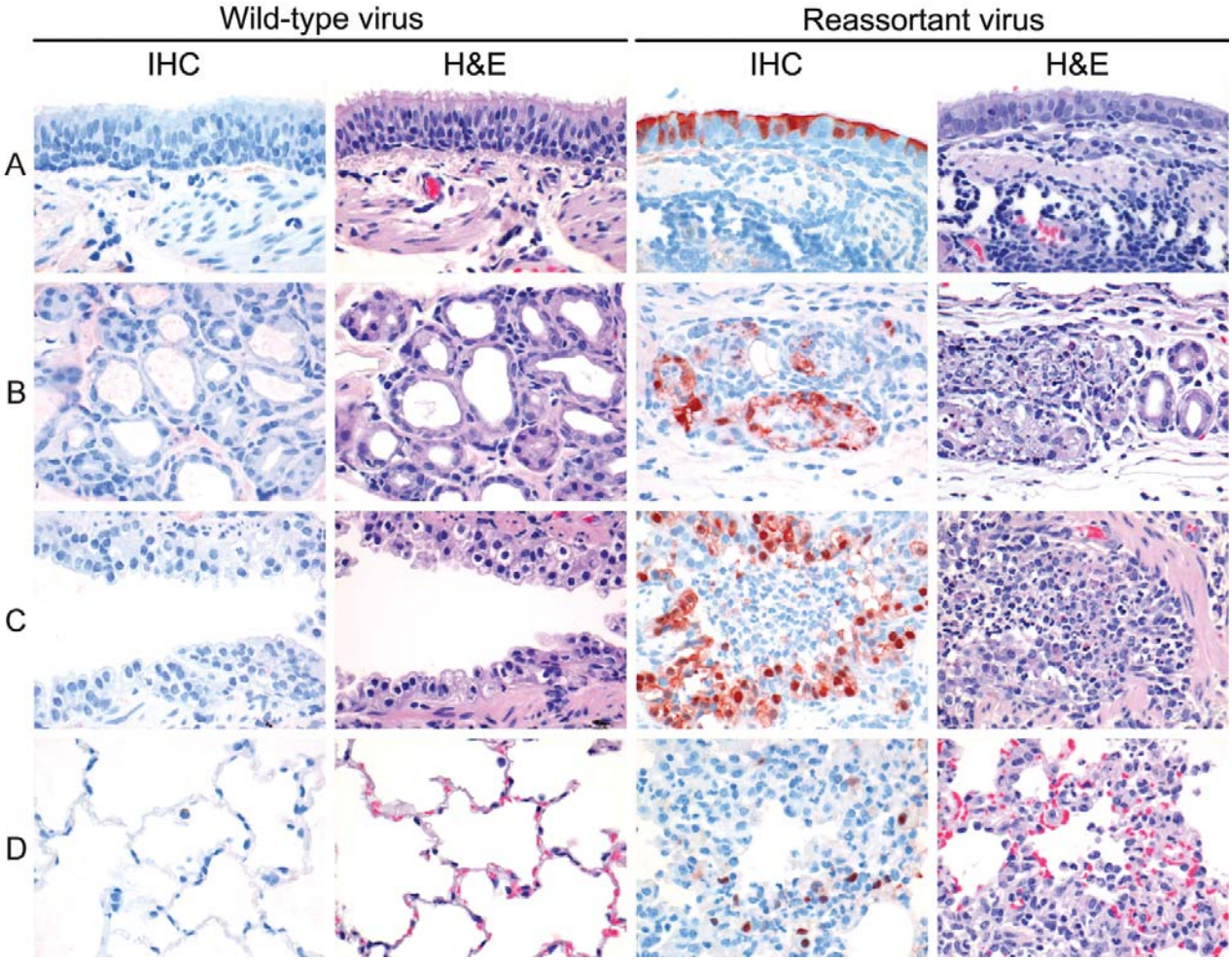

Figure 4. Examples of virus antigen expression and severity of lesions in different tissues of the lungs of ferrets. A) Bronchial surface; B) bronchial submucosal gland; C) bronchiole; D) alveolus. Two of 3 ferrets inoculated with wild-type pandemic (H1N1) 2009 virus had neither virus antigen expression (first column) nor associated lesions (second column) in the lung at day 3 postinoculation. In contrast, all 3 ferrets inoculated with reassortant pandemic (H1N1) 2009–seasonal influenza (H3N2) virus neuraminidase had virus antigen expression in bronchi, bronchial submucosal glands, bronchioles, and alveoli (third column), associated with epithelial degeneration and necrosis and infiltration of inflammatory cells, predominantly neutrophils (fourth column). IHC, immunohistochemistry; H&E, hematoxylin and eosin stain. Original magnification ×400.

in ferrets and humans (*28*), and the ferret model has further been used successfully for studies on virus transmission through respiratory droplets or aerosols (*18*,*29*).

All reassortants were transmitted between ferrets through aerosol or respiratory droplets. These results demonstrate that some reassortants between pandemic (H1N1) 2009 and seasonal influenza A (H3N2) were viable, remained transmissible, and were more pathogenic than the wild-type pandemic (H1N1) 2009 virus and emphasize the importance of monitoring reassortant viruses in surveillance programs because reassortment events may affect pathogenicity.

Although viruses with the NA gene (with or without the PB1 gene) of seasonal influenza A (H3N2) were identified here as potentially fit virus reassortants, reassortant viruses with other gene constellations may have selective advantages in humans as well. The 1968 influenza virus A (H3N2) pandemic also continued to reassort after the pandemic year, resulting in viruses during 1969–1971 with a different N2 gene than those earlier in the pandemic (*30*). Reassortants of influenza virus A (H1N2) with the HA of seasonal influenza A (H1N1) and the NA of seasonal influenza A (H3N2) viruses have been isolated from humans during previous influenza seasons, thereby confirming that reassortant influenza viruses with such an HA/NA combination can emerge in humans (*15*,*16*). Moreover, influenza (H1N2) viruses frequently have been detected in pigs around the world (*31*). Therefore, we recommend that reassortant of pandemic (H1N1) 2009 influenza viruses be monitored closely in surveillance programs, particularly when changes in pathogenicity or transmission in humans become apparent.

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Common Reservoirs for *Penicillium marneffei* Infection in Humans and Rodents, China

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Human penicilliosis marneffei is an emerging infectious disease caused by the fungus *Penicillium marneffei*. High prevalence of infection among bamboo rats of the genera *Rhizomys* and *Cannomys* suggest that these rodents are a key facet of the *P. marneffei* life cycle. We trapped bamboo rats during June 2004–July 2005 across Guangxi Province, China, and demonstrated 100% prevalence of infection. Multilocus genotypes show that *P. marneffei* isolates from humans are similar to those infecting rats and are in some cases identical. Comparison of our dataset with genotypes recovered from sites across Southeast Asia shows that the overriding component of genetic structure in *P. marneffei* is spatial, with humans containing a greater diversity of genotypes than rodents. Humans and bamboo rats are sampling an as-yet undiscovered common reservoir of infection, or bamboo rats are a vector for human infections by acting as amplifiers of infectious dispersal stages.

Penicillium marneffei is the only pathogenic species of *Penicillium* within this grouping of >270 species. This unique feature is due to the ability of *P. marneffei* to exhibit temperature-dependent dimorphic growth as an intracellular macrophage-associated fission yeast at 37°C. Before the HIV pandemic in Asia during the early 1990s, human penicilliosis was an exceedingly rare infection (*1*). Since then, however, this mycosis has become widely recognized as a co-infection in patients with HIV/AIDS, with an incidence that rivals that seen for *Cryptococcoccus neoformans* and *Mycobacterium tuberculosis* (*1*). The organism is endemic across a narrow band of tropical Southeast Asia, with human- and rodent-associated infections occurring in northeast India, Thailand, the Guangxi region of China, Vietnam, Taiwan, and Hong Kong (*2*–*4*). Within these regions, *P. marneffei* has emerged as a major threat to public health; in Guangxi Province alone, ≈16% patients with AIDS are infected with the pathogen, and >100 new cases are reported from The First Affiliated Hospital of Guangxi Medical University per year (C. Cao, unpub. data). Although unproven, humans are assumed to become infected by inhaling aerosolized infectious conidia originating from thus far unidentified environmental sources (*1*).

Despite the growing cost of this infection to human health across this region, the reservoir for human infections remains enigmatic. One clue to the potential source of infection is that *P. marneffei* maintains a close association with rodent species, particularly bamboo rats. Across Thailand and Vietnam, *P. marneffei* is commonly recovered from species of *Cannomys* and *Rhizomys* bamboo rats, with prevalences of infection approaching 100%. The type isolate of the pathogen was identified from a sample from an infected *Rhizomys sinensis* rat in 1956 (*5*). The observation that *P. marneffei* is the only species of *Penicillium* to have evolved a pathogenic lifestyle strengthens the hypothesis that small mammals are an obligate phase in the life cycle of *P. marneffei*.

As with other dimorphic fungal pathogens that infect rodents, such as *Coccidioides* spp., infection in bamboo rats is assumed to lend a selective benefit by creating a nutrient-rich patch for sporulation and widespread aerosol-dispersal after the eventual death of the host (*6*). However, identifying penicilliosis infections in rodents as the ultimate sources of penicilliosis infections in humans requires, as a first step, a demonstration that the genotypes of sylvatic and human-associated isolates are similar or identical.

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To this end, we ascertained the sylvatic prevalence of infection by trapping hoary bamboo rats (*Rhizomys pruinosis*) from across a region to which the infection is endemic, Guangxi Province in southern China, a region in which the observed case-rate for human penicilliosis marneffei is rapidly increasing (*1*,*2*). From these rodents, *P. marneffei* was isolated and genotyped by using a panel of highly polymorphic microsatellite loci. We also collected a panel of isolates from human infections across this region and then compared the distribution of genetic diversity within and between bamboo rats and humans across Guangxi and, more widely, Southeast Asia. These analyses were then used to identify the distribution of genetic diversity within and between hosts, identifying its major hierarchical components and identifying common genotypic features.

Materials and Methods

Study Area and Isolate Sources

All isolates were collected in Guangxi region of southern China on the southeastern corner of the Yunnan-Guizhou Plateau, situated from 20.54°N to 26.23°N and from 104.08°E to 112.04°E. This region borders Vietnam to the southwest and is surrounded by Guangdong, Guizhou, Yunnan, and Hunan Provinces in China. The region has a terraced topography sloping from the northwest to the southeast, with hilly land constituting 85% of its total area and plains constituting 15%. The region has a subtropical humid monsoon climate, with average daily temperatures of 16°C–23°C. The rainy season lasts from April until September, with an annual rainfall of 1,500 mm–2,000 mm.

Farmers trapped 43 adult hoary bamboo rats (*R. pruinosus*) from 8 different districts across Guangxi Province. The 15 female and 28 male captured rats were euthanized and aseptically dissected as described (*7*). *P. marneffei* was recovered from the main organs of the rats (lungs, liver, and spleen) by injection onto Sabouraud dextrose agar and brain–heart infusion agar and cultured at 25°C and 37°C, respectively, for 3–4 weeks. Both media were supplemented with chloramphenicol (0.05 mg/mL). Species identification of *P. marneffei* was based on conversion of yeast to hyphae at 25°C, secretion of a characteristic bright red pigment, and morphologic identification of colonies and conidia formation. In addition to the rodent isolates, 40 isolates were collected from human patients (including 36 persons positive for HIV) across Guangxi Province. The clinical specimens included blood, skin biopsy samples, pus from subcutaneous abscesses, lymph node biopsy samples, and bronchoalveolar lavage pellets. The linear geographic distance among the sites ranged from 133 km to 503 km, with an average distance of 224 km (Table 1; Figure 1).

Table 1. Sampling information for *Penicillium marneffei* isolates, Guangxi Province, People's Republic of China

Sampling site location	Coordinates	No. isolates	
		From rats	From humans
Liuzhou	24.275°E, 109.385°N	0	9
Hezhou	24.415° E, 111.547°N	10	5
Guigang	23.1159°E, 109.633°N	0	4
Hechi	24.71°E, 108.06°N	0	2
Nanning	22.815°E, 108.27°N	10	11
Guiling	25.219°E, 110.32°N	9	5
Bose	23.889°E, 106.626°N	9	4
Luchuan	22.62°E, 110.149°N	5	0

Multilocus Microsatellite Typing of Isolates

DNA was extracted from 7-day-old cultures of each *P. marneffei* isolate as described (*8*). Six microsatellite-containing loci were chosen from the panel described by Fisher et al (*9*). These loci (PM5, PM6, PM19, PM22, and PM23) were selected because of their high discriminatory power within a previously genotyped cohort of isolates obtained

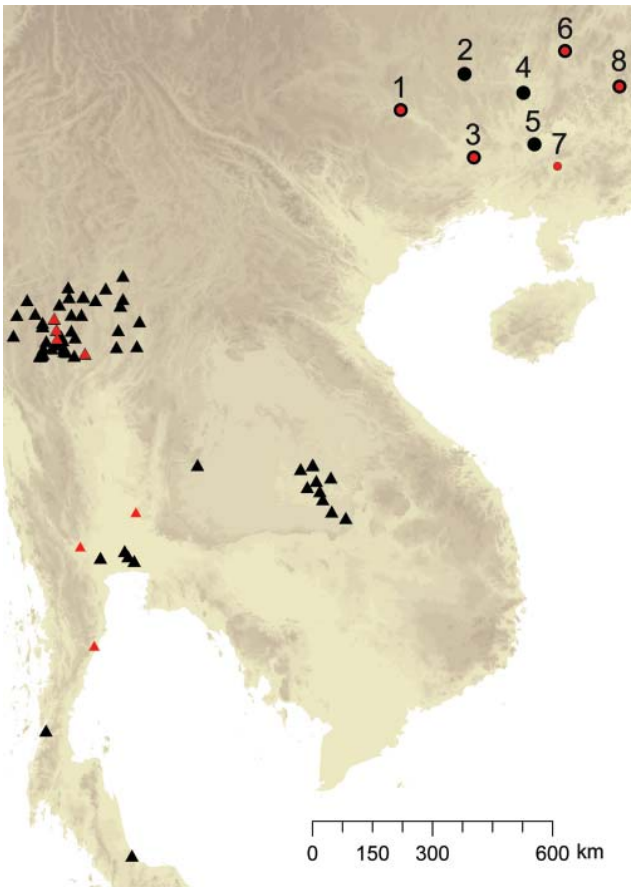

Figure 1. Spatial distribution of sampling sites for *Penicillium marneffei*, Guangxi Province, People's Republic of China. 1, Bose; 2, Hechi; 3, Nanning; 4, Liuzhou; 5, Guigang; 6, Guiling; 7, Luchan; 8, Hezhou; Black signifies origin of human-associated isolates, and red signifies origin of bamboo rat–associated isolates; both types were found in some sites.

from humans and bamboo rats in Thailand (*10*); the loci vary in the length of the microsatellite-containing repeat region. The 6 loci were amplified according to published PCR protocols (*9*). Subsequently, the PCR products were subjected to electrophoresis through a capillary sequencer with a POP6 gel and a ROX-500 internal size standard (Applied Biosystems, Foster City, CA, USA). Alleles were scored by using Genotyper software (Applied Biosystems), and multilocus genotypes for each isolate were then generated by scoring length polymorphisms at the 6 microsatellite-containing loci. Microsatellite types were subsequently identified for newly typed isolates by comparing this novel dataset from China against previously genotyped isolates from Thailand (*10*).

Genetic Data Analysis

Multilocus microsatellite types (MLMTs) were manipulated and analyzed by using the GenAlEx software add-in for Excel (*11*). The 86 Chinese isolates were coded into 2 populations according to host, human or rat, and were compared against 186 *P. marneffei* MLMTs from Thailand, also coded into 2 populations according to host. Isolates were further partitioned into 2 broad geographic regions, China or Thailand. Basic data exploration was undertaken to calculate allele and genotype frequencies and to calculate diversity statistics. The number of identical genotypes shared between hosts (human and rat) was then determined for each region by assessing which MLMTs were common to both host species. Subsequently, the genetic distances between the MLMT genotypes of *P. marneffei* from different hosts were calculated and visualized by using the neighbor-joining tree algorithm in GenAlEx (*11*).

The distribution of genetic variation across Southeast Asia between isolates of *P. marneffei* was estimated by performing an analysis of molecular variance (AMOVA) (*12*). AMOVA is a statistical technique that estimates the extent of genetic differentiation between individuals and populations directly from molecular data. The technique treats the raw molecular data as a pairwise matrix of genetic distances between all possible combinations of *P. marneffei* isolates, with submatrices corresponding to the different hierarchical data partitions (here, the genetic differences between *P. marneffei* infecting different host individuals, host species, and geographic regions). The data are then analyzed within a nested analysis of variance framework. Means squares are computed for each hierarchy of data, enabling significance testing between the following: 1) individual *P. marneffei* genotypes within hosts; 2) genotypes distributed between hosts (humans and bamboo rats); and 3) genotypes distributed between region (China and Thailand). Randomized distributions of the data are generated through random permutations, and the rejection of the null hypothesis (Ho = no significant component of variation occurs between the hierarchical divisions) then demonstrates the existence of population subdivision, either at the level of geography or host.

Subsequently, the presence of fine-scale geographic substructure within regions was determined by the use of Mantel tests. Mantel tests work by creating 2 pairwise matrices from each collection of isolates corresponding to 1) the pairwise genetic distances between isolates, and 2) the pairwise spatial distances between isolates in kilometers. The observed correlation between these genetic and geographic distances within China and for each host population (human and rat) were calculated and compared against 1,000 randomized datasets. The observed correlations were then considered significant (>0) if they exceeded 950 of the randomized datasets.

Results

Prevalence of *P. marneffei* in *R. pruinosus* Rats across Guangxi

Our survey demonstrated that 100% of the 43 adult *R. pruinosus* rats captured in Guangxi Province were positive for *P. marneffei* (Table 1). All the *P. marneffei*–positive *R. pruinosus* rats appeared healthy and, at necropsy, no visible pathologic changes were observed in any of the internal organs of the rats. The strains were most frequently isolated from lung, liver, and spleen tissues; however, no isolate was recovered from the embryonic tissue of pregnant rats (n = 15). This finding suggests that vertical transmission of infection within *R. pruinosis* rats does not occur. Forty isolates were obtained from human, including 36 patients who were positive for HIV. The strains were most frequently recovered from blood cultures (100%), followed by bone marrow aspirate (91%), skin biopsy specimens and pus of subcutaneous abscess (84%), lymph node biopsy specimens (34%), and bronchoalveolar lavage pellets (2.5%).

MLMT Analyses of *P. marneffei* in Guangxi

A single PCR amplification product was observed for all 83 isolates at each locus. Because *P. marneffi* has a haploid genome, this finding suggests that none of the cultures were composed of a mixture of *P. marneffei* strains and that hosts were therefore infected with single-genotype infections. All 6 MLMTs were polymorphic; 65 alleles were found in the pooled *P. marneffei* populations (human and rat), ranging from 3 alleles for locus PM25 to 11 for locus PM5 (Table 2). The numbers of alleles, haploid gene diversity, and distribution of private alleles were significantly different between human- and rat-associated isolates of *P. marneffei* in Guangxi (Table 2). Human-associated *P. marneffei* isolates in Guangxi were more polymorphic (Table 3) and showed higher haploid genetic diversity (Table 3) and numbers of unique alleles (Table 3).

Table 2. Microsatellite loci scored in *Penicillium marneffei* isolates, Guangxi Province, People's Republic of China

Populations sampled	Locus	No. samples	Mean no. alleles	Mean effective no. alleles
Human	*PM5*	40	11	7.767
	PM6	40	6	3.419
	PM19	40	8	6.838
	PM22	40	5	3.433
	PM23	40	5	1.810
	PM25	40	3	1.831
Rat	*PM5*	43	7	5.945
	PM6	43	4	3.332
	PM19	43	5	3.332
	PM22	43	5	2.039
	PM23	43	3	1.208
	PM25	43	3	2.158
Total		41.5	5.417	3.592

MLMT analyses of the 83 isolates from Guangxi recovered 59 distinct multilocus microsatellite barcode types (MTs). A total of 38 MTs were found infecting humans, and 22 were infecting bamboo rats. A single MT haplotype was found to co-occur in both human and rats (Figure 2). The probability of observing infections with identical genotypes in different hosts by chance alone can be approximated as $\approx 5.46 \times 10^{6}$; this occurrence is therefore statistically highly unlikely to occur, and we conclude that these 2 hosts were co-infected from a single clonally reproducing individual of *P. marneffei*. Although the bamboo rats in question were trapped in Hezhou, the human isolate was recovered from Hechi, 355 km distant; this observation could indicate either that the patient traveled to and acquired infection in the environs of Hezhou, or infectious *P. marneffei* conidia can disperse over that physical distance.

We then tested whether *P. marneffei* isolates from humans and rats represented genetically unique subpopulations of the total genetic diversity by using AMOVA (Table 4). Although isolates recovered from Thailand and China are clearly genetically isolated populations, only 2% of the total diversity was partitioned between host species within these regions. Within Guangxi, however, this minor component of diversity was significant ($p = 0.008$), which suggests that humans and bamboo rats in Guangxi are not infected by *P. marneffei* in a completely random manner and that some underlying genetic structure exists.

To test whether this component of variation between humans and bamboo rats was related to spatial factors, we tested the extent of correlation between geographic distance and genetic distance across 3 components of *P. marneffei* diversity in Guangxi by using Mantel tests for the following data partitions: 1) human and bamboo rat isolates together; 2) human isolates alone; and 3) bamboo rat isolates alone. Combined human and bamboo rat data showed a significant spatial component to the distribution of genetic diversity across Guangxi ($p = 0.001$). However, this effect is not observed for isolates collected from humans ($p = 0.323$) but is observed for isolates collected from bamboo rats ($p = 0.001$). This result shows that the spatial signal in the dataset is attributable to the rodent-associated isolates.

The finding that rodent isolates are more spatially structured than human-associated isolates is confirmed by assessing the proportion of identical genotypes that are to be found infecting either human or rodent hosts within the different sample sites in Guangxi (Figure 2). This analysis showed that although 9 MLMT haplotypes infected >1 bamboo rat within a sample site, only a single MLMT haplotype was shared among human patients within 1 sample site (Hezhou). That no MTs were shared by bamboo rats between trapping sites is strong evidence that *P. marneffei* genotypes are spatially patchy within Guangi Province. Furthermore, although no MLMT haplotypes were shared between bamboo rats from different sample sites, 2 MLMT haplotypes (Human18, Guigang and Human32, Nanning; Human22, Hechi and Rat29, 30, 34, and 35, Hezhou) were shared between humans or bamboo rats from different sample sites. Together, these data show that humans are exposed to a greater diversity of *P. marneffei* genotypes relative to the bamboo rats, despite occupying a similar geographic region.

Discussion

The body of current evidence suggests that bamboo rats play a key role in the life cycle of *P. marneffei.* Efforts to detect live *P. marneffei* in the soil environment have generally failed, although *P. marneffei* DNA has been detected in soils in Thailand that have a known association with animals, such as elephants (*13*). *P. marneffei* is consistently and reproducibly isolated from several species of bamboo rat across its known range and, within China, Li et

Table 3. Difference in allele numbers, haploid gene diversity, and distribution of private alleles between human- and rat-associated isolates of *Penicillium marneffei* in Guangxi Province, China, and Thailand

Location and population sampled	No. samples	No. haplotypes	Mean no. alleles (SE)	Mean effective no. alleles (SE)	Mean haploid genetic diversity (SE)	Mean no. private alleles (SE)
China						
Human	40	38	6.333 (1.145)	4.183 (1.036)	0.674 (0.076)	1.167 (0.543)
Rat	43	22	4.500 (0.619)	3.002 (0.677)	0.575 (0.094)	0.333 (0.211)
Thailand						
Human	163	51	6.667 (1.430)	1.840 (0.224)	0.410 (0.079)	1.167 (0.833)
Rat	23	11	3.833 (0.401)	1.967 (0.389)	0.404 (0.094)	0.000 (0.000)
Total	269	116	5.333 (0.524)	2.748 (0.364)	0.516 (0.047)	

al. (*14*) and Deng et al. (*15*) have shown that infection in these rodents exhibits a high prevalence of infection in *R. pruinosus*. In this study, we used a sample of 43 animals from 5 sample sites across a wide geographic region spanning 500 km to show that infection is prevalent at 100% in all sampled populations. Therefore, as in Thailand and India, *P. marneffei* in China appears to be strongly associated with bamboo rats (*7*,*15*–*17*), underscoring the likely role of these rodents in the life cyle of this mycosis.

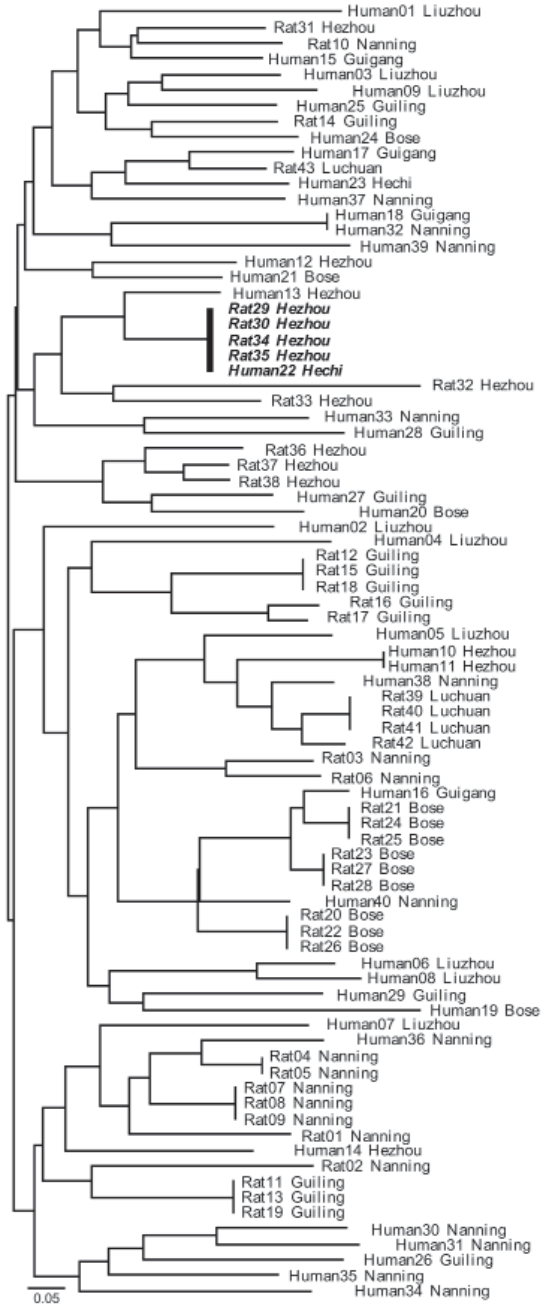

Figure 2. Neighbor-joining tree of the relationship between multilocus microsatellite type genotypes of human and bamboo rat associated *Penicillium marneffei* isolates, Guangxi Province, People's Republic of China. Identical genotypes shared between humans and rats are in **boldface**. Scale bar indicates nucleotide substituitons per site.

To address this question, we ascertained whether *P. marneffei* isolates from bamboo rats in China were the same as, or different from, those infecting humans across this region. Our use of molecular genotyping clearly showed little difference in allele frequencies between these 2 host-associated populations of *P. marneffei*, and in 1 case direct sharing of a common genotype was observed, showing that human and bamboo rat infections in China are highly similar. This finding parallels reports from Thailand and India (*7*,*10*), where little difference between human and rodent isolates was observed. Taken together, these observations strongly suggest that all bamboo rat associated isolates are potentially able to cause infections in humans.

However, as was observed in Thailand (*10*), Chinese bamboo rat isolates represent a more spatially heterogeneous population of *P. marneffei* compared with human infections and exhibit lower gene-diversity indices and lower relatedness across large spatial regions and higher clonality (as shown by identical genotypes) within a sampled site. From a strictly genetic standpoint, the spatially clustered clonal isolates infecting the bamboo rat populations relative to the higher diversity, nonclustered isolates observed in human infections could be explained by 2 hypotheses: The first hypothesis is that humans travel more widely than bamboo rats, and therefore sample a greater number of *P. marneffei*–infected environments. The second hypothesis is that bamboo rat and human infection reflect different aspects of the sylvatic reservoir of *P. marneffei*. In this second hypothesis, rodents become infected by spores that are locally produced, reflecting the innate spatial clustering of sylvatic spore dispersal. However, some aerosolized conidia may be dispersed beyond forests, thus generating a relatively more homogeneous, but less dense, population of infectious conidia that are able to infect persons with HIV outside of the naturally occurring ecologic niche for *P. marneffei*.

Conclusions

We were not able to discriminate between our 2 hypotheses; however, we did identify the type of questions that we need to be asking. First, we need to establish whether infected humans are widely exposed by traveling within potentially *P. marneffei*– and bamboo rat–associated habitats: the answer to this is likely to be no because 1) there is no recognized epidemiologic association between infection and travel to bamboo rat habitats (*18*); and 2) the infected population tends to be urbanized sex workers and drug users who have HIV. In this case, if humans are acquiring their infections within urban environments by inhaling aerosolized conidia, then conidia should be detectable by the use of high-throughput air sampling and molecular-probe detection of *P. marneffei*. In support of

Table 4. Analysis of molecular variance among and within sampled populations of *Penicillium marneffei**

Source	df	Estimated variance (%)	p value
Regions (China and Thailand)	1	0.974 (39)	>0.001
Among species (humans and rats)	2	0.059 (2)	>0.002
Within species	265	1.446 (58)	>0.001
Total	268	2.478 (100)	

*%, percentage of the total variance component across the specified hierarchy; p, probability that the observed variance is greater than that expected for the null hypothesis *Ho*, generated by 999 random permutations of the data.

this technique, a broad-scale sampling of soils in Thailand that used quantitative PCR (*13*) showed that *P. marneffei* could be patchily detected in animal-associated soils; such technology could be readily adapted to be used with air-sampling technology.

Finally, if bamboo rats are a major amplification reservoir for *P. marneffei* by the pathogen being sequestered within bamboo rats and becoming transmissible upon host death, then domestic or pest animal hosts may be involved. However, we know little about the host range of *P. marneffei*. Therefore, given the widespread increase in the prevalence of this infection across Southeast Asia, we assert that there is a pressing need to revisit the epidemiology of this highly enigmatic infection and that the occurrence of realized and potential amplifiers of human infection need to be reassessed.

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Dr Cao is an associate professor at the First Affiliated Hospital of Guangxi Medical University, People's Republic of China. Her research interests include the biology and molecular epidemiology, antifungal drug sensitivity, and gene function of *P. marneffei*.

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Phocine Distemper Virus in Seals, East Coast, United States, 2006

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In 2006 and 2007, elevated numbers of deaths among seals, constituting an unusual mortality event, occurred off the coasts of Maine and Massachusetts, United States. We isolated a virus from seal tissue and confirmed it as phocine distemper virus (PDV). We compared the viral hemagglutinin, phosphoprotein, and fusion (F) and matrix (M) protein gene sequences with those of viruses from the 1988 and 2002 PDV epizootics. The virus showed highest similarity with a PDV 1988 Netherlands virus, which raises the possibility that the 2006 isolate from the United States might have emerged independently from 2002 PDVs and that multiple lineages of PDV might be circulating among enzootically infected North American seals. Evidence from comparison of sequences derived from different tissues suggested that mutations in the F and M genes occur in brain tissue that are not present in lung, liver, or blood, which suggests virus persistence in the central nervous system.

In 1988, harbor seals (*Phoca vitulina*) and gray seals (*Halichoerus grypus*) died in large numbers off the coast of northern Europe (*1*). A virus was first isolated in April 1988, when widespread abortions and deaths among harbor seals were reported in the Kattegat area between Denmark and Sweden. The infection spread to the North, Wadden, and Baltic seas, killing 17,000–20,000 seals in northwestern Europe in 8 months. The virus subsequently was classified as a species of the genus *Morbillivirus* (family *Paramyxoviridae*) (*2*,*3*), *Phocine distemper virus* (PDV). The virus is believed to have originated in harp seals in which the infection is enzootic (*4*). Migrations of harp seals into the North Sea may have initiated the epizootic in harbor seals. Gray seals in the northeastern Atlantic Ocean also were infected, but disease was not as severe as in harbor seals (*5*).

A more recent outbreak occurred in Europe in 2002 (*6*). An estimated 30,000 harbor and gray seals died during this epizootic (*7*,*8*). The origin of this second epizootic 14 years after the first remains unknown. PDV may have jumped species into terrestrial carnivores, particularly mink, and reinfected seals (9), but this hypothesis remains unproven. Phylogenetic analysis of the hemagglutinin (H) genes of PDV, together with those of other morbilliviruses, suggests that the reemergent 2002 PDV is more closely related to a putative recent ancestral PDV than to the 1988 isolates (*10*). Millions of seals of various species inhabit the waters surrounding North America; populations of most species are believed to be stable or increasing, and no epizootics on the scale of those reported in Europe have been reported. PDV disease in the United States was first reported in harbor seals on the east coast during the winter of 1991–92 (*11*), and serologic testing of gray and harbor seals suggested that a PDV-like strain or strains were circulating enzootically in the region (*12*). This circulation was attributed to an increased number of harbor seals (mainly immature animals) overwintering in southern New England (*13*). During the spring of 2006, deaths among seals (harbor, gray, and hooded) also increased along the coasts of Maine and Massachusetts. This increase was considered an unusual mortality event. Both dead and sick seals appeared nonemaciated. Live-stranded seals were weak and had generalized body tremors and spasms. Affected seals were taken to the Marine Science Education and Research Center (University of New England, Biddeford, ME, USA); investigations indicated that the pathologic changes were consistent with morbillivirus infection. Recent advances in

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virus isolation and genetic sequencing methods have provided us with better insight into PDV epizootiology in Europe and in North America.

Materials and Methods

We isolated the 2006 virus from liver tissue of a harbor seal and confirmed it as PDV. To determine the phylogenetic relationship and possible origin of the isolate, we compared the virus RNA sequences and deduced amino acid sequences for the virus cell receptor attachment protein hemagglutinin (H) with those from various PDVs from both the 1988 and 2002 epizootics in Europe. We also investigated whether any differences in sequences between the PDV/USA2006 and the 2002 and 1988 viruses were likely to have occurred through sequencing errors, their tissue of origin, or adaption to Vero cells. Sequence information, when available for the phosphoprotein (P) membrane fusion (F), and internal matrix (M) protein genes also were compared for various viruses from outbreaks in Europe, the United States, and Canada during 1988–2006.

Cells and Tissues

Vero and VeroDogSLAM (VDS) cells were grown in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum. A blood sample from an infected seal from the 1988 epizootic was obtained from Albert Osterhaus, Erasmus University (Rotterdam, the Netherlands). Brain tissue from a harbor seal (designated PDV/3541UK) that was found off the coast of Scotland at the end of the 2002 epizootic and was PCR positive for PDV in the brain but not other tissues (lung, spleen, and lymph nodes) was obtained from Paul Jepson, Institute of Zoology, Zoological Society of London.

Reverse Transcription–PCR and DNA Sequencing

Total RNA was extracted from infected cells and tissues by using TRIzol reagent (Invitrogen). cDNA synthesis was conducted by using oligo-dT primers and the SuperScript First-Strand Synthesis kit (Invitrogen). PCR was performed by using the High Fidelity Taq kit (Invitrogen). Morbillivirus universal P gene and β-actin primers (*14*) and further PDV primers to the H, F, M, and P genes designed to previously published PDV sequences are given in the Table. DNA sequencing was performed by using a BigDye 3.1 Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with primers listed in the Table. Completed PCR products were sent to the Genomics Core Facility, Queens University (Belfast, UK) for chromatographic preparation.

Results

Isolation and Identification of USA 2006 as PDV

Signaling lymphocyte activation molecule (SLAM) is a receptor for both vaccine and wild-type strains of measles virus and for canine distemper and rinderpest morbilliviruses (*15*,*16*). VDS cells have been used successfully to isolate PDV from experimentally infected ferrets (*Mus-*

Table. Phocine distemper virus primers for reverse transcription–PCR and DNA sequencing*

Gene	Primer sequence, 5′ → 3′	Gene	Primer sequence, 5′ → 3′
P forward	ATGTTTATGATCACAGCGGT	F4 reverse	CCCGTAAACTTGGTCCAA
P reverse	ATTGGGTTGCACCACTTGTC	F5 forward	ATAATATAGGGTCACAGG
M forward	ATACTGCATTAACCCTGG	F5 reverse	CCTGTGACCCTATATTAT
M reverse	TTAGGTTGTTGGTCTTGGTAG	F6 forward	TTGTGCTTCTATCTTGTG
M1 forward	ATAACGATGATCTTGGCC	F6 reverse	CACAAGATAGAAGCACAA
M1 reverse	GGCCAAGATCATCGTTAT	F7 forward	GAATCCTCTGATCAAATC
M2 forward	ACACAGCTCAGAGATTCC	F7 reverse	GATTTGATCAGAGGATTC
M2 reverse	GGAATCTCTGAGCTGTGT	F8 forward	TGCAAGCTGGCACATCAG
M3 forward	ACTGGTGTTCGCCCTTGG	F8 reverse	CTGATGTGCCAGCTTGCA
M3 reverse	CCAAGGGCGAACACCAGT	H forward	CGAGGTTGAGGAAAGAAG
M4 forward	TTAAATTCCCAGTTCTTG	H reverse	CTCAATCTCGGTGGGTAC
M4 reverse	CAAGAACTGGGAATTTAA	H1 forward	AGGCAGTGCATCATCAAG
M5 forward	AGCCACTTGAATCTACGG	H1 reverse	CTTGATGATGCACTGCCT
M5 reverse	CCGTAGATTCAAGTGGCT	H2 forward	CAATCCTCTTGCTGACAC
F forward	GAGATTTGTGCACCTTTC	H2 reverse	GTGTCAGCAAGAGGATTG
F reverse	GC ATTGTTCTTGTAAAAGGC	H3 forward	AGATGGCTAGGTGATATG
F1 forward	TCATAGTCTCGATTCACC	H3 reverse	CATATCACCTAGCCATCT
F1 reverse	GGTGAATCGAGACTATGA	H4 forward	CACCGGGGTTTCATAAAG
F2 forward	TTATCAACAATTGGAATC	H4 reverse	CTTTATGAAACCCCGGTG
F2 reverse	GATTCCAATTGTTGATAA	H5 forward	GGATTATTATGAGGGTAC
F3 forward	TGCAGGTGCAGCTCTAGG	H5 reverse	CAATAGCATGATCACTCC
F3 reverse	CCTAGAGCTGCACCTGCA	H6 forward	GGAGTGATCATGCTATTG
F4 forward	TTGGACCAAGTTTACGGG	H6 reverse	GTACCCTCATAATAATCC

*H, hemagglutinin; P, phosphoprotein; F, fusion; M, matrix.

tela putorius furo) (*17*), and we recently confirmed that this molecule also is used as a receptor for PDV (M. Melia et al., unpub. data). The USA 2006 virus was isolated by inoculating homogenized liver tissue from a harbor seal onto VDS cells, which resulted in syncytia formation (Figure 1). Reverse transcription–PCR (RT-PCR) was initially conducted by using previously published morbillivirus universal P gene primers (*14*). The PCR product was sequenced and aligned with morbillivirus sequences for this target region and showed 100% homology with PDV (data not shown).

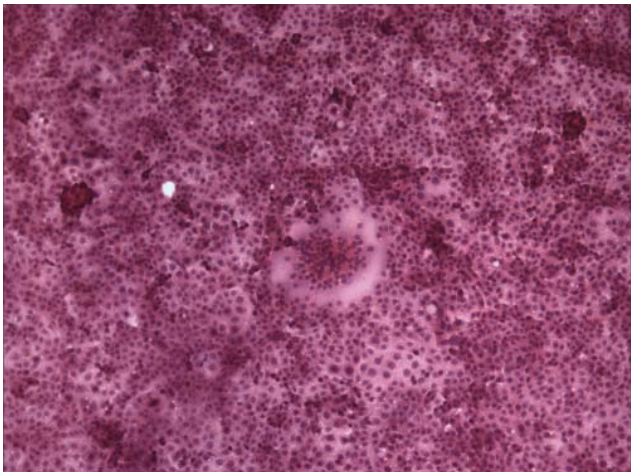

Figure 1. VeroDogSLAM cells 30 h postinfection inoculated with tissue (liver) homogenate from a dead adult female harbor seal from Maine that died in July 2006. Syncytia are seen in the monolayer, which is stained with hematoxylin and eosin (original magnification ×100).

Origin of Sequence Data

Selected gene sequences from the PDV/Ulster 88, PDV/NL88, PDV/DK 88, and PDV/DK2002 viruses from Europe were available for comparison with the PDV/USA2006 strain (*10*,*18*–*21*). In addition, we included partial P gene sequences obtained from tissues and nasal swabs of northern seas otters (*Enhydra lutris*) from an unusual mortality event in south-central Alaska in 2006, as well as samples from a harp seal found in the Gulf of St. Lawrence (Canada) in 1991 and from a hooded seal on the New Jersey, USA, coast in 1998 (*22*,*23*).

Because of improvements in techniques of sequence determination from those available in 1988, we initially resequenced the PDV/Ulster88 virus H, M, and F genes. We have designated this "new" sequence PDV/Ulster88n. For the same reason, and to determine whether adaptation to Vero cells changed the virus sequence, particularly in the cell receptor attachment H protein, we obtained RNA directly from a blood sample used to isolate the PDV/NL88 strain and conducted RT-PCR directly. We designated this "new" sequence for the H, F, and M genes as PDV/NL88n. The sequence obtained from the central nervous system tissue of the seal from the 2002 epizootic is designated PDV/3541UK.

Nucleotide and Deduced Amino Acid Sequence Comparison

Previous studies have shown that the H protein is the most variable and that comparison of the PDV/2002 and PDV88 sequences from Europe showed 8 minor amino acid changes (*10*,*21*). The sequences for the PDV/Ulster88 and PDV/NL88 viruses had been obtained from isolates in Vero (African green monkey kidney) cells, unlike the 2002 sequences, which were obtained directly from lung tissue. To determine whether errors may have occurred in the PDV/Ulster88 sequence, we initially compared the original PDV/Ulster88 and new PDV/Ulster88n H gene sequences. Changes in PDV/Ulster88 compared with the consensus sequence for all the viruses at bases 910, 911, 1134, and 1135 are not mirrored in the PDV/Ulster88n sequence, which indicates that these are likely to have resulted from sequencing errors and therefore do not reflect real differences in the more recent isolates. Similarly, to determine whether changes in the original PDV/NL88 strain are likely to have resulted from adaption to Vero cells, we compared the original PDV/NL88 H sequence with PDV/NL88n, which was amplified directly from a blood sample. Differences of PDV/NL88 from the consensus at bases 23 and 1711 are not reflected in the PDV/NL88n sequence, which indicates that these are likely to have resulted from tissue culture adaptation and/or sequencing errors.

The newly isolated PDV/USA2006 virus has 11 aa changes in the H gene (GenBank accession no. 1375698), compared with the PDV/DK2002 strain at codons 176, 200, 218, 221, 276, 327, 399, 432, 561, 561, and 576. However, the sequences at codons 200, 218, 276, 399, and 432 are in common with the PDV/NL88n strain. Similarly, silent mutations at nt 180, 564, and 1728 are the same as PDV/NL88n. With the exception of codon 564, all other differences of PDV/USA2006, compared with PDV/DK2002, reflect the consensus sequence. All of these observations suggest that the similarities of PDV/USA2006 to the 1988 virus may be due to circulation of multiple lineages. A phylogenic analysis for the H gene is shown in Figure 2, panel A.

Few amino acid changes are found in the P (GenBank accession no. 1375683) and M (Figure 3) genes of PDV/USA2006, compared with the other viruses. Complete P gene sequences were available for the DK2002, NL2002, Ulster, NL and DK 88 viruses. Partial sequences from the 1991 Canadian and 1998 USA viruses, as well as the sea otter 2006 virus, also were used for comparison (*22*,*23*). The USA/2006 P gene differs from the more recent 2002

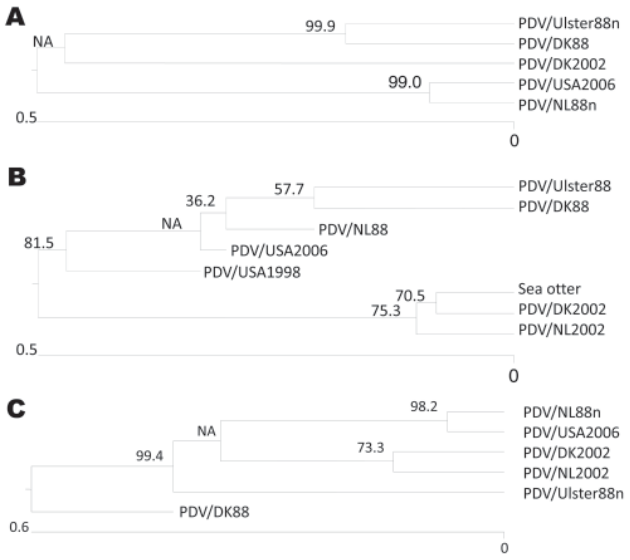

Figure 2. Phylogenic relationship of PDV/USA2006 to viruses from the 1988 and 2002 epizootics in Europe based on hemagglutinin (H) and phosphoprotein (P) gene sequences. A) Phocine distemper virus (PDV) H gene sequences used for the alignments were from PDV/DK2002 (lung), GenBank accession no. FJ648456; PDV/DK88 (isolated in Vero cells), GenBank accession no. Z36979; PDV/Ulster88n (isolated in Vero cells), PDV/NL88n (blood), GenBank accession no. D10371 (minus described changes in this study); and PDV/USA2006 (isolated in VeroDogSLAM cells), GenBank accession no. 1375698. B) PDV P gene sequences used for the alignments were from PDV/DK2002 (lung), GenBank accession no. –af52587; PDV/NL2002 (lung) GenBank accession no. af52588; PDV/DK88 (isolated in Vero cells), GenBank accession no. –x75960; PDV/Ulster88 (isolated in Vero cells), GenBank accession no. D10371; PDV/NL88 (isolated in Vero cells), GenBank accession no. af525289; PDV/USA1998, GenBank accession no. ay3323389; and PDV/USA2006 (isolated in VeroDogSLAM cells), GenBank accession no. 1375683. Unrooted neighbor-joining phylogenetic trees in A and B were constructed by using the MegAlign version 7.1 package (DNASTAR, www.dnastar.com) with the ClustalW method (www.clustal.org). C) Unrooted neighbor-joining phylogenetic tree for concatenated H and P squences constructed by using ClustalV. Scale bars denote number of nucleotide substitutions per site along the branches. Percentage bootstrap values, indicating the significance of clusters, are shown.

viruses at codon 152 but is the same as all three 1988 isolates. Other minor variations between strains at the sequence level align PDV/USA2006 most closely to PDV/NL88. A phylogenic analysis based on the P gene is shown in Figure 2, panel B, and a concatenated tree based on both the H and P genes (for viruses where both sequences are available) is shown in Figure 2, panel C. The M and F gene sequence of PDV/USA2006 were compared with the sequences of PDV/Ulster88n, PDV/NL88n, PDV/DK88, and PDV/3541UK. The PDV/USA2006 M and F protein amino acid sequences (Figures 3, 4) reflect at least 1 of the other viruses at all positions. PDV/Ulster 88 M sequence differs from the other viruses at codon 84, whereas PDV/NL88n differs at codons 178, 310, 331, and 333. The first 233 nt of the PDV/3541UK M gene could not be amplified. However, alignment of the remaining sequence showed amino acids to be in common with ≥1 of the other viruses. In the F gene, an amino acid change occurred in the initiation codon from Met to Val in PDV/3541UK, compared with the other viruses, and silent mutations occurred at codons 20, 22, 51, 156, 198, 440, and 528.

Discussion

We confirmed that at least some of the deaths in seals that occurred around Maine and Massachusetts in 2006 resulted from PDV infection and conducted sequence alignments with other strains derived during 1988–2006. Müller et al. reported 8 aa changes in the H gene between the 1988 and 2002 European strains of PDV (*21*). We have shown that 4 base changes in PDV/Ulster88, compared with the 2002 isolates, probably resulted from errors by resequencing this virus (PDV/Ulster88n) by using current technology. Furthermore, the PDV/NL88 strain when reisolated directly from a blood sample (PDV/NL88n) also showed fewer differences to 2002 strains, which in this case may have been due to adaptation to Vero cells, although sequencing errors cannot be ruled out. Phylogenetic analysis of the individual H and P gene sequences, as well as combined concatonated sequences (Figure 2), suggests that PDV/USA2006 is more closely related to PDV/NL88n than to the 2002 European viruses. Although sequence information was limited, we found that the 2006 sea otter virus P gene sequence was identical to that of the PDV/DK2002 virus. Therefore, the two 2006 viruses, 1 each from the US Atlantic and Pacific coasts, might have had different origins, but

Figure 3. Amino acid alignment of the matrix protein of phocine distemper virus (PDV) strains PDV/USA2006, PDV/Ulster88n, PDV/NL88n, and PDV/UK3541. The first 233 nt of the matrix gene of PDV/3541UK, as indicated by a dotted line, are undetermined. Large boxes indicate amino acid changes and small boxes the position of silent mutations.

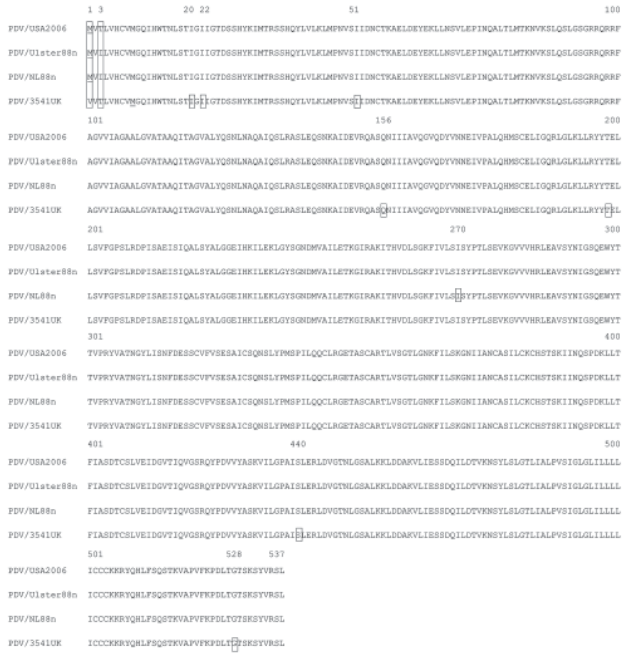

Figure 4. Amino acid alignment of the fusion protein of phocine distemper virus (PDV) strains PDV/USA2006, PDV/Ulster88n, PDV/NL88n, and PDV/UK3541. Large boxes indicate amino acid changes and small boxes the position of silent mutations.

this possibility requires further investigation. The limited sequence information for the P gene of the 1991 virus from Canada and the 1998 virus from the United States is identical to that of the P gene of PDV/DK2002. More sequence information for these North American viruses, particularly for the H gene, might give further insight into their origin and evolution.

Unlike the 2002 viruses, from which RNA sequences were obtained directly from tissues, the PDV/USA2006 strain was isolated into VDS cells, which might explain the differences, particularly in the H gene. Sequencing viruses isolated in these cells is preferable to sequencing them isolated in Vero cells because of expression of the SLAM receptor. The SLAM binding site of the H protein has been shown to be conserved across all known morbilliviruses (*24*). We found that the residues were conserved in all of the PDVs tested, including the 1988 strains that had been isolated in Vero cells. Nielsen et al. (*10*) suggested that the higher identity of the H protein in 1988 strains than in PDV/DK2002 could have been due to passaging of the former in Vero cells. By comparing directly amplified PDV/NL88n sequences from a blood sample, we demonstrated that the similarities with the USA/2006 isolate are unlikely to have resulted from tissue culture adaption of the latter.

The M and F amino acid sequences are in common with ≥1 of the other viruses. The first 233 nt of the M gene of PDV/3541UK could not be amplified despite successful amplification of the F gene and the housekeeping mRNA β-actin. Therefore, mutations may remain to be identified, and those mutations may account for the lack of primer specificity in this region.

A total of 8 mutations were found in the PDV/3541UK F sequence, compared with those of PDV/USA2006, PDV/Ulster88n, and PDV/NL88n. Seven of these changes were silent. The substitution of Met to Val in the first codon may be particularly noteworthy. Perhaps, in PDV/3541UK, GUG can be used as the initiation codon or initiation may take place at the next available Met. In the latter case, a fully functional F protein would not be produced in the brain of this animal. Truncations, mutations, and deletions in the cytoplasmic domain of the F protein occur in the persistent measles central nervous system complications (subacute sclerosing panencephalitis and measles inclusion body encephalitis) (*25*). Sequencing of more F genes from seal brain tissue is necessary to determine whether the observed substitutions are a common feature and whether they are associated with persistent infection.

In this study we confirmed that the virus isolated from the US 2006 unusual mortality event in seals was PDV. The similarity of this isolate to the PDV/NL88n virus suggests that PDV/USA2006 may have reemerged independently of the 2002 PDVs and that multiple lineages of PDV may be circulating enzootically among the large populations of North American seals. Multiple lineages of canine distemper virus occur worldwide and affect different carnivore species (*26*). The Maine 2006 unusual mortality event in seals never progressed to a full-blown epizootic as occurred in Europe, perhaps because of differences in the pathogenicity of the US 2006 virus itself, or more likely, because several PDV viruses are circulating among populations of seals that are large enough to maintain them enzootically without large-scale die-offs. Contributing to the early detection of PDV in the affected seals were range extension and rapidly increasing populations of harbor and gray seals (*13*) that may have had a high prevalence of immunologically naive individuals, coupled with an active surveillance program to rehabilitate stranded seals. The findings of increased base substitutions in the F gene and lack of an amplifiable product at the start of the M gene from brain tissue of an animal in 2002, compared with those obtained from lung, liver, or blood, raises the possibility that mutations could occur in the central nervous system and might be associated with persistent infection.

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Leptospirosis in Hawaii, USA, 1999–2008

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Although infrequently diagnosed in the United States, leptospirosis is a notable reemerging infectious disease throughout developing countries. Until 1995, when the disease was eliminated from the US list of nationally notifiable diseases, Hawaii led the nation in reported annual incidence rates. Leptospirosis remains a notifiable disease in Hawaii. To ascertain the status of leptospirosis in Hawaii since the most recent US report in 2002, we reviewed 1999–2008 data obtained from case investigation reports by the Hawaii State Department of Health. Of the 345 case reports related to in-state exposures, 198 (57%) were laboratory confirmed. Our findings indicate a change in seasonal disease occurrence from summer to winter and in the infective serogroup from Icterohemorrhagiae to Australis. Also, during the past 20 years, recreational exposures have plateaued, while occupational exposures have increased. Ongoing surveillance is needed to clarify and track the dynamic epidemiology of this widespread zoonosis.

Leptospirosis is considered the most globally widespread zoonotic illness; it has been classified as an emerging or reemerging infectious disease by the World Health Organization (*1*) and the US Centers for Disease Control and Prevention (CDC) (*2*). Most frequently recognized as a disease of the developing world (*3*), leptospirosis was removed from the US list of nationally reportable infectious diseases in 1995 (*4*). Before the disease's removal from national surveillance, Hawaii consistently led the nation in reported annual incidence rates (*5*). The state of Hawaii continues to include leptospirosis as a notifiable illness. The last published US population-based surveillance report was from Hawaii and covered data obtained during 1974–1998 (*5*). This study serves as an update for leptospirosis in Hawaii during 1999–2008.

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Methods

We reviewed leptospirosis case investigation reports by Hawaii Department of Health (HDOH) investigators submitted during 1999–2008. These reports were (and still are) generated for all reported leptospirosis cases in the state. A standardized case investigation form was used, which includes demographic, epidemiologic, clinical, and laboratory information obtained from patient interviews, medical record reviews, and laboratory reports. Research for this study was approved by the HDOH Institutional Review Board.

For exposure source to be assessed, incubation periods estimated, and exposures classified, patients were asked about high-risk activities that occurred during the 21 days before symptom onset. These included exposure to animals, mud, or potentially contaminated freshwater sources involving occupational activities (e.g., farming, ranching), recreational activities (e.g., freshwater swimming, hiking), or habitational activities (around the home; e.g., gardening, trapping rats). If exposure was continuous or if persons had been exposed multiple times, the incubation period was considered indeterminate. Ascertainment of exposure classification involved placing cases into 3 mutually exclusive exposure categories: occupational, recreational, or habitational. If exposure activities involved >1 category, the exposure classification was considered indeterminate. Outbreaks were defined as ≥2 epidemiologically linked cases.

A patient with a confirmed case had a clinically compatible illness plus a ≥4-fold increase in microscopic agglutination test (MAT) titer between acute- and convalescent-phase serum specimens or isolation of *Leptospira* spp. from a clinical specimen (*6*,*7*). All other cases were classified as either probable (clinically compatible illness with MAT titer ≥200 in ≥1 serum specimens without a 4-fold increase

in titer [*8*]) or suspected (clinically compatible illness with less supportive laboratory evidence of infection [e.g., MAT titer <200, positive macroscopic slide agglutination test result, reactive immunoglobulin (Ig) M ELISA, or positive indirect hemagglutination assay results]). Only laboratory-confirmed cases in patients whose disease was contracted through exposure within the state of Hawaii were included in this analysis. MATs were conducted by CDC from January 1999 through November 2004, and by HDOH from December 2004 through December 2008.

All isolates were sent to CDC for definitive serogroup identification. To determine the presumptive infecting serogroup for serologically confirmed cases, MAT titers were examined. The highest and most recent titer was presumed to be the infecting serogroup. If >1 serogroup had the same high titer, the identification was labeled indeterminate.

To calculate mean annual incidence rates (overall and by age, sex, and Hawaii island on which patient was exposed), the numerator was the number of cases for the specified groups over the 10-year observation period divided by 10. The denominator was the overall or relevant group-specific population estimate from the 2000 US Census (*9*). Data from our earlier 25-year study period, 1974–1998, were used for trend analyses (*5*).

We calculated frequencies, tests for trends, and tests for difference using Epi Info version 3.3.2 (CDC, Atlanta, GA, USA); p values ≤0.05 were considered significant. All statistical tests were 2-tailed.

Results

HDOH received 356 leptospirosis case reports; 345 were related to exposures within the state of Hawaii. The 11 cases from exposures occurring out of state included 2 from Guam; 2 from Thailand; and 1 each from Panama, the Federated States of Micronesia, Borneo, Okinawa, Malaysia, Singapore, and Texas. Of the case reports related to in-state exposures, 198 (57%) were laboratory confirmed, 116 (34%) were probable, and 31 (9%) were suspected.

The number of confirmed cases reported per year ranged from 11 to 27 (median 20), and the estimated mean annual incidence rate was 1.63 per 100,000 population. Mean monthly reported cases were highest from October through February (Figure 1). The observed seasonal disease occurrence for the recent 10-year study period was significantly different from that of the previously reported 25-year study period; summer cases predominated in the latter (p<0.01) (*5*).

Case-patients were predominately male (91%), and ages ranged from 3 to 76 years (median 38 years). The highest age-specific rate was among persons 20–29 years of age, and the lowest was among children 0–9 years of age. Most cases and the highest incidence rates were related to exposures on the islands of Kauai and Hawaii (Table 1). In addition, cases were most consistently reported from the northeast, windward sides of the islands: Hanalei (n = 8) and Wailua (n = 12) on Kauai, Waipio Valley (n = 12) and Hilo (n = 17) on Hawaii, and Maunawili Falls (n = 13) on Oahu (Figure 2).

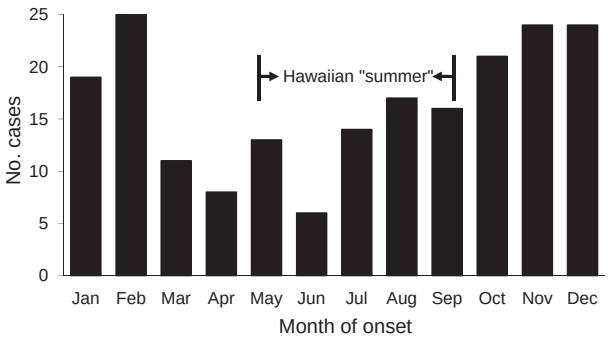

Figure 1. Month of onset for 198 laboratory-confirmed leptospirosis cases, Hawaii, USA, 1999–2008.

We were able to determine exposure classifications for 177 (89%) of the 198 confirmed cases. Recreational exposures accounted for 79 (45%) and were mostly related to freshwater swimming, hiking, and camping. Occupational exposures accounted for 78 (44%), mostly relating to farming, specifically, taro farming. Exposures around the home accounted for 20 (11%), most commonly, gardening. After

Table 1. Sex, age, and island of exposure for 198 case-patients with laboratory-confirmed leptospirosis, Hawaii, USA, 1999–2008

Variable	No. (%) case-patients	Estimated mean annual incidence rate*
Sex		
M	181 (91)	2.97
F	17 (9)	0.28
Age group, y		
0–9	1 (1)	0.06
10–19	24 (12)	1.46
20–29	48 (24)	2.87
30–39	31 (16)	1.69
40–49	38 (19)	2.05
50–59	36 (18)	2.55
60–69	15 (8)	1.68
70–79	5 (3)	0.64
Island		
Hawaii	98 (49)	6.59
Kauai	47 (24)	8.06
Oahu	44 (23)	0.50
Maui	4 (2)	0.34
Molokai	1 (1)	1.38
Unknown	4 (2)	

*No. cases/100,000 population. Rate = no. case-patients observed over 10 years for the specified category divided by 10 divided by specified subgroup population estimate from 2000 US Census data (*9*).

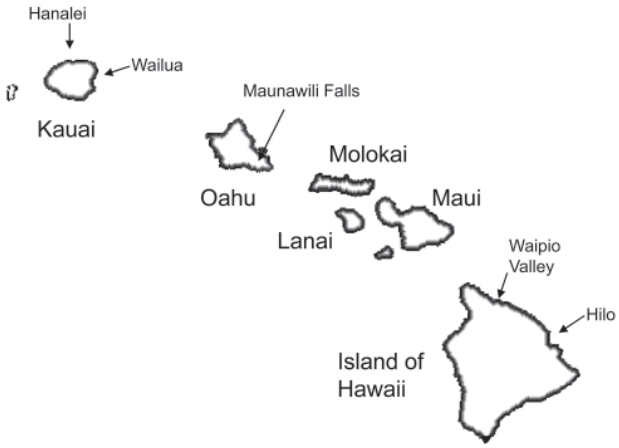

Figure 2. Exposure locations associated with the greatest number of leptospirosis cases, Hawaii, USA, 1999–2008.

categorizing cases into 5-year intervals and comparing the results with reports from 1989 through 1998 (*5*), we found that recreational exposures remained relatively stable over the past 20 years (1989–2008), while occupational exposures actually increased, but the difference was not significant (p = 0.08) (Figure 3). After stratification by island, a significant increase in occupational exposures was shown for the island of Hawaii (p = 0.04). No other trends for exposure classification were significant.

Most cases occurred sporadically. One outbreak (≥2 epidemiologically linked cases), which involved 2 landscapers, occurred on Kauai in 1999; both cases were laboratory confirmed. Another outbreak (2 epidemiologically linked cases: 1 confirmed, 1 probable) was associated with flooding of the University of Hawaii campus on October 31, 2004, when heavy rains caused an adjacent stream to overflow its banks (*10*).

For case-patients with known exposure dates, the median incubation period was 9 days (range 1–21 days). The median duration of illness was 14 days (range 3–90 days). A total of 118 (73%) of 161 case-patients, for whom treatment information was available, were hospitalized.

The most frequent signs and symptoms among patients who sought treatment were fever, myalgias, headache, nausea, and vomiting. Abnormal urinalysis results were common; specimens from 78 (73%) of 107 and 71 (68%) of 105 case-patients showed hematuria and proteinuria, respectively. Results of liver function tests were frequently abnormal as well; laboratory results for 109 (74%) of 147 case-patients showed elevated alanine aminotransferase levels (>40 U/L), and 85 (63%) of 134 showed elevated total bilirubin levels (>1 mg/dL). The most common hematologic anomaly was thrombocytopenia (<140 × 10^9/L), which was observed for 97 (66%) of 146 case-patients (Table 2). Initial clinical impression was recorded for 151 (76%) of 198 patients. The most common initial diagnosis was leptospirosis for 114 (75%) of 151 patients.

During the 10-year reporting period, 1 death occurred among 198 patients with confirmed cases (case-fatality rate 0.5%). A 23-year-old man who attended college on the mainland had been exposed through recreational activities while at home in Hawaii during winter break 2003. Symptoms developed after he returned to school, and he died in January 2004.

Of the 198 patients with confirmed infection, 152 (77%) received a diagnosis on the basis of serologic testing with the MAT, 18 (9%) cases were confirmed with culture isolates, and 28 (14%) were confirmed by MAT and isolates. Forty-three isolates obtained during 2000–2008 were characterized at CDC by molecular and serologic techniques. Isolates were grouped into 4 clades based on MAT results and pulsed-field gel electrophoresis: 19 (44%) unknown serovar (serogroup Australis), 17 (40%) serovar Icterohemorrhagiae (serogroup Icterohemorrhagiae), 4 (9%) serovar Ballum (serogroup Ballum), and 3 (7%) of unknown serovar (serogroup Bataviae). Cross-agglutination absorption assay identified the unknown serovar from serogroup Australis as a new serovar closely related to Lora (*11*).

The most common infecting serogroups (identified definitively by isolate or presumptively by MAT) were Australis (n = 50) and Icterohemorrhagiae (n = 51). Analysis for linear trend, after cases were categorized into 5-year intervals and compared with confirmed cases reported during 1974–1998 (*5*), showed a significant increase in infections attributed to serogroup Australis and a decrease in infections caused by serogroup Icterohemorrhagiae (p<0.0001 for each).

Discussion

The most recent 10-year reporting period has demonstrated a statistically significant shift in the seasonal occurrence of leptospirosis from the drier summer months (*5*) to

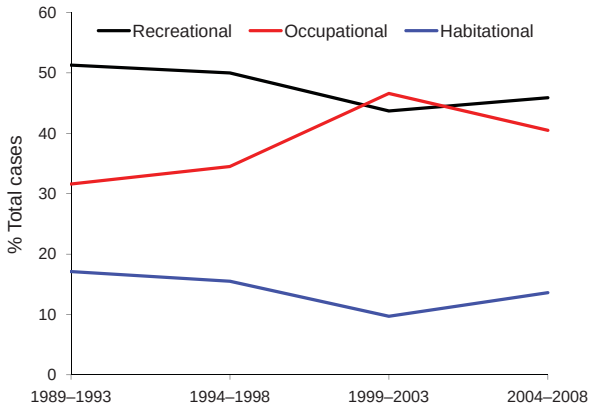

Figure 3. Trends in exposure classification for laboratory-confirmed leptospirosis cases, Hawaii, USA, 1989–2008.

Table 2. Clinical findings for 198 case-patients with laboratory-confirmed leptospirosis, Hawaii, USA, 1999–2008

Sign, symptoms, and laboratory result	No. (%) patients affected	No. patients with data available
Sign or symptom		
Fever	187 (98)	191
Myalgia	162 (88)	185
Headache	156 (87)	179
Nausea	117 (68)	173
Vomiting	101 (59)	172
Arthralgia	73 (46)	157
Diarrhea	79 (46)	171
Backache	50 (34)	146
Jaundice	53 (33)	163
Oliguria or anuria	32 (21)	152
Conjunctival suffusion	30 (19)	156
Nuchal rigidity	23 (14)	159
Pneumonia	13 (8)	154
Hepatosplenomegaly	8 (6)	140
Laboratory results		
Renal		
Hematuria	78 (73)	107
Proteinuria	71 (68)	105
Elevated creatinine (>1.5 mg/dL)	60 (51)	118
Elevated blood urea nitrogen (>20 mg/dL)	68 (50)	136
Hepatic		
Elevated alanine aminotransferase (>40 U/L)	109 (74)	147
Elevated total bilirubin (>1 mg/dL)	85 (63)	134
Hematologic		
Thrombocytopenia (<140 × 10^9/L)	97 (66)	146
Elevated leukocyte count (>10 × 10^9 cells/L)	74 (48)	155
Decreased hematocrit (<34%)	56 (38)	146

the wetter winter months. Climatologists have characterized the Hawaiian archipelago as having only 2 seasons: summer (May through September) and winter (October through April). Rainfall and widespread rainstorms are most common during the winter months (*12*).

During the earlier reporting period, 1974–1998, recreationally associated exposures predominated and increased over time; therefore, the summer predominance was attributed to the greater likelihood of recreational exposure in the summer. During 1989–2008, the frequency of recreational exposures plateaued while frequency of occupational exposures seemed to increase. This observed change in exposure history might allow seasonal climatic effect to have a greater influence on the epidemiology of the disease. In addition, taro farming, a recognized high-risk occupation (*5*), which had been on the decline, has experienced a resurgence relating to renewed interest in the cultural importance to native Hawaiians and an awareness of taro's nutritional value (*13*). In 2000, Hawaii produced 7 million pounds of taro, the largest crop yield since 1977 (*14*).

The island distribution of leptospirosis cases remains virtually unchanged since our earlier report (*5*). Kauai, the island with the highest annual rainfall and second most rural island, had and continues to have the highest incidence rate, followed by Hawaii, the most rural island. As in our earlier report, cases were most consistently reported from the wetter, windward, northeast sides of each island. Notably, climatic changes have been documented for the Hawaiian archipelago with significant trends in increasing temperatures (*15*), decreasing rainfall (*16*), and increasing rain intensity (*17*) over the past 30 years. The effects of climate change on ecosystems are complex, but the potential for influencing infectious disease patterns has been well described (*18*,*19*). Temperature and climate changes may affect the host animal's environment, making transmission to humans more likely. Increase in rain intensity with resultant flooding is a well-recognized climatic risk factor for transmission of *Leptospira* spp. (*20*). Flooding was responsible for 1 of the 2 outbreaks during the study period (*10*).

The predominance of men among case-patients is well recognized (*21–24*) and is virtually unchanged from our earlier report (*5*). This predominance has been explained by the tendency of more men to participate in high-risk outdoor exposure activities. The low reported age-specific case rates in children <10 years of age and highest rates among adults 20–50 years of age are also consistently reported (*22–24*) and similar to our earlier findings (*5*).

Our findings corroborate other large case series that show that the most common clinical manifestation of leptosporisis are nonspecific signs or symptoms, such as fever, headache, and mylagias (*5*,*22*,*25–27*). The case-fa-

tality rate (0.5%) is lower than that reported from Brazil (*25*), Barbados (*28*), Guadeloupe (*22*), and the Andaman Islands (*26*), but similar to the rates found in our earlier study (*5*) and in a recent case series from France (*27*). The low case-fatality rate in this series may be explained by early recognition and initiation of supportive therapy and antimicrobial drugs. Other case series may be biased toward recognition and inclusion of only the most severely ill, hospitalized patients, which leads to higher case-fatality rates. A recent population-based case-control study from Brazil (*29*) showed that pulmonary involvement was the strongest independent predictive factor for death caused by severe leptospirosis. Pulmonary findings were infrequent among case-patients in this study, the earlier Hawaii series (*5*), and the France series (*27*).

The changing temporal trend in the infecting serogroup first identified in our earlier study has continued; most of the current leptospirosis isolates are in the Australis serogroup. This documented trend over the past 35 years from the previously predominant serogroup Icterohemorrhagiae to the now predominant Australis may reflect the influence of different host animals, the effects of climatic and land use changes, or both. Serogroup Icterohemorrhagiae has been associated with rats (*Rattus norwegicus* and *R. rattus*), and Australis has been associated with swine, including feral swine or wild boars (*Sus scrofa*) (*30*,*31*). Recent reports from Germany have shown high seroprevalence of Australis serogroup (serovar Bratislava) in urban feral swine (*32*) and documented increased size in the feral swine population and habitat changes leading to epidemiologic linkages between leptospirosis occurrence and feral swine exposure (*33*). Hawaii has also experienced an increase in the feral swine population, with a concordant sharp increase in the number of feral swine encroaching on urban residential areas (*34*,*35*). Researchers at the University of Hawaii are currently investigating the possible influence of feral swine exposure on human disease in Hawaii by undertaking a leptospirosis seroprevalence study of feral swine.

Annual reported leptospirosis incidence rates in the United States ranged from 0.02 to 0.05 per 100,000 population from 1974 through 1994, the last year leptospirosis was included in the list of nationally notifiable diseases (*5*). If we include probable and suspected cases, as was done nationally, our mean estimated annual incidence rate during this 10-year study period would increase from 1.63 to 2.85 per 100,000 population, ≈100× greater than that reported nationally. Compared with other locales for which annual leptospirosis incidence rates are available, Hawaii would be considered in the moderate range category (1–10/100,000 population) (*36*). Countries in this range include Cuba (2.47/100,000 population) and Costa Rica (6.72/100,000 population) (*3*). Countries categorized as having high rates (>10/100,000 population) include Barbados (10.03/100,000 population), Trinidad and Tobago (12.04/100,000 population), and Seychelles (43.21/100,000 population) (*3*). Additional countries or regions considered to have high rates (for which data are not available) are Vietnam and French Polynesia (*36*). Although leptospirosis is a notifiable disease in Hawaii, case reporting is based on passive surveillance and likely underestimates true disease occurrence. During a 1-year period in 1988 and 1989, an active surveillance study was conducted on the islands of Hawaii and Kauai, which resulted in a 5-fold increase in case identification (*37*). A recent retrospective analysis of serum obtained from febrile patients during a dengue fever outbreak in Hawaii, 2001–2002, also identified a substantial number of leptospirosis cases that otherwise would have gone undiagnosed (*38*).

Conclusions

Future field studies using geographic information system technology to link climatic and environmental phenomena, such as rainfall occurrence and environmental isolates with human and animal infection, could offer valuable insights. Given the potential effects of climate and land use changes, public health officials must remain alert to the occurrence and changing epidemiology of emerging and reemerging infectious diseases. Without national surveillance, the occurrence of leptospirosis outside of Hawaii or other regions that have leptospirosis surveillance may go largely unrecognized, and thus, unmonitored. Ongoing surveillance activities, such as ecologic, animal, and laboratory studies are necessary to clarify and track the dynamic epidemiology of this widespread, reemerging zoonotic illness.

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Next-Generation Sequencing of *Coccidioides immitis* Isolated during Cluster Investigation

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Next-generation sequencing enables use of whole-genome sequence typing (WGST) as a viable and discriminatory tool for genotyping and molecular epidemiologic analysis. We used WGST to confirm the linkage of a cluster of *Coccidioides immitis* isolates from 3 patients who received organ transplants from a single donor who later had positive test results for coccidioidomycosis. Isolates from the 3 patients were nearly genetically identical (a total of 3 single-nucleotide polymorphisms identified among them), thereby demonstrating direct descent of the 3 isolates from an original isolate. We used WGST to demonstrate the genotypic relatedness of *C. immitis* isolates that were also epidemiologically linked. Thus, WGST offers unique benefits to public health for investigation of clusters considered to be linked to a single source.

Genotyping of microorganisms typically relies on comparison of genomic features (e.g., fragment size, repeats, single-nucleotide polymorphisms [SNPs]) between strains and/or against a database of feature profiles (e.g., PulseNET and mlst.net) for a population of the microbe of interest. Such genotyping tools are useful for molecular epidemiologic studies, microbial forensics, and phylogenetic applications. Molecular epidemiology methods may differ in genotyping specificity in linking cases to sources in an epidemiologic investigation; may be less than optimal (e.g., use of pulse-field gel electrophoresis to identify sources of foodborne outbreak often includes nontarget isolates); may not be sensitive enough to detect minor mutations in closely related strains in a forensic investigation (e.g., identifying markers in nearly identical strains of *Bacillus anthracis*); or may not have the resolution necessary to clearly elucidate population structure (e.g., use of nonphylogenetically informative characters such as amplified fragment-length polymorphism fragments or variable-number tandem repeats to establish clades of organisms).

Next-generation sequencing technology (next gen) provides rapid, relatively cost-effective whole-genome sequence typing (WGST). Although these technologies are relatively novel, they are quickly being adapted for use in the fields of genomics, transcriptomics, and phylogenetics and have been highly successful for resequencing, gene expression, and genomic profiling projects (*1*). Recently, next gen sequencing has been described as a viable genotyping tool in the fields of infectious disease epidemiology and microbial forensics (*2*,*3*).

Coccidioidomycosis is an invasive fungal infection caused by the dimorphic fungus *Coccidioides* spp. and is endemic to the southwestern United States (*4*). Organ donor–transmitted coccidiodomycosis was first reported almost 5 decades ago and is a rare but serious complication of solid organ transplantation; death rate associated with disseminated disease in this patient population is high (72%)

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(*5*). In these cases, donor-transmitted coccidioidomycosis was recognized because recipients underwent transplantation in a coccidioidomycosis-nonendemic area and had no prior travel history to a coccidioidomycosis-endemic area. No genotyping methods were used to confirm the genetic relationship between isolates recovered from the donor and recipient in any of these studies.

We describe the use of WGST to genotypically link *C. immitis* isolates recovered from a transplant-related cluster of coccidioidomycosis in an area to which it is endemic. Results show that isolates recovered from the transplantation patients were essentially genetically indistinguishable, thereby identifying the donor as the common source for these infections.

Methods

Patients and Isolates

In early 2009, coccidioidomycosis was diagnosed for 3 patients (X, Y, and Z); all had recently received transplanted organs in Los Angeles, California, USA, where this fungus is endemic. Later serologic investigations showed that the donor's postmortem serum was positive for immunoglobulin M antibodies to *Coccidioides* spp.; however, no isolate was available from the donor. Isolates B7709, B7556, and B7557 were available from patients X, Y and Z, respectively, for further molecular analyses.

Whole-Genome Sequencing

Genomic DNA extracted from the 3 isolates was plated onto potato dextrose agar plates for 5 days for a sterility check. DNA fragment libraries for each of the cluster-associated *C. immitis* strains were constructed for sequence analysis on the SOLiD sequencing platform (Life Technologies, Foster City, CA, USA) according to the manufacturer's instructions. Libraries were prepared in equimolar ratios, and sequencing was conducted to 50 bp by using SOLiD V3 chemistry as described (*2*).

WGST Analysis

The whole-genome sequence (WGS) data for each isolate was aligned to the most recent version of the *C. immitis* RS3 strain sequence (AAEC02000000) (*6*) by using the software program BFAST (*7*) with the following exclusion criteria: 1) indel-containing reads; 2) reads aligning to multiple locations; and 3) reads with mapping and alignment scores <20 and <100, respectively. Because *C. immitis* has a high level of repetitive DNA (17% of genome) (*8*) that could confound SNP analysis, reads that matched >1 location on the RS3 genome were identified and removed before SNP analysis.

The alignment files were then used to identify putative SNPs among the 3 outbreak isolates. An SNP caller application (*9*) was used to identify putative SNPs. To be called an SNP, the position had to have a minimum of 5× coverage. After eliminating any bases with a quality score <20 (as reported by SOLiD) or a mapping score <40 (calculated by BFAST), 90% of the reads had to agree. Identified SNPs were then visually evaluated by viewing the WGS alignment in SolScape, a short-read sequence-alignment viewer developed in house (J. Pearson et al., unpub. tool available on request). Any SNPs identified between the 3 cluster isolates were confirmed by Sanger sequencing by using standard methods.

An additional in-house analysis tool, In Silico Genotyper (S. Beckstrom-Sternberg et al., unpub. data; tool available upon request) was used to identify SNPs between the cluster isolates and 10 additional publically available *C. immitis* WGS datasets: CimmH538, CimmRm2394, CimmRm3703, CimmRS3 (*6*); and RMSCC-3505, -3693, -2395, -3474, -3705, -3377 (*10*). SNP calls were required to have a minimum of 5× coverage, at least 1 read on each strand, have 95% of reads contain the alternate base, and have a SNP quality score of ≥20, as calculated by SAMtools (*11*). These SNPs were then used for phylogenetic analysis of the 13 combined *C. immitis* sequences. Only SNP loci common to all taxa were included in the analysis. In an attempt to remove SNP loci that might be more subject to genome rearrangements, horizontal gene transfer, and potential repeat induced point mutation processes (*8*), SNP loci falling within genomic regions repeated within the reference genome (RS3) were also excluded from the phylogenetic analysis. Repeat regions were identified by using a pairwise self-comparison of the reference genome (RS3) in MUMmer version 3.22 (*12*). Phylogenetic trees were generated by the maximum-parsimony algorithm in MEGA4 (*13*) with bootstrapping of 1,000 replicates. Loci with missing data were removed before analysis.

Results

Whole-Genome Sequencing

The generated sequence data (50-bp reads) alignment of the 3 outbreak isolates resulted in average coverage depths of 40.8×, 48.6×, and 33.6× for isolates B7709 (patient X), B7556 (patient Y), and B7557 (patient Z), respectively (Figure 1). The overall percentage of the *C. immitis* RS (revision 3) genome (≈28.9 Mb) coverage by the 3 datasets was similar, ranging from 94.6% to 95.0%. Supercontig 3 had the lowest total base coverage (89.8%–90.3%); supercontig 6 had the highest (96.6%–96.8%).

SNP Analysis

The initial SNP analysis identified 17 candidate SNPs among the 3 transplant isolates. After further evaluation, including manual inspection of alignments and coverage

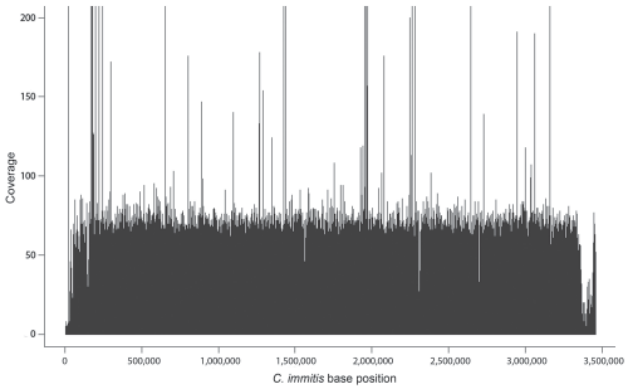

Figure 1. Example coverage plot of sequenced genome of *Coccidioides immitis*. Plot shows base coverage (y-axis) of supercontig 6 from isolate from patient Z, who had coccidioidomycosis. Average depth of coverage for this supercontig was 48.63× over 3,385,806 bases (x-axis) for a total of 164,650,400 bases sequenced.

and, finally, Sanger sequence confirmation, only 3 loci were determined to be polymorphic (see Figure 2 for sequences alignment showing 1 of these SNPs). The isolate from patient X contained 1 SNP, and the isolate from patient Y contained the other 2 SNPs. Comparative SNP analysis of the 13 *C. immitis* genomes showed 32,695 shared SNPs among all taxa. Approximately half (17,080) of these were parsimony informative in that multiple taxa contained alternate allele states; the remaining SNPs (15,615) were considered autapomorphic in that only 1 strain showed the alternate allele state. Of the 32,695 shared SNPs, the cluster isolates differed from the reference genome by an average of 8,541 SNPs.

Phylogenetic Analysis

Maximum-parsimony analysis that used all SNPs common to all 13 taxa is shown in Figure 3. The consistency index (0.63) for the tree indicates a moderate level of homoplasy among these SNPs. However, the high bootstrap values indicate strong support for the outbreak isolates and the central and southern California isolate branch points. Branch lengths indicate that the outbreak isolates are more closely related to the isolates from central California than to the isolates from southern California.

Discussion

Multiple donor transplant–related coccidioidomycosis cases have been reported (*5*,*15*). In most of these studies, none of the recipients were from *C. immitis*–endemic areas, and the organ donor had either lived in or visited a *C. immitis*–endemic area. For organ transplant recipients living in such areas, coccidioidomycosis is most often believed to occur by primary infection with *Coccidioides* spp. after environmental exposure or from reactivation of latent infection. However, whether additional cases of donor-derived infections are occurring in endemic areas is not clear because the cases are difficult to recognize as such. Molecular epidemiologic tools may help differentiate donor-derived infections from primary or latent infections.

In our investigation, the recipients and the donor were from a *C. immitis*–endemic region, and we used next gen sequencing to conduct WGST to better elucidate the relationship between the isolates recovered in the investigation. Our analyses demonstrate that the *C. immitis* isolates from 3 transplant recipients originated from the same source, the organ donor. Although a molecular clock has not been established for *Coccidioides* spp., we can infer that the minor SNP differences resulted from limited mutation since

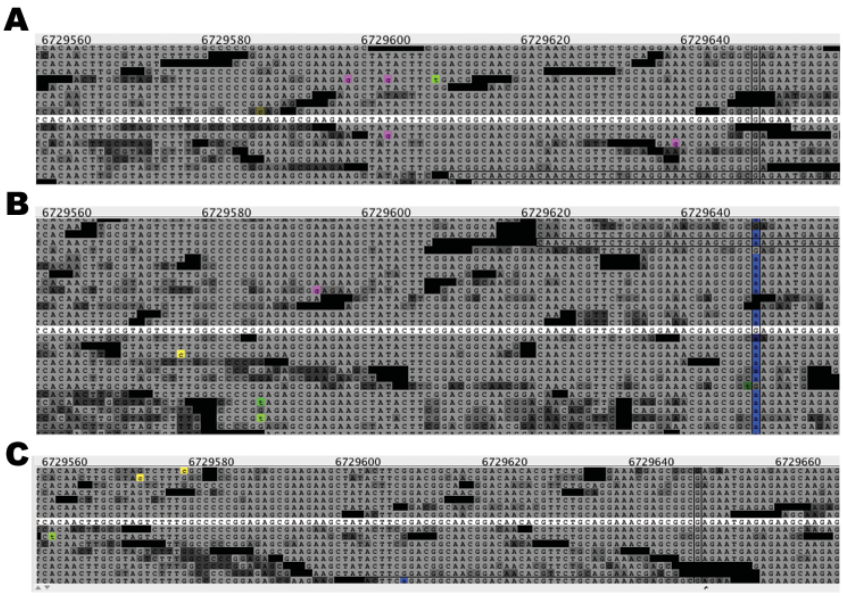

Figure 2. Alignment of *Coccidioides immitis* whole-genome sequence reads flanking a confirmed single-nucleotide polymorphism (RSv3 supercontig 1, position 6729646, highlighted in blue in panel B) among the 3 cluster isolates. Isolates from patients X, Y, and Z, who had coccidioidomycosis, are shown in panels A, B, and C, respectively. The alignment was created by using SolScape, a short-read sequence-alignment viewer developed in house (J. Pearson et al., unpub. data; tool available upon request). Reference sequence position is given at the top of each panel; actual reference sequence is highlighted in white at the center of each panel. Bases differing from the reference sequence are highlighted in pink, green, or yellow.

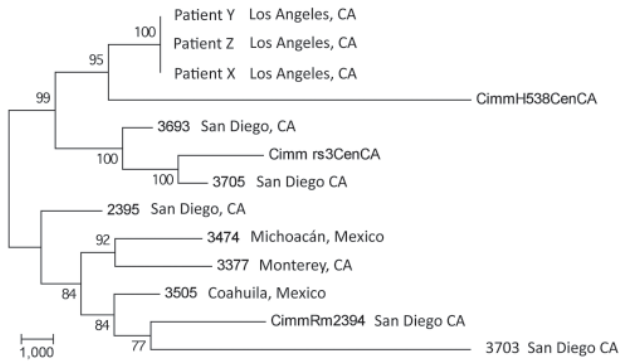

Figure 3. Maximum-parsimony phylogenetic analysis of 13 *Coccidioides immitis* genomes. MEGA4 (*13*) was used to conduct maximum-parsimony analysis of all single-nucleotide polymorphism (SNP) loci common to the 3 transplant isolate genomes and the 10 publicly available *C. immitis* genome sequences (*6,10*). A total of 32,695 SNP positions were identified in the final dataset, of which 17,080 were parsimony informative. The percentages of replicate trees in which the associated taxa clustered in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale; branch lengths were calculated by using the average pathway method (*14*) and are in the units of the number of changes over the whole sequence. The consistency index of the tree is 0.63. Scale bar indicates nucleotide substitutions per site.

divergence. Estimated mutation rates in these eukaryotic microbes (≈10^{-9} per base per year) (*16*) limit the possibility of these isolates being direct descendants in clonal lineages.

Previously, only microsatellite-based methods have proven useful for molecular epidemiologic studies of *Coccidioides* spp., which provide adequate separation across geographically diverse samples (*17*) and identifying clonal isolates (genotypically identical) recovered from the same patient (*18*). However, microsatellite methods can be biased in that they may fail to detect genomic changes outside these loci. By using WGST, we firmly established genetic linkage between isolates recovered from patients X, Y and Z, with a total of only 3 SNP differences among the 3 isolates. By comparison, when other *C. immitis* genomes are included in the WGST analysis, we noted 8,700–32,700 SNP differences (Figure 3). We can argue that the recipients may have been infected independent of their receipt of organ transplant, and subsequently, disseminated coccidioidomycosis developed after transplant-associated immunosuppressive therapy. This explanation is plausible given that all 3 recipients lived in an area endemic for *C. immitis*, although less probable given that all 3 received organs from the same donor. However, WGST analyses established that the 3 isolates shared a common ancestry, thereby unequivocally establishing that the isolates originated from 1 donor.

SNPs are highly informative for phylogenetic and epidemiologic analyses. WGST focuses on the SNP differences between all sequenced strains. Although 1 canonical SNP may be all that is required to identify a clonal species, subpopulation, and/or isolate (*19*), the massive number of potential SNPs in a genome provides incredible resolution of nonclonal species as well. By exploring all shared SNPs between a particular group of isolates (e.g., across a species), we are able to not only identify identical or closely related isolates, but also to better understand the population structure for further analyses (e.g., phylogeography) (*20*). As with other genotyping techniques, genotyping fungi (and other eukaryotes) by using SNPs is challenging because of genetic recombination rather than the genetic stability of more clonal microorganisms (i.e., bacteria and viruses) (*21*). Although *Coccidioides* spp. have asexual reproduction, allowing for some clonality, it has extensive recombination, probably from cryptic sexual reproduction (*8*,*22*). The effects of recombination on phylogenetic analyses of *Coccidioides* spp. and similar microbes can be overcome by use of large SNP datasets and appropriate algorithms (*21*). The use of WGST, therefore, provides the highest degree of phylogenetic and genotyping robustness by enabling interrogation of all possible informative SNPs along with other genetic variation (e.g., insertions, deletions, gene changes). The focus of this WGST investigation was limited to SNP analysis, primarily because of sequence coverage of the chosen sequencing method, similar to what has been described as the dirty genome approach (*23*).

Use of WGS for molecular epidemiology has been limited to a handful of studies involving primarily viral pathogens, including linkage of hepatitis C virus strains in humans and wild boars (*24*); genotyping of HIV strains by using near full-length genomes (*25*); and molecular epidemiology of influenza A (H5N1) virus in waterfowl outbreaks (*26*). A more recent study used next gen sequencing to link hospital-associated isolates of methicillin-resistant *Staphylococcus aureus* in Thailand (*3*). We have used WGST to help confirm that the cluster reported here represented donor-transmitted infection and not a primary or latent infection in the transplant recipients. With the wide-scale use of next gen technology for microbe sequencing, we anticipate that WGST will be used more frequently for future public health and forensic applications. The costs per sample are rapidly declining (because of ability to index multiple samples in a single lane [*27*]) and the amount of sequence data per run is greatly increasing (because of improved chemistry) on existing next gen platforms. Third-generation sequencing promises faster turnaround times and exponentially greater read lengths and sequence coverage. These advances will enable sequencing of entire global repositories of pathogens for future WGST analysis. The major challenges to universal acceptance and use of WGST for infectious disease epidemiology are the costs of instrumentation and the development and availability of appropriate bioinformatic tools for data analysis, along

with available server/computing capacity. Although the former will depend on the marketplace, the latter is already being addressed by development of novel analysis tools (*7*,*9*,*11*,*28*), global databases (*10*), and access to shared server systems and parallel computing networks (*29*,*30*). These findings also lead us to envision a use for WGS in clinical medicine much sooner than originally anticipated, perhaps within the next 5 years.

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Arbovirus Prevalence in Mosquitoes, Kenya

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Few studies have investigated the many mosquito species that harbor arboviruses in Kenya. During the 2006–2007 Rift Valley fever outbreak in North Eastern Province, Kenya, exophilic mosquitoes were collected from homesteads within 2 affected areas: Gumarey (rural) and Sogan-Godud (urban). Mosquitoes (n = 920) were pooled by trap location and tested for Rift Valley fever virus and West Nile virus. The most common mosquitoes trapped belonged to the genus *Culex* (75%). Of 105 mosquito pools tested, 22% were positive for Rift Valley fever virus, 18% were positive for West Nile virus, and 3% were positive for both. Estimated mosquito minimum infection rates did not differ between locations. Our data demonstrate the local abundance of mosquitoes that could propagate arboviral infections in Kenya and the high prevalence of vector arbovirus positivity during a Rift Valley fever outbreak.

Emerging zoonotic diseases threaten the health and security of human and animal populations throughout the world (*1*). Because arthropod-borne viruses, or arboviruses, can be spread by competent mosquito vectors across great distances, they pose substantial risk to other regions in which the disease is currently nonendemic (*1*). Zoonotic arboviruses circulate in sylvatic and peridomestic cycles involving wild animals and nearby humans. Often these arboviruses remain undetected by health care systems (*2–4*). Kenya has had multiple arbovirus outbreaks in the past 2 decades resulting in economic and public health distress, including yellow fever in 1992 (*5*,*6*) and 1995 (*7*), chikungunya fever in 2004 (*8*), and Rift Valley fever (RVF) in 1997 (*9*) and 2006 (*10*). Much remains unknown about the true prevalence of arboviruses in Kenya and the mosquito vectors responsible for virus maintenance and transmission. We investigated the local abundance of mosquitoes in Kenya that are infected with RVF virus (RVFV) and West Nile virus (WNV); mosquitoes were collected near human habitation during a period of prolonged heavy rainfall.

Rift Valley fever virus, family *Bunyaviridae*, genus *Phlebovirus*, is a vector-borne virus endemic to Africa and the Middle East (*11*). Recent outbreaks of RVF have resulted in substantial human illness and livestock losses in Kenya (*9*,*10*,*12*). Domestic ungulates are a principal source of transmissible RVFV, and human infection has been associated with direct animal contact, specifically with cattle, sheep, and goats (*2*,*9*,*12*). It is unclear which, if any, animal species maintain RVFV during interepidemic periods, and it is possible that RVFV is maintained solely within arthropod vectors during these periods (*13*).

West Nile virus, family *Flaviviridae*, genus *Flavivirus*, is a vector-borne virus that is maintained in nature between mosquitoes and birds (*11*). Humans and other mammals are incidental hosts and do not play a role in the natural preservation of WNV (*11*). Because most WNV infections are self-limiting and subclinical, human infections in Kenya are often misdiagnosed (*14*). As a result, the true prevalence of WNV in the country is probably underestimated (*15*). Further clarification of the true presence and circulation of WNV in mosquito vectors could enhance human WNV case detection in the region.

Few studies have investigated the many mosquito species that harbor arboviruses in Kenya (*16–21*). Entomologic surveys have demonstrated that mosquitoes that usually

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facilitate outbreaks of arboviral diseases, specifically *Aedes* spp., *Anopheles* spp., and *Culex* spp., flourish in Kenya (*16*,*18*,*19*,*22*–*26*). At least 40 different mosquito species can harbor RVFV, although their ability to transmit RVFV varies (*14*,*20*,*21*,*27*–*29*). Furthermore, although many species are susceptible to RVFV infection, studies of mosquito vectors in northeastern Kenya have shown that the proportion of positivity in individual species differs greatly (5.9% *An. squamosus*, 30% *Ae. ochraceus*, 42% *Ae. mcintoshi*) (R. Sang, pers. comm.). RVFV can also be transovarially transmitted in at least 1 mosquito species, *Ae. mcintoshi* (*17*). The isolation of WNV from a non–blood-feeding male *Cx. univattatus* mosquito trapped in northwestern Kenya indicates that WNV also transmits transovarially in that region (*23*).

Materials and Methods

Sampling

To evaluate the temporal profile of vector mosquitoes in North Eastern Province, Kenya, trapping was performed during the dry season (August 2006) and during the rainy season (December 2006–January 2007). Mosquitoes collected during December 2006 and January 2007 were trapped during an epizootic/epidemic of RVF. Homestead trapping locations adjacent to homesteads in the regions were randomly selected from previously prepared census lists and were restricted to only those homes where animals (cows, goats, or sheep) were housed alongside human habitats. Each household had only 1 CDC light trap (John W. Hock Company, Gainesville, FL, USA) located next to animal structures; trap was set 1 time for 12 hours, 6:00 PM–6:00 AM.

Mosquito sampling was conducted in 2 areas within Masalani Division, Ijara District, North Eastern Province, where human surveillance had taken place 8 months before the RVF outbreak (*2*) (Figure 1). Traps were located in the rural village of Gumarey (1°40′12″S, 40°10′48″E) and the town of Sogan-Godud (1°41′24″S, 40°10′12″E). The population of Gumarey consists of seminomadic herders who live in traditional grass huts near their livestock. Sogan-Godud is more urban with a marketplace and contains a greater proportion of tin-roofed permanent dwellings. The centroids of these 2 locations are 5 km apart, and the borders are within 500 m of each other. Both locations had persistent local flooding during the extensive El Niño/Southern Oscillation associated heavy rains during 2006–2007, and both are within 10 km of the Tana River. Persons seropositive for RVFV from both locations were documented in early 2006; seroprevalence rates were greater in rural Gumarey (20% vs. 6%) (*2*). During that initial study, all homesteads were identified and their locations identified by Global Positioning Satellite. Spatially referenced data on individual residence and homestead exposure features were maintained and analyzed by using ArcGIS version 9.2 (ESRI, Redlands, CA, USA).

Figure 1. Location of Masalani Division of Ijara District, North Eastern Province, Kenya.

Mosquito Preparation

Mosquito genera were identified in Kenya by local entomologists on the basis of microscopic morphologic appearance. Only female mosquitoes were included in this study; male mosquitoes were not further tested. Single leg specimens were preserved in RNAlater (Ambion, Austin, TX, USA) and transported to Case Western Reserve University (Cleveland, OH, USA) for processing. DNA and RNA were extracted from mosquito legs by using a column purification kit (QIAGEN, Valencia, CA, USA) with the following modifications: each mosquito leg was placed into a microcentrifuge tube containing 150 μL of RNeasy lysis buffer and finely ground with a disposable RNase/DNase-free pestle. After homogenization, samples were processed according to established protocols through either individual QIAGEN RNeasy columns or 96-well plates, washed, and eluted in RNase-free water. The DNase step was omitted so that DNA and RNA could be collected from samples. Individual RNA samples were combined in pools of ≤12 mosquitoes (median 10, mean 8.7), based on homestead

trap for cDNA synthesis and PCR or quantitative reverse transcription–PCR (qRT-PCR).

Primers and Generation of Standard Controls

To verify the quality of the RNA and the integrity of the cDNA products after reverse transcription, mosquito 18S rRNA primers were designed to amplify within a region conserved in many *Culicidae* spp. mosquitoes (*30*). These mosquito primers were designed against the 18S rRNA gene sequences for *Aedes* spp. (GenBank accession no. AB085210) and *Culex* spp. (GenBank accession no. U48385) mosquitoes to amplify an optimally sized product (124 bp) for qRT-PCR. WNV primers were based on the New York 1999 WNV isolate (GenBank accession no. AF196835.2) described by Lanciotti et al. (*11*). These primers have been shown to detect Old and New World WNV strains, including a strain isolated in Kenya in 1998 (*11*,*31*). RVFV primers, which amplify a conserved region of the large segment (90 bp), were used as described by Bird et al. (*32*) (Table 1).

An RVFV standard control was generated by amplifying RVFV vaccine strain rMP-12 in Vero E6 cells for 72 h and then extracting viral RNA from supernatant and cell lysate by using the PureLink Total RNA Purification System (Invitrogen, Carlsbad, CA, USA). A WNV standard control was generated by using confirmed WNV-positive samples received from the Ohio Department of Health. Mosquito 18S rRNA-, WNV-, and RVFV-positive controls were generated by using the primers listed in Table 1 and cloned by using the pCR 8/GW/TOPO TA cloning kit (Invitrogen). All inserts were verified by sequencing of the plasmids.

cDNA Synthesis, PCR, and qRT-PCR Conditions

Two-step qRT-PCR was performed on all pooled samples. First-step total cDNA synthesis was performed on RNA extracted from mosquito leg tissue by using random hexamer primers. The reaction mixture was incubated at 65°C for 5 min, chilled on ice, and combined with 4 μL 5× First-Strand Buffer, 1 μL 0.1M dithiothreitol, 1 μL RNase inhibitor, and 0.5 μL SuperScript III Reverse Transcriptase (Invitrogen). The final reaction mixture was incubated at 25°C for 10 min, 50°C for 50 min, and heat inactivated at 70°C for 15 min.

After cDNA synthesis, 1 μL of total cDNA was added to the qRT-PCR mixture containing 0.2 μmol/L forward primer and 0.2 μmol/L reverse primer (18S and WNV testing), 12 μL FastStart Universal SYBR Green Master mix (Roche, Indianapolis, IN, USA), and 12 μL sterile, nuclease-free water. The qRT-PCR was conducted in an Applied Biosystems 7300 instrument (Applied Biosystems, Foster City, CA, USA) with a heating cycle of 50°C for 2 min and 95°C for 10 min; followed by 45 cycles of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s; and an additional dissociation step of 60°C for 1 min. All samples, which registered a cycle threshold value <35 cycles and had a lower cycle threshold value than negative controls, were considered positive for their respective targets. All pools were further PCR tested for RVFV by using 2 μL cDNA, 0.5 μmol/L each forward/reverse RVFV primer, 10.5 μL sterile, nuclease-free water, and 12.5 μL JumpStart ReadyMix Taq (Sigma-Aldrich, St. Louis, MO, USA). PCR cycling parameters were 94°C for 5 min, with 30 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a 10-min 72°C extension. PCR products were run on a 2% agarose gel with SYBR Safe (Invitrogen) for band visualization (Figure 2). An initial sampling of RVFV PCR products was cloned by using the above-mentioned methods and sequenced for confirmation. Mosquito minimum infection rates (MIR) for RVFV and WNV were calculated on the basis of maximum-likelihood estimation by using the PoolScreen 2.0 program (University of Alabama at Birmingham, Birmingham, AL, USA) (*33*–*35*).

Results

A total of 74 trapping events occurred at 38 different homestead locations in the study villages. Because of the annual drought, no mosquitoes were recovered in the traps set in August. Overall, 12,080 mosquitoes were collected: 9,701 mosquitoes during the 7 trapping nights in December (December 12–19, 2006) and 2,379 mosquitoes during the 6 trapping nights in January (January 19–26, 2007). The most abundant mosquitoes trapped were of the genus *Culex*. For the entire trapping period 7,853 *Culex* spp., 3,488 *Anopheles* spp., 682 *Mansonia* spp., and 57 *Aedes* spp. mosquitoes were trapped and identified. Traps caught an average of 199 mosquitoes per trap, with an average of 141 *Culex* spp. mosquitoes.

To estimate location-specific risk for arbovirus transmission during the December 2006–January 2007 sampling period, 920 mosquitoes collected in the field were pooled for PCR detection of RVFV and WNV. These 920 exophilic mosquitoes were trapped at 30 different homesteads

Table 1. Oligonucleotide primer pairs used in assay during Rift Valley fever outbreak, Kenya, 2006–2007*

Target	Forward sequence, 5′ → 3′	Reverse sequence, 5′ ← 3′	Product size, bp	GenBank accession no.
Mosquito 18S rRNA	GATCAAGTGGAGGGCAAGTC	AAGGAGTAGCACCCGTGTTG	124	AB085210.1
RVFV	TGAAAATTCCTGAGACACATGG	ACTTCCTTGCATCATCTGATG	90	DQ375404.1
WNV	CAGACCACGCTACGGCG	CTAGGGCCGCGTGGG	103	AF196835.2

*RVFV, Rift Valley fever virus; WNV, West Nile virus.

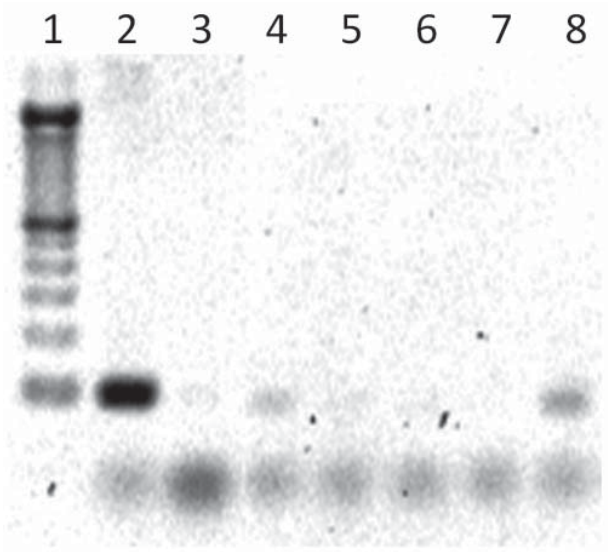

Figure 2. PCR gel showing positive Rift Valley fever virus bands (90 bp). Lane 1, molecular mass ladder; lane 2, Rift Valley fever virus MP-12 positive control; lane 3, negative control; lane 4, pool 103 (positive); lane 5, pool 86 (negative); lane 6, pool 104 (negative); lane 7, pool 87 (negative); lane 8, pool 105 (positive).

adjacent to animal structures, yielding 105 pools based on trap location (homestead) per trapping night with an average of 10 mosquitoes (range 1–12 mosquitoes) per pool. In 23 Gumarey homesteads, 552 mosquitoes were trapped and divided into 65 total pools in the laboratory (1–12 individual mosquito legs/pool, based on trap night). A total of 368 mosquitoes were trapped at 7 Sogan-Godud homesteads and divided into 40 pools for testing (1–11 individual mosquito legs/pool, based on trap night). Most mosquitoes tested were morphologically identified as C*ulex* spp. (n = 654, 71%) (Figure 3). The remaining mosquitoes were identified as *Anopheles* spp. (n = 107, 12%), *Mansonia* spp. (n = 101, 11%), and *Aedes* spp. (n = 58, 6%). Synthesis of total cDNA was successful; 99% of samples amplifyed 18S, and the remaining 1% was removed from further testing.

In total, of the 105 trap-night pools, 18% (95% confidence interval [CI] 11.3%–26.8%) had positive results by PCR for WNV and 22% (95% CI 14.5%–31.1%) for RVFV (Table 2). Of the 65 pools from Gumarey, 14% (95% CI 6.5%–24.7%) and 30% (95% CI 18.6%–41.8%) had positive results for WNV and RVFV, respectively. Of the 40 pools from Sogan-Godud, 25% (95% CI 12.7%–41.2%) and 10% (95% CI 2.8%–23.7%) had positive results for WNV and RVFV, respectively. A comparison of positive results for RVFV in mosquito pools across villages was significantly different (p = 0.0279); a comparison of positive results for WNV across village pools was not (p = 0.1932). Three percent of mosquito pools tested had positive results for both WNV and RVFV.

Figure 4 shows the area distribution of homesteads, mosquito traps, and local abundance of RVFV-positive and WNV-positive trap pools. When analyzed based on the 30 homestead locations, 10 (33%; 95% CI 17.3%–52.8%) homesteads with tested mosquitoes were positive for WNV, versus 15 (50%; 95% CI 31.3–68.7%) for RVFV (Table 3). Most (5/7; 71%) Sogan-Godud homesteads were positive for WNV (95% CI 29.0%–96.3%), compared with 5/23 (22%; 95% CI 7.5%–43.7%) for Gumarey, although MIRs did not differ (Table 3). Homestead WNV positivity significantly differed between villages (p = 0.0256); RVFV positivity of homesteads did not (p = 1.000). RVFV homestead positivity rates were similar between the 2 locations; 12/23 (52%; 95% CI 30.6–73.2) mosquito pools in Gumarey homesteads had positive results, versus 3/7 (43%; 95% CI 9.9–81.6) in Sogan-Godud.

In terms of the general population, by using geographic information systems analysis of spatially referenced census data (Figure 5), we confirmed that >30% of Sogan residents and >40% of Gumarey residents lived within 100 meters of an identified RVFV-positive mosquito trap site. Forty-eight percent of Sogan residents lived within 100 meters of a WNV-positive trap site; only 19% of Gumarey residents lived within 100 meters of a WNV-positive site.

Of the mosquitoes trapped during this study, *Culex* spp. was the predominant genus, although *Aedes* spp., *Anopheles* spp., and *Mansonia* spp. mosquitoes were also recovered during nocturnal light trapping. In the pools that contained only 1 genus of mosquito, positivity varied. A total of 63 pools were composed solely of *Culex* spp. mosquitoes (specifically *Cx. quinquefasciatus*), 9 of which were positive for RVFV. Additionally, 1 of 4 pools containing only *Aedes* spp. mosquitoes were RVFV positive, 3 of 8 *Anopheles* spp.–only mosquito pools had positive results for RVFV, and 3 of 8 *Mansonia* spp.–only mosquito pools had positive results for RVFV. WNV-positive pools composed of only 1 genus included 3 of 4 *Aedes* spp.–only mosquito pools and 15 of 63 *Culex* spp.–only mosquito pools. All *Aedes* spp. mosquitoes collected were trapped in December but were absent in the traps in January (Figure 3). The temporal distribution of these mosquitoes correlates with previous studies showing that *Aedes* spp. mosquitoes predominate in the initial weeks after substantial flooding and then curtail after the first month of flooding, at which time *Culex* spp. and *Anopheles* spp. mosquitoes emerge as the predominant species (*17*,*36*). It is believed that the dramatic proliferation of transovarially infected *Aedes* spp. mosquitoes immediately after flooding re-introduces virus into an epizootic/epidemic cycle, after which *Culex* spp. mosquitoes propagate the virus in an epizootic/endemic cycle among humans and animal species (*17*).

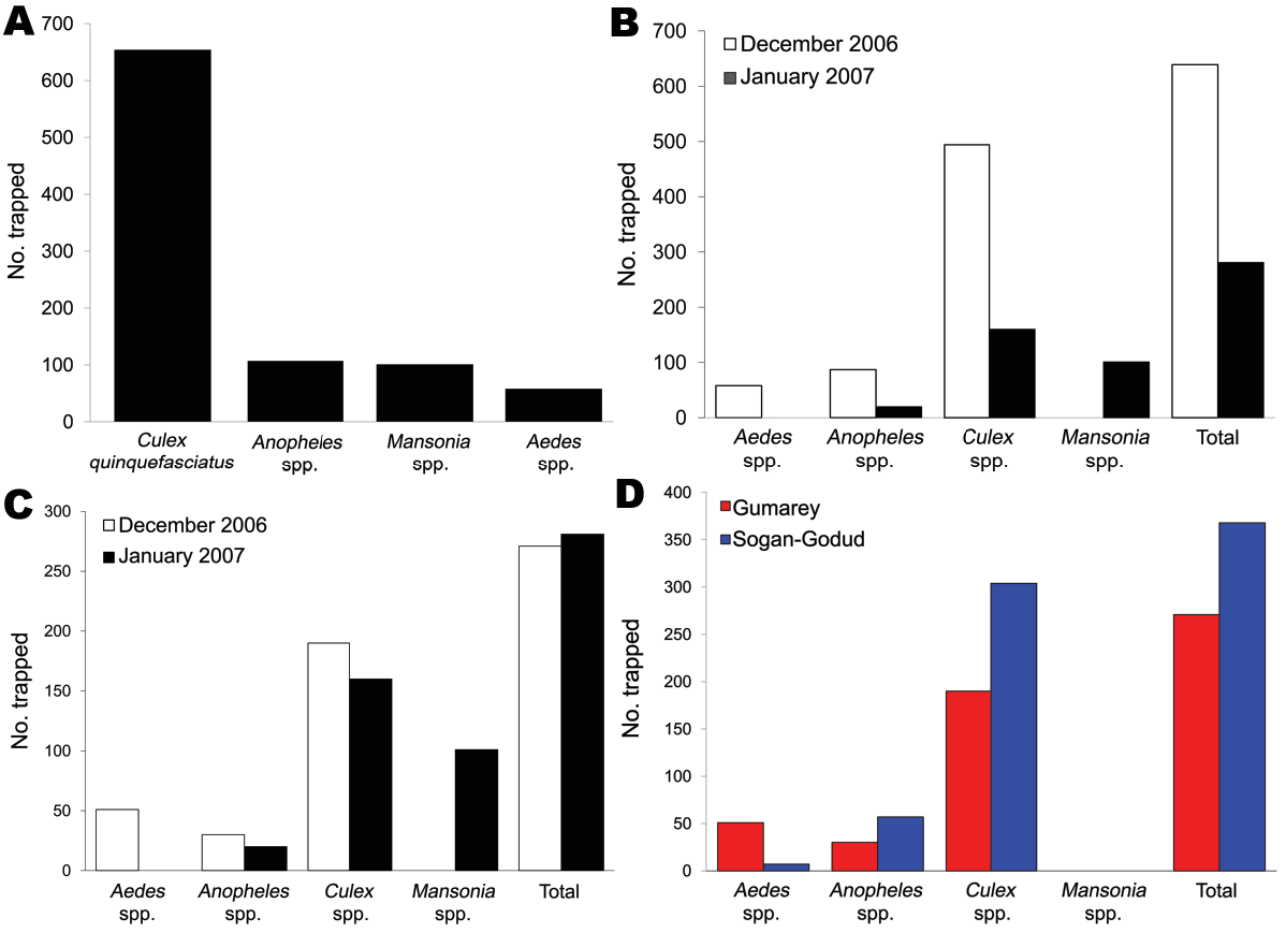

Figure 3. Identification of mosquitoes trapped, Gumarey and Sogan-Godud, Masalani Division of Ijara District, Kenya, 2006–2007. A) Mosquito species trapped during sampling effort. B) Mosquitoes trapped by date. *Aedes* spp. mosquitoes were found in traps only in December 2006 and *Mansonia* spp. mosquitoes only in January 2007. C) Temporal comparison of mosquitoes trapped in Gumarey. D) Mosquitoes trapped by study area, December 2006.

Discussion

A substantial proportion of the mosquito population collected within our study area consisted of RVFV- or WNV-infected potential vectors. The close proximity of these infected mosquitoes to amplifying hosts and susceptible animals and humans during an RVFV epizootic/epidemic warrants further investigation of transmission dynamics. RVFV RNA in mosquitoes collected within the area was high, and the substantial presence of WNV RNA in these mosquito samples was unexpected. The presence of WNV in mosquitoes from Sogan-Godud and Gumarey in our study corroborates recent documentation of the widespread presence of WNV in Kenya and the ability of mosquito populations, including *Cx. quinquefasciatus*, to acquire and transmit WNV (*23*).

The previous isolation of WNV from male *Culex* spp. mosquitoes in Rift Valley Province suggests a natural transovarial transmission cycle among some mosquito vectors but is unlikely to contribute greatly to virus maintenance between enzootic periods (*23*). Additionally, although human epidemics and outbreaks of WNV have not been reported, the presence of the virus in local mosquitoes suggests that the virus is maintained in a natural cycle yet to be elucidated and that the actual incidence of WNV in human populations in the region could be underestimated. Improved field diagnostics are necessary for rapid and accurate diagnosis of circulating arbovirus threats and expedient translation into preventive public health practices.

The isolation of RVFV and WNV RNA from mosquito leg samples confirms that these viruses were disseminated within the bodies of the mosquitoes tested. These results also confirm that single mosquito leg samples are sufficient for PCR/qRT-PCR detection of RVFV and WNV, respectively. Positive results from testing of the mosquito legs also diminish concern about false-positive results from testing whole mosquitoes, which might contain recent bloodmeals with substantial viral content. Our study confirms that RVFV disseminates to the legs of wild *Cx. quinquefas-*

Table 2. PCR results for RVFV and WNV in mosquito pools, by pool, Kenya, 2006–2007*

Virus and location	No. positive/ no. tested (%)	MIR estimate, %	95% CI
Total			
WNV	19/105 (18)	2.3	1.3–3.6
RVFV	23/105 (22)	2.8	1.7–4.2
Gumarey			
WNV	9/65 (14)	1.8	0.75–3.40
RVFV	19/65 (30)	3.9%	2.3–6.3
Sogan-Godud			
WNV	10/40 (25)	3.0%	1.4–5.7
RVFV	4/40 (10)	1.1%	0.29–2.90

*RVFV, Rift Valley fever virus; WNV, West Nile virus; MIR, minimum infection rate; CI, confidence interval.

ciatus mosquitoes and suggests that these mosquitoes, promiscuous feeders, could play a role in the maintenance or transmission of RVFV in disease-endemic regions (*20*,*21*). Other vector competence studies have shown that RVFV does disseminate in *Cx. quinquefasciatus* mosquitoes but have yet to show that they are efficient vectors for RVFV (*20*,*21*,*37*). Although identification of viral RNA in the legs of *Cx. quinquefasciatus* as well as the other mosquitoes tested supports dissemination of virus, no conclusions can be made from these results regarding the role of these mosquitoes in maintaining these arboviruses in this environment or their ability to transmit virus. Additional studies are required to determine vector competence of *Cx. quinquefasciatus* and other mosquito species tested for these 2 viruses.

During this RVFV outbreak, we documented >1 arbovirus circulating in local mosquitoes. During an arbovirus outbreak, other viruses may be circulating concomitantly without recognition and serve as alternative causes of fever. Additional arthropod surveillance studies during RVFV outbreaks in Kenya have found arboviruses in mosquitoes, including flaviviruses and alphaviruses, which can cause febrile illness in humans (*38*). Because diseases from arboviral infections can be nonspecific in humans and animals, it is necessary, even during large outbreaks, to document the true cause of disease with detailed testing. Cases of other arboviral infections could be missed if suspected cases are attributed to the epidemic arbovirus without accurate diagnosis.

Although MIRs for RVFV were similar in the 2 villages studied, rural Gumarey was more likely to have RVFV-positive pools than was Sogan-Godud. This finding concurs with previous human seroprevalence studies that found that risk for being RVFV seropositive is 4× greater for those living in Gumarey than for those in Sogan-Godud (*2*). Gumarey residents were more likely to report greater contact with animals and mosquitoes (*2*). Continued research to identify village level and landscape factors responsible for increased human transmission is necessary. Although RVFV can be transmitted to humans by the bite of an infected mosquito, alternative forms of human exposure, such as aerosol and direct contact, may be more critical for transmission during epidemics (*2*,*28*,*36*). More research must be conducted to elucidate the most common and most effective routes of RVFV transmission to humans during epidemic and interepidemic periods.

Few research studies have documented the presence of WNV and the vectors responsible for its transmission in Kenya. The identification of WNV in North Eastern Province indicates a greater prevalence of the virus than was expected. WNV has not been previously reported in mosquitoes from these 2 villages, and study results imply regional variance in infection rates. Further studies may elucidate a difference between these 2 villages with regard

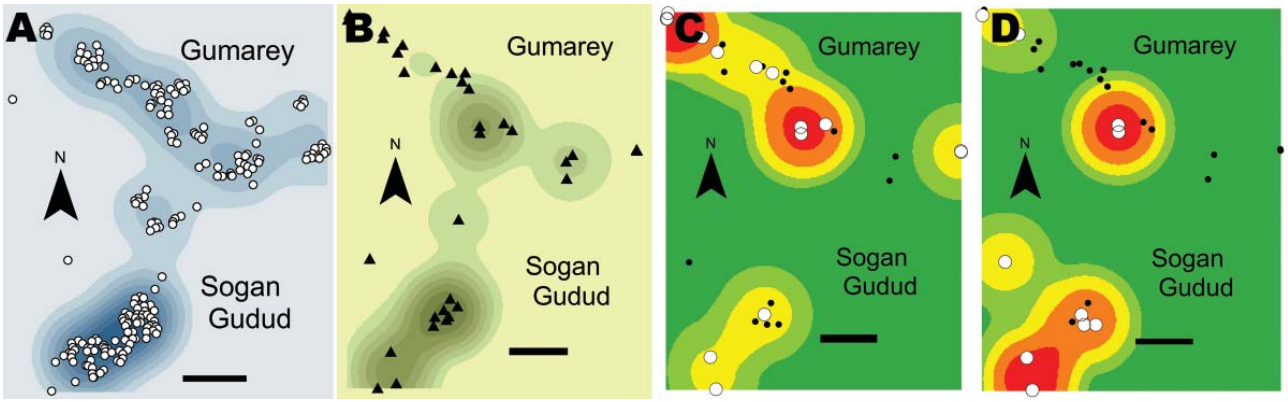

Figure 4. Distribution of human population and infected and uninfected mosquitoes across the selected study areas, Gumarey and Sogan-Godud, Masalani Division of Ijara District, Kenya. A) Area homestead locations (circles) and relative area density of human population (contours, 500-m kernel density; darker color indicates higher values). B) Study trap locations (triangles) and area density of mosquitoes (contours for average mosquitoes per trap, 500-m kernel density). C) Homestead locations of mosquito pools testing positive (white circles) and negative (black circles) for Rift Valley fever virus. Relative local density of positive pools per 500 m is indicated by contours. D) Homestead locations of mosquito pools testing positive (white circles) and negative (black circles) for West Nile virus. Relative density of positive pools is indicated by contours.

Table 3. PCR results for RVFV and WNV in mosquito pools, by homestead source, Kenya, 2006–2007*

Virus and location	No. positive/ no. tested (%)	MIR estimate, %	95% CI
Total			
WNV	10/30 (33)	2.0	0.87–4.20
RVFV	15/30 (50)	4.6	2.2–8.9
Gumarey			
WNV	5/23 (22)	1.4	0.39–3.30
RVFV	12/23 (52)	5.3	2.4–1.0
Sogan-Godud			
WNV	5/7 (71)	7.5	1.4–24
RVFV	3/7 (43)	2.4%	0.37–12.00

*RVFV, Rift Valley fever virus; WNV, West Nile virus; MIR, minimum infection rate; CI, confidence interval; HH, homestead.

to resident reservoirs (birds) or undiscovered amplifying hosts, especially if data are collected during outbreak conditions of flooding and mosquito proliferation. The spatial overlap of human population density with mosquito abundance (Figure 4) and the proximity of humans to infected mosquitoes (Figure 5), suggest that RVFV and WNV transmission during epizootic/epidemic periods could be high in both villages. Additional exposure-modifying factors, including the relative contribution of aerosol transmission of RVFV and the effects of housing construction, sleep and work habits, and the role of personal protective measures need to be further elucidated (*2*).

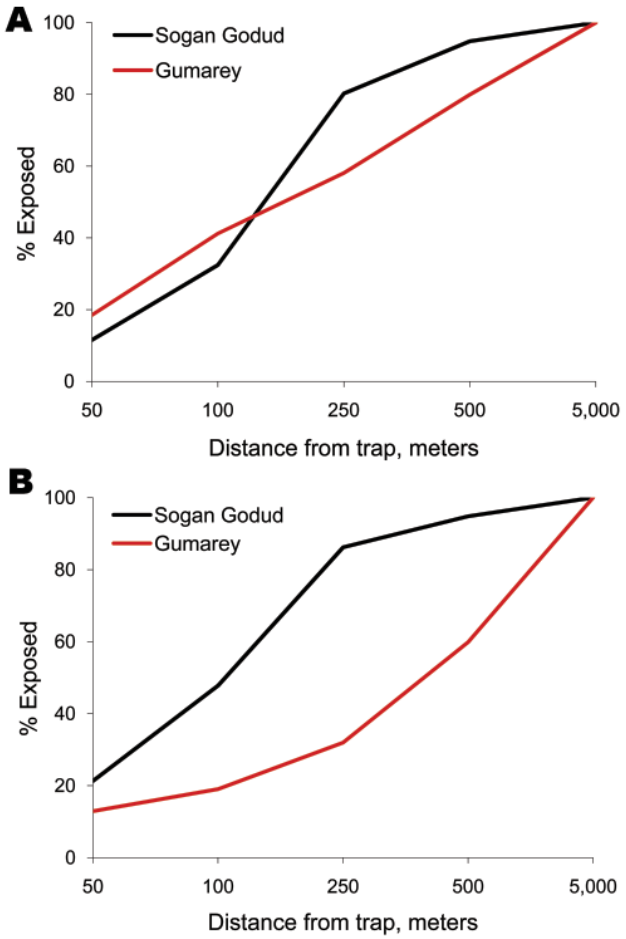

Figure 5. Cumulative proportion of residents within range of Rift Valley fever virus (RVFV)–positive (A) and West Nile virus (WNV)–positive (B) mosquito pools, by village, Gumarey and Sogan-Godud, Masalani Division of Ijara District, Kenya.

Our study has several limitations. Mosquito sampling during the outbreak was not stratified, and pooling of collected mosquitoes was not randomized (*39*). Mosquito sampling was conducted only at homesteads where specific animals, those known to be reservoirs of RVFV, were housed closely with humans. This sampling method may have underestimated the WNV MIR detected. This type of targeted sampling, however, can provide earlier detection of arboviruses and greater understanding of transmission and maintenance factors of these viruses (*39*). Although only 920 mosquitoes were tested for WNV and RVFV, a fraction of the total mosquito population collected, it has been shown that testing of mosquito pools versus testing of all samples can yield suitable results, thereby conserving time and resources (*39*,*40*). The choice of screening pools for arboviruses offers many benefits, especially during an outbreak. The potentially limiting factors of cost and time are avoided, while mosquito positivity is accurately identified (*33*,*39*).

In conclusion, we found high MIR for RVFV and WNV for many mosquitoes, some potentially efficient vectors, in our study region during the 2006–07 RVF outbreak in northeastern Kenya. MIRs did not differ between villages, although RVFV pool positivity and human seroprevalence (as measured in a previous homestead-based study during an interepidemic period) were higher in the rural village of Gumarey (*2*). Our data demonstrate the local abundance of mosquitoes infected with arboviruses in Kenya and highlights simultaneous arbovirus circulation. A greater understanding of how these arboviruses are maintained in nature will improve targeted prevention in regions where disease is endemic and curtail introduction to new areas. Our current inability to quickly detect arboviral infections in endemic communities has led to inaccurate risk assessments, underdiagnosis of clinical cases, and ineffective control measures. Better detection methods in vector, animal, and human populations and recognition of arboviral risk zones and circulation may alter current perceptions about these diseases. These methods could also lead to improved surveillance and better estimates of the true impact of arboviral disease on animal and human populations.

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Dr LaBeaud is an assistant scientist and associate physician at the Children's Hospital Oakland Research Institute. Her research interests include infectious disease epidemiology, domestic and international arbovirology, and emerging infections.

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New Delhi Metallo-β-Lactamase from Traveler Returning to Canada[1]

Gisele Peirano, Jasmine Ahmed-Bentley, Neil Woodford, and Johann D. Pitout

An *Escherichia coli* isolate with New Delhi metallo-β-lactamase was isolated from a patient with pyelonephritis and prostatitis who returned to Canada after recent hospitalization in India. The patient was successfully treated with ertapenem and fosfomycin. This patient highlights the role of international travel in the spread of antimicrobial drug resistance and bla_{NDM-1}.

The *Enterobacteriaceae*, particularly *Escherichia coli* and *Klebsiella pneumoniae*, are among the most common causes of serious hospital- and community-acquired bacterial infections in humans. Resistance to antimicrobial agents in these species has become increasingly prevalent. Of special concern is the development of resistance to the carbapenems; this development is caused by bacterial carbapenemases. These drugs are often the last line of effective therapy for treating infections caused by multidrug-resistant *Enterobacteriaceae*. Three types of β-lactamases inactivate the carbapenems: *K. pneumoniae* carbapenemases, metallo-β-lactamases (MBLs), and oxacillinases. The 2 most reported MBLs are the VIM and IMP types, which until recently have been mostly associated with *Pseudomonas aeruginosa* and *Acinetobacter* spp., although VIM-2 has spread among *Enterobacteriaceae* in Greece and, to a lesser extent, Italy (*1*).

Recently, a new type of MBL, New Delhi metallo-β-lactamase (NDM-1), in bacteria (*K. pneumoniae* and *E. coli*) recovered from a patient from Sweden who was hospitalized in New Delhi, India, was described (*2*).We characterized a carbapenem-resistant *E. coli* isolate from the urine of a patient with pyelonephritis and prostatitis who returned to Canada after recent hospitalization while visiting India.

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The Study

A 32-year-old man was admitted to the medical ward of a hospital in Mysore, southwestern India, during 2010, with hyperglycemia and upper urinary tract infection (UTI). His underlying diabetes mellitus was stabilized, but his UTI did not improve after 5 days of ciprofloxacin. He was transferred to a hospital in Alberta, Canada. Prostatitis with pyelonephritis was diagnosed, and the patient was treated with ertapenem, 2 g/day. Culture of a clean-catch urine sample taken before the ertapenem was started yielded *E. coli* MH01 at $>10^5$ CFU/mL urine. The patient improved clinically, and a urine culture taken after 7 days of therapy showed no bacterial growth. The patient received 1 dose of 3 g fosfomycin after completing the ertapenem.

Antimicrobial drug susceptibility was determined with the VITEK 2 instrument (Vitek AMS; bioMérieux Vitek Systems, Hazelwood, MO, USA). MICs of the following drugs were determined: amoxicillin/clavulanic acid, piperacillin/tazobactam, cefoxitin, ceftriaxone, ceftazidime, aztreonam, meropenem, ertapenem, amikacin, gentamicin, tobramycin, ciprofloxacin, and trimethoprim/sulfamethoxazole. Additional susceptibility tests for imipenem, meropenem, ertapenem, tigecycline, and colistin were performed by using Etest (AB BioDisk, Solna, Sweden) according to the manufacture's instructions. Results were interpreted by using Clinical and Laboratory Standards Institute (CLSI) criteria for broth dilution (*3*). Fosfomycin susceptibility was determined by using CLSI disk methods (*3*).

The sample with *E. coli* was screened for MBLs with the MBL Etest according to the manufacturer's instructions. Isoelectric focusing was performed on freeze–thaw extracts on polyacrylamide gels as described (*4*). PCR amplification for bla_{VIM}, bla_{IMP}, bla_{NDM}, $bla_{CTX\text{-}Ms}$, bla_{OXAs}, bla_{TEMs}, and bla_{SHV} was conducted on the isolate by using a GeneAmp 9700 ThermoCycler instrument (Applied Biosystems, Norwalk, CT, USA) and PCR conditions and primers as described (*4*–*6*). The $bla_{CTX\text{-}M}$ was sequenced by using PCR conditions and primers as described (*4*), and the bla_{NDM} was sequenced by using the following primers and conditions: NDM-F1: 5′-CAGCGCAGCTTGTCG-3′, NDM-R1: 5′-TCGCGAAGCTGAGCA-3′. The PCR program consisted of an initial denaturation step at 95°C for 5 min; followed by 30 cycles of DNA denaturation at 95°C for 1 min, primer annealing at 52°C for 1 min, and primer extension at 72°C for 1 min; followed by a final extension at 72°C for 5 min.

The *qnrA*, *qnrS*, and *qnrB* genes were amplified in MH01 by using multiplex PCR (*7*). The *aac(6′)-Ib* and *qepA* genes were amplified in a separate PCR by using primers and conditions as described (*8*,*9*). The variant *aac(6′)-Ib-cr*

[1]Data from this study were presented at the 50th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, September 13, 2010, Boston, MA, USA.

was further identified by digestion with *BstF5*I (New England Biolabs, Ipswich, MA, USA).

Multilocus sequencing typing (MLST) was performed on MH01 by using 7 conserved housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*). The MLST protocol, including allelic type and sequence type assignment methods, is detailed at http://mlst.ucc.ie/mlst/dbs/Ecoli.

MH01 was assigned to 1 of the 4 main *E. coli* phylogenetic groups (A, B1, B2, D) by using a multiplex PCR-based method (*10*). Plasmid sizes were determined by using protocols and conditions described (*11*) and assigned to plasmid families by PCR-based replicon typing (*12*). Conjugation experiment was performed by mating-out assays with a selection agar containing different β-lactams (IMP 2 μg/mL, ceftazidime 4 μg/mL respectively) and by using *E. coli* C600N as recipient.

When we used Vitek 2, *E. coli* MH01 was resistant to amoxicillin/clavulanic acid, piperacillin/tazobactam, cefoxitin, ceftriaxone, ceftazidime, aztreonam, meropenem, ertapenem, amikacin, gentamicin, tobramycin, ciprofloxacin, and trimethoprim/sulfamethoxazole. The MICs detected by Etest were meropenem 32 μg/mL, imipenem 32 μg/mL, ertapenem >32 μg/mL, tigecycline 0.5 μg/mL, and colistin 0.125 μg/ml. The zone size for fosfomycin was 26 mm. MH01 was susceptible only to tigecycline and fosfomycin; CLSI has not published colistin MICs for *Enterobacteriaceae*.

E. coli MH01 was positive for MBL production by MBL Etest. Isoelectric focusing showed that *E. coli* MH01 produces 2 β-lactamases with isoelectric points of 5.2 and 8.9; PCR with sequencing identified these enzymes as NDM-1 and CTX-M-15, respectively. The isolate was positive for *aac(6′)-Ib* (but not *aac(6′)-Ib-cr*) and belonged to MLST clone 101 and phylogenetic group B1. *E. coli* MH01 harbored 4 plasmids of 75 kb, 165 kb, 300 kb, and 400 kb. *E. coli* (MH01A) transconjugant with an MBL phenotype was obtained, and plasmid analysis showed that it harbored a 75-kb plasmid. PCR confirmed that the transconjugant contained bla_{NDM} that was untypeable by PCR-based replicon typing. The $bla_{CTX\text{-}M\text{-}15}$ was identified on the 165-kb plasmid that belonged to incompatibility groups IncA/C and IncFII. These results were similar to those obtained by Poirel et al. (*13*).

Conclusions

Kumarasamy et al. (*5*) recently provided evidence that NDM-producing *Enterobacteriaceae* (mostly *K. pneumoniae* and *E. coli*) are widespread in the Indian subcontinent. They also found that many patients in the United Kingdom infected with bacteria that produce NDM-1 had been hospitalized on the Indian subcontinent. The patients sought care for a variety of hospital- and community-associated infections; UTIs were the most common clinical infections. NDM-producing *Enterobacteriaceae* also have recently been isolated from patients residing in the United States (*14*), Netherlands (*15*), and Australia (*5*); all patients had received medical care while visiting India.

Our findings add Canada to the growing list of countries from which these bacteria have been isolated. An *E. coli* isolate with NDM-1 and belonging to the same sequence type has been reported from Australia from a patient previously hospitalized in Bangladesh (*13*). Isolation of the same clone in 2 patients in different countries without any obvious contact underscores the probable acquisition of these bacteria during receipt of medical care in the subcontinent and suggests that *E. coli* ST101 with NDM-1 may be widespread throughout the region. The recent pandemic caused by *E. coli* clone ST131, which produces CTX-M types of β-lactamases, highlights the ability of certain clones to spread rapidly. *E. coli* ST101 with NDM-1 may have the potential to cause a similar pandemic.

The worldwide spread of *Enterobacteriaceae*-producing NDMs has serious implications for the empiric treatment of hospital- and community-associated infections because of the multiresistant nature of these bacteria, which severely limits treatment options. Worse, few antimicrobial drugs being developed have activity against gram-negative bacteria. If the emerging public health threat of international travel in the spread of antimicrobial resistance is ignored, the medical community may face carbapenem-resistant *Enterobacteriaceae* that cause common infections such as UTIs.

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School Closures and Student Contact Patterns

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To determine how school closure for pandemic (H1N1) 2009 affected students' contact patterns, we conducted a retrospective questionnaire survey at a UK school 2 weeks after the school reopened. School closure was associated with a 65% reduction in the mean total number of contacts for each student.

During pandemic (H1N1) 2009, several countries closed schools (*1*–*6*) to slow virus transmission. The effects of such school closures on student contact patterns have not been directly quantified. We report these effects for students from a UK secondary school.

The Study

We retrospectively surveyed 128 students at a coeducational, state secondary school in an urban area of West Midlands, UK, where attack rates for pandemic (H1N1) 2009 were high and (as of March 2010) levels of unemployment were among the highest in Great Britain (*7*). The head teacher selected 1 class from each of years 7–10 (equivalent to US grades 6–9, student ages 11–15 years) to participate. The school had closed for 1 week in mid-June 2009, reopened for 2 days, then closed for another week. Questionnaires were completed during class ≈2 weeks after the school reopened the second time. An electronic version of a similar questionnaire pilot tested at another school had been found comprehensible and acceptable to participants. The London School of Hygiene and Tropical Medicine ethics committee approved the study; the Health Protection Agency approved it as part of wider outbreak investigations not requiring additional approval.

Students reported how many times they visited specified public places before the school closure and how many times they visited these places during closure (children had been advised to not visit public places only if they were symptomatic). Students also provided information about persons who looked after them during closure.

For typical school days before and during closure, students reported the number of different persons spoken to (contacted) in the following groups: contacts who attended their school (contacts from the same class [classmates], the same year but a different class [yearmates], and the same school but a different year [schoolmates]) and others (age stratified to reflect the UK school system). Students were asked whether they were ill during closure and whether being ill affected their contact patterns.

Questionnaires were returned by 107 (84%) of 128 students. Approximately 100 students (range 99–103, depending on place visited) stated how frequently they would visit public places while school was open, and 46 stated how many times they visited these places during school closure; 45 (98%) of 46 visited ≥1 place. Fewer students visited shops, places of worship, parks, and playing fields at least 1×/week when school was closed than when open (Figure 1). For other places, frequency of visits did not differ.

Among those who provided information about caregivers, 93 (95%) of 98 reported that ≥1 adult looked after them during school closure; 49% reported having 2 caregivers (range 1–5). Among caregivers for whom further information was available, 125 (69%) of 182 would have seen the student on a typical school day, 54 (31%) of 173 typically worked outside the home, and 12 (34%) of 35 took time off work to care for the student during school closure.

Among students, 73 provided number of contacts on a typical day during school closure; 35 also provided information for a typical school day, and another 6 only provided information for a typical school day. We therefore conducted unpaired and paired analyses on data from 79

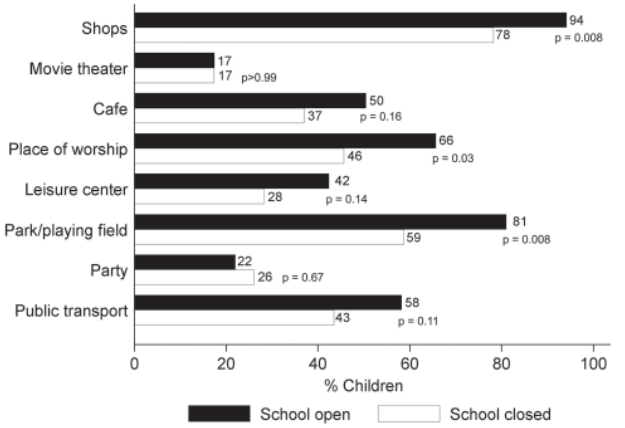

Figure 1. Visits to public places during open and closure periods of a UK secondary school, June–July 2009. Percentage of students visiting public places ≥1×/week while the school was open (n = 99–103, depending on the place) and while it was closed (n = 46). Numbers after bars show percentages in each group; p values are from Fisher exact tests comparing the proportions during the open versus closed periods.

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and 35 respondents, respectively. Students who provided contact data were most likely to be in years 7 or 9 but were otherwise similar to those who did not.

The mean totals of reported contacts were 70.3 (SD 40.8) and 24.8 (SD 22.5) during typical school days and closure, respectively (Figure 2). School closure was therefore associated with a reduction of 45.5 (95% confidence interval [CI] 33.8–57.2) in students' typical daily number of contacts, a 65% relative reduction (95% bootstrap CI 52%–73%). The corresponding absolute and relative reductions in numbers of contacts with other students were 37.0 (95% CI 27.0–46.9) and 65% (95% bootstrap CI 52%–74%), respectively. The absolute and relative reductions in the numbers of contacts made with adults (including teachers) were 8.5 (95% CI 4.9–12.1) and 63% (95% bootstrap CI 45%–75%), respectively. No apparent change was found for number of contacts with adults outside school (34%, 95% bootstrap CI –6% to 63%).

The greatest reductions in the numbers of contacts were for students from the same school (Figure 2), e.g., ≈80% reduction in numbers of contacts with classmates and yearmates. Absolute reductions in numbers of contacts with persons not attending the school were small; the relative reductions had wide confidence intervals and rarely showed evidence of a genuine reduction (Figure 2). Paired analysis of data for 35 students with information for contacts during both periods produced similar results as unpaired analysis. Among 40 respondents who reported illness during closure and self-assessed whether they consequently contacted fewer persons, 53% stated that their contacts were reduced, 33% stated that they were not, and 15% were unsure.

Conclusions

Closing this school was associated with a 65% reduction in face-to-face conversational contacts made by secondary school students, primarily because of reductions in contact with students from the same school. Our estimated reductions exceed estimates from analyses of surveillance data for seasonal influenza-like illness in France (24% reduction in child-to-child transmission during school holidays compared with in-school days) (*8*) and a study conducted in Belgium (19% reduction in total contacts made by children and adolescents during Easter holidays) (*9*). Our estimate of a 65% reduction in total contacts is similar to that from a survey at a primary school in Germany, in which students reported 72% fewer contacts on Sundays than on weekdays but in which all classmates were considered contacts (*10*). Consistent with findings of other studies (*11–13*), most students visited public places during closure, although certain places were visited less frequently while the school was closed than when open.

Our study has several limitations. Our definition of contact excluded nonconversational contacts (e.g., passengers on public transport), which may enable transmission, and some conversations may not involve close contact. We did not collect data about duration or intensity of contact or whether persons were contacted multiple times. Our

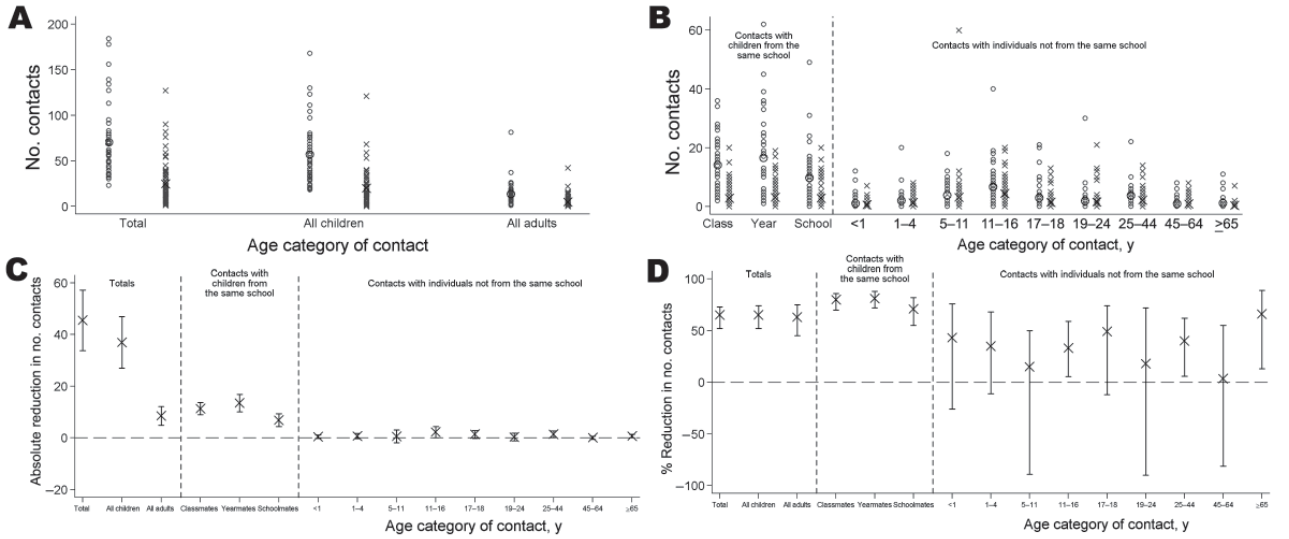

Figure 2. Number of contacts made by students with persons in different categories and the changes associated with school closures. A) total contacts overall and with students and adults; B) contacts with persons in different categories at school and in different age groups outside school; C) absolute reductions in numbers of contacts with persons in different groups associated with school closure; D) relative reductions in numbers of contacts with persons in different groups associated with school closure. In (A) and (B), large black markers indicate the mean number of contacts; small gray markers indicate individual data points; circles indicate data for when the school was open (n = 41), crosses indicate data for when the school was closed (n = 73). In (C) and (D), error bars indicate 95% confidence intervals.

use of a typical day does not capture variation in student behavior.

For logistical reasons, a 2–3 week delay occurred between school reopening and completion of questionnaires, providing potential for recall bias and underestimation of numbers of contacts during school closure (although closure was an unusual event that children are likely to remember well). Prospective data collection was impossible and has limitations, including greater effort required from participants and therefore potentially lower response rates. The data refer to a convenience sample from 1 secondary school during what was often perceived as a mild pandemic and may not be generalizable to other situations (e.g., primary schools, different socioeconomic settings, infections with high case-fatality rates, or different seasons).

Most students provided data only for the closure period, and few did so for a typical school day (probably because of the order of questions). The primary analysis therefore ignored the pairing in the data. Ignoring the pairing would not affect point estimates but would reduce their precision. Paired analysis of 35 students who provided data for both periods produced similar results to the unpaired analysis.

Other issues must also be considered when deciding whether to close schools (*14*). Subject to the limitations described above, reactive school closures may substantially reduce the numbers of contacts made by students and may potentially reduce transmission of infection in some settings.

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Unusual Transmission of *Plasmodium falciparum*, Bordeaux, France, 2009

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Plasmodium falciparum malaria is usually transmitted by mosquitoes. We report 2 cases in France transmitted by other modes: occupational blood exposure and blood transfusion. Even where malaria is not endemic, it should be considered as a cause of unexplained acute fever.

Unusual forms of parasitic infection, such as those acquired by blood transfusion (*1*,*2*) or accidental exposure to infected blood (*3*), may be challenging to diagnose in areas where these infections are not endemic (*4*). We report 2 cases of *Plasmodium falciparum* malaria transmitted by routes other than mosquito vectors: occupational blood exposure and blood transfusion.

The Patients

Patient 1, a 36-year-old woman who worked as a technician in a clinical laboratory, was admitted to Bordeaux Hospital, France, on June 2, 2009. She had a 7-day history of high fever (up to 39°5 C), rigors, headache, and jaundice. Hematologic tests showed decreased platelets ($33{,}000 \times 10^9$ platelets/L) and increased serum C-reactive protein (130 mg/L; reference <5 mg/L). Although the patient denied having traveled abroad in the past 5 years, she lived near an international airport, and her clinical signs were typical of malaria. Consequently, thick and thin blood smears were performed and indicated *P. falciparum* parasitemia of 0.1%. Serum bilirubin was 86 μmol/L (reference 3–18 μmol/L). By 6 hours after hospital admission, her condition had dramatically worsened, with hemodynamic collapse (blood pressure 60/32 mm Hg) associated with macroscopic hemoglobinuria; parasitemia increased to 6%. All viral and bacteriologic testing results were negative. Treatment was intravenous quinine formate (loading dose 17 mg/kg, followed by maintenance dose of 8.3 mg/kg 3×/d). After 5 days, her medication was switched to oral quinine for another 2 days. Parasitemia was absent 6 days after starting quinine, and the patient was discharged 2 days later. Blood smears were negative 21 days after discharge.

Patient 1 later recalled that 2 weeks before hospital admission she had been injured by a broken, blood-contaminated, malaria diagnostic (QBC) test tube at work. Because she considered the incident trivial, she did not inform her workplace of it. Had she done so, the standard blood exposure protocol would have been automatically triggered. The source of the blood was subsequently traced to a patient returning from the Congo, for whom *P. falciparum* parasitemia of 4% had been diagnosed when the blood sample was taken. Genotyping of blood samples from patient 1 and the presumed source traveler were performed. *P. falciparum* isolates were genotyped at the 7A11, C4M79, Pf2802, and Pf2689 microsatellite loci and at the highly polymorphic loci of the merozoite surface protein 1 and 2 antigen genes by fluorescent end-labeled nested PCR and restriction fragment length polymorphism analysis (*5*–*7*). Results showed that the 2 infections had the same molecular signature and complete homology and were confirmed by genotypic analysis of resistance markers. A threonine variant on codon K76T point mutation of *P. falciparum* chloroquine resistance transporter and an identical level of resistance to antifolate drugs (*P. falciparum* dihydrofolate reductase–thymidylate synthase point mutations at positions 51, 59, and 108) were found (Table) (*5*,*7*). A diagnosis of severe malaria as a result of occupational percutaneous blood exposure was therefore retained.

Patient 2 was a 15-day-old girl who was referred from Dakar, Senegal, where she had been born on March 11, 2009. She was the third child of a Lebanese family who had no known genetic illness and who lived in an air-conditioned house. The pregnancy had been uneventful, and the infant was delivered at term. Her blood group was B Rh+. At day 14 after birth, pallor and jaundice suggested severe neonatal anemia. Blood testing indicated decreased hemoglobinemia (5.2 g/dL; reference 9–14 g/dL) and reticulocytosis ($260{,}000 \times 10^9$ cells/L; reference $25{,}000$–$85{,}000 \times 10^9$ cells/L). Serum C-reactive protein was <5 mg/L. The patient received a 60-mL whole blood transfusion from a

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Table. Genotyping results for 2 *Plasmodium falciparum* isolates, France, 2009*

Locus	Secondary case		Index case	
	Type	Allele	Type	Allele
Microsatellites†				
7A11	NA	119 bp	NA	119 bp
C4M79	NA	203 bp	NA	203 bp
Pf2802	NA	139 bp	NA	139 bp
Pf2689	NA	87 bp	NA	87 bp
Antigen genes‡				
msp-1	Ro33	149 bp	Ro33	149 bp
msp-2	FC27	413 bp	FC27	413 bp
Drug resistance genes§				
pfcrt codon 76	Mutated	ACA	Mutated	ACA
pfdhfr				
Codon 16	Wild-type	GCA	Wild-type	GCA
Codon 51	Mutated	ATT	Mutated	ATT
Codon 59	Mutated	CGT	Mutated	CGT
Codon 108	Mutated	AAC	Mutated	AAC
Codon 164	Wild-type	ATA	Wild-type	ATA

*NA, not applicable; *msp*, merozoite surface protein; *pfcrt*, *P. falciparum* chloroquine resistance transporter; *pfdhfr*, *P. falciparum* dihydrofolate reductase.
†Size of amplified DNA fragment expressed.
‡Allelic family and size of amplified DNA fragment expressed.
§Single-nucleotide polymorphism. *pfcrt* codon 76 point mutation was genotyped by nested PCR and restriction fragment-length polymorphism analysis. *pfdhfr* point mutations at positions 16, 51, 59, 108, and 164 were genotyped by nested PCR and primer expansion. Underlining indicates nucleotide positions that can be mutated.

compatible donor in Dakar on March 26; the next day, she was transferred to Bordeaux Hospital.

At the time of admission, hematologic testing confirmed severe regenerative hemolytic anemia (hemoglobin 9.5 g/dL, reticulocytes 320,000 × 10^9 cells/L, and serum haptoglobin <0.08 g/L [reference 0.80–2.15 g/L]). Abdominal and transfontanellar echography showed no abnormalities. PCR specific for parvovirus B19 and cytomegalovirus was negative. Serologic testing for malaria, thick and thin blood smear, and specific PCR for *P. falciparum pBRK1–14* in the newborn and her mother were negative. Investigation for ABO blood incompatibility or immunologically related hemolysis produced negative results. Screening of both parents and the newborn for inherited hemolysis excluded an erythrocyte membrane abnormality. Hemoglobinopathy and erythrocyte enzyme (glucose 6-phosphate dehydrogenase, pyruvate kinase, and hexokinase) deficiencies were also ruled out. The newborn and her parents were followed up weekly; no pertinent clinical findings or abnormal PCR and blood smear were found until 7 weeks after birth, when febrile thrombocytopenia (92,000 × 10^9 platelets/L) and neutropenia (540 × 10^9 cells/L) were detected.

At 7 weeks after birth, blood smears indicated *P. falciparum* parasitemia of 6%. Treatment with intravenous quinine formate at 8.3 mg/kg 3×/d for 4 days, followed by oral mefloquine (20 mg/kg) for 1 day led to prompt improvement. Follow-up at 3 and 5 months of age showed resolution of anemia and no relapse of malaria. Test results for hemolysis remained negative, and glucose 6-phosphate deshydrogenase and erythrocyte pyruvate kinase levels 6 months after transfusion were within normal limits. Although it could not be confirmed, neonatal anemia was attributed to fetal–maternal hemorrhage.

We contacted the clinic in Dakar, where the blood sample but not the donor could be traced. However, a thick and thin blood smear examination subsequently performed in Dakar was positive for *P. falciparum*, clearly supporting a diagnosis of transfusion-transmitted malaria.

Conclusions

In non–malaria-endemic countries, accidental bloodborne inoculation of *P. falciparum* after direct occupational exposure of health care or laboratory personnel has been rarely reported (*8–10*). The course of such occupationally acquired malaria may be critical if the inoculating injury is neglected or unrecognized, as it was by patient 1. This case emphasizes the need for laboratories handling blood to ensure that every accidental injury, however trivial it may seem, is declared and managed. As occurred with patient 1, a delayed diagnosis may also delay delivery of appropriate care for preventing severe or complicated illness (*9*,*10*).

Whereas most non–malaria-endemic countries, including France, are implementing selective screening strategies for blood product donors (*11*), prevention of transfusion-transmitted malaria in endemic areas such as Senegal remains a challenge because such screening is not routinely performed. For patients such as patient 2, malaria is rarely diagnosed in non–malaria-endemic countries (*12*,*13*). For this patient, the chronology of events and exposure to blood from a contaminated donor are highly suggestive of transfusion-transmitted malaria. In the absence of immunologic indicators that the mother had been exposed to *P. falciparum*, vertical transmission is extremely unlikely, and the risk for mosquito transmission is low because of the family's living environment and the time of year. Nevertheless, without gene sequencing of parasites from the donor, transfusion-transmitted malaria cannot be demonstrated unequivocally.

Neonates are assumed to be able to counteract natural infection with malaria because of the predominance of fetal hemoglobin, which is not suitable for complete erythrocyte schizogony of *P. falciparum* (*14*). For patient 2, transfusion with infected adult blood may have compromised this capacity, enabling the infection to follow its natural course. In Senegal, blood transfusion recipients are routinely given antimalaria treatment (*15*). Unfortunately, such treatment cannot be extended to neonates because of lack of a validated and convenient therapeutic antimalaria regimen for this age group.

The above-mentioned types of malaria transmission are unusual. However, in non–malaria-endemic countries,

a recent history of blood transfusion or an episode of accidental inoculation of blood may account for malaria infection in persons who are not otherwise at risk.

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Transmission of *Armillifer armillatus* Ova at Snake Farm, The Gambia, West Africa

Dennis Tappe, Michael Meyer, Anett Oesterlein, Assan Jaye, Matthias Frosch, Christoph Schoen, and Nikola Pantchev

Visceral pentastomiasis caused by *Armillifer armillatus* larvae was diagnosed in 2 dogs in The Gambia. Parasites were subjected to PCR; phylogenetic analysis confirmed relatedness with branchiurans/crustaceans. Our investigation highlights transmission of infective *A. armillatus* ova to dogs and, by serologic evidence, also to 1 human, demonstrating a public health concern.

Pentastomes are an unusual group of vermiform parasites that infect humans and animals. Phylogenetically, these parasites represent modified crustaceans probably related to maxillopoda/branchiurans (*1*). Most documented human infections are caused by members of the species *Armillifer armillatus*, which cause visceral pentastomiasis in West and Central Africa (*2*–*4*). An increasing number of infections are reported from these regions (*5*–*7*). Close contact with snake excretions, such as in python tribal totemism in Africa (*5*) and tropical snake farming (*2*), as well as consumption of undercooked contaminated snake meat (*8*), likely plays a major role in transmission of pentastome ova to humans.

The Study

In May 2009, a 7-year-old female dog was admitted to a veterinary clinic in Bijilo, The Gambia, for elective ovariohysterectomy. The owner of the dog, a snake farm operator, reported late abortions during several pregnancies of the animal. The dog had been kept on the farm premises, where adult snakes (African rock pythons, *Python sebae*) had died several months before of infection with adult *A. armillatus* pentastomes (Figure 1, panel A). During the dog's surgery, hundreds of pentastomid larvae were seen on the enteral serosa, bladder, uterus, and in the omentum (Figure 1, panels B, C). In April 2010, a male stray dog, 6 months of age, was admitted to the veterinary clinic for elective neutering. Coiled pentastomid larvae were found in the vaginal processes of the testes during surgery. Adult and larval parasite specimens were preserved in 100%

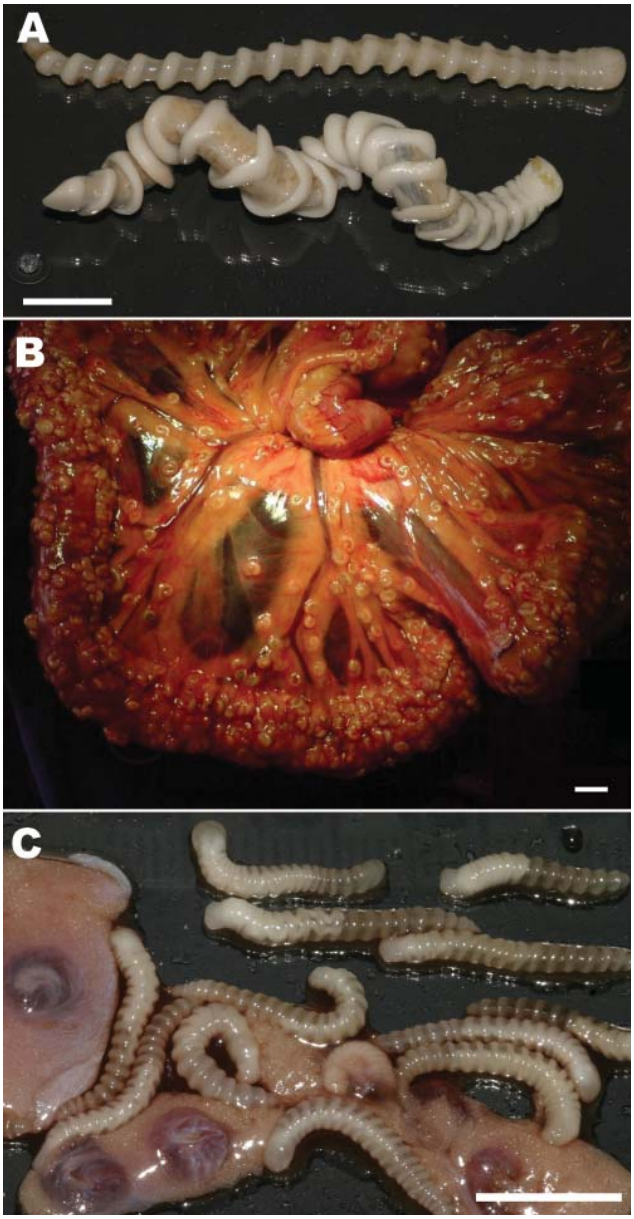

Figure 1. Adult and larval *Armillifer armillatus* parasites. A) Ventral view of 2 adult *A. armillatus* parasites recovered from the lungs and trachea of a deceased rock python; a gravid female (bottom) and a pre-adult female (top) are shown. The parasites showed 20 and 18 marked body rings, and had a length of 10 cm and 9 cm and a body width of 5–8 mm and 3–5 mm, respectively. B) Heavily parasitized omentum of a female stray dog, showing typical encapsulated C-shaped larval stages of *A. armillatus* parasites. C) Larvae from the omentum. The larvae had a length of 18–19 mm and a body width of 2 mm and showed 20–22 rings. Scale bars = 1 cm.

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ethanol for further parasitologic, histologic, and molecular examinations.

To investigate the extent of infection and determine whether transmission to humans on the snake farm grounds had occurred, we collected serum specimens from the 46-year-old male Caucasian snake farm owner, his 28-year-old wife, his 3 children, and the infected female dog. All human patients were asymptomatic, and informed consent was obtained. Serum samples were transferred to the Institute of Hygiene and Microbiology (University of Würzburg, Würzburg, Germany) for analysis by ELISA and Western blot based on larval parasite antigens.

DNA of the pentastome specimens (2 adults from the snakes, 1 larva from each dog) was extracted by using the QIAGEN Tissue Kit (QIAGEN, Hilden, Germany) and subjected to 18S rRNA and cytochrome c oxidase (*cox*) gene PCR with primers Pent629F (5′-CGGTTAAAAAGCTCGTAGTTGG-3′) and Pent629R (5′-GGCATCGTTTATGGTTAGAACTAGGG-3′ [*9*]) and primers Cox1-F (5′-CTGCGACAATGACTATTTTCAAC-3′) and Cox1-R

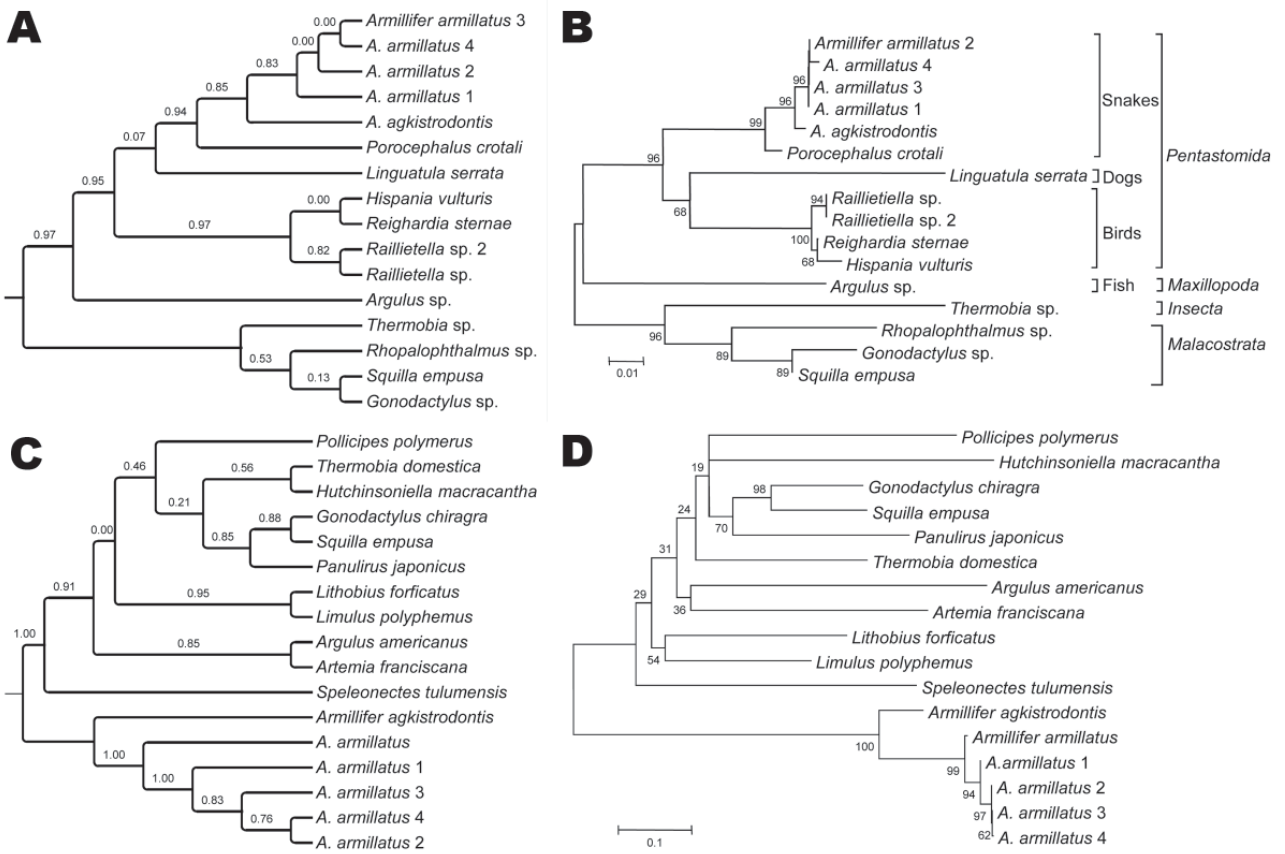

Figure 2. Molecular phylogeny of 4 *Armillifer armillatus* specimens based on partial 18S rRNA and partial cytochrome c oxidase (*cox*) gene sequences. Panels A and C show cladograms based on maximum-likelihood (ML); panels B and D show minimum-evolution (ME). In the cladograms, the approximate likelihood ratios are given next to the branches to indicate the statistical support for the respective branches. In panels B and D, the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches, and the evolutionary distances are given as scale bars as the number of base substitutions per site. The phylogram of the partial 18S rRNA sequences (B) depicts respective host species for the parasitic Pentastomida. The bird parasites *Raillietiella* sp., *Reighardia sternae*, and *Hispania vulturis* belong to the order Cephalobaenida, whereas snake parasites *Armillifer* spp. and *Porocephalus* spp. and dog parasites *Linguatula* spp. belong to the order Porocephalida. Note that not all species have entries for 18S rRNA and *cox* genes in GenBank. GenBank accession numbers of the species depicted can be found in the Table. MUSCLE (www.ebi.ac.uk/Tools/muscle/index.html) and RevTrans 1.4 (www.cbs.dtu.dk/services/RevTrans/) were used for the alignment of partial 18S rDNA and *cox* sequences, respectively. Poorly aligned regions were removed with Gblocks. MODELTEST (darwin.uvigo.es/software/modeltest.html) was used for the selection of the substitution models. ML analyses of the 18S rDNA sequences were performed with PhyML 3.0 (http://atgc.lirmm.fr/phyml/) under the TN93 + I + Γ substitution model (6 rate classes and NNI algorithm for tree searching). ML analyses of the *cox* sequences were performed under the generalized time reversible + I + Γ substitution model. Approximate likelihood ratios were used to estimate the branch supports of the inferred ML phylogeny, which was visualized with TreeGraph2. MEGA4 (www.megasoftware.net) was used for ME analysis of the 18S rDNA sequences under the TN93 + I + Γ substitution model, and with maximum composite likelihood for *cox* sequences. The close neighbor interchange algorithm was used for tree searching, with pair-wise deletion of sequence gaps and considering differences in the substitution pattern among lineages.

(5′-ATATGGGAAGTTCTGAGTAGG-3′ [*10*]). After sequencing and BLAST (www.ncbi.nlm.nih.gov/blast) analysis of the partial 18S rRNA gene amplicons, the sequences showed high homology with *A. agkistrodontis* and *Porocephalus crotali* parasites. However, no *A. armillatus* 18S rRNA gene entry existed in GenBank; *cox* gene sequences showed high homology with *A. armillatus*, followed by *A. agkistrodontis*. The 4 amplified partial 18S rRNA gene sequences were 100% identical, as expected for the same species, but heterogenicity was seen in the *cox* sequences (99.7% identity). When phylogenetic trees were constructed, the nearest neighbor of *A. armillatus* was *A. agkistrodontis* in both models for both genes, followed by *P. crotali* and *Linguatula serrata* (18S rRNA only as no *cox* entry existed). All pentastomes analyzed clustered together and formed their own branch, with *Argulus* sp. being the nearest maxillopodan/branchiuran neighbor in both models by using 18S rRNA sequence data, and *Speleonectes tulumensis* (Remipedia) crustaceans when *cox* sequences were used (Figure 2). The 18S sequence of *A. armillatus* was submitted to GenBank (accession no. HM756289).

The crude parasite antigen ELISA was set up in a similar manner to an in-house *Echinococcus multilocularis* ELISA (*11*) by using larvae from the canine omentum. Because no serum specimens from persons with proven *Armillifer* spp. infections were available as positive controls, a stored serum sample was used from a patient with a histologically confirmed *L. serrata* tongueworm infection (*12*). Ten serum samples from healthy German blood donors served as negative controls, and a standardized threshold index of 1.0 was calculated (*11*). In addition, the serum of the infected female dog was tested, as well as 10 serum samples from uninfected dogs from Germany. The crude larval antigen was also used in a Western blot (2 μg/slot), and all serum samples from The Gambia and the serum sample from the *Linguatula* spp.–infected patient were analyzed. Of the serum samples tested, only the serum sample from the snake farm owner was positive for pentastomiasis in the ELISA, index 1.2. All other persons had indices below the threshold index (0.71–0.30). The control serum sample from the patient with linguatuliasis exhibited an index of 1.3.

When tested by Western blot, the serum of the farm owner demonstrated a banding pattern similar to the *L. serrata*–positive control serum. Both serum samples exhibited 97-kDa and 37-kDa bands, and the serum from the patient with linguatuliasis had an additional 50-kDa band (not shown). All human serum samples were negative for various helminthic diseases. When dog serum samples were tested, only the sample from The Gambia showed a positive reaction in the ELISA, with an index of 1.0.

Conclusions

Pentastomiasis is a parasitic zoonosis with an increasing number of recognized human infections in West Africa. Our investigation highlights the local transmission of infective *A. armillatus* ova to dogs and, by serologic evidence, also to 1 human, and thus demonstrates a public health concern. Possibly because of their eating habits (e.g., consumption of dead snakes), dogs seem to be at high risk and could function as sentinel animals. In this study, we set up serologic assays for pentastomiasis based on raw larval *A. armillatus* antigens and screened the farm workers for past infection. The infection of 1 person, the snake farm owner, could be demonstrated by ELISA and Western blot for human serum samples. In 1982, an indirect immunofluorescence assay based on *A. armillatus* larvae was used for a survey in the Ivory Coast; results indicated a low seroprevalence (*13*).

In most human cases, pentastomiasis is asymptomatic and is an incidental finding during surgery or autopsy, and diagnosis largely relies on parasitologic and histopathologic examination (*2*,*14*,*15*). Recently, PCRs have been developed for canine pentastomiasis (*9*), but DNA sequences in the databases are limited to a few species of pentastomes only.

We have provided partial 18S rRNA gene sequences of *A. armillatus* pentastomes and used PCR for the diag-

Table. Sequences used for phylogenetic inferences of *Armillifer armillatus,* The Gambia, 2009–2010

Species and/or genera used	GenBank accession no.
18S rRNA gene PCR	
A. armillatus (samples 1–4)	HM756289 (this study)
A. agkistrodontis	FJ607339.1
Porocephalus crotali	M29931.1
Linguatula serrata	FJ528908.1
Raillietiella sp.	AY744887.1, EU370434.1
Reighardia sternae	AY304521.1
Hispania vulturis	AY304520.1
Argulus sp.	DQ531766.1
Thermobia sp.	AY338726.1
Rhopalophthalmus sp.	AM422488.1
Gonodactylus sp.	L81947.1
Squilla empusa	L81946.1
Cytochrome oxidase gene PCR	
A. armillatus	AY456186.1
A. agkistrodontis	FJ607340.1
Speleonectes tulumensis	AY456190.1
Argulus americanus	AY456187.1
Artemia franciscana	NC_001620.1
Lithobius forficatus	AF309492.1
Limulus polyphemus	NC_003057.1
Thermobia domestica	AY639935.1
Panulirus japonicus	NC_004251.1
Gonodactylus chiragra	NC_007442.1
Pollicipes polymerus	NC_005936.1
Hutchinsonella macracantha	AY456189.1
S. empusa	NC_007444.1

nosis of pentastomiasis from a clinical sample (*9*). We also constructed phylogenetic trees for all pentastome species infecting humans and animals from which sequence data were available. Phylogenetic analysis showed that pentastomes formed their own branch in proximity to the Branchiura and Remipedia, a finding which is consistent with results of a previous study by others who investigated *A. armillatus* as a sole member of the pentastomes, in comparison with pancrustaceans (*1*). The nearest phylogenetic relatives of *A. armillatus* are *A. agkistrodontis* and *P. crotali*, 2 species of the Porocephalida, followed by *L. serrata* and by the members of the Cephalobaenida (pentastomes that infect birds). The phylogenetic trees constructed here indicate a coevolution of the pentastomes and other maxillopodan/branchiuran parasites with their vertebrate hosts (birds, snakes, mammals, and fish).

We demonstrated that the serum of a patient with linguatuliasis markedly cross-reacted on the ELISA and Western blot based on *Armillifer* spp. antigens. To prevent further infections, personal hygiene measures were implemented, such as thorough hand cleansing after handling snakes and avoidance of contact with snake excretions. Public health institutions have been informed, and future studies will address the extent of seroprevalence in the local population.

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Characteristics of Patients with Oseltamivir-Resistant Pandemic (H1N1) 2009, United States

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During April 2009–June 2010, thirty-seven (0.5%) of 6,740 pandemic (H1N1) 2009 viruses submitted to a US surveillance system were oseltamivir resistant. Most patients with oseltamivir-resistant infections were severely immunocompromised (76%) and had received oseltamivir before specimen collection (89%). No evidence was found for community circulation of resistant viruses; only 4 (unlinked) patients had no oseltamivir exposure.

During April, 2009–June, 2010 the United States had enhanced surveillance for oseltamivir resistance among pandemic influenza A (H1N1) 2009 viruses. We describe characteristics of patients infected with oseltamivir-resistant and oseltamivir-susceptible pandemic (H1N1) 2009 virus.

The Study

During April 2009–June 2010, the Centers for Disease Control and Prevention (CDC) requested state public health laboratories to submit specimens for antiviral susceptibility testing by 2 routes. In the first route, the first 5 influenza specimens of any type or subtype collected every 2 weeks from each laboratory underwent virus isolation for comprehensive antiviral testing, including testing by neuraminidase inhibition (NI) assay, sequencing viruses with elevated 50% inhibitory concentration (IC_{50}) values, and pyrosequencing for adamantine resistance–conferring M2 mutations. In the second route, the first 5 additional clinical specimens from pandemic (H1N1) 2009 virus–infected patients that were collected each week by these laboratories were submitted and screened for the oseltamivir-resistant conferring neuraminidase H275Y mutation by using pyrosequencing. Patients with oseltamivir-resistant pandemic (H1N1) 2009 infection had demographic and clinical information collected by using a standard form.

Oseltamivir resistance was determined by either NI or pyrosequencing for the H275Y mutation. NI was performed on virus isolates with a chemiluminescent substrate; viruses with elevated IC_{50} values for oseltamivir were identified as resistant, based on previously set criteria (*1*,*2*). All oseltamivir-resistant viruses had H275Yconfirmed by pyrosequencing (*1*). Original clinical specimens collected from surveillance were screened by pyrosequencing for H275Y, without NI. NI testing was performed at CDC, and pyrosequencing for H275Y was performed at CDC and state laboratories in Wisconsin, New York, and California. All oseltamivir-resistant viruses referenced here were reported on FluView (*3*). Four patients, identified in June and August 2009, were reported previously (*4*,*5*).

A comparison group of hospitalized patients infected with oseltamivir-susceptible pandemic (H1N1) 2009 was identified from the Influenza Hospitalization Network (FluSurv-NET). FluSurv-NET includes 10 states that participate in the Emerging Infections Program, a population-based surveillance for hospitalized patients with influenza infection (California, Colorado, Connecticut, Georgia, Maryland, Minnesota, New Mexico, New York, Oregon, Tennessee), plus 6 states (Iowa, Idaho, Michigan, North Dakota, Oklahoma, South Dakota) added in response to the 2009 pandemic, as previously described (*3*,*6*). The counties within FluSurv-NET represent 26 million persons (8.5% of the US population) (*6*). The 16 states participating in FluSurv-NET collected demographic and clinical information for all hospitalized patients with laboratory-confirmed influenza infection within their catchment counties (*6*). We identified patients hospitalized in FluSurv-NET who had specimens submitted to national antiviral resistance surveillance by using Link Plus software to link antiviral resistance surveillance and FluSurv-NET data by patient county of residence, age, and sex and specimen collection date.

We considered identical matches on all 4 variables as a high probability match, e.g., a patient from FluSurv-NET who had a pandemic (H1N1) 2009 virus specimen submitted to national antiviral resistance surveillance who had an oseltamivir-susceptible pandemic (H1N1) 2009 virus infection. We validated our linking methods with Oregon data (n = 41); all 4 patients identified as high probability matches were true matches. For validation purposes, we identified 4 specimens

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that were matched on county, age, and sex but not on specimen collection date up to 7 days, e.g., moderate probability matches; 1 patient was hospitalized, 2 were outpatients, and 1 specimen was from a medical examiner (patient not hospitalized). The Oregon surveillance specimens that were neither high nor moderate probability matches were surveillance specimens from outpatients and cluster investigations (M. Vandermeer, pers. comm.).

Overall, 6,740 virus isolates and specimens were submitted to surveillance systems; 37 (0.5%) viruses were oseltamivir resistant (*3*); 18 were identified by NI, contained the H275Y mutation, and were susceptible to zanamivir and resistant to adamantanes; the 19 remaining viruses were detected by pyrosequencing for H275Y. Oseltamivir-susceptible viruses exhibited IC_{50} values ranging from 0.05 to 1.44 nmol/L. Oseltamivir-resistant viruses exhibited a median IC_{50} value of 80.08 nmol/L (range 6.24–116.48 nmol/L).

Most patients infected with oseltamivir-resistant pandemic (H1N1) 2009 viruses were hospitalized (81%), had a severe immunocompromising condition (76%), and had been exposed to oseltamivir before collection of the specimen tested for antiviral resistance (89%) (Table); 9 (30%) had received oseltamivir as chemoprophylaxis, and 21 (70%) had received oseltamivir as treatment. Four patients with oseltamivir-resistant pandemic (H1N1) 2009 virus infection had no documented exposure to oseltamivir before collection of the specimen for testing, including exposure to family members receiving oseltamivir. No epidemiologic links were found between the 4 patients.

Among the 28 patients infected with oseltamivir-resistant pandemic (H1N1) 2009 virus, with a severe immunocompromising condition and a complete case form, 24 (86%) had a malignancy reported, 23 had a hematologic malignancy and were receiving chemo- or immunosuppressive therapy at the time of their infection, and 10 (38%) were recipients of a hematopoietic stem cell transplant (SCT). One patient had AIDS and a lymphoma of the central nervous system. Among the 3 immunosuppressed patients without a malignancy, 2 were recipients of solid organ (renal) transplants, and another had received SCT <6

Table. Characteristics of patients infected with oseltamivir-resistant and -susceptible pandemic (H1N1) 2009 viruses from national influenza antiviral resistance surveillance and enhanced hospital influenza surveillance, April 2009–June 2010*†

	Oseltamivir-resistant infections		Oseltamivir-susceptible infections	
Characteristic	Total from national surveillance, n = 37	Total from FluSurv-NET states,‡ n = 17	National surveillance cases from FluSurv-NET counties, n = 401	National surveillance cases matched in FluSurv-NET, n = 65
Median age, y (range)	18 (1–74)	21 (5–74)	22 (0–89)	31 (0–82)
Female sex	18 (49)	6 (35)	177 (50)	38 (58)
Hospitalized	30 (81)	16 (100)	65 (16)	65 (100)
ICU admission	13/30 (43)	7/12 (58)	–	14 (23)
Deaths, all cause§	7/31 (23)	2/12 (17)	–	3 (5)
Oseltamivir exposure¶	31 (89)	16 (94)	–	6 (14)
Underlying medical condition	33 (89)	17 (100)	–	49 (75)
Severe/Immunosuppression#	28 (76)	17 (100)	–	7 (11)**
Pregnancy	1 (2.7)	0	–	5 (7.7)
Asthma and CLD	10 (27)	2 (18)	–	21 (34)
CVD	8 (22)	4 (24)	–	5 (8)
Diabetes mellitus	5 (14)	2 (13)	–	12 (18)
Chronic kidney disease	4 (11)	3 (19)	–	5 (8)
No underlying medical conditions	4 (11)	0	–	13 (21)
Median time from oseltamivir initiation to specimen collection, d (range)	11 (2–37)	14 (3–37)	–	1.5 (1–4)††

*Values are no. (%) except as indicated. FluSurv-NET, Influenza Hospitalization Network; ICU, intensive care unit; CLD, chronic lung disease; CVD, cardiovascular disease, excluding hypertension; –, not applicable.
†Missing data were excluded from analysis; denominators are included where they varied from cohort size.
‡Of the 16 oseltamivir-resistant cases from states participating in FluSurv-NET, 5 had county information and were from FluSurv-NET counties. None were high probability matches to a FluSurv-NET hospitalized patient and none were from Oregon. FluSurv-NET captured only 1 hospitalization for each patient infected with pandemic (H1N1) 2009 virus during the year. Repeat hospitalizations were not recorded, although many sites noted repeat hospitalizations for patients with immunosuppressive conditions (L. Kamimoto, pers. comm.).
§Among oseltamivir-resistant cases, 4 deaths were reported to national surveillance as directly caused by influenza. Cause of death was not recorded for patients infected with oseltamivir-susceptible pandemic (H1N1) 2009 virus.
¶Patients with oseltamivir exposure received oseltamivir, either as chemoprophylaxis or treatment, before the collection date of the pandemic (H1N1) 2009 virus specimen tested for antiviral resistance.
#For patients with oseltamivir-resistant pandemic (H1N1) 2009, severe immunosuppression was defined as any of the following: receiving treatment for any cancer within 6 months before onset of influenza illness, currently receiving immunosuppressive medication, including systemic corticosteroids, as part of prevention strategies for transplant (bone marrow or solid organ) rejection, or for management of pulmonary or autoimmune conditions, or having a diagnosis of AIDS, not just HIV infection. For patients within FluSurv-NET, we included any patient with a medical record of the syndromes above or if immunosuppressed or immunosuppression was recorded in the medical chart.
**Among the 7 hospitalized patients from FluSurv-NET with oseltamivir-susceptible pandemic (H1N1) 2009 and an immunosuppressive condition, 3 (43%) were receiving chronic systemic corticosteroids; 1 for systemic lupus erythematosus and the other 2 for unknown reasons. The immunosuppressive condition was not known for 4 patients, but immunosuppression was recorded from the medical record.
††n = 6.

months before influenza illness (reason for SCT could not be confirmed).

Among 1,982 national surveillance oseltamivir-susceptible specimens from the 16 FluSurv-NET states, 1,607 (81%) had county information; among these, 401 (25%) specimens were from FluSurv-NET counties, and 65 patients from FluSurv-NET were high probability matches to patients identified in antiviral resistance surveillance data (Table). Compared with patients with oseltamivir-resistant pandemic (H1N1) 2009 infections identified in national surveillance, few (11%) FluSurv-NET patients with an oseltamivir-susceptible pandemic (H1N1) 2009 virus infection had severely immunosuppressive conditions, and few (14%) had oseltamivir exposure before collection of the specimen for testing, none were reported to have received oseltamivir as chemoprophylaxis. Among all 8,740 FluSurv-NET hospitalized patients with pandemic (H1N1) 2009 during this period, 10% had an immunosuppressive condition. Patients with oseltamivir-resistant infections had specimens for testing collected a median of 11 (range 2–37) days after oseltamivir initiation, and results may reflect testing due to clinical suspicion of resistance. Among the 6 FluSurv-NET patients with specimens collected after oseltamivir was begun, the median time between oseltamivir initiation and specimen collection was shorter.

Conclusions

Infections with oseltamivir-resistant pandemic (H1N1) 2009 viruses were rare in the United States during April 2009–June 2010. Few patients had no oseltamivir exposure before resistant virus was detected, and none had epidemiologic links to another patient. Thus, evidence for community transmission of oseltamivir-resistant pandemic (H1N1) 2009 viruses was rare (*7*). Patients with severe immunocompromising conditions with prior exposure to oseltamivir were most likely to have an oseltamivir-resistant infection. Infections were most frequently reported in patients with hematologic cancers who were undergoing immunosuppressive treatment, chemotherapy, or SCT. Other studies have also reported a high frequency of patients with hematologic malignancies or SCT and oseltamivir exposure among patients with oseltamivir-resistant pandemic (H1N1) 2009 virus infections (*8*,*9*). Oseltamivir resistance should be considered among patients with severe immunocompromising conditions and pandemic (H1N1) 2009 in the setting of oseltamivir treatment or chemoprophylaxis failure.

Although the number of patients with oseltamivir-resistant pandemic (H1N1) 2009 virus infections was small in the United States during this period, this is the largest case series published and confirms findings from reports with smaller samples (*8–10*). Although all patients in our comparison group of patients with oseltamivir-susceptible pandemic (H1N1) 2009 were hospitalized, most patients in the oseltamivir-resistant group were also hospitalized. Finally, we do not have a comparison group of patients with immunocompromising conditions and oseltamivir-susceptible pandemic (H1N1) 2009 virus infections; thus, risk factors for infection with oseltamivir-resistant infection among patients with immunocompromising conditions cannot be determined. The finding of oseltamivir-resistant pandemic (H1N1) 2009 viruses associated with oseltamivir treatment highlights the need for new antiviral agents and new treatment strategies.

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Primary Amebic Meningoencephalitis Caused by *Naegleria fowleri*, Karachi, Pakistan

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We report 13 cases of *Naegleria fowleri* primary amebic meningoencephalitis in persons in Karachi, Pakistan, who had no history of aquatic activities. Infection likely occurred through ablution with tap water. An increase in primary amebic meningoencephalitis cases may be attributed to rising temperatures, reduced levels of chlorine in potable water, or deteriorating water distribution systems.

Primary amebic meningoencephalitis (PAM) is a fatal disease caused by the thermotolerant free-living ameba *Naegleria fowleri*. Found worldwide in moist soil and freshwater, these amebae proliferate during summer when ambient temperature increases. The organism enters the nasal cavity when water contaminated with amebae is aspirated. Subsequently, it invades the central nervous system through the olfactory neuroepithelium and causes a fatal infection that clinically resembles acute bacterial meningitis. We report 13 cases of *N. fowleri* PAM in a period of 17 months in the coastal city of Karachi, Pakistan.

The Study

In June 2008, a 30-year-old, previously healthy man was referred to the Aga Khan University Hospital with a 2-day history of high-grade fever, severe headache, and seizures. He was comatose with a fixed and dilated left eye pupil. Magnetic resonance imaging showed basal meningeal enhancement. A lumbar puncture found an opening pressure of 44 cm H_2O. Cerebrospinal fluid (CSF) analysis showed low glucose (<5 mg/dL), high protein level (1,028 mg/dL), and lymphocytic pleocytosis (900 cells/mm^3 with 85% lymphocytes). Gram stain and latex agglutination (LA) test results were negative for bacteria. Wet film of CSF showed motile amebic trophozoites, but CSF volume was insufficient for amebic culture. A preliminary diagnosis of PAM was made, and therapy was begun with intravenous amphotericin B plus oral rifampin and fluconazole. The patient died 4 days after admission.

In September 2008, a previously healthy 25-year-old man was admitted with a 24-hour history of fever, vomiting, and neck rigidity. CSF analysis indicated hypoglycorrhachia, elevated protein level, and neutrophilic pleocytosis. Gram stain and LA test results were negative for bacteria, and wet preparation of CSF showed motile amebic trophozoites. Ptosis of the left eye and a fixed dilated pupil developed on the day of admission, and he was intubated for airway protection. Despite therapy with intravenous amphotericin B, oral fluconazole, and rifampin, his condition deteriorated, and he died 14 days after admission. CSF was cultured on nonnutrient agar on a lawn of *Escherichia coli* American Type Culture Collection (Manassas, VA, USA) 29522 in Page ameba saline (*1*). Cultured amebae produced flagellates in distilled water at 37°C within 15–30 min.

On the basis of this apparent upsurge of cases, a laboratory policy was instituted at the Aga Khan University Hospital to perform wet mounts of all processed CSF samples that were consistent with bacterial meningitis but had negative Gram stain and LA test results. Although no new cases of PAM were detected in 2008, 11 were identified from April through November 2009. Case-patients were referred from tertiary-care hospitals in Karachi (Table).

Twelve of 13 patients were male; ages ranged from 16 to 64 years (mean ± SD 31.0 ± 15.33 years). All were residents of Karachi but lived in different districts (online Appendix Figure, www.cdc.gov/EID/content/17/2/256-appF.htm). Only 1 patient acknowledged a history of swimming. All patients' conditions were treated with amphotericin B (1.5 mg/kg/d) and rifampin (600 mg/d). Most patients also received either fluconazole or itraconazole. All required intubation and ventilation within 24 hours of hospital admission, and treatment for PAM was started within 48–72 hours of admission. Nonetheless, only 2 patients survived for >5 days after admission; the mean ± SD time from symptom onset to death was 6.38 ± 3.15 days (range 3–15 days).

CSF samples from all patients (except the first) were positive for amebic culture. Furthermore, CSF samples collected from 3 patients in July 2009 were confirmed by real-time PCR for *N. fowleri* DNA at the Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA. Briefly, primers NaeglF192 (3′-GTG CTG AAA CCT AGC TAT TGT AAC TCA GT-5′) and NaeglR344 (5′-CAC TAG AAA AAG CAA ACC TGA AAG G-3′) were used to amplify a 153-bp fragment, detected by the hexachlorofluorescein (HEX)–labeled probe NfowlP

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Table. Clinical features and CSF analysis results for 13 patients with *Naegleria fowleri* meningoencephalitis, Karachi, Pakistan, 2008–2009*

Characteristic	Patient nos., in order of treatment													Descriptive statistics†
	1	2	3	4	5	6	7	8	9	10	11	12	13	
Age, y	31	25	30	36	60	30	64	18	22	16	18	18	35	Mean 31.0 ± 15.33
Date treatment sought	2008 Jul	2008 Sep	2009 Apr	2009 May	2009 Jul	2009 Jul	2009 Jul	2009 Jul	2009 Aug	2009 Aug	2009 Oct	2009 Oct	2009 Nov	
Duration of illness before seeking treatment, d	2	1	2	4	4	1	2	5	2	2	2	3	3	Mean ± SD 2.5 ± 1.19
Duration of survival after symptom onset, d	6	15	3	6	7	9	7	6	5	3	3	7	6	Mean ± SD 6.38 ± 3.15
Fever	+	+	+	+	+	+	+	+	+	+	+	+	+	Present in 13/13 (100%)
Headache	+	+	+	+	+	+	+	–	+	+	+	+	+	Present in 12/13 (92.3%)
Seizures	+	–	+	–	+	–	+	–	+	+	+	+	–	Present in 8/13 (61.5%)
CSF analysis														
Glucose, mg/dL†	<5	42	2	<5†	26	<5†	<5‡	30	<5‡	<5‡	80	50	<5‡	Undetectable in 53.8%
Protein, mg/dL	1,028	418	909	774	504	1,147	1342	320	998	330	371	179	1,296	Mean ± SD 739.69 ± 405.29
Leukocytes, cells/mm^3	900	900	5,976	2,000	2,500	7,500	5,200	6,000	840	6,500	150	185	11,750	Mean ± SD 3,877.0 ± 3,565.37
Neutrophils, %	15	90	90	90	60	80	90	90	90	96	75	25	95	Neutrophilic pleocytosis, 84.6
Lymphocytes, %	85	10	10	10	40	20	10	10	10	4	25	75	5	Lymphocytic pleocytosis, 15.4
CSF amebic culture	NA	+	+	+	+	+	+	+	+	+	+	+	+	NA
CSF PCR for *N. fowleri*	NA	NA	NA	NA	+	+	+	NA	NA	NA	NA	NA	NA	NA

*All patients were male except patient no. 11, who was female. CSF, cerebrospinal fluid; +, present; NA, no data available.
†Statistics derived in SPSS software version 16.0 (LEAD Technologies, Charlotte, NC, USA).
‡Glucose <5 mg/dL= undetectable.

(5′-HEX-AT AGC AAT ATA TTC AGG GGA GCT GGG C-BHQ1-3′). PCR was performed in an Mx3000P real-time thermocycler (Stratagene, La Jolla, CA, USA), with 2 initial incubations at 50°C for 2 min (incubation for uracil-DNA-glycosylase activity) and 95°C for 2 min (activation of Platinum *Taq* DNA-polymerase), respectively, followed by 40 cycles of 95°C for 15 s and 63°C for 60 s. Fluorescence was measured after each 63°C incubation. Results were analyzed by using Mx3000P version 2.0 software (*2*).

Domestic tap water was obtained for amebic culture from 2 patients' homes (second and seventh patients in the series). Water samples (100 mL) were passed through a Millipore filter (Millipore Corp., Billerica, MA, USA), which was then inoculated face down on a nonnutrient agar plate with a lawn of *E. coli* as described above. Amebae were isolated from both water samples. However, only cultured amebae from the seventh patient's sample were analyzed by real-time PCR, and *N. fowleri* DNA was detected in the sample.

Conclusions

We report an increase of *N. fowleri* PAM cases in Karachi, Pakistan. Most cases were in healthy young adults with acute, fatal meningitis. Although enhanced case detection after instituting measures to improve the diagnosis of PAM may have contributed toward the rise in cases, sporadic previous reports from this region indicate that this is not the sole reason (*3*,*4*).

PAM is associated with freshwater swimming (*5*), and outbreaks have also been associated with poorly chlorinat-

ed swimming pools (*6*). However, all but 1 of the patients in this case series denied recent freshwater swimming or recreational water activities. Nevertheless, because all patients were Muslim, they routinely performed ritual ablution, which involves taking water into the nostrils. Infection acquired through this route has been reported (*7*). Because patients used domestic tap water for ablutions, we tested water from 2 patient's homes and found *N. fowleri* amebae by culture. Additionally, *N. fowleri* DNA was identified by real-time PCR in a water sample from 1 patient's home. PAM, resulting from aspiration of untreated ground water containing *N. fowleri* amebae, has been reported in 2 children from Arizona (*8*).

The presence of *N. fowleri* DNA in the ground water supply has been described (*9*). *N. fowleri* infections have also been described in children from Australia after exposure to water transported through overland pipes (*10*). The presence of *N. fowleri* amebae n Karachi's municipal water supply may have several explanations. Karachi lacks an indigenous water source, and water obtained from 2 suburban freshwater lakes is not adequately filtered or chlorinated (*11*). Moreover, frequent leaks in water and sewage pipes can cause seepage of sewage into the water supply (*12*), which may be a potential reservoir for *N. fowleri* amebae (*13*).

Demonstrating *N. fowleri* amebae in the water supply, however, does not explain the sudden increase in the number of cases. *N. fowleri* amebae are thermotolerant, and outbreaks have been linked to steep rises in temperature (*14*). Recent temperature records from Karachi have also shown a temperature surge, and a further rise is expected in future summers (*15*). Our patients were also exposed to higher average monthly temperatures (Figure). Several factors may have precipated this outbreak: an increase in the number of pathogens (due to rising temperatures), changes in the ecosystem of the lakes, and further deterioration in the quality of water treatment and distribution systems.

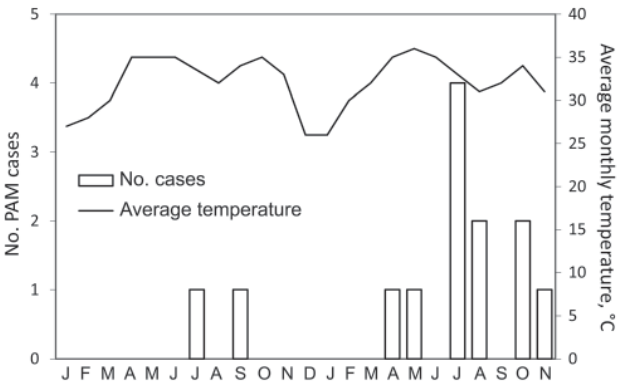

Figure. Primary amebic meningoencephalitis (PAM) cases seen in 2008 and 2009 by month (white bars) and average monthly peak temperatures (black line), Karachi, Pakistan. Karachi monthly temperatures obtained from http://www.wunderground.com/global/PK.html.

This report highlights the emergence of fatal *N. fowleri* infection in a megacity. Changing climatic conditions may have contributed toward this sudden upsurge, which has serious consequences for the public at large. Urgent epidemiologic investigation into relevant environmental factors is needed to identify reasons for this sudden rise in PAM cases.

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etymologia

Naegleria fowleri

[nə′gliəriə fau(ə)l′·ər·ī]

From F.P.O. Nägler, an early 20th century bacteriologist, and Malcolm Fowler, an Australian physician. In 1912, A. Alexeieff proposed a new genus for questionable amoeboid forms, which he named Nägleria in honor of Nägler's work in identifying amoebae that pass through a biflagellate stage. Fifty-three years later, a report from Australia described human meningoencephalitis caused by an amebo-flagellate, later recognized as a member of *Naegleria*. In 1970, the pathogen was designated *Naegleria fowleri* after Fowler, who obtained one of the first isolates from human brain tissue.

Source: Alexeieff A. Sur less caractères cytologiques et la systématique des amibes du groupe limax (*Naegleria* nov gen et *Hartmannia* nov. gen) et des amibes parasites des vertebras (Proctamoeba nov. gen). Bull de la Soc Zool de France. 1912; 37:55; Calkins GN. Genera and species of ameba. In: Transactions of the Fifteenth International Congress on Hygiene and Demography, Vol. II, Washington, September 23–28, 1912. Washington: General Printing Office; 1913; Fowler M, Carter RF. Acute pyogenic meningitis probably due to *Acanthamoeba* sp.: a preliminary report. BMJ. 1965;2:740–2. DOI: 10.1136/bmj.2.5464.734-a; Marciano-Cabral F. Biology of *Naegleria* spp. Microbiol Rev. 1998;52:114–33.

Alert System to Detect Possible School-based Outbreaks of Influenza-like Illness

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To evaluate the usefulness of school absentee data in identifying outbreaks as part of syndromic surveillance, we examined data collected from public schools in Miami-Dade County, Florida, USA. An innovative automated alert system captured information about school-specific absenteeism to detect and provide real-time notification of possible outbreaks of influenza-like illness.

Information about school absenteeism is commonly used as part of syndromic surveillance for detecting disease outbreaks in the United States. For example, health officials from the New York City Department of Health and Mental Hygiene evaluated school absentee percentage data for 2001–02 and identified moderate increases in influenza-associated absenteeism (*1*). However, absence is not always related to illness; thus, understanding why students miss school can be difficult because specific reasons are not usually recorded (*2*).

The Miami-Dade County Health Department (MDCHD) is Florida's largest county health department and serves the Miami metropolitan area of ≈2.5 million persons. Approximately 350,000 students are enrolled in 436 schools in the Miami-Dade County Public Schools system (MDCPS), which includes public, charter, vocational, and alternative schools. Each school is required to enter students' attendance information daily into an MDCPS database, the Automated Student Attendance Recordkeeping System. MDCHD has access to this database through a secure file transfer protocol that provides file access over a reliable data stream. Since 2007, MDCHD has automatically received these electronic raw data that contain students' demographic and geographic information, which includes gender, race/ethnicity, age, school code, and ZIP code (*3*). After the emergence of pandemic (H1N1) 2009 virus in April 2009, MDCHD designed an automated school-based absentee surveillance system (SBASS) at the beginning of the 2009–10 school year. This system had an alert function to monitor trends in absentee activity and potentially link absenteeism with influenza outbreaks. We assessed this innovative SBASS as an adjunct to traditional disease reporting.

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The Study

We evaluated absentee data for MDCPS during September 8–October 21, 2009. Eighty-seven charter and special education schools were excluded because of consistently unstable absenteeism levels. On the basis of MDCPS's previous year's mean of 4.9% absenteeism, we used 8.0% as the threshold level (*4*,*5*). The mean and standard deviation were estimated in countywide and individual school levels. Alerts were automatically generated 1) for an absentee rate >8% and 2) when the percentage was at least 1.0 SD beyond the mean of the previous 30 days in countywide or an individual school, compared with their own value. At 1.0 SD, a warning was signaled, and when the standard deviations were 1.96 and 2.58 beyond the mean, yellow and red alerts were triggered, respectively. These cutoffs were set to alert at the 95th and 99th percentiles, assuming the percentage of absenteeism was normally distributed.

Only the yellow and red alerts were applied to countywide absenteeism trends by age group; however, all alerts were applied to the individual school trends. Combining absentee rates with an alert status helped exclude schools with percentage of absenteeism >8% without an alert that, on the basis of historical data, typically have high absentee rates because of low attendance. SAS version 9.13 (SAS, Cary, NC, USA), Visual Basic (Microsoft, Redmond, WA, USA), and ArcGIS 9.3 (www.esri.com) were used to design an SBASS that created 4 reports. These comprised 1) a figure with the percentage of countywide absentee trends by age group (<5, 6–11, 12–14, 15–17, and ≥18 years of age); 2) a table with the countywide absentee percentages by mean, ratio, standard deviation, and alert status (red alert, yellow alert, or warning); 3) a list of the alerted schools; and 4) a geographic information system map with the alerted school locations.

The applied epidemiology and research team of the MDCHD Epidemiology, Disease Control and Immunization Services (EDC-IS) performed daily school absentee surveillance. Protocol dictated contacting attendance offices when the system detected an alert. The school calendar was used to ascertain dates that would have high absenteeism because of teacher planning days, early release days, holidays, and other events. Daily school absentee reports were sent to MDCPS offices through email.

If clustering of influenza-like illness (ILI) was identified, MDCHD initiated an investigation. ILI clusters were

deemed outbreaks when ≥2 students or staff with a clear association, such as classmates or sharing of similar activities, had symptoms of fever along with cough or sore throat within a specified period.

In our report, passive surveillance and direct reporting refer to school-initiated reporting of public health events to MDCHD. Direct reporting comprises public and private schools; the SBASS comprises public schools only.

The SBASS gave 61 red alerts, 28 yellow alerts, and 67 warnings during the study period (Table). After active investigation, 9 of 89 alerted schools were confirmed to have influenza outbreaks, and 71 persons with ILI were identified (Figure). Two of these 9 schools had simultaneously initiated reporting of outbreaks directly to MDCHD. Additionally, MDCHD received reports of suspected ILI activity from 24 public schools, none of which were confirmed outbreaks. Thus, 2 (8%) of 26 schools that directly reported to MDCHD had confirmed ILI outbreaks. Regardless of how ILI outbreaks were detected, all were investigated in accordance with EDC-IS protocol.

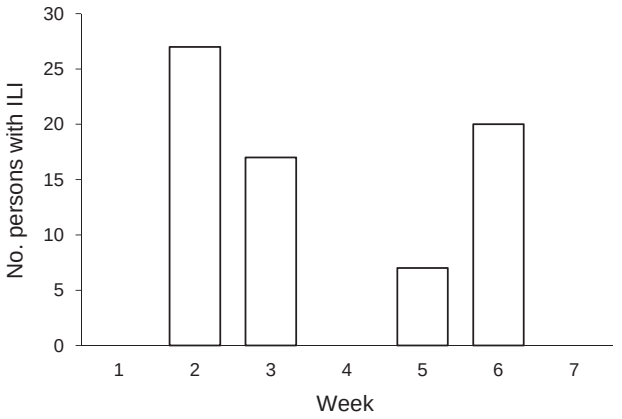

Figure. Epidemic curve of persons with influenza-like illness (ILI) identified through a school-based absentee surveillance system, Miami-Dade County Public Schools, Miami, Florida, USA, September 8–October 21, 2009.

Conclusions

The SBASS detected all influenza-related outbreaks among public schools and proved useful in conjunction with traditional surveillance methods. Pandemic (H1N1) 2009 was a novel disease with unknown implications; therefore, implementation of an aggressive surveillance approach was needed to better characterize and understand its public health effects, particularly among school-aged children. Schools are ideal settings for detecting influenza outbreaks, and the epidemiology of influenza has shown that children play an important role in the acquisition and spread of ILI (*6*).

As of July 13, 2009, a total of 90% of positive influenza specimens in Florida tested positive for pandemic (H1N1) 2009 virus. Subsequently, the Florida Department of Health declared that identified clusters of ILI were assumed to be pandemic (H1N1) 2009 (*7*).

The inherent design of the SBASS sets it apart from other school-absentee systems, which use only percentages to determine absentee rates. Major advantages of an SBASS include an ability to identify schools with higher than normal absenteeism. The system assesses absenteeism against a historic baseline for each school. Schools with consistently high levels alone did not trigger an alert; only schools with higher than normal levels generated alerts and required follow-up. Use of an SBASS has also helped in the development of stronger partnerships between MDCHD and the school system. Frequent communication increased public health awareness and emphasized the vital role schools play in preventing and controlling disease. Additionally, the SBASS geographic information system mapping feature enabled better detection of geographic clustering when multiple schools had alerts.

Limitations of an SBASS still include an inability to capture reasons for absenteeism and its exclusion of private school attendance information. Furthermore, manual entry on the part of schools' attendance offices may lead to a lag in data submission time, and data may contain typographical errors. Future studies should aim to extend the study

Table. Influenza-like illnesses identified through an SBASS, Miami-Dade County, Florida, USA, September 8–October 21, 2009*

Week	Dates	No. red alerts	No. yellow alerts	No. warning alerts	No. schools with outbreaks identified through SBASS	No. ILI identified through SBASS
1	Sep 8–Sep 11	3	2	17	0	0
2	Sep 14–Sep 18	8	2	16	1	27
3	Sep 21–Sep 25	9	11	10	2	17
4	Sep 28–Oct 2	9	4	7	0	0
5	Oct 5–Oct 9	16	4	11	2	7
6	Oct 12–Oct 16	16	5	6	1	20
7	Oct 19–Oct 21	0	0	0	3	0
Total†	Sep 8–Oct 21	61	28	67	9	71

*SBASS, school-based absentee surveillance system.
†3 d were excluded due to school closures. October 20–21, high schools were excluded for participation in Florida's Comprehensive Assessment Test and only elementary schools were counted.

period and compare influenza trends over multiple years. Research using an SBASS to detect other infectious disease outbreaks, not only in the event of a known source as was the case with pandemic (H1N1) 2009, should also be considered.

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New Avian Influenza Virus (H5N1) in Wild Birds, Qinghai, China

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Highly pathogenic avian influenza virus (H5N1) (QH09) was isolated from dead wild birds (3 species) in Qinghai, China, during May–June 2009. Phylogenetic and antigenic analyses showed that QH09 was clearly distinguishable from classical clade 2.2 viruses and belonged to clade 2.3.2.

In May 2005, highly pathogenic avian influenza (HPAI) virus (H5N1) caused a disease outbreak in wild birds in the Qinghai Lake region of the People's Republic of China (*1*). Subsequently, this virus (QH05, clade 2.2) disseminated from Asia to Europe and Africa, which has led to great concern and energetic debates about the role of migratory birds in influenza epidemics (*1*–*5*). In 2006, this virus was detected in migratory birds in Qinghai (*6*,*7*). In 2007, viruses similar to QH05 were isolated from surveyed anseriformes in Qinghai and showed only a short evolutionary distance from earlier viruses (*8*). Genetic diversity of avian influenza viruses (H5N1) was not detected in wild birds in Qinghai before 2008 (*7*,*8*). We report evidence that a second lineage of viruses, in addition to clade 2.2, has emerged in wild birds in Qinghai.

The Study

During May 8–June 15, 2009, a total of 273 wild birds died in the wetlands of Gengahai Lake, Qinghai Province, China (Figure 1) during an influenza outbreak. We obtained 224 great crested grebes (*Podiceps cristatus*), 16 great black-headed gulls (*Larus ichthyaetus*), 15 brown-headed gulls (*Larus brunnicephalus*), 9 bar-headed geese (*Anser indicus*), 5 ruddy shelducks (*Tadorna ferruginea*), 3 great cormorants (*Phalacrocorax carbo*), and 1 common coot (family *Rallidae*) (Figure 1).

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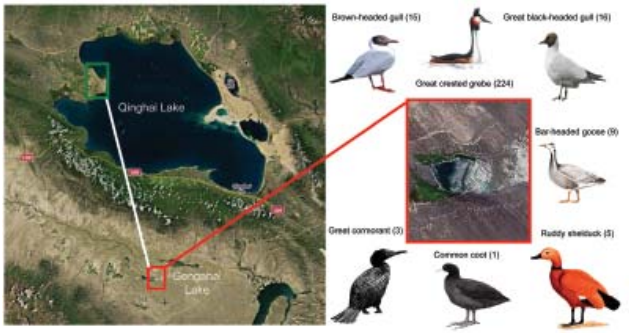

Figure 1. Location in Qinghai, China, of dead birds that were tested for avian influenza virus (H5N1), with images and common names of bird species tested. Red box indicates Gengahai Lake, where dead birds were detected, and green box indicates Bird Islet of Qinghai Lake; the distance between them is 90 km. Numbers of dead birds of each species are indicated in parentheses.

To determine the pathogenesis of this outbreak, we obtained organs, including lung and brain, and cloacal swabs from 13 birds at different times. Tissue samples were inoculated into 10-day-old, embryonated, specific pathogen–free eggs for virus isolation. Hemagglutinin and neuraminidase subtypes were determined as described (*9*). Eleven avian influenza viruses (H5N1) were isolated from 3 species of wild birds: 4 from great crested grebes, 5 from great black-headed gulls, and 2 from brown-headed gulls. Results of virus isolation for samples from a bar-headed goose and a shelduck were negative. Samples from great cormorants and a common coot were not obtained and tested.

We sequenced genomes of the 11 viruses and found that the viruses (QH09) were closely related and showed 100% homology at the nucleotide level. Sequences of the entire genome of QH09 (representative strain A/great crested grebe/Qinghai/1/09) are available in GenBank (accession nos. CY063315–CY063322).

Hemagglutinin of QH09 virus had a series of basic amino acids (PQRERRRKR) at the cleavage site. Neuraminidase of this virus had a deletion of 20 aa at residues 49–68 in the stalk region. No amino acid substitutions conferred resistance to adamantane or neuraminidase inhibitors. Unlike many isolates related to Qinghai Lake strains of clade 2.2, QH09 virus does not have an E627K substitution in basic polymerase. Nonstructural protein 1 had a deletion of 5 aa at residues 80–84, which is commonly observed in HPAI viruses (H5N1) that are circulating in Southeast Asia.

Seven gene segments of QH09, except for the acidic polymerase (PA) gene, showed the greatest homology with 2 clade 2.3.2 viruses, A/little egret/Hongkong/8863/07(H5N1) and A/whooper swan/Hokkaido/1/08(H5N1); identities were 97.0%–99.5%. The strain that showed the greatest

[1]These authors contributed equally to this article.

homology with QH09 PA was A/chicken/Yamaguchi/7/04 (H5N1), which was the precursor of the PA gene of the A/bar-headed goose/Qinghai/2/05 virus (*1*) (Table). Homology of the 8 gene segments of QH09 with QH05-like viruses was 92%–96.3%.

Phylogenetic analysis showed that 7 of 8 gene fragments of QH09, except for the PA gene, mapped with clade 2.3.2 viruses A/little egret/Hongkong/8863/07 and A/whooper swan/Hokkaido/1/08 and were in a different cluster than clade 2.2 viruses isolated in Qinghai during 2005–2007 (Figure 2, panel A). The PA gene of QH09 had the same lineage as A/chicken/Yamaguchi/7/04 (Figure 2, panel B).

Antigenic analyses by hemagglutination inhibition (HI) assay with chicken antiserum against a QH05 virus (A/bar-headed goose/Qinghai/3/05) (clade 2.2) and QH09 virus showed that the cross-reactive HI titer of QH09 virus (64) was 16-fold lower than that of homologous QH05 virus (1,024). The cross-reactive HI titer of QH05 virus (128) was also 16-fold lower than that of homologous QH09 virus (2,048).

Conclusions

Genetic and antigenic characterization of HPAI viruses (H5N1) from wild birds in Qinghai in 2009 suggests that these viruses are closely related to clade 2.3.2 and are clearly distinguishable from the classical QH05 clade 2.2. Previously reported QH05-like virus represented ≥4 genotypes, and no 2 QH05-like viruses were identical at the amino acid or nucleotide sequence levels (*1*,*6*). Although isolates of QH09 were obtained from 3 species at different times, 100% homology of the 11 isolates of QH09 was observed, which suggests that wild birds in Qinghai in 2009 were newly infected by 1 strain of virus.

Clade 2.3.2 viruses from wild nonpasserine bird species were reported in Hong Kong in 2007–2008 (*10*). The fact that a similar virus was isolated from a whooper swan (order Anseriformes) in Japan in 2008 showed that clade 2.3.2 was dispersed by migration of wild birds (*11*). Our results indicated that QH09 virus is a reassortant containing 7 gene segments of clade 2.3.2 viruses detected in wild birds

Table. Percentage homology of influenza viruses closely related to avian influenza virus (H5N1) QH09, Qinghai, China*

Gene	Virus	% Homology
HA	LE/Hong Kong/8863/07	97.1
NA	WS/Hokkaido/1/08	98.9
PB2	LE/Hong Kong/8863/07	98.7
PB1	WS/Hokkaido/1/08	98.8
PA	CK/Yamaguchi/7/04	97.2
NP	WS/Hokkaido/1/08	98.3
M	LE/Hong Kong/8883/07	98.9
NS	LE/Hong Kong/8863/07	99.5

*HA, hemagglutinin; NA, neuramindase; PB, basic polymerase; PA, acidic polymerase; NP, nucleoprotein; M, matrix; NS, nonstructural.

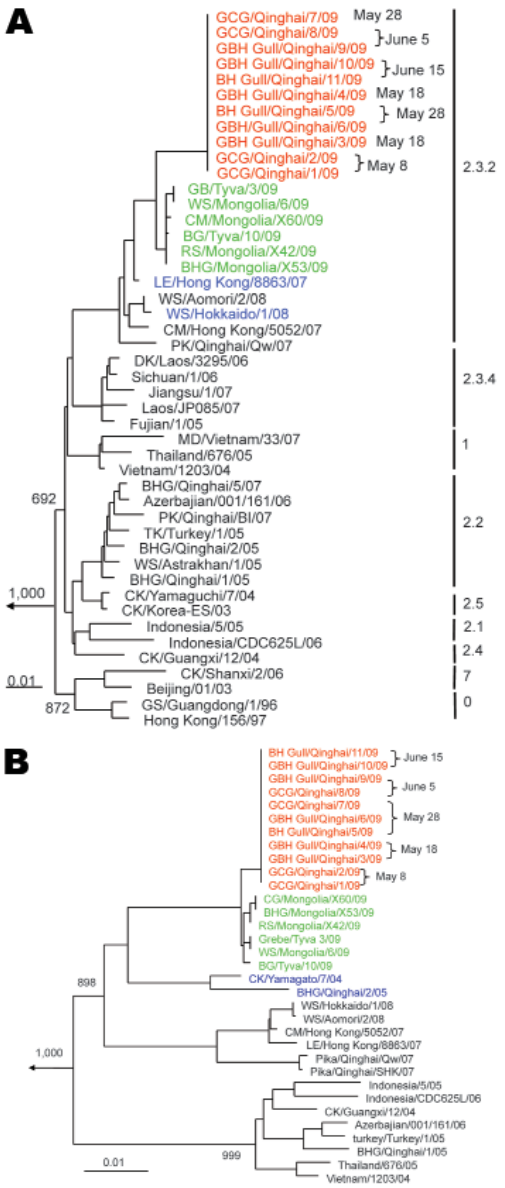

Figure 2. Phylogenetic trees of hemagglutinin genes (nt 29–1,728) (A) and acidic polymerase genes (nt 25–2,151) of avian influenza viruses (H5N1) (B). Clade numbers are indicated on the right in panel A. Trees were constructed by using the PHYLIP program of ClustalX software version 1.81 (www.clustal.org), the neighbor-joining algorithm, and rooted to A/chicken/Pennsylvania/1/83(H5N2). Bootstrap analysis was performed with 1,000 replications. Viruses obtained in this study are shown in red, previously detected viruses that are closely related to avian influenza virus (H5N1) QH09 are shown in blue, and closely related viruses that were detected after the Qinghai wild bird outbreak in 2009 are shown in green. Dates of virus isolation are shown. Scale bars indicate nucleotide substitutions per site. GCG, great crested grebe; GBH, great black-headed gull; BH, brown-headed gull; GB, grebe; WS, whooper swan; CM, common magpie; BG, bean goose; RS, ruddy shelduck; BHG, bar-headed goose; LE, little egret; PK, pike; DK, duck; MD, Muscovy duck; TK, turkey; CK, chicken; GS, goose; CG, common goldendye.

and the PA gene of CK/Yamaguchi/7/04-like virus, which contributed the PA gene to 1 QH05 virus (*1*).

Similar genotypes of QH09-like clade 2.3.2 viruses were also detected in great-crested grebes and black-headed gulls in Russia in 2009 (*12*). Bar-headed geese, whooper swans, and other anseriforme birds in Mongolia were infected with QH09-like clade 2.3.2 viruses (*13*). Therefore, QH09-like clade 2.3.2 virus is likely adapted to wild birds and is similar to clade 2.2 viruses, and its presence in Qinghai suggests that wild birds have spread this virus to other regions.

The possibility that wild birds in Qinghai in 2009 were infected by domestic fowl that harbored clade 2.3.2 virus is low because of the location of Qinghai Province and the scarcity of poultry in this region. In addition, no influenza outbreak in poultry occurred in this region in 2009. Therefore, it is likely that wild birds spread the virus.

How wild birds transmit HPAI virus (H5N1) is not clear. The ecology, epidemiology, genetics, and evolution of this virus are not fully understood. QH05-like clade 2.2 viruses and clade 2.3.2 viruses have been detected in wild pikas (*14*). However, whether the 2 virus clades have propagated in other mammalian hosts is unknown.

Qinghai Lake is located near multiple avian flyways. Although there are no reports of detection of clade 2.3.2 virus in wild birds near Qinghai Lake, the finding of clade 2.3.2 virus in the Gengahai wetlands of Qinghai increases concerns about a potential pandemic and the likelihood that avian influenza virus (H5N1) will again spread and increase its genetic diversity. Therefore, determining movements of wild migratory waterfowl from Qinghai Lake and their virologic status is needed to assess potential avian vectors of HPAI virus (H5N1).

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Blastomycosis in Man after Kinkajou Bite

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We report transmission of *Blastomyces dermatitidis* fungal infection from a pet kinkajou to a man. When treating a patient with a recalcitrant infection and a history of an animal bite, early and complete animal necropsy and consideration of nonbacterial etiologies are needed.

Blastomycosis is caused by inhalation of conidia of the dimorphic fungus *Blastomyces dermatitidis*. This fungus causes pneumonia; disseminated infection; or rarely, cutaneous disease through contact with a wound (*1*). It is endemic to southern, south-central, and midwestern states in the United States, particularly in areas bordering the Mississippi and Ohio Rivers (*2*). Outbreaks among humans have been linked to recreational activities near rivers or streams in disease-endemic areas (*3,4*).

Blastomycosis can also affect other mammals (*5*). Zoonotic transmission of blastomycosis is rare but has been reported in association with dog bites (*6,7*), cat scratches (*8*), and animal necropsies (*9*). We report zoonotic transmission of blastomycosis by a bite from a pet kinkajou.

The Study

On September 21, 2009, a 37-year-old male zoologist in Indianapolis, Indiana, visited his physician with a 3-day history of swelling and tenderness of the third digit of his right hand. He reported having been bitten on the affected finger on August 29 by his pet kinkajou. At the time, the animal was severely ill with respiratory signs and died shortly after biting the patient. The wound initially healed after treatment with antimicrobial ointment. At the physician visit, the patient was prescribed 2 weeks of doxycycline and amoxicillin/clavulanate and instructed to return if no improvement was noted.

On September 24, the patient returned with worsening pain. He was hospitalized the next day with fever (101.0°F), nausea, headache, and continued finger tenderness. Ascending lymphangitis and swollen, tender, axillary lymph nodes were noted. Except for his leukocyte count (15,100 cells/mL), laboratory values were within reference ranges. The patient was treated with intravenous vancomycin and ampicillin/sulbactam, wound incision, and drainage. Results of blood and wound cultures were negative. By September 27, the patient's fever and lymphangitis subsided. He was discharged and received amoxicillin/clavulanate and ciprofloxacin.

The next week the patient returned to the emergency department with erythematous nodules along his right basilic vein and recurrent ascending lymphadenitis. An infectious disease consultant noted fusiform swelling of the right middle finger, nodular erythematous fluctuant areas of the right wrist, swollen and tender axilla, and a nodular area on the right ankle. A punch biopsy sample from the right hand showed acute inflammation and suppurative granulomas of deep soft tissue. Initial stainings were negative for parasites, fungi, and acid-fast bacilli. Tissue and blood cultures for aerobic, anaerobic, acid-fast, and fungal organisms were negative, as were serologic results for *Bartonella* spp. and *Brucella* spp.

The patient was readmitted to a local hospital on October 12 with lesions on the right ankle and swelling of the left ankle. Results of complete blood count with differential, metabolic profile, and liver function studies were unremarkable; additional blood cultures were obtained. The patient was treated with azithromycin, ciprofloxacin, and streptomycin. Axilla aspirate was cultured. On October 16, the patient's condition improved, and he was discharged. Blood cultures obtained during hospitalization were negative. However, a mold was found growing in the axillary fungal culture.

Tissue from the punch biopsy sample and mold cultures were sent to the Indiana State Department of Public Health Laboratories, where *B. dermatitidis* was isolated from culture on October 21. The patient was prescribed itraconazole (200 mg 2×/d) for 6 months; he improved rapidly, and his infection resolved completely.

Before this illness, the patient had been healthy and had no recent history of travel or recent history of camping, digging, or gardening. None of the >60 other exotic animals he cared for had a history of *B. dermatitidis* infection.

Kinkajous (*Potos flavus*), which are native to South and Central America, are members of the family Procyonidae. *Leishmania* spp. (*10*), herpesvirus (*11*), and *Salmonella* spp. (*12*) have been reported in association with kinkajous, but only *Kingella potus* has been reported as a zoonotically transmitted infection resulting from a kinkajou bite (*13*).

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The kinkajou in this report was a 9-year-old wild-born female (birth location unknown) brought to the United States at ≈2 years of age by an animal facility in Texas. It was acquired by the patient in November 2008 from an educational organization in Chicago.

The kinkajou lived in a large walk-in enclosure in the patient's basement with its male cage mate, which never displayed similar illness and remains alive. The enclosure included plastic platforms and a nest box with regularly cleaned T-shirt bedding, hanging hammock-style fleece sleep bags, and wooden branches collected from outside that were treated regularly with a bleach scrub. The kinkajou's diet included fresh fruits, vegetables, and monkey crunch biscuits (Mazuri, Lincoln, NE, USA). It was not handled outside the home, did not roam outside its enclosure, and had no contact with other animals besides its cage mate. Its medical history was unremarkable. The patient reported that the kinkajou showed increased respiratory distress during the 3 days before its death.

The initial necropsy, performed in early September 2009, showed white lesions on the kinkajou's lungs, which suggested bacterial pneumonia. However, no bacterium was cultured. No histopathologic examinations for rabies were performed at that time. Immediately after necropsy, the carcass was frozen. After the patient's diagnosis of blastomycosis, the carcass was thawed, and lung and oral mucosal tissue samples were sent to the Centers for Disease Control and Prevention, Atlanta, GA, USA, for culture and molecular, histopathologic, and immunohistochemical (IHC) analysis.

The Centers for Disease Control and Prevention received tissue samples from the kinkajou and patient punch biopsy samples. Sections of skin from the patient showed extensive epidermal ulceration with superficial and deep perivascular inflammatory cell infiltrates comprising predominantly lymphocytes and macrophages (Figure 1). A fibrinopurulent exudate containing neutrophils, erythrocytes, and necrotic cellular debris tracked from the deep dermis to the edge of the ulcer. Large, ovoid yeast cells with double-contoured walls, ≈10–15 μm, were identified in this exudate by using the Grocott methenamine silver staining technique and IHC for *B. dermatitidis*. Polyclonal antibody (Meridian Diagnostics, Cincinnati, OH, USA) used in this assay is broadly reactive with multiple fungal species, including *B. dermatitidis*, making it useful for detection, but not speciation, of yeasts in tissues.

Kinkajou lung tissue was processed for culture and histopathologic and molecular analysis. Staining of lung with hematoxylin and eosin (Figure 2) showed numerous intraalveolar yeasts with double-contoured walls diffusely filling alveolar spaces and associated with inflammatory cell infiltrates (macrophages, lymphocytes, and plasma cells). Yeasts were also closely associated with surfaces of the tongue, palate, and buccal mucosae. Use of the Gomori methenamine silver staining technique and IHC for *B. dermatitidis* showed many yeasts in the lungs and fewer in the liver and on epithelial surfaces of the oral cavity.

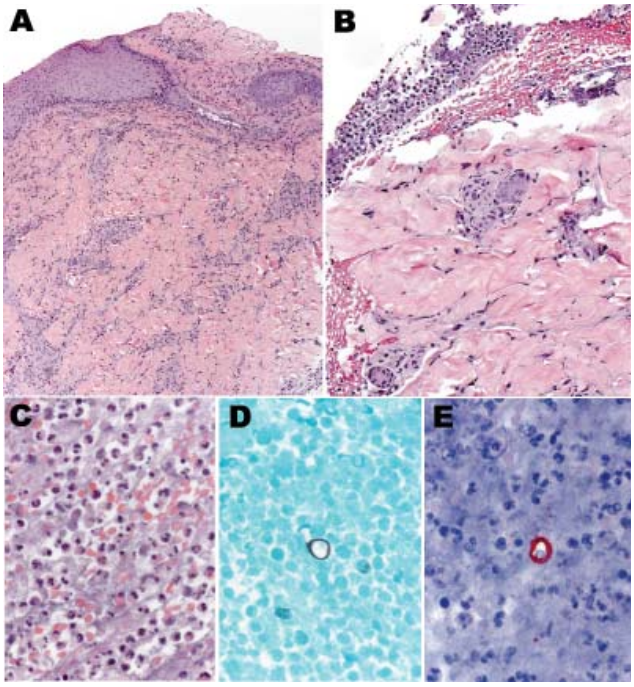

Figure 1. Histologic appearance of the cutaneous lesion of a man with blastomycosis. Ulcerated epidermis (A) showing superficial and deep perivascular infiltrates, predominantly mononuclear inflammatory cells. Fibrinopurulent exudate (B) adjacent to the ulcer, comprising neutrophils, erythrocytes, and necrotic cellular debris (C), and occasional large yeasts morphologically compatible with *Blastomyces dermatitidis* infection (D and E). Hematoxylin and eosin stain (A, B, and C), Grocott methenamine silver stain (D), and immunoalkaline phosphatase with antibody against *B. dermatitidis* and naphthol fast red with hematoxylin counterstain (E). Original magnifications ×12.5 (A), ×25 (B), and ×100 (C–E).

DNA was amplified from patient tissue and kinkajou lung by using non-nested PCR and primers BlastoI and BlastoII as described (*14*). Pairwise sequence alignment showed that the *B. dermatitidis* BAD-1 promoter region sequences from the patient isolate and the kinkajou tissue were indistinguishable.

Conclusions

The successive timing of the kinkajou's illness and the patient's symptom onset suggests that the source of the patient's infection was the kinkajou bite. Because asymptomatic animal infections are not known to occur (*15*), we believe that the kinkajou likely acquired the infection while living with the patient.

Immediate necropsy and histopathologic analysis should be conducted for any animal that bites a human and

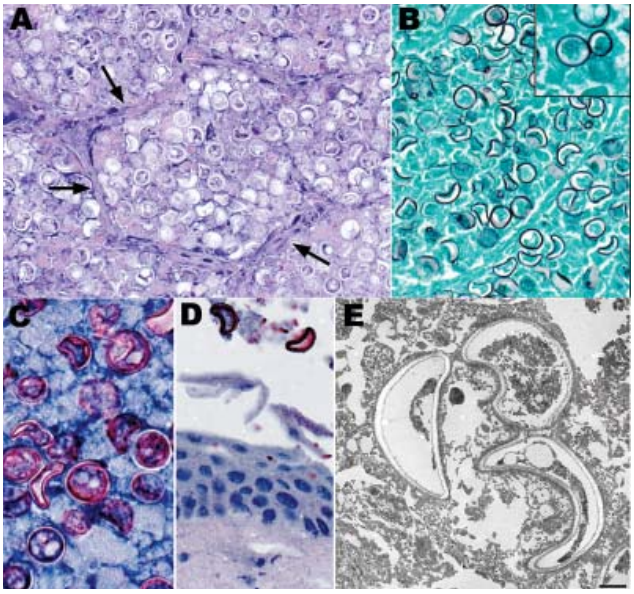

Figure 2. Histopathologic and electron microscopic appearance of *Blastomyces dermatitidis* in kinkajou (*Potos flavus*) tissues. A) Lung showing *B. dermatitidis* yeast forms filling alveolar spaces. Alveolar septa are indicated by arrows. B) Lung showing yeast forms of *B. dermatitidis*. Inset shows broad-based budding of a yeast form, a major diagnostic feature. C) Lung showing *B. dermatitidis* yeast. D) Oral mucosa showing 2 yeast forms of *B. dermatitidis* closely associated with the mucosal surface. E) Transmission electron micrograph showing 3 yeast forms of *B. dermatitidis* in lung tissue. Note the thick cell walls and crescent shapes of the yeast (scale bar = 2 μm). Hematoxylin and eosin stain (A), Grocott methenamine silver stain (B and inset), and immunoalkaline phosphatase with antibody against *B. dermatitidis* and naphthol fast-red with hematoxylin counterstain (C, D). Original magnifications ×400 (A, B, D) and ×630 (Inset, C).

then dies, particularly when a lesion develops at the bite site. This report emphasizes the need for early and complete animal necropsy and consideration of nonbacterial etiologies when treating a patient with a recalcitrant infection and a history of an animal bite.

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Novel HIV-1 Recombinant Forms in Antenatal Cohort, Montreal, Quebec, Canada

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Near full-length genomes of 4 unclassified HIV-1 variants infecting patients enrolled in an antenatal cohort in Canada were obtained by sequencing. All 4 variants showed original recombination profiles, including A1/A2/J, A1/D, and A1/G/J/CRF11_cpx structures. Identification of these variants highlights the growing prevalence of unique recombinant forms of HIV-1 in North America.

HIV-1 displays extensive genetic diversity. Group M includes 9 subtypes and >45 circulating recombinant forms (CRFs) (*1*). In western and central Africa, where the highest levels of HIV-1 genetic heterogeneity are observed, most subtypes cocirculate along with CRFs and unique recombinant forms (URFs). This diversity may complicate diagnosis and treatment of HIV infection and represents a challenge for vaccine design. In North America, the HIV-1 epidemic is dominated by subtype B; non-B subtypes are infrequently reported (*2*,*3*). Nonetheless, recent studies have shown a growing prevalence of non-B variants (*4*,*5*). In 2005, Akouamba et al. reported high levels of HIV-1 genetic diversity among participants in the Centre Maternel et Infantile sur le SIDA (CMIS) antenatal cohort of Centre Hospitalier Universitaire (CHU) Sainte-Justine, Montreal, Canada (*6*). Of these patients, 44 of 103 were infected with non-B subtypes, including 4 variants that failed to group within known subtypes in phylogenetic analyses (*6*). We performed near full-length genomic sequencing to characterize these 4 unassigned variants.

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The Study

All 4 patients were newcomers to Canada from sub-Saharan Africa who received prenatal care at CMIS during 1999–2003 (*6*). Patient TV721 emigrated from the Democratic Republic of Congo, TV749 from Congo, and TV725 and TV919 from Rwanda. HIV-1 viral loads at study entry, measured by the Versant HIV-1 RNA 3.0 assay (bDNA) (Bayer, Pittsburgh, PA, USA), with a limit of detection of 50 RNA copies/mL plasma, were 164–23,369 RNA copies/mL plasma. CD4+ T cell counts ranged from198 cells/mm^3 to 816 cells/mm^3 (Table). Standardized clinical follow-up, including antiretroviral prophylaxis and treatment, was provided to all women and their children. This study was conducted according to the guidelines of the Ethics Review Board of CHU Sainte-Justine.

Viral RNA was extracted from serum and amplified by using custom-designed primers and the QIAGEN OneStep reverse transcription–PCR (QIAGEN, Mississauga, ON, Canada) (sequences available on request). For each isolate, 14–20 amplicons spanning the complete genome were generated and subcloned into pCR 2.1 TOPO (Invitrogen, Carlsbad, CA, USA). For each amplicon, we sequenced 3–10 independent clones (Beckman-Coulter, Palo Alto, CA, USA). Chromatograms were edited with Chromas version 1.45 (Technelysium, Southport, Queensland, Australia). Overlapping segments were aligned by using ClustalX version 1.81 (*7*) and assembled manually. Consensus sequences were generated by selecting the most frequent nucleotide at each position. We performed bootscan analyses according to the neighbor-joining method and Kimura 2-parameter distances using a 300-nt window and 10-nt increments (Simplot 3.5.1) (*8*). These parameters allow accurate localization of recombination breakpoints in HIV-1 recombinants (*9*). We computed phylogenetic reconstructions based on the neighbor-joining method and Kimura 2-parameter distances by using MEGA4 (*10*) to confirm recombinant structures. Bootstrap values >80% were considered significant.

Complete HIV-1 genomic sequences were obtained from patients TV721 (9,794 nt) and TV749 (9,791 nt). Genomic coverage of 79.3% and 91.6% was achieved for patients TV725 (7,763 nt) and TV919 (8,905 nt), respectively. On the basis of HXB2 numbering (*1*), missing regions were located between positions 545–1411 and between 6946–7930 for patient TV725, and between positions 7138 and 7834 for patient TV919. Screening of HIV-1 genomic sequences from patient TV721 with the HIVdb Genotypic Resistance Interpretation Algorithm (http://hivdb6.stanford.edu/asi/deployed/HIVdb.html) showed minor resistance mutations to protease inhibitors (L10I) and integrase inhibitors (I203M). Mutations associated with minor resistance to protease inhibitors (L10I) and non-nucleoside reverse transcription inhibitors (E138A) were detected in

Table. Virologic and immunologic parameters in patients participating in antenatal cohort, Canada*

Patient	Age, y	CD4 count, cells/mm^3	Viral load, RNA copies/mL	Antiretroviral treatment
TV721	33.2	210	164	AZT-3TC-NVP
TV725	30.1	816	739	None
TV749	34.1	198	23,369	AZT-3TC-NFV
TV919	34.0	420	1,910	AZT-3TC-NFV

*AZT, zidovudine; 3TC, lamivudine; NVP, nevirapine; NFV, nelfinavir.

sequences from patient TV919, and a mutation conferring high-level resistance to delavirdine (P236L) was detected in patient TV725. In contrast, sequences from patient TV749 did not show mutations associated with resistance to antiretroviral agents (*11*). Previous subtyping, based on phylogenetic analyses of *pol* gene sequences, showed that sequences from patients TV721 and TV749 grouped together (100% bootstrap) but only loosely with clade J references (61% bootstrap); sequences from patients TV725 and TV919 grouped outside all major clades (*6*). Bootscan analysis showed complex recombinant structures for all 4 full-length or near full-length genomes (Figure). Sequences

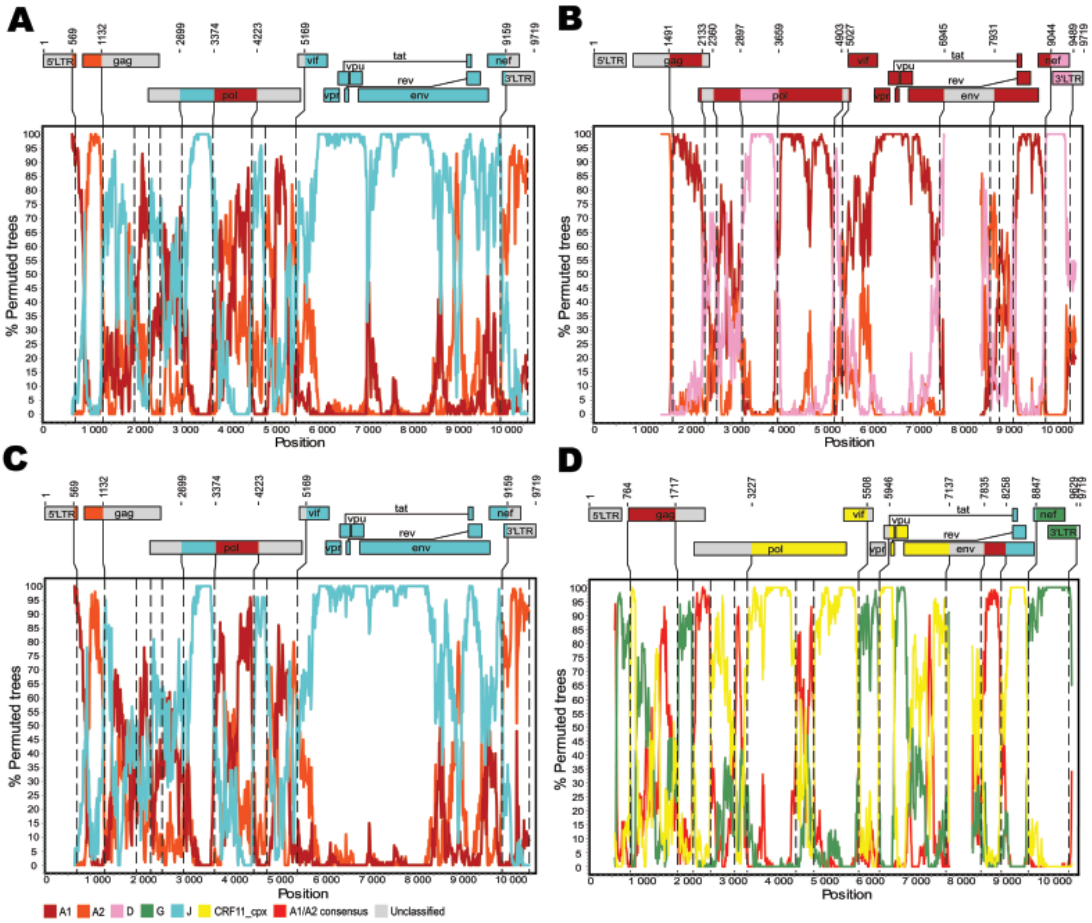

Figure. Genetic organization and recombination breakpoints in HIV-1 genomic sequences isolated from patients TV721 (A), TV725 (B), TV749 (C), and TV919 (D). Nucleotide sequences were submitted to GenBank (accession nos. HM215249–HM215252). Similarity plots were produced with Simplot version 3.5.1 (http://sray.med.som.jhmi.edu/SCRoftware/simplot) by using windows of 500 nt and increments of 50 nt to guide the choice of reference sequences used for bootscanning (*8*). Bootscan analyses were then performed according to the neighbor-joining method and Kimura 2-parameter distances. The size of the sliding window was set at 300 nt with 10-nt increments (*9*). Reference sequences used were subtype A1: A1.AU.03 (DQ676872), A1.KE.94 (AF004885), A1.RW.92 (AB253421); subtype A2: A2.CD.97 (AF286238), A2.CY.94 (AF286237); subtype D: D.CD.83.ELI (K03454), D.CM.01 (AY371157), D.TZ.01 (AY253311); subtype G: G.BE.96 (AF084936), G.KE.93 (AF061641), G.NG.92 (U88826); subtype J: J.CD.97 (EF614151), J.SE.94 (AF082394), J.SE.93 (AF082395); and CRF11_cpx: 11_cpx.CM.95 (AF492624). Phylogenetic reconstructions based on the neighbor-joining method and the Kimura 2-parameter distance model were computed by MEGA4 (*10*) and used to confirm the structures of the recombinants. Bootstrap values >80% (500 replicates) were considered significant. Vertical dashed lines indicate the position of recombination breakpoints. Numbering of residues is based on the sequence of HIV-1 HXB2 (GenBank accession no. K03455).

derived from patients TV721 and TV749 comprised regions from subtype J but were also similar to subtypes A1 and A2 (Figure). Examination of the homology, position, and sharing of recombination breakpoints suggests that HIV-1 isolates infecting patients TV721 and TV749 may be closely related recombinants, perhaps resulting from common-source transmission or representing a novel CRF or URF. Reciprocal bootscan analyses that included subtypes A1, A2, and J supported this assessment (data not shown). However, a review of the medical files and case histories of patients TV721 and TV749 did not confirm epidemiologic relatedness. Although multiple CRFs and URFs contain segments related to subtypes A and J, sequences from patients TV721 and TV749 are 2 of only 3 full-length HIV-1 genomes reported that exclusively comprise sequences related to subtypes A and J (*12*). The genomic structure of 98BW21.17 resembles that of sequences from patients TV721 and TV749 in terms of chimerism, but the location of recombination breakpoints is distinct (data not shown).

Sequences from patients TV725 and TV919 grouped outside all major clades in phylogenetic analyses of *pol* gene sequences (*6*). Bootscan analysis of the HIV-1 strain infecting patient TV725 clearly demonstrated that this variant comprised segments most closely related to subtypes A1 and D (Figure). Sequences from patient TV725 display a recombination pattern resembling that of CRF35_AD, the only other A1/D intersubtype recombinant described, which was recently identified in Kabul, Afghanistan (*13*). CRF35_AD and TV725 share A1 backbones, and recombination points bordering clade D segments are comparatively close (positions 2166–2444 for CRF35_AD and 2133–2360 for the isolate infecting patient TV725; positions 2901–3538 for CFR35_AD and 2897–3659 for TV725). However, they differ with respect to a clade D–related segment at positions 9044–9489 in TV725 (Figure). Finally, analysis of TV919 sequences showed complex A1/G/J mosaicism and similarities with CRF11_cpx, in terms of clade composition and localization of recombination breakpoints (*14*). Including the CRF11_cpx reference sequence 95CM1816 in bootscan analysis highlighted the similarities between sequences from patient TV919 and CRF11_cpx (Figure), which extends from positions 3227 to 7137 and includes segments corresponding to subtypes A1, G, and J. The most distinctive difference between the isolate infecting patient TV919 and CRF11_cpx started at position 7835; the former sequentially clustered with subtypes A1, J, and G, CRF11_cpx closely associated with subtypes J, A1, and E. Thus, the isolate infecting patient TV919 represents a novel A/G/J/CRF11_cpx recombinant. The HIV-1 isolates that infected patients TV721, TV725, TV749, and TV919 had more recombination breakpoints and unclassified regions than most CRFs and URFs, highlighting their unique recombination profiles and structural complexity.

Conclusions

We identified novel HIV-1 recombinants infecting pregnant women in Montreal. None of the 4 patients transmitted HIV-1 to their children. No evidence was found that these particular variants currently circulate within the Canadian population. Thus, the HIV-1 isolates infecting patients TV721, TV725, TV749, and TV919 must be construed as URFs. In North America, only 1 URF, also isolated in Montreal, was characterized by near full-length genomic sequencing (*5*). Given the ongoing movement of the population from areas where the disease is endemic into regions in which subtype B predominates, reports of novel HIV-1 recombinants are likely to increase and include complex mosaic genomes. Biological properties of recombinant subtypes might differ from those of other clades, particularly in terms of HIV disease progression (*15*) and drug resistance. In terms of public health, antenatal cohorts represent unique sentinel sites to monitor the emergence of novel HIV-1 variants, including complex mosaic recombinants, in countries where their prevalence is low.

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Eschar-associated Spotted Fever Rickettsiosis, Bahia, Brazil

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In Brazil, Brazilian spotted fever was once considered the only tick-borne rickettsial disease. We report eschar-associated rickettsial disease that occurred after a tick bite. The etiologic agent is most related to *Rickettsia parkeri, R. africae, and R. sibirica* and probably widely distributed from São Paulo to Bahia in the Atlantic Forest.

Brazilian spotted fever (BSF), caused by *Rickettsia rickettsii*, was at one time considered the only tick-borne rickettsial disease in Brazil (*1*). Its transmission in 5 southern states is primarily associated with *Amblyomma cajennense, A. aureolatum*, and *Rhipicephalus sanguineus* ticks; however, many other rickettsiae of unknown pathogenicity are carried by ticks in Brazil (*1,2*). We describe an eschar-associated rickettsiosis in a traveler from the state of Bahia, Brazil; this disease seems to have been caused by the same *Rickettsia* sp. that caused a similar disease in São Paulo in 2009 (*3*).

The Case

In April 2007, a 30-year-old man from Bahia sought care for a 6-day febrile illness that began 9 days after he found a tick attached to his right wrist while hiking and camping in the Chapada Diamantina National Park in Paty Valley (12°48′26″S, 41°19′53″W), a semiarid region in Bahia. Primary signs and symptoms were fever (39–40°C), severe myalgia, and swelling and pain at the site of the tick bite. Two days after onset of illness, the man noticed a scab forming on his right wrist and painful swelling in his right axillary region, followed 2 days later by a generalized rash and painful ulcerative lesions in the mouth. The patient sought medical care, and an outpatient physician prescribed acetaminophen and cefadroxil, which did not reduce symptoms.

On day 6 of his illness, the patient sought care from an infectious disease specialist, who noted a 2.5-cm eschar on the patient's wrist (Figure 1, panel A); disseminated papular rash on his face, trunk, and upper extremities (Figure 1, panel B); and several small erosions on his tongue, buccal mucosa, and lips (Figure 1, panels C, D). The mucosal erosions were painful, and some skin papules formed small pustules (Figure 1, panel E). In the right axilla was a tender, enlarged, 3-cm lymph node. Results of a hemogram and blood biochemistry were unremarkable except for a high level (425 U/L) of lactic dehydrogenase. A rickettsial disease was considered, and the patient was given doxycycline (100 mg 2×/d) for 14 days. The fever and generalized rash resolved within 2 days, and the eschar healed completely within 2 weeks after initiation of therapy.

Acute-phase and convalescent-phase serum samples were evaluated by microimmunofluorescence assay for antibodies to spotted fever group rickettsiae (SFGR) (*4*). Before antimicrobial drug therapy was started, biopsy specimens of the papule and the scab from the eschar were collected, preserved in 10% formol, and evaluated by routine histopathology, immunohistochemical staining, and PCR (*4,5*).

Serum collected on day 6 of the illness was nonreactive with *R. rickettsii* and *R. parkeri* antigens (class-specific immunoglobulin G [Ig] and IgM <32 for both assays, cutoff ≥64). Subsequent testing determined IgG/IgM titers on day 12 to be 128/<32 against *R. parkeri* and 128/32 against *R. rickettsii* antigens and on day 19 to be 128/64 and 512/32, respectively.

Hematoxylin and eosin–stained sections of the papule biopsy specimen demonstrated lymphohistiocytic perivascular inflammatory cell infiltrates in the superficial to middle dermal layers. Immunohistochemical staining for SFGR showed rare antigens in a few small foci of perivascular inflammation.

The sequences for *omp*A (632-bp, GenBank accession no. GQ853063) from the scab and *glt*A (382-bp, GenBank accession no. GQ900666) from the papule specimen each had 100% identity to homologous gene sequences of SFGR detected recently in an eschar specimen from a patient from Peruibe, São Paulo (*3*). The sequences from both organisms were most related to SFGR strain S previously reported from Armenia (*6*) but were not identical to *R. sibirica*, *R. parkeri*, and *R. africae* (Figure 2). The nucleotide sequence of a 928-bp *sca*4 fragment (GenBank accession no.

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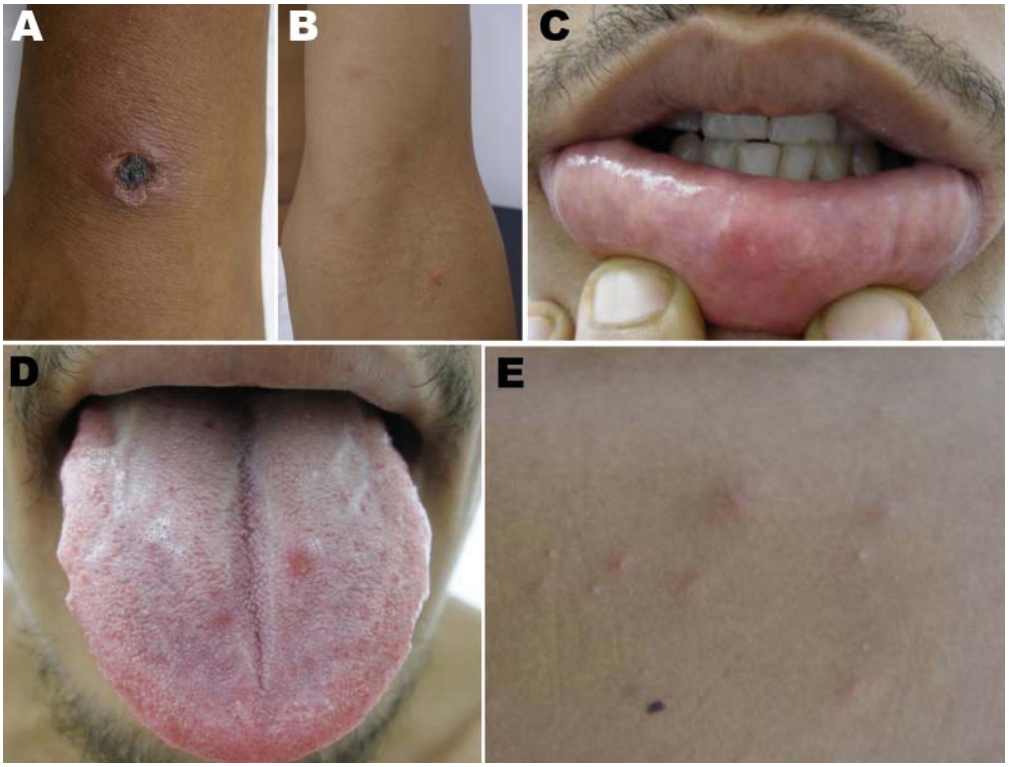

Figure 1. Lesions on day 6 of illness of patient with eschar-associated rickettsial disease, Bahia, Brazil, 2007. A) Eschar on right wrist; B) papular skin rash on left elbow; C) ulcerated lesion on lower lip; D) erosions on tongue mucosa; E) vesicular papular lesions on trunk.

GQ853064) had 99% identity to the homologous fragment of *R. parkeri* (GenBank accession no. AF155059), and the conserved 17-kDa protein gene amplicon (GenBank accession no. GQ853062) was similar to those of many SFGR.

Conclusions

During the past decade, many newly identified tick-borne rickettsiae from South America have been described (*1*,*2*), including *R. parkeri*, *R. massiliae*, *R. amblyommii*, *R. bellii*, and other *Rickettsia* spp. of unknown pathogenicity. We describe another confirmed case of a novel eschar-associated SFGR disease in Brazil.

Development of an eschar is a characteristic manifestation of rickettsioses caused by *R. parkeri,* 364D *Rickettsia*, and *R. massiliae* (*4*,*7*). Possible eschar formation in association with Rocky Mountain spotted fever has been reported (*8*), but this manifestation does not seem to be a hallmark of disease caused by *R. rickettsii* or of other rickettsioses in Brazil and South America (*2*). BSF has been most often confirmed solely by serologic testing; however, atypical clinical manifestations, including eschar formation and lymphadenopathy, have been described (*9*–*12*). Lymphadenopathy and ulcers on the oral mucosa, as found for this patient, have been found in patients with rickettsiosis caused by *R. parkeri* and African tick bite fever (caused by *R. africae*) (*4*,*13*) but not in the index case-patient from São Paulo (*3*), who seemed to have less severe clinical manifestations than the patient described in this report.

In the scientific literature from Brazil, the earliest reference to an eschar in a suspected case of BSF was in 1932 (*12*). Subsequent eschar-associated cases have been identified in regions where BSF is endemic (e.g., the states of Minas Gerais, Rio de Janeiro, and Espirito Santo) (*9*–*11*) and in regions where it is not endemic (e.g., states of Santa Catarina, situated along the Argentina border, and Bahia [*14*], where the case reported in this article occurred). Furthermore, clinical descriptions of eschar-associated rickettsioses in Brazil have been reported from BSF-endemic areas with large populations of *A. dubitatum* ticks but no known *A. triste* ticks, which are recognized vectors of *R. parkeri* in southern Brazil (*15*). Although *A. dubitatum*, a human biting tick that is highly prevalent in many BSF-endemic areas (*2*), is a potential candidate for transmission of *R. parkeri* to humans in Brazil, this tick species and its vertebrate hosts, capybaras, have not yet been described in the Paty Valley, Bahia, where the patient acquired the rickettsial infection. Unfortunately, the ticks causing both cases in São Paulo and Bahia were not available for identification.

The taxonomic status of the etiologic agent of this novel rickettsiosis in Brazil cannot be definitively determined until it is isolated. On the basis of the available genetic information presented here and elsewhere (*3*), the pathogen detected in the cutaneous lesion of the patients from Bahia and São Paulo is equally distant from *R. africae*, *R. parkeri*, and *R. sibirica*. Each of these 3 SFGR is

among species long accepted by International Committee of Systematics of Prokaryotes, and this status is consistent with their long evolutionary divergence and differences in their vectors and geographic distributions. Molecular confirmation can and must therefore be used to identify new rickettsial agents because they cannot be identified by clinical case presentations or serologic analyses. Additional efforts will be required to establish the full genetic diversity and range of tick and animal reservoirs of SFGR in Brazil and to determine the prevalence and clinical presentations of different rickettsioses in humans. Clinicians should be alert for tick-borne infectious diseases resulting from ecotourism activities, especially in parks and ecologic reserves in the areas of the Atlantic Forest and other areas of Brazil where many rickettsiae-infected ticks have been identified and most BSF cases have been reported.

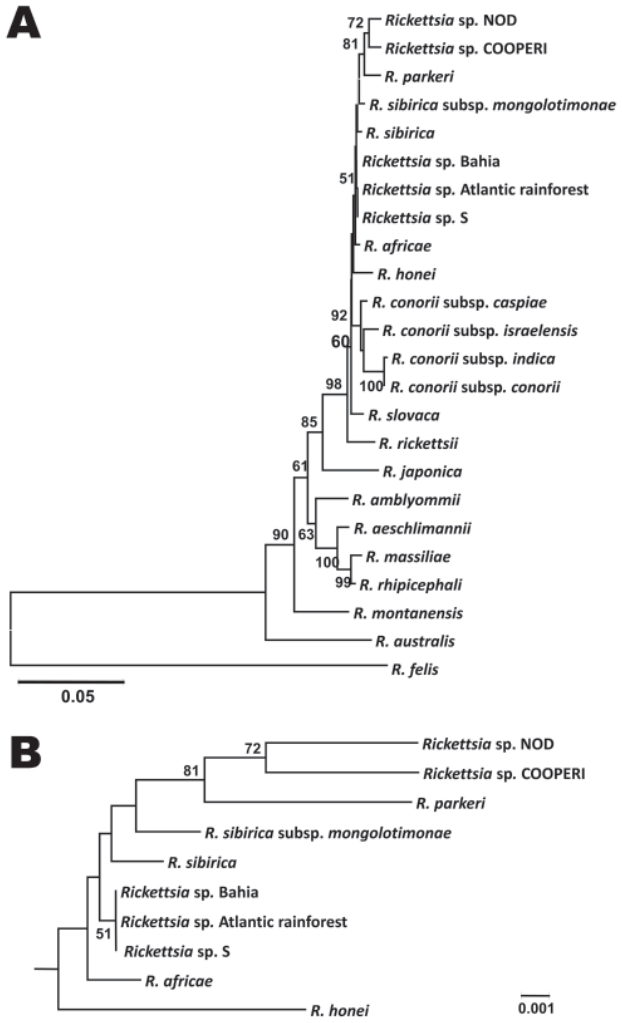

Figure 2. Genetic relationships of the spotted fever group rickettsiae (SFGR) detected in tissue of patient with eschar-associated rickettsial disease, Bahia, Brazil, 2007. Sequence comparison was conducted with MEGA version 4 (www.megasoftware.net). The phylogenetic optimal tree was inferred by using the neighbor-joining method, and distances were evaluated by implementing the Kimura 2-parameter model of substitution (sum of branch length = 0.58588522). In total, 323 nt sites of *glt*A and 401 nt sites of *omp*A were concatenated and evaluated; primer sequences and sites containing gaps and deletions were excluded from the analysis. Statistical reliability of the tree is based on 1,000 bootstrap replicates; only bootstrap values >50 are shown above the branches. The corresponding sequences of reference species and isolates were obtained from the National Center for Biotechnology Information GenBank database. A) Genetic association of *Rickettsia* sp. Bahia and other previously characterized SFGR; B) expanded tree of relationships among new SFGR to *R. africae*, *R. parkeri*, *R. sibirica*, *Rickettsia* sp. S and Atlantic Forest. Scale bars indicate nucleotide substitutions per site.

Addendum

Since submission of this article, recent investigation in Brazil has identified *A. ovale* ticks as potential vectors for the spotted fever group *Rickettsia* sp. described here (*16*).

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Pandemic (H1N1) 2009–associated Pneumonia in Children, Japan

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To describe clinical aspects of pandemic (H1N1) 2009 virus–associated pneumonia in children, we studied 80 such children, including 17 (21%) with complications, who were admitted to 5 hospitals in Japan during August–November 2009 after a mean of 2.9 symptomatic days. All enrolled patients recovered (median hospitalization 6 days). Timely access to hospitals may have contributed to favorable outcomes.

We describe the clinical aspects of pandemic (H1N1) 2009 virus infection in children who developed spontaneous pneumomediastinum (*1*) or plastic bronchitis (*2*). In Mexico, 18 persons, including 5 children, had pandemic (H1N1) 2009–associated pneumonia (*3*). However, active surveillance to collect data on pneumonia cases among children infected with pandemic (H1N1) 2009 virus has not been conducted in Japan.

The Study

Active procurement of specimens from pediatric inpatients with pandemic (H1N1) 2009–associated pneumonia was organized by the Laboratory of Molecular Epidemiology for Infectious Agents at Kitasato University. Clinical data and respiratory specimens were provided by pediatric departments at 5 institutions during August 9–November 6, 2009. Pandemic (H1N1) 2009–associated pneumonia was diagnosed from influenza-like illnesses associated with infiltrates on chest radiographs and laboratory-confirmed pandemic (H1N1) 2009 virus (*3*). Each patient's pediatrician informed us of any major complication that followed the pneumonia.

First, patients were divided into 2 groups: those who had and did not have complications. The group having no complications then was divided into 2 age-defined subgroups (cutoff, 6 years). Each subgroup was further divided into subgroups: hospital admission 1–3 days after symptom onset or admission >4 days after symptom onset. Information about clinical features; routine laboratory findings at hospital admission; and if available, serum immunoglobulin E concentration was obtained from patients' medical charts. Tachypnea was defined by using criteria in Japanese guidelines adopted in 2007 for managing respiratory infectious diseases (*4*) in children. Chest radiographic findings taken at time of hospital admission were classified by extent of pulmonary infiltrates (localized vs. diffuse) and infiltrate distribution (bilateral vs. unilateral; upper, middle, or lower lung field) (*4*).

Nasopharyngeal swabs (n = 79) or an endotracheal aspirate were sent to the laboratory for microbiologic identification. Pandemic (H1N1) 2009 virus in specimens was determined by real-time reverse transcription–PCR (RT-PCR) (*1*,*2*). Additionally, comprehensive real-time RT-PCR was performed to confirm respiratory co-infection with any of 12 viruses (*5*). Multiplex real-time PCR also was performed to detect 6 respiratory bacteria (*6*).

Patient demographic characteristics, symptoms, physical findings, treatments, and clinical courses were compared between groups with and without complications by using the χ^2 test. Neutrophil and lymphocyte counts were analyzed by using box-and-whisker plots. A p value <0.05 indicated a significant difference between patient groups.

The study comprised 80 pediatric inpatients who received treatment at 5 medical institutions for pandemic (H1N1) 2009–associated pneumonia over a 3-month period. Family members were informed about the purpose of the study, and children's parents provided informed consent.

We compared patients by presence or absence of complications (Table 1). Complications included pleural effusion (5 patients), pneumomediastinum (6), atelectasis (6), myositis (2), and plastic bronchitis (1). No patients had organ dysfunction or encephalopathy.

The median age of pneumonia patients was 7 years; 57 (71%) were male; 26 (33%) had asthma, 4 (5%) had atopic dermatitis without asthma, and 1 (1%) had DiGeorge syndrome. Forty-nine (61%) patients were previously healthy. Mean time from onset of illness to admission was 2.9 days; 61 (76%) patients were admitted early to the hospital (within 3 days after symptom onset). Respiratory distress, inspiratory retraction, and low percutaneous oxygen saturation (≤93% while breathing room air) were significantly

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Table 1. Demographic characteristics and clinical features of children hospitalized with pandemic (H1N1) 2009–associated pneumonia, Japan, August–November 2009*

Variable	Total	Group A, no complications, n = 63	Group B, complications, n = 17	p value, A vs. B
Sex, M/F	57 (71.3)/23 (28.8)	46 (73.0)/17 (27.0)	11 (64.7)/6 (35.3)	0.71
Median age, y (range)	7 y (9 mo–14y)	7 y (9 mo–14 y)	6 y (4 y–12 y)	0.41
≤1 y	4 (5)	4 (6.3)	0	
2–5 y	14 (17.5)	11 (17.5)	3 (17.6)	
≥6 y	62 (77.5)	48 (76.2)	14 (82.3)	
History of asthma	26 (32.5)	21 (33.3)	5 (29.4)	0.76
Admission ≤3 d/≥4 d after symptom onset	61 (76.3)/19 (23.8)	45 (71.4)/18 (28.6)	16 (94.1)/1 (5.9)	0.10
Major symptoms, physical findings				
Cough	66 (82.5)	53 (84.1)	13 (76.5)	0.71
Respiratory distress	29 (36.3)	17 (27.0)	12 (70.6)	<0.01
Fever ≥38°C	74 (92.5)	58 (92.1)	16 (94.1)	0.53
Tachypnea	57 (71.3)	42 (66.6)	15 (88.2)	0.15
Inspiratory retraction	39 (48.8)	27 (42.9)	12 (70.6)	0.04
Rhonchi	48 (60.0)	40 (63.5)	8 (47.1)	0.22
SpO_2 ≤93	39 (48.8)	25 (39.7)	14 (82.4)	<0.01
Treatment and clinical course				
O_2 supplementation	49 (61.3)	34 (54.0)	15 (88.2)	0.01
Mean duration of O_2 administration, d (range)	3.5 (1–11)	2.9 (1–6)	4.7 (1–11)	0.02
Treatment with oseltamivir	67 (83.8)	51 (80.9)	16 (94.1)	0.35
Treatment with antimicrobial drugs†	63 (78.8)	46 (73.0)	17 (100)	0.04
Isoproterenol inhalation	6 (7.5)	0	6 (35.3)	<0.01
Median duration of hospitalization, d (range)	6 (3–18)	6 (3–9)	8 (5–18)	<0.01

*Values are no. (%) except as indicated. SpO_2, percutaneous oxygen saturation while breathing room air.
†Parenteral infusion of sulbuctum/ampicillin or cefazoin was carried out for 3–5 d.

more frequent among patients with than without complications ($p<0.01$).

Infiltrates were more often localized (64 patients) than diffuse (16 patients). Unilateral localized infiltrates occurred more commonly in a lower lung field than in upper or middle fields, and unilateral infiltrates were more common in the right than left lung.

Clinical laboratory results are shown in Table 2. The neutrophil count was significantly higher in patients with complications than in others (Figure 1). Lymphopenia (<1,000 cells/μL) was characteristic in children with complications and in children who had no complications and were ≥6 years of age and admitted to the hospital on day 1–3 of illness (Figure 2). Lymphocyte count was significantly higher in the corresponding group with admission ≥4 days after onset. Serum immunoglobulin E concentration was high (>170 IU/mL) in both groups admitted on day 1–3, regardless of whether complications were present.

PCR detected bacteria in nasopharyngeal specimens from 41 (51%) patients. Organisms present included *Strep-*

Table 2. Laboratory test results for children hospitalized with pandemic (H1N1) 2009–associated pneumonia, Japan, August–November 2009*

Characteristic	Group A, no complications, n = 63		Group B, complications, n = 17
	Age ≤5 y, n = 15	Age ≥6 y, n = 48	Age ≥6 y, n = 14
Leukocytes, cells/μL (range)†	6,400 (3,600–14,400)	7,400 (2,400–17,100)	14,200 (5,100–22,700)
Neutrophils‡	4,929 (2,227–8,256)	6,081(1,248–15,287)	12,849 (4,182–22,042)
Lymphocytes§	1,593 (74–7,638)	608 (214–2,064)	560 (295–1,889)
Eosinophils	0 (0–102)	16 (0–918)	23 (0–145)
Monocytes	240 (37–1,685)	359 (99–1,271)	337 (0–714)
CRP, mg/dL (range)¶	1.0 (0.1–2.9)	2.4 (0.05–11.95)	3.5 (1–7.83)
LDH, IU/L (range)¶	304 (230–415)	248 (182–575)	248 (193–353)
CK, IU/L (range)¶	101 (40–328)	110 (29–2,240)	148 (57–1,524)
IgE, IU/mL (range)#	61 (7.3–311)	443 (34–4,680)	1,058 (43–4,011)

*CRP, C-reactive protein; LDH, lactate dehydrogenase; CK, creatine kinase; Ig, immunoglobulin.
†Reference ranges by age group: ≤1 y, 7,000–15,000; 2–5 y, 7,000–11,000; ≥6 y, 6,500–10,000.
‡Reference range by age group: ≤1 y, 4,000–8,000; 2–5 y, 2,500–5,500; ≥6 y, 3,000–5,000.
§Reference range by age group: ≤1 y, 4,000–11,000; 2–5 y, 3,000–7,000; ≥6 y, 2,500–4,500.
¶Reference upper limits for CRP, LDH, and CK levels are 0.3, 400, and 200, respectively.
#Reference ranges for IgE by age group: 1–3 y, <30; 4–6 y, <110; ≥7 y, <170. Serum IgE data were analyzed when available.

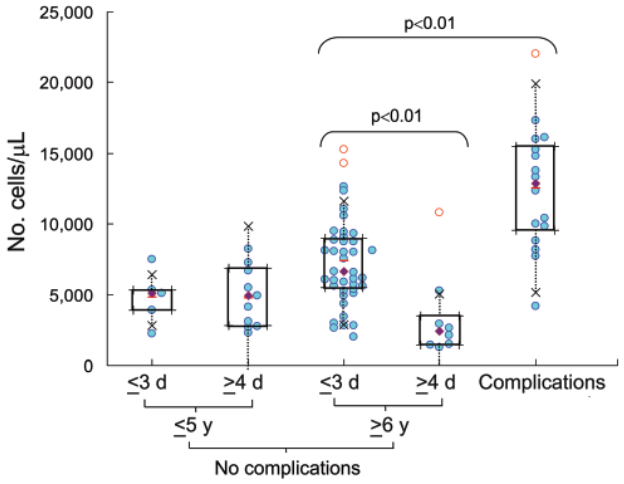

Figure 1. Neutrophil counts (cells/μL) in blood samples from 5 groups: patients with complications, patients ≥6 years of age without complications who had early or late hospital admission, and patients ≤5 years of age without complications who had early or late hospital admission. Data were analyzed by using box-and-whisker plots. Lower limit, median, and upper limit shown within each box correspond to the 25%, 50%, and 75% percentile, respectively; half of the patients considered fall within each box. Dotted lines extending from each box represent 1.5× the quartile deviation. Open red circles, outlying cases; closed diamonds, medians; horizontal bars, means.

tococcus pneumoniae (25 patients), *Haemophilus influenzae* (28), and *Mycoplasma pneumoniae* and *S. pyogenes* (1 each); some patients had multiple organisms. In addition, rhinovirus was detected in 2 patients and enterovirus in 1.

Forty-nine (61%) patients required oxygen administration (mean duration 3.5 days) (Table 1). Oxygen supplementation was provided significantly more often to children who had than who did not have complications (15 [88%] vs. 34 [54%]; p<0.05). A total of 67 (84%) patients received oseltamivir, and 63 (79%) received antimicrobial drugs. Median time from onset of symptoms to initiation of oseltamivir treatment (4 mg/kg/d for 5 days) based on 20 applicable patients was 2 days, showing no differences between groups. Isoproterenol inhalation was needed only for patients with complications. In 1 patient who had an asthma attack, plastic bronchitis developed and the patient required invasive mechanical ventilation for 5 days.

All children recovered, with a median hospital stay of 6 days (Table 1). Hospitalization was longer for patients with than without complications (median 8 days vs. 6 days; p<0.01).

Our study has several limitations. Our PCR data from nasopharyngeal swabs cannot distinguish pathogens from colonizing organisms and cannot reliably guide decisions regarding antimicrobial drug treatment. Various reports have described invasive secondary bacterial infection with *Staphylococcus aureus* diagnosed from lower respiratory tract or blood specimens (*7*,*8*); such cultures were not obtained from all of the patients in our study. Moreover, pneumonia may have been underdiagnosed in our patients considering limited sensitivity of chest radiography compared with computed tomography (*9*).

Conclusions

Pediatricians should be aware that early diagnosis of influenza can enable prompt antiviral treatment of severe illness. All Japanese citizens have ready access to medical institutions through the national health insurance system. On November 13, 2009, the Japan Pediatric Society reported surveillance data concerning 60 pandemic (H1N1) 2009–associated deaths in children (*10*). Main causes of death were sudden death and rapidly progressive severe pneumonia. Testing practices, access, and policies regarding early administration of antiviral agents have protected many children from life-threatening pandemic (H1N1) 2009.

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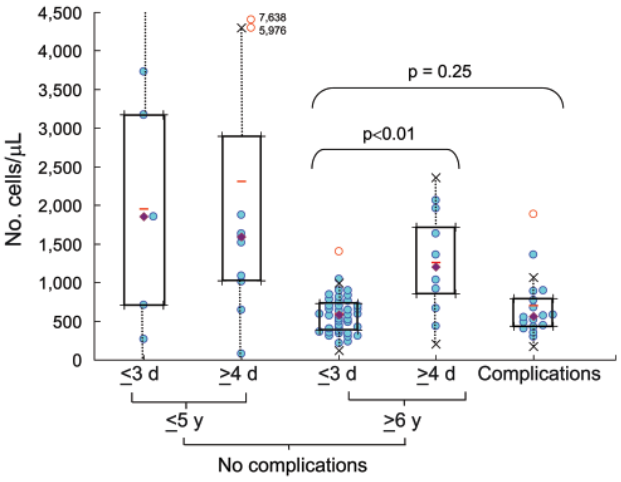

Figure 2. Lymphocyte counts (cells/μL) in blood samples from 5 groups (patients with complications, patients ≥6 years of age without complications who had early or late hospital admission, and patients ≤5 years of age without complications who had early or late hospitalization). Data were analyzed by using box-and-whisker plots. Lower limit, median, and upper limit shown within each box correspond to the 25%, 50%, and 75% percentile, respectively; half of the patients considered fall within each box. Dotted lines extending from each box represent 1.5× the quartile deviation. Open red circles, outlying cases; closed diamonds, medians; horizontal bars, means.

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Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus, Mexico

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During May 2009–April 2010, we analyzed 692 samples of pandemic (H1N1) 2009 virus from patients in Mexico. We detected the H275Y substitution of the neuraminidase gene in a specimen from an infant with pandemic (H1N1) 2009 who was treated with oseltamivir. This virus was susceptible to zanamivir and resistant to adamantanes and oseltamivir.

In March and early April 2009, a new strain of influenza A virus that contained genes from the Eurasian–North American triple reassortant and classical swine lineage viruses emerged in North America (*1*,*2*). By May 21, 2010, a total of 214 countries and overseas territories or communities had reported laboratory-confirmed pandemic (H1N1) 2009, which resulted in at least 18,097 deaths in patients with PCR-confirmed illness (*3*). In Mexico 72,533 cases (1,228 deaths) were PCR confirmed by the second week of May 2010 (*4*).

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To control influenza A virus infections, the US Food and Drug Administration has approved the use of matrix 2 (M2) blockers, amantadine and rimantadine, and the neuraminidase (NA) inhibitors (NAIs), oseltamivir and zanamivir (*5*). However, for pandemic (H1N1) 2009, therapeutic options are limited to the NAIs because this virus has a swine virus–origin M2 gene, which contains a mutation associated with resistance to adamantanes (*6*). NAI resistance in pandemic (H1N1) 2009 viruses has been rare; nevertheless, 285 oseltamivir-resistant cases were reported worldwide as of April 14, 2010 (*7*). All oseltamivir-resistant viruses have the H275Y substitution that confers resistance to oseltamivir but not to zanamivir. Spread of oseltamivir-resistant seasonal influenza A virus (H1N1) was first detected in 2007, and this virus has now become the predominant lineage of influenza A virus (H1N1) in humans (*8*,*9*). This finding raises strong concerns that the H275Y mutation could become dominant in pandemic (H1N1) 2009 as well. We report oseltamivir-resistant pandemic (H1N1) 2009 detected through virologic surveillance in Mexico.

The Study

We aimed to determine the drug susceptibility of pandemic (H1N1) 2009 in Mexico. We randomly selected 692 independent clinical samples (452 cell culture supernatants and 199 nasopharyngeal swab specimens [NPS]) or viral isolates, mostly from patients hospitalized in Mexico and from a few symptomatic patients with highly suspected oseltamivir- resistant infections (31 NPS and 10 lung biopsy specimens from patients who died). The study was conducted during July 2009–May 2010. All samples were received during May 2009–April 2010 at the Institute of Epidemiologic Diagnosis and Reference (InDRE [Mexico City, Mexico]); all were positive for pandemic (H1N1) 2009 by real-time reverse transcription–PCR (RT-PCR), according to the procedure recommended by the Centers for Disease Control and Prevention (Atlanta, GA, USA) and the World Health Organization.

Samples were collected from patients in all Mexican states. Patients did not differ significantly by sex, and persons 10–29 years of age were most commonly affected, similar to the number of incident cases of acute respiratory infection in Mexico (Table 1). Viral RNA was extracted by using either MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Rotkreuz, Switzerland) or QIAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany).

An endpoint RT-PCR was performed for all 692 samples screened for the H275Y molecular marker by using the Superscript III RT-PCR system (Invitrogen, Carlsbad, CA, USA) and FLUAN1–721F and FLUAN1–924R primers spanning position 275 of the NA gene (Table 2). Direct sequencing of these PCR products was performed by using a sequencing primer (FLUAN1–904R, Table 2) and the

Table 1. Characteristics of patients with pandemic (H1N1) 2009 reported to the Institute of Epidemiologic Diagnosis and Reference, by age group, Mexico, May 2009–April 2010

	No. (%) patients		
Age group, y	Female	Male	Total
0–1	9	6	15
1–4	30	23	53
5–9	40	34	74
10–19	67	83	150
20–29	66	65	131
30–39	25	34	59
40–49	25	24	49
50–59	19	19	38
≥60	5	6	11
Unknown	51	61	112
Total	337 (48.6)	355 (51.3)	692

BigDye Terminator version 3.1 cycle sequencing reaction kit on an ABI PRISM 3130xl DNA analyzer (Applied Biosystems, Foster City, CA, USA).

All the sequences obtained indicated that the H→Y mutation at the NA 275 residue was absent, except in 1 NPS from an 8-month-old girl (A/Mexico/InDRE797/2010). This patient received oseltamivir treatment from the evening of January 22 through January 27. She had no known history of travel, contact with a person treated with this drug, or diagnosed immunodeficiency. Her clinical record mentioned 2 respiratory events in the 2 months before the influenza diagnosis: broncholitis, which required hospitalization, and readmission to the hospital 2 weeks after discharge because of influenza-like illness and severe pneumonia. In addition to oseltamivir, the child required other antimicrobial drugs (ceftriaxone and vancomycin) and mechanical ventilation. She slowly recovered.

The clinical specimen was collected, and pandemic (H1N1) 2009 was laboratory confirmed on January 27, 2010. In this sample and in isolating the virus we further analyzed, we discarded any of the 9 previously described mutations (*5*,*10*) associated with resistance to NAIs (V116, I117, E119, Q136, K150, D151, D198, I223, and N295 [N2 numbering]) by the full sequence of the NA gene by using 2 overlapping RT-PCR products (Figure 1, Table 2). The complete NA sequence of the A/Mexico/InDRE797/2009 virus obtained from NPS and MDCK isolate (GenBank accession no. CY057074) confirmed the H275Y substitution and showed no other NA mutations known to be associated with NAI resistance (Figure 2, panel A). Pyrosequencing analysis performed on the clinical specimen from the 8-month-old patient showed oseltamivir-resistant H275Y and wild-type H275 virus variants (Figure 2, panel B). The A/Mexico/InDRE797/2009 virus was tested at the Centers for Disease Control and Prevention by using an NA inhibition assay (NA-Star kit, Applied BioSystems) and showed an ≈120-fold increase in oseltamivir 50% inhibitory concentration over that of a sensitive control (27.3 nmol/L vs. 0.23 nmol/L; this result was consistent with H275Y and H275 variants. No change in zanamivir susceptibility was detected (0.32 nmol/L vs. 0.30 nmol/L), which was in accord with the NA sequencing analysis. We also sequenced the M2 gene and confirmed the S31N substitution that confers M2 blocker resistance. Sequencing of the HA gene showed that the substitution D222G, potentially associated with severe clinical outcome (*12*), was not present in this isolate (data not shown).

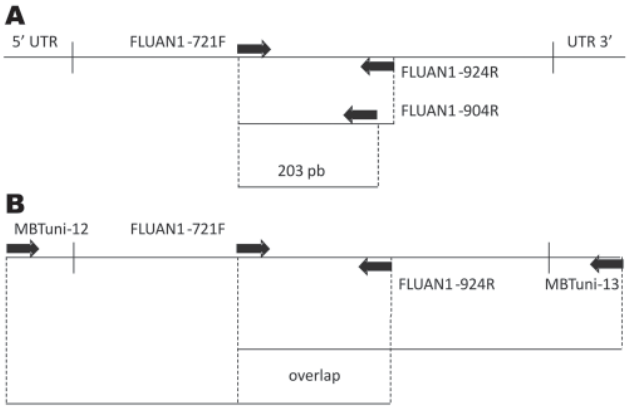

Figure 1. Reverse transcription–PCR (RT-PCR)/sequencing primers scheme for the neuraminidase (NA) gene. A) Primer position for screening RT-PCR protocol. B) Primer position and the 2 overlapping RT-PCR products for the complete NA sequence. UTR, untranslated region.

In addition, 24 virus isolates collected during December 2009–January 2010 were tested in the NA inhibition assay. All were sensitive to both NAIs, with 50% inhibitory concentrations of 0.12–0.29 nmol/L and 0.17–0.36 nmol/L for oseltamivir and zanamivir, respectively.

No other molecular markers of NAI resistance, such as at residues Q136, K150, or D151, which might confer

Table 2. Primer sets used in reverse transcription–PCR and Sanger sequencing of isolates for pandemic (H1N1) 2009, Mexico, May 2009–April 2010*

Primer	Sequence, 5′ → 3′	Target/position, nt
FLUAN1–721F	GTAATGACCGATGGACCAAG	NA/721
FLUAN1–924R	CTGGTTGAAAGACACCCAC	NA/924
FLUAN1–904R	GTCGATTCGAGCCATGCCAG	NA/904
MBTuni-12†	ACGCGTGATCAGCAAAAGCAGG	NA/5′ UTR
MBTuni-13†	ACGCGTGATCAGTAGAAACAAGG	NA/3′ UTR

*NA, neuraminidase; UTR, untranslated region.
†Primer sets previously published in 2009 (*11*).

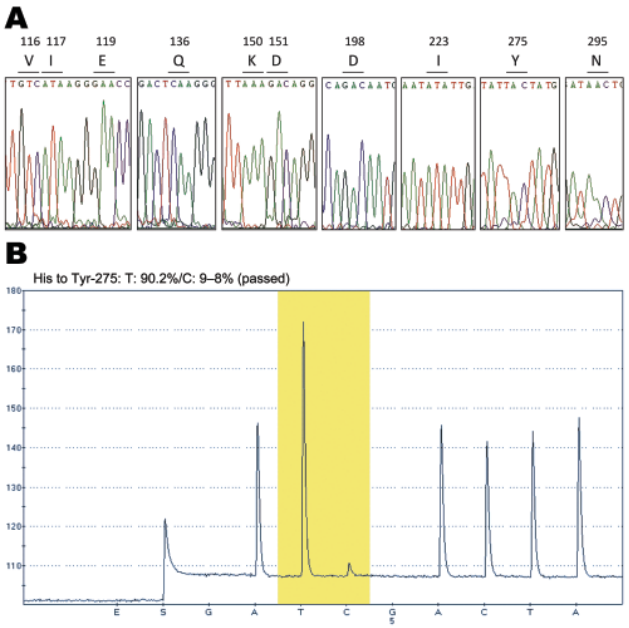

Figure 2. DNA sequence electropherograms for neuraminidase (NA) gene sequences. A) Analysis of molecular markers (V116, I117, E119, Q136, K150, D151, D198, I223, H275, and N295) for oseltamivir and/or zanamivir resistance among the pandemic (H1N1) 2009 virus isolates. The oseltamivir resistance–conferring mutation CAC (histidine) to TAC (tyrosine) at position 275 was detected in the InDRE797 sample. B) Detection of the H275Y mutation in the NA of the viruses by single-nucleotide polymorphism analysis at NA275 position (yellow area).

zanamivir resistance, were observed in the isolate from the 8-month-old girl. The functional NI assay confirmed the oseltamivir resistance and susceptibility to zanamivir of the virus. Both variants (H275Y and wild type) were present in the clinical specimen and its matching virus isolate, which is not unusual (*13*). The proportion of resistant virus (1 [0.14%] of 692 analyzed cases) is lower than has been described, perhaps because of the limited analysis. Analysis of additional samples will enable detection of additional cases. The M2 S31N substitution, the adamantane resistance marker, also was present, as is expected in this virus.

Conclusions

Concern exists that an oseltamivir-resistant variant of pandemic (H1N1) 2009 virus may emerge and spread in a manner similar to that of oseltamivir-resistant seasonal influenza A virus (H1N1) (*14*). Because pandemic (H1N1) 2009 virus is already resistant to adamantanes, oseltamivir resistance would leave zanamivir as the only antiviral treatment option. Consequently, close monitoring of the antiviral susceptibility of pandemic (H1N1) 2009 strains is critical for controlling the spread of this virus (*15*).

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Dr Ramirez Gonzalez is a researcher at the Institute of Epidemiologic Diagnosis and Reference. His primary research interests are the molecular epidemiology of viral pathogens.

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Comparison of Pandemic (H1N1) 2009 and Seasonal Influenza Viral Loads, Singapore

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Mean viral loads for patients with pandemic (H1N1) 2009 were ≈1 $\log_{10}$ times lower than those for patients with seasonal influenza within the first week after symptom onset. Neither pandemic nor seasonal influenza viral loads correlated with clinical severity of illness. No correlation was found between viral loads and concurrent illness.

Although clinical characteristics of pandemic (H1N1) 2009 have been well documented (*1*,*2*), fewer specific virologic comparisons with seasonal influenza have been studied in hospitalized patients (*3*). Studies of other influenza virus infections in humans suggest that host immune responses play a major role in determining clinical outcomes (*4*,*5*). We describe the initial viral loads for patients infected with pandemic (H1N1) 2009 and seasonal (H1 and H3) influenza viruses and their correlation with various aspects of signs and symptoms at admission to the National University Hospital (NUH) in Singapore.

The Study

The study consisted of patients seen at NUH during May–November 2009 as emergency admissions, outpatients, or inpatients whose nasopharyngeal swabs submitted for routine diagnostic testing were positive for seasonal influenza virus A (H1 and H3) or pandemic influenza A virus (H1N1) 2009. From samples taken before treatment was begun, we identified 578 patients with pandemic (H1N1) 2009 and 88 patients with seasonal influenza (11 H1 and 77 H3). Clinical characteristics of some of these patients have been described elsewhere (*2*). Local ethics approval (ref. no. B/09/360) was granted for this study.

Age, sex, and clinical information (i.e., days after onset of symptoms, comorbidities, clinical severity) were obtained from patient records. Comorbidities were defined as ≥1 of the conditions listed in Table 1. Clinical severity was defined as follows: mild, patients well enough to be treated as outpatients; moderate, patients ill enough to warrant hospital admission; severe, hospitalized patients who died or who required intensive or high-dependency care. In-house quantitative assays (online Technical Appendix, www.cdc.gov/EID/content/17/2/285-Techapp.pdf) were performed on archived samples previously tested as positive for pandemic (H1N1) 2009 and reported elsewhere (*6*).

Viral loads of hemagglutinin (HA) and nucleoprotein (NP) for pandemic (H1N1) 2009 ranged from 10^2 to 10^9 RNA copies/mL of virus transport medium (mean 10^5–10^7 RNA copies/mL). Seasonal influenza viral loads ranged from 10^3 to 10^{10} RNA copies/mL (mean 10^6–10^8 RNA copies/mL for seasonal influenza subtype H3 and mean 10^5 to 10^7 RNA copies/mL for seasonal influenza H1). Viral loads decreased with time after onset of symptoms from date the patient sought care at NUH in patients with pandemic or seasonal influenza (Figure 1).

Because of the small number of patients with seasonal influenza H1, further analysis for seasonal influenza was limited to H3. Patients infected with pandemic (H1N1) 2009, compared with those having seasonal influenza H3, were younger ($p<0.0001$), and a higher proportion had comorbidities ($p = 0.0068$; Table 1).

For the 578 pandemic influenza cases, the multiple analysis of variance showed that viral loads were associated with number of days after symptom onset from date of presentation ($p<0.0001$) and with age ($p = 0.0112$) (Figure 2, panel A; Table 2). For the 77 seasonal influenza H3

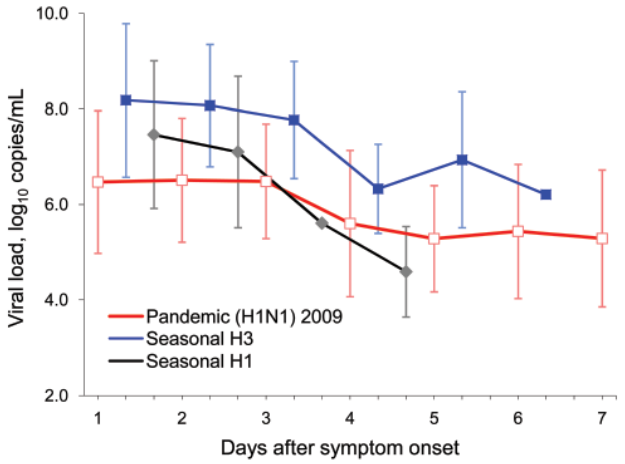

Figure 1. Viral loads (in RNA copies/mL) in patients with pandemic (H1N1) 2009 (NP) and seasonal H1 and H3 (MP) influenza at time patient sought hospital care against days after symptom onset. Vertical bars indicate ±1 SD. Line plots are slightly offset with respect to each other along the time axis to allow the SD bars to be seen clearly. NP, nucleoprotein; MP, matrix protein.

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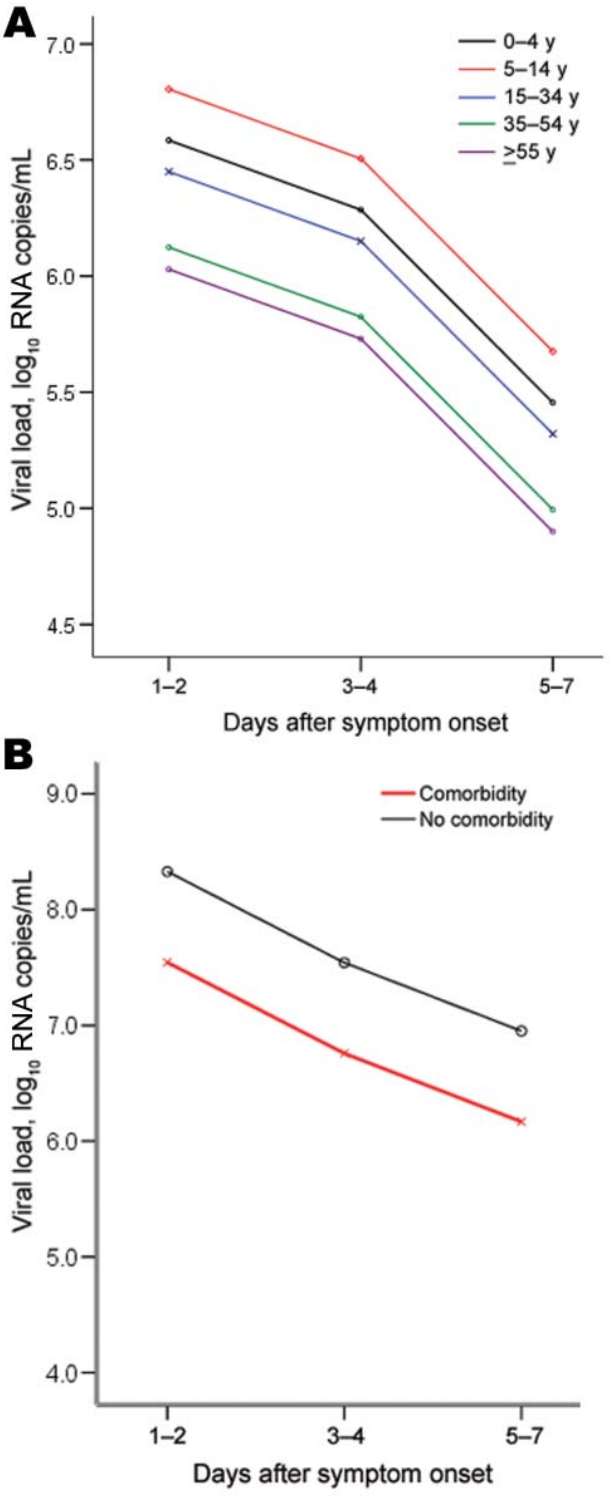

Figure 2. A) Profile plot and multivariate comparisons of the estimated nucleoprotein viral loads of pandemic (H1N1) 2009, by patient age group, against days from symptom onset in the final multiple analysis of variance model. B) Profile plot and comparisons of the estimated matrix protein viral loads of seasonal influenza H3 by the presence or absence of comorbidities against days from symptom onset in the final analysis of variance model.

cases, the analysis of variance showed that days after onset of symptoms from date of presentation ($p = 0.0223$) and presence of any comorbidities ($p = 0.0249$) significantly affected viral loads (Table 2). Viral loads for seasonal influenza were lower in patients with than without comorbidities (Figure 2, panel B).

Conclusions

One of our most striking findings was that the mean viral loads of patients visiting NUH were ≈1 $\log_{10}$ higher for seasonal than for pandemic influenza (Figure 1). This difference persisted even after we adjusted for age. Another study demonstrated that within the first 3 days after symptom onset, historical mean viral loads of seasonal influenza exceed those of the contemporary pandemic virus by 1–2 $\log_{10}$ (*3*). However, a limitation of that study is its use of viral load data for seasonal influenza that was historical rather than obtained contemporaneously with the data for pandemic (H1N1) 2009.

Approximately 30%–50% of influenza case-patients may be asymptomatic (*7*), and although the correlation between viral load and clinical symptoms is not well established, a viral load threshold may exist below which most persons have no clinical symptoms (although individual variation will always exist). Our analysis suggests that if such a threshold exists, it is lower for novel than for seasonal influenza viruses. For a direct virus-mediated pathologic process, this hypothesis may be understandable, given the lower prevalence of preexisting (and therefore potentially partially protective) cross-reactive immunity for this novel virus (*8–10*).

Viral loads for both pandemic (H1N1) 2009 and seasonal influenza tend to decrease with time after symptom onset (Figure 1). Larger studies are needed to confirm the more rapid decline of seasonal influenza H1 than of H3 viral loads. In addition, younger age groups had significantly higher viral loads for pandemic (H1N1) 2009 (Figure 2, panel A), which may not be surprising given that this Southeast Asian population appears to have little or no preexisting specific or cross-reacting antibodies to this novel virus (*9*,*10*).

Two findings are perhaps the most surprising of this analysis. First, we found no significant correlation between pandemic (H1N1) 2009 or seasonal influenza viral loads and clinical severity of illness (Table 2). Second, pandemic (H1N1) 2009 viral loads in infected patients with and without preexisting comorbidities did not differ significantly, although a significant difference was found for seasonal influenza (Figure 2, panel B; Table 2). We offer some possible explanation for these findings but note that these influenza viral loads have been measured in respiratory samples. These samples are peripheral types of specimens that may not necessarily directly affect, or be directly affected

Table 1. Comparison of baseline characteristics between patients with pandemic (H1N1) 2009 and seasonal influenza H3 infection, Singapore, May–November 2009

Characteristic	Pandemic (H1N1) 2009, no. (%), n = 578	Seasonal influenza H3, no. (%), n = 77	p value
Age, y			<0.0001
0–4	69 (11.9)	7 (9.1)	
5–14	144 (24.9)	11 (14.3)	
15–34	250 (43.3)	28 (36.4)	
35–54	72 (12.5)	13 (16.9)	
≥55	43 (7.4)	18 (23.4)	
Female sex	275 (47.6)	41 (53.2)	0.3959
Comorbidities*	262 (45.3)	22 (28.6)	0.0068
Asthma	120 (20.8)	7 (9.1)	0.0137
Chronic lung disease	15 (2.6)	3 (3.9)	0.4584
Cardiac disease	21 (3.6)	4 (5.2)	0.5214
Chronic renal failure	21 (3.6)	2 (2.6)	1.0000
Chronic liver disease	11 (1.9)	0	0.6275
Cerebrovascular disease	9 (1.6)	2 (2.6)	0.3776
Neoplasms	22 (3.8)	3 (3.9)	1.0000
Diabetes	41 (7.1)	5 (6.5)	1.0000
Pregnancy	39 (6.7)	2 (2.6)	0.2115
Immunocompromised	27 (4.7)	2 (2.6)	0.5621
Receipt of steroid medication	23 (4.0)	1 (1.3)	0.3429
Autoimmune disease	14 (2.4)	1 (1.3)	1.0000
Neurocognitive disease	12 (2.1)	1 (1.3)	1.0000
Neuromuscular disease	2 (0.3)	0	1.0000
Premitigation phase	104 (18.0)	51 (66.2)	<0.0001
Clinical severity†			0.0462
Severe cases‡	23 (4.9)	1 (3.8)	
Hospitalized cases§	222 (46.8)	6 (23.1)	
Outpatient only	229 (48.3)	19 (73.1)	

*Patient had ≥1 of the conditions listed.
†Analysis was limited to patients in whom influenza were diagnosed during the mitigation phase (n = 474 for pandemic and n = 26 for H3 seasonal influenzas). Singapore switched from premitigation (i.e., containment) to mitigation management protocols on July 8, 2009, which altered how patient treatment with oseltamivir was initiated. However, this transition does not affect the results shown above because none of the patients were undergoing treatment when these first diagnostic samples were taken.
‡Patients requiring intensive or high-dependency care or who died.
§Patients requiring hospitalization because of clinical conditions but not intensive or high-dependency care.

by, many of the preexisting comorbidities that involve nonrespiratory systems, unless their management involves, for example, some sort of immunosuppressive therapy.

A main limitation of this study is that these viral load measurements were performed on only 1 acute diagnostic sample from each patient at admission before treatment with oseltamivir; therefore, determining how these viral loads would have changed later during the natural course of the infection was not possible. Also, some of the patient categories (Tables 1, 2) contained relatively few patients, e.g., the relatively low number of severe cases (Tables 1, 2), which may have limited the statistical significance of some correlations. Finally, although influenza viral loads in various types of respiratory samples are now often reported (*3*,*6*), these are heterogeneous, peripheral samples, and such viral loads may vary considerably in the same patient during a single day, depending on individual host immune responses.

If human illness caused by influenza virus infections is mediated by host immune responses (*4*,*5*), then a more vigorous, primary immune response in the immunologically naive, otherwise healthy younger population against the pandemic (H1N1) 2009 virus may also contribute to the degree of clinical illness. The interplay between a direct viral pathologic process and a host immune-mediated pathologic process is probably unique to each person. Some recent studies investigating cytokine responses in persons with acute pandemic (H1N1) 2009 infections had contrasting findings (*11*–*13*), although postmortem investigations of some fatal cases of pandemic (H1N1) 2009 infection found substantial inflammation, which supports an immune-mediated pathologic process for at least in these cases (*14*). Similarly, for the more well-established seasonal H3 influenza (to which most persons have had many years of exposure) more well-established, robust, yet sufficiently individually different patterns of homologous and heterologous immune responses may contribute more (compared with similar responses to pandemic [H1N1] 2009) to the different degrees of clinical illness in infected persons with different combinations of comorbidities.

Table 2. Analysis of pandemic (H1N1) 2009 (HA and NP) and seasonal H3 (MP) viral loads with clinical parameters, Singapore, May–November 2009*

	Pandemic influenza				Seasonal H3 influenza		
Characteristic	No.	HA viral load, log_{10} copies/mL, mean (SD)	NP viral load, log_{10} copies/mL, mean (SD)	MANOVA p value†	No.	MP viral load, log_{10} copies/mL, mean (SD)	ANOVA p value
Time from symptom onset, d				<0.0001			0.0223
1–2	416	6.49 (1.44)	6.49 (1.38)		53	8.12 (1.43)	
3–4	114	6.18 (1.39)	6.16 (1.40)		20	7.27 (1.31)	
5–7	48	5.33 (1.21)	5.31 (1.23)		4	6.76 (1.22)	
Age, y				0.0112‡			0.9652‡
0–4	69	6.46 (1.40)	6.45 (1.39)		7	7.66 (0.75)	
5–14	144	6.62 (1.36)	6.65 (1.26)		11	7.93 (1.38)	
15–34	250	6.34 (1.48)	6.33 (1.43)		28	7.88 (1.66)	
35–54	72	5.85 (1.46)	5.88 (1.41)		13	7.99 (1.04)	
≥55	43	5.88 (1.41)	5.83 (1.50)		18	7.63 (1.66)	
Sex				0.3018‡			0.3883‡
F	275	6.23 (1.49)	6.26 (1.38)		41	7.68 (1.52)	
M	303	6.42 (1.41)	6.39 (1.43)		36	8.00 (1.35)	
Comorbidities				0.9967‡			0.0249‡
Yes	262	6.35 (1.49)	6.35 (1.44)		22	7.23 (1.53)	
No	316	6.31 (1.42)	6.31 (1.39)		55	8.07 (1.35)	
Clinical severity§							
Severe	23	5.97 (1.76)	5.98 (1.84)				
Hospitalized	222	6.44 (1.49)	6.42 (1.43)		7¶	7.55 (1.06)	
Outpatient	229	6.29 (1.45)	6.30 (1.40)		19	7.25 (1.54)	

*HA, hemagglutinin; NP, nucleoprotein; MP, matrix protein; MANOVA, multiple analysis of variance; ANOVA, analysis of variance.
†Wilks Lambda statistics.
‡Effect of days from symptom onset adjusted.
§Analysis included patients who sought care during the mitigation phase only (n = 474 for pandemic and n = 26 for H3 seasonal influenzas). Singapore switched from premitigation (i.e., containment) to mitigation management protocols on July 8, 2009, which altered how patient treatment with oseltamivir was initiated. However, this transition does not affect the results shown above because none of the patients were undergoing treatment when these first diagnostic samples were taken.
¶Includes 1 severe case.

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Pandemic (H1N1) 2009, Abu Dhabi, United Arab Emirates, May 2009–March 2010

Gulfaraz Khan, Jamal Al-Mutawa, and Muhammad Jawad Hashim

To ascertain characteristics of pandemic (H1N1) 2009 virus infection, we reviewed medical records for all suspected or confirmed cases reported in Abu Dhabi during May 2009–March 2010. Overall case-fatality rate was 1.4/100,000 population. Most patients who died had ≥1 risk factor, and female decedents were considerably younger than male decedents.

The outbreak of pandemic (H1N1) 2009 influenza virus was first noted in Mexico in March 2009 (*1*) but quickly spread worldwide. On June 11, 2009, the World Health Organization declared the first influenza pandemic in >40 years, triggering governments around the world to make pandemic (H1N1) 2009 a top public health priority (*2*). Although numerous published studies from around the world have described experiences with the pandemic, few have been from the Middle East. In this study, we present data from Abu Dhabi, the largest of the 7 states in the United Arab Emirates. Abu Dhabi is also the country's capital and has a population of ≈2 million (*3*).

The Study

By May 1, 2009, Abu Dhabi had procedures in place for reporting suspected or confirmed cases of pandemic (H1N1) 2009 (*4*). The state government made reporting mandatory, and data were recorded by Health Authority Abu Dhabi (HAAD). All health care facilities in Abu Dhabi were provided with the case definition of pandemic (H1N1) 2009 virus infection along with reporting guidelines (revised September 8, 2009) (*4*). Briefly, influenza-like illness (ILI) was defined as fever (>37.8°C) with cough and/or sore throat in the absence of known causes other than influenza. Pandemic (H1N1) 2009 was confirmed by using real-time reverse transcription–PCR according to protocol (*5*). Laboratory testing was recommended only for patients with severe illness (ILI with signs such as hypotension, dyspnea, tachypnea, abnormal radiographic appearance of the lungs) or patients with mild illness who had risk factors (e.g., pregnancy, age <5 years, chronic disease). All patients who had symptoms of influenza but negative test results by PCR for pandemic (H1N1) 2009 or who were not tested were grouped into the ILI category for statistical analysis.

Data was analyzed using PASW Statistics version 18 (SPSS Inc, Chicago, IL, USA). One-way analysis of variance was used to compare differences in mean age between the 3 groups (ILI, confirmed pandemic [H1N1] 2009 infections, pandemic [H1N1] 2009–associated deaths) and the χ^2 test (2-sided) to compare gender and national origin of patients.

From May 1, 2009, through March 23, 2010, a total of 2,806 patients with confirmed or suspected pandemic (H1N1) 2009 infection were reported to HAAD. The first ILI case was recorded on May 3; the number of cases peaked in August (Figure 1). The first laboratory-confirmed case occurred on May 20, with the first pandemic (H1N1) 2009–associated death on September 1. Laboratory-confirmed pandemic (H1N1) 2009 cases showed a bimodal distribution, with the first peak in August and the second peak in October (Figure 1). The lack of a second peak in the ILI group is probably due to the change in testing recommendations issued by HAAD on September 8 (testing only patients with severe illness or with risk factors).

Of the 2,806 patients reported, 1,872 (67%) had ILI, 908 (32%) had laboratory-confirmed pandemic (H1N1)

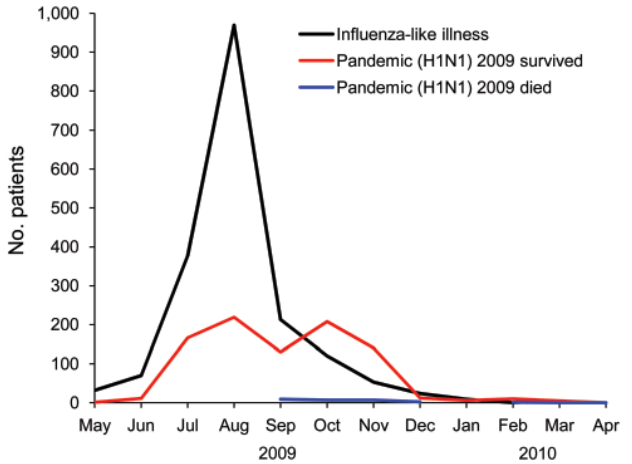

Figure 1. Distribution of cases of influenza-like illness (ILI), laboratory confirmed-pandemic (H1N1) 2009 in patients who survived, and pandemic (H1N1) 2009 in patients who died, Abu Dhabi, United Arab Emirates, May 1, 2009–March 23, 2010. Of the 2,806 cases reported to Health Authority Abu Dhabi, 1,872 were ILI (pandemic [H1N1] 2009 negative or status unknown), 908 were confirmed pandemic (H1N1) 2009 infections in patients who survived, and 26 were pandemic (H1N1) 2009 infections in patients who died. Patients with ILI and survivors of confirmed pandemic (H1N1) 2009 are plotted by date patient first sought care. Pandemic (H1N1) 2009 fatalities are plotted by date of death.

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Table 1. Number of cases of influenza-like illness, laboratory-confirmed pandemic (H1N1) 2009, and pandemic (H1N1) 2009–associated deaths, Abu Dhabi, United Arab Emirates, May 1, 2009–March 23, 2010*

Illness	Total no. cases	Incidence†	Mean age, y		
			All patients	Male patients	Female patients
Influenza-like illness	1,872	97.7	23.0	22.5	23.7
Pandemic (H1N1) 2009					
Survived	908	47.4	21.6	20.7	22.7
Died	26	1.4	43.8	52.9	31.5

*Based on Heath Authority Abu Dhabi statistics for 2009 (*3*). The emirate of Abu Dhabi has a total population of 1,915,903.
†Per 100,000 population.

2009 infection and survived, and 26 (0.9%) had pandemic (H1N1) 2009 infection and died (Table 1). Of the 2,806 patients, 60% (1,679) were male; the preponderance of male patients most likely reflects the substantially higher population of male than female residents in Abu Dhabi (*3*). Of the 1,872 patients with ILI, 646 had laboratory-confirmed negative results for pandemic (H1N1) 2009; the remaining patients were not tested. Almost half (439/896, or 49%) of all laboratory-confirmed cases occurred in children and young adults <20 years of age (Figure 2). For 12 laboratory-confirmed cases, the precise age of the patient was not known. Most (21/26, 81%) decedents were 21–60 years of age; 1 reason may be that the overall population of Abu Dhabi is skewed toward younger age groups. Men who died of pandemic (H1N1) 2009 were significantly older (mean age 52.9 years, 95% confidence interval [CI] 44.0–61.7) than their female counterparts (mean age 31.5 years, 95% CI 18.9–44.1; Mann-Whitney U test, p = 0.007) (Table 2). However, these findings have to be interpreted with caution because our sample of patient deaths is small.

Abu Dhabi has a high expatriate population. According to HAAD 2009 statistics (*3*), 78.8% of the population consists of persons who are not citizens of the United Arab Emirates. Patients in our study represented >50 different nationalities, the top 5 being Emirati, Indian, Filipino, Egyptian, and Pakistani. To have sufficient numbers for a meaningful statistical analysis, we grouped all reported cases into United Arab Emirate nationals (n = 1,708) or expatriates (n = 1,098). Analysis of these 2 groups showed no significant age difference (1-way analysis of variance, p = 0.357) between the Emiratis and the expatriates in terms of ILI, pandemic (H1N1) 2009 survivors, and pandemic (H1N1) 2009 decedents.

Of the 26 decedents, 15 were male; 12 were United Arab Emirate nationals, and 14 were expatriates. Calculating case-fatality rates (CFR) with laboratory-confirmed cases as the denominator is, in this type of study, inaccurate and misleading. Because not all persons with symptoms seek medical attention or are tested, pandemic (H1N1) 2009–confirmed cases are likely to be underestimated and CFR, in turn, to be grossly overestimated (*6*,*7*). We chose to represent mortality estimates per 100,000 persons because the number of fatal cases and the population are accurately known. This information gave an estimated CFR of 1.4 deaths per 100,000 persons. The mean age of decedents was 43.8 years compared with 21.6 years for persons with laboratory-confirmed pandemic (H1N1) 2009 infection who survived (1-way analysis of variance, p<0.01). The most common initial symptoms were fever, cough, and breathing difficulty (Table 2). All patients with pandemic (H1N1) 2009 who died received oseltamivir; however, complete details of antiviral treatment were available for only 21/26 cases. For 20 patients, treatment was started before or on the day of laboratory confirmation (mean –3.2 days). Mean duration of antiviral treatment was 8.3 days (range 2–28 days). Most (20/26; 77%) patients with pandemic (H1N1) 2009 who died had ≥1 underlying risk factor (*8*), most commonly pregnancy, diabetes, malignancy, and hypertension. Twelve decedents each had 1 risk factor, 3 had 2, 3 had 3, and 2 had 4. Mean duration from hospital admission to death was 27.5 days (Table 2).

Ages of decedents with pandemic (H1N1) 2009 infection differed significantly by gender; female patients were considerably younger (mean 31.5 years) than male patients (mean 52.9 years). Even after excluding pregnant women from the equation (*9*), female decedents remained significantly younger than male decedents. In contrast, ages of survivors of pandemic (H1N1) 2009 infection did not differ significantly by sex.

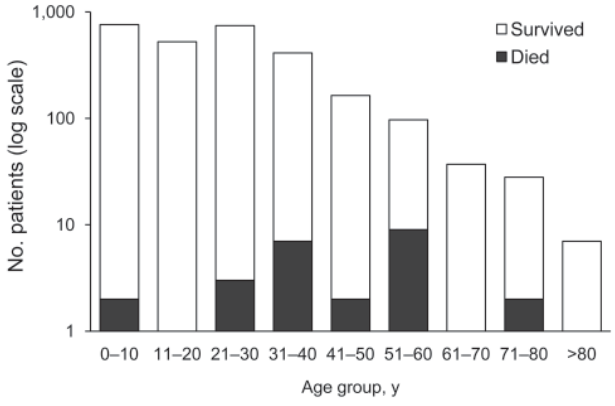

Figure 2. Age group distribution of patients with influenza-like illness and laboratory-confirmed pandemic (H1N1) 2009 infection, Abu Dhabi, United Arab Emirates, May 1, 2009–March 23, 2010.

Table 2. Characteristics of 26 patients who died of pandemic (H1N1) 2009 infection, Abu Dhabi, United Arab Emirates, May 1, 2009–March 23, 2010

Characteristic	Value
Gender, no. (%)	
M	15 (59.7)
F	11 (42.3)
Nationality, no. (%)	
United Arab Emirates	12 (46.2)
Expatriates	14 (53.8)
Age, y	
Mean	43.8
Median	47.0
Range	0.67–83.00
Signs and symptoms, no. (%)	
Fever	19 (73.1)
Cough	14 (53.8)
Breathing difficulty	14 (53.8)
Other,* with or without above symptoms	17 (65.4)
Underlying conditions, no. (%)	
Pregnancy	6 (54.5)
Diabetes	9 (34.6)
Malignancy	7 (26.9)
Cardio/cerebrovascular disease	2 (7.7)
Hypertension	5 (19.2)
Asthma	2 (7.7)
Other	4 (15.4)
Not recorded	6 (23.1)
Duration of oseltamivir treatment, d†	
Mean	8.3
Median	5.0
Range	2–28
Time from laboratory confirmation of pandemic (H1N1) 2009 infection to start of oseltamivir treatment, d‡	
Mean	−3.2
Median	−2.0
Range	−16 to 2
Duration from hospitalization to death, d	
Mean	27.5
Median	21
Range	1–86

*Sore throat, lung infiltration, diarrhea, headache, chest pain, abdominal pain.
†Although all patients with pandemic (H1N1) 2009 were treated with oseltamivir, the exact duration of treatment was known for only 12.
‡The exact date of laboratory confirmation of pandemic (H1N1) 2009 and start of oseltamivir treatment was known for only 21 of the 26 patients.

Conclusions

In this study from the United Arab Emirates, we report the epidemiologic and clinical features of pandemic (H1N1) 2009 infection in Abu Dhabi. The characteristics are similar to those reported in other parts of the world (*10*–*12*). Children were most at risk for pandemic (H1N1) 2009 infection; older adults (>60 years) appeared to be least affected, probably because of cross-protective immunity from exposure to antigenically related influenza viruses earlier in life (*13*,*14*). Twenty-six persons died, most of whom were 21–60 years of age (*7*,*10*). This number translates to an overall incidence of pandemic (H1N1) 2009–associated death in Abu Dhabi of 1.4/100,000 population, which is relatively low compared with some studies (*11*,*15*).

Our findings are subject to limitations. For example, like most epidemiologic studies based on surveillance systems, data are often incomplete, and therefore resulting analysis can be subject to bias. Nonetheless, we believe the aggressive approach implemented by the Abu Dhabi government (e.g., body temperature scans at airports, isolation of persons suspected to have pandemic [H1N1] 2009, tracing of contacts of persons with confirmed cases, and providing oseltamivir prophylaxis) played an important role, not only in delaying the onset and spread of pandemic (H1N1) 2009, but also in reducing deaths.

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Usefulness of Published PCR Primers in Detecting Human Rhinovirus Infection

Cassandra E. Faux, Katherine E. Arden, Stephen B. Lambert, Michael D. Nissen, Terry M. Nolan, Anne B. Chang, Theo P. Sloots, and Ian M. Mackay

We conducted a preliminary comparison of the relative sensitivity of a cross-section of published human rhinovirus (HRV)–specific PCR primer pairs, varying the oligonucleotides and annealing temperature. None of the pairs could detect all HRVs in 2 panels of genotyped clinical specimens; >1 PCR is required for accurate description of HRV epidemiology.

Human rhinoviruses (HRVs) cause more asthma exacerbations than any other known factor, in addition to causing most colds and influenza-like illnesses. The prevalence of HRV in published reports varies considerably. A novel HRV clade identified in 2006, now known as HRV species C (HRV-C) (*1*), can be identified only by PCR. Since 1988, seasonality and clinical outcomes and numerous different primer pairs have been used to identify HRV; how well these methods perform on new HRV types is uncertain. Given the likely variation in the preparation of RNA, the quality and formulations of commercial reverse transcription (RT)-PCR enzymes and reaction mix components and changes in thermal cyclers since 1988, not surprisingly many, perhaps most, of these assays are not being used in the manner they were originally described. For example, the first HRV-specific primers reported (*2*) have subsequently been used with different RNA preparation methods, amounts of reverse transcriptase, cDNA priming strategies, dNTP concentrations, annealing temperatures (T_Ms), and cycling conditions (*3*,*4*).

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The Study

We conducted a preliminary comparison of the relative sensitivity of a cross-section of published HRV-specific PCR primer pairs (most of which were first published before HRV-C was reported), independent of most variables described above, by testing a panel of 57 clinical specimen nucleic acid extracts from combined nose and throat swabs from preschool children with colds and influenza-like illnesses in Melbourne, Australia. The study was approved by the Royal Children's Hospital Human Research Ethics Committee. The panel included representatives of the 3 HRV species (Figure), human enteroviruses (HEVs), and extracts negative for picornaviruses. The HRVs had been previously detected by using a nested primer pair (online Appendix Table, www.cdc.gov/EID/content/17/2/294-appT.htm) (*5*). We used 10 different HRV primer pairs and also retested specimens by using the original primer pair with our standard reagents and equipment (*5*). We applied the published T_M when possible. The original descriptions of primer pairs 7 and 10 (online Appendix Table) lacked T_M information, and after in-house calculations, we used T_Ms of 50°C and 58°C, respectively. We also deliberately standardized the reagents (OneStep RT-PCR kit, QIAGEN, Doncaster, Victoria, Australia) and thermal cyclers used (Veriti, Applied Biosystems, Foster City, CA, USA) for conventional PCR and the RotorGene 3000 real-time cycler (QIAGEN). Because primer pair 1 had a published history of detecting types from all HRV species, we chose it to genotype HRV-positive samples by sequencing the amplified products. Other pairs were used if pair 1 was unsuccessful.

We found that no primer pair detected the same HRVs and HEVs typed when the original pair (*5*) or pair 1 (online Appendix Table) was used. Five primer pairs, including real-time PCR (rtPCR) pair 5, did not amplify the HEVs, a positive feature for HRV-specific studies. Only 2 primer pairs amplified anything from a specimen that was positive for both HRV and HEV, a problem for accurate estimation of the frequency of co-detections. The original primer pair screen detected 3 untypeable picornaviruses, which were not detected by any other pair or by repeat testing using the same pair. Only the second-round amplicon of the 3 nested sets of nested primer pairs (2, 3, and 9) was considered because the second round increased the total number of positive specimens over the first round. The longest amplicon, produced by primer pair 7, was also a valuable genotyping target, but it detected only 14 of the original 27 HRV-positive specimens in this population.

We next selected 4 frequently published primer pairs (1, 5, 7, and 8) to examine 44 picornavirus-positive specimens (39 HRVs, 3 HEVs, and 2 untypeable picornaviruses) from nonhospitalized children with acute asthma exacerbation (*6*). As before, primer pair 1 detected the greatest num-

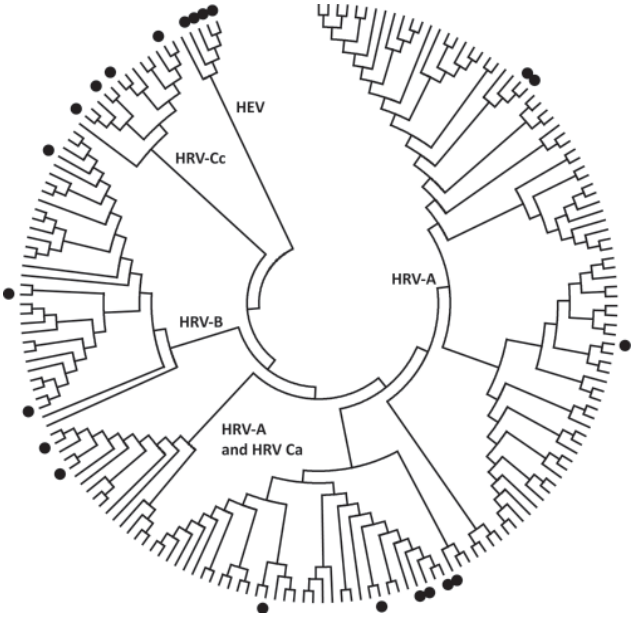

Figure. Distribution of human rhinovirus (HRV) and human enterovirus (HEV) sequences used for primer pair studies. The HRV and HEV genotypes from the testing panel (indicated by filled circles) were aligned with the central 154 nt of the 5′ untranslated region (UTR) region of all complete HRV genomes and poliovirus-1. HRV-Ca and HRV-Cc refer to HRV-Cs with 5′ UTR sequences that have phylogenetic origins from either HRV-As or HRV-Cs, respectively. The tree was constructed by neighbor joining of maximum composite likelihood distance implemented in MEGA (www.megasoftware.net).

ber of HRV- and HEV- positive specimens and all positive specimens detected by other primer sets (n = 41), followed by pair 7 (n = 40), pair 5 (n = 36), and pair 8 (n = 31). Most notably, primer pair 7 performed better than it had in the previous population, detecting only 1 fewer HRV than primer pair 1 and 9 more HRVs than pair 8. No species-specific bias was apparent, but generally, a specimen with a lower RNA concentration, as indicated by the cycle threshold from primer pair 5, was less likely to be detected or typed by using other primer pairs. Primer pairs 5 and 8 did not detect the 3 HEVs (HEV-68). We noted in both populations that primer pair 1 sometimes amplified a region of human genomic DNA from chromosome 6 (GQ497714), for which amplicon size was indistinguishable from that expected due to HRV.

It was not possible to use the precise conditions reported for the 10 compared assays; 1 was published >2 decades ago and used phenol chloroform extraction. Some of the original enzyme formulations or reagents are no longer available, and production processes have changed in the interim. Thermal cyclers have also changed. There was no consensus on enzymes and reaction mixes used. In addition, the previously published primers were used in assays divided between those using 1-step RT-PCR and those using a separate RT cDNA synthesis step. A review of studies that detected HRVs with adequately described conditions during 2009–2010 found that fewer used a single-tube RT-PCR approach than a 2-step system. We conducted single-tube RT-PCR to maintain the benefits of the so-called closed amplification system of rtPCR. Thus, we chose to use a single common set of reagents as the fairest way to compare the primer pairs examined in this study. We believe the nature of this relative comparison best reflects performance for the likely end users: clinical microbiology laboratories or researchers.

We compared primers rather than assay function using clinical material instead of cultured virus, plasmid or synthetic RNA standards, or screening contemporary or archived extracts, which are sometimes of low viral load. When picornavirus epidemiology is the primary research focus, we recommend using ≥2 primer pairs to maximize the detection of HRVs. Under our conditions, pairs 1–4 returned the highest number of positive results, and the rtPCRs behaved similarly but with reduced sensitivity. The rtPCR that used pair 5 did not amplify known HEVs.

Many possible reasons could cause discrepant virus testing results between different sites, including changes to specimen integrity resulting from transport and variable amplification resulting from low viral loads. The effects of viral load can be seen in this study: specimens in population 1 that were positive with multiple (>6 separate pairs) primer pairs had a mean cycle threshold of 33.3 (combining results from both rtPCRs), whereas those with <6 positive results had means of 39.3 cycles. Most (29/33) specimens with <3 positive primer pairs were negative by rtPCR. Amplification variability can also be attributed to the substantial nucleotide sequence diversity between HRVs and the different temporal and clinical characteristics of the 2 specimen populations we used. Population diversity is a feature of HRV studies in the literature.

Conclusions

Our selection of published primer pairs includes those from studies that have informed our current understanding of HRV epidemiology. Finding such a high degree of variability in performance was thus noteworthy. Inefficient HRV detection by PCR may be a serious problem for research studies. Comparison of data between different HRV studies is confounded as are data from studies seeking to determine the effects of other respiratory viruses. The prevalence, seasonality, transmission, and clinical effects of HRV types and species require reexamination with tools that have been comparatively validated to ensure their sensitivity.

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Ms Faux conducted this study as a research scientist in the Queensland Paediatric Infectious Diseases Laboratory, Sir Albert Sakzewski Virus Research Centre. Her main field of research was the detection and characterization of newly identified and classic respiratory viruses in children.

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Surveillance for West Nile Virus in Dead Wild Birds, South Korea, 2005–2008

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To investigate the possibility of West Nile virus (WNV) introduction into South Korea, the National Veterinary Research and Quarantine Service has conducted nationwide surveillance of WNV activity in dead wild birds since 2005. Surveillance conducted during 2005–2008 found no evidence of WNV activity.

Wild birds are considered the principal hosts of West Nile virus (WNV). In the United States, surveillance of birds for WNV is used to quickly detect outbreaks and take action against its spread. The sampling of sick or dead birds can indicate WNV in a region before human and equine cases occur (*1*). This approach is considered the most effective method for detecting WNV in a specific region. During 1999, mass deaths among wild birds indicated the emergence and rapid spread of WNV in North America.

Although WNV has not yet been detected in South Korea, the perceived threat of its arrival has been highlighted by reports of WNV infection in a dead cinereous vulture (*Aegypius monachus*) in the Vladivostok region of Russia, which is adjacent to the Korean peninsula (*2*), and in several samples from cinereous vultures and cattle egrets (*Bubulcus ibis*) in the Russian Far Eastern Region during 2002–2004 (*3*). A variety of migratory birds, such as Mandarin ducks (*Aix galericulata*), cinereous vultures, bean geese (*Anser fabalis*), and white-fronted geese (*Anser albifrons*), fly from Russia to South Korea during the winter for the breeding season (*4*–*6*). Furthermore, Saito et al. recently reported that test results on several migrating birds captured in Japan were positive for flavivirus antibodies (*7*). This finding suggests that the threat of WNV in South Korea is increasing because many migratory birds share flyways over South Korea and Japan (*8*). Therefore, spread of the virus by migratory birds from WNV-infected areas, such as Russia, into uninfected hosts throughout the Korean peninsula is likely.

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The Study

A wide variety of bird species from all regions of South Korea were tested, and particular attention was paid to susceptible species and birds with neurologic signs. Carcasses of wild birds submitted to the Conservation Genome Resource Bank for Korean Wildlife, Seoul National University, Seoul, South Korea, were used for this study. The study also included samples from dead wild birds submitted to the Animal Disease Diagnostic Center of the National Veterinary Research and Quarantine Service of the Ministry of Food, Agriculture, Forestry and Fisheries of South Korea.

Investigation focused on the presumed peak period of mosquito vector activity (April–October) and included samples from dead wild birds. A total of 715 wild birds (belonging to 72 species) from all regions of South Korea were found dead and were examined during 2005–2008. All carcasses underwent postmortem examination, during which samples were obtained for diagnosis. In 2005, a total of 51 samples were tested; 167 samples were tested in 2006, 239 in 2007, and 258 in 2008. Taxonomic families of the collected birds and their migratory status are shown in the online Appendix Table (www.cdc.gov/EID/content/17/2/297-appT.htm). Samples from *Ae. monachus*, *A. fabalis*, and *A. albifrons* birds, which are known to migrate from the Russian Eastern Region to South Korea (*4*,*5*), were included. Samples of dead wild birds such as *Corvidae* spp. and raptors (*Accipitridae* and *Strigidae* spp.), which have been identified as potential sources of WNV for resident birds (*9*,*10*), were also included.

Carcasses were subjected to necropsy, and brains and kidneys were obtained. Organs were homogenized in phosphate-buffered saline (10% suspension) and centrifuged. Ten 50% tissue culture infectious doses of a stock WNV were used as a control for antigen detection. WNV RNA in samples was investigated by reverse transcription–PCR with primers (Table). Information on the RNA extraction and the reverse transcription–PCR used is available in the online Technical Appendix (www.cdc.gov/EID/content/17/2/297-Techapp.pdf).

During 2005–2008, we analyzed 1,309 organ samples (639 brain and 670 kidney) from dead birds for WNV RNA. WNV was not detected in these samples. Diagnostic examination of wild birds as a part of the nationwide surveillance has not detected patterns or clusters of birds with evidence of neurologic disease or viral encephalitides suggestive of

Table. Oligonucleotide primers used for reverse transcription–PCR of West Nile virus in dead wild birds, South Korea, 2005–2008

Primer	Sequence, 5′ →3′	Orientation*	Genome position†	Product size, bp
WN233	TTGTGTTGGCTCTCTTGGCGTTCTT	S	233	408
WN640	CAGCCGACAGCACTGGACATTCATA	AS	640	408
AmWN1401	ACCAACTACTGTGGAGTC	S	1401	445
AmWN1845	TTCCATCTTCACTCTACACT	AS	1845	445
AmWN1485	GCCTTCATACACACTAAAG	S (nested PCR)	1485	248
AmWN1732	CCAATGCTATCACAGACT	AS (nested PCR)	1732	248

*S, sense; AS, antisense.
†Genbank accession no. NC_009942.

WNV infection. Several cases of mass die-offs among wild birds were the result of chemical poisoning (*11*).

Conclusions

Our surveillance of wild birds conducted during 2005–2008 supports the hypothesis that WNV has not reached South Korea and corroborates findings of previous reports. In a study conducted at the National Institute of Health, Korea Centers for Disease Control and Prevention, 2,275 pools of mosquitoes were tested for WNV RNA; results for all samples obtained during 2006–2008 were negative (*12*). The study reported that 27 cerebrospinal fluid samples and 57 serum specimens obtained from patients who were suspected of having Japanese encephalitis and dengue fever were also negative for WNV. In another surveillance study of mosquitos and crows in Japan, a country near South Korea, no WNV RNA was detected. This study included mosquitoes obtained in a park in Tokyo during 2002–2006 and 329 captured or dead crows obtained during1994–2006 (*13*). In addition, antibodies against WNV antibodies were not detected in 18 crows sampled during 1995–2003. The first human WNV infection in Japan was confirmed in a person who returned from the United States in 2005 (*14*). However, no indigenous human or equine cases have been reported.

Although our surveillance found no evidence of WNV in South Korea, WNV could be introduced into this country in the near future. Moreover, several species of mosquitoes with the ability to transmit WNV have been identified in South Korea. Turell et al. reported that mosquitoes captured in Paju County, Gyeonggi Province, South Korea, were highly susceptible to WNV infection when they fed on viremic chickens (*15*).

Introduction of WNV into South Korea would undoubtedly become a major public health problem. An outbreak similar to the one that occurred in New York during 1999 could result in the disease becoming endemic to the country. Continued surveillance of dead wild birds is essential to enable prompt detection of WNV. Additionally, WNV surveillance programs in South Korea should continue to examine cases of viral encephalitis in horses and mass deaths among birds. Temperature increases caused by climate change should also be taken into account, and vigilant monitoring of emerging arboviruses, in addition to WNV, will be required. Finally, increased cooperation between the government and other agencies, such as wildlife conservation organizations and horse-racing authorities, is needed for early detection of WNV disease and development of effective veterinary and public health strategies.

This study was supported by a grant from the National Veterinary Research and Quarantine Service, Republic of Korea.

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Ode to Rickettsiae[1]

Veranja Liyanapathirana

Oh! dear Rickettsiae.

Why did you become such a difficult bug?

You and your cousin (once removed) *Orientia*,

So tiny: yet so powerful

I cannot grow you, because you need special care.

My poor home can not offer a cocoon for you to grow

My poor home has no safety nets to contain you.

You are an illusion to me.

I see you in the darkened room, indirectly as a bright green star,

Illuminated on the glassy shrines.

I try to look for you, for your DNA

You still manage to evade me.

Some day my dear, some day,

I will build a house, with walls so strong,

So I can grow you and nurture you within.

Some day my dear, some day soon,

I will find a little DNA, amplify and see.

Till then, let us meet, in the darkened room, you dressed in your finest green,

Brightly illuminated like the Milky Way.

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[1]This short poem describes my frustrations at trying to establish laboratory diagnosis of rickettsial infections in a place where there is no cell culture facility and no class 3 containment facility.

Hantavirus Infection in Istanbul, Turkey

To the Editor: More than 20 serotypes of hantavirus have been identified, and 11 infect humans. Puumala virus (PUUV), Dobrava virus (DOBV), and Seoul virus cause different forms of hemorrhagic renal syndrome (*1*,*2*). DOBV is endemic to Turkey and countries in the Balkan region. Approximately 10,000–12,000 cases of infection with PUUV and DOBV occur in European Russia each year (*3*). Initial case reports identified a hantavirus epidemic (laboratory confirmed) in February 2009 that involved 12 persons in Bartin and Zonguldak in western Turkey near the Black Sea. The hantavirus responsible for this epidemic was a PUUV subtype (*4*). We report a man infected with DOBV in Turkey who died 2 days after admission to an intensive care unit (ICU).

The patient was a 22-year-old man who lived near Istanbul, Turkey. He was admitted to the Silivri State Hospital in March 2010 because of fatigue, diffuse pain, nausea, and vomiting. Approximately 2 hours after admission, ecchymotic rashes developed on his upper extremities and spread to other areas. His general condition worsened, and 15 hours later, he was transferred to the ICU of the Emergency Service of Gulhane Military Medical Academy Haydarpasa Training Hospital. His medical history did not include exposure to rodents or any travel.

At admission to the ICU, his general condition was poor, and his speech was garbled and incoherent. He had a body temperature of 37.2°C, a pulse of 140 beats/min, an arterial blood pressure of 90/60 mm Hg, diffuse hemorrhagic foci, and a disseminated ecchymotic rash. Laboratory test results showed the following: 13,200 leukocytes/mm^3, 92% polymorphonuclear leukocytes, hemoglobin 11.6 mg/dL, 385,000 platelets/mm^3, alanine aminotransferase 62 IU/mL, aspartate aminotransferase 170 IU/mL, creatine phosphokinase 2,115 IU/L, lactate dehydrogenase 1,109 IU/L, urea 65 mg/dL, creatinine 3.78 mg/dL, prothrobin time 24.8 s, activated partial thromboplastin time 116.3 s, potassium 2.9 mEq/L, C-reactive protein 326 mg/dL, and erythrocyte sedimentation rate 132 mm/h.

Subsequently, urinary output decreased and respiratory functions worsened. He then lost consciousness and was subjected to mechanic ventilation. Lumbar puncture was not performed because of risk for bleeding (high international normalized ratio values for blood coagulation and thrombocytopenia). Cranial computed tomographic scan did not show any pathologic changes. Treatment with ceftriaxone, 4 g/day intravenously, was initiated, and the dose was adjusted according to creatinine clearance because of suspected meningococcemia. A single dose of prednisolone, 80 mg intravenously, was given concomitantly. Bacterial growth was not observed in cultures of urine and blood samples.

The Hanta Profile 1 EUROLINE Test (Euroimmun, Luebeck, Germany) was used to detect immunoglobulin (Ig) G and IgM against 3 hantavirus serotypes (PUUV, DOBV, and Hantaan virus). Results of a hantavirus IgM immunoblot test were positive for DOBV. The QIAamp viral RNA Mini Extraction Kit (QIAGEN, Hilden, Germany) was used for extraction of viral RNA. PUUV and DOBV RNA in serum and urine samples were investigated by using an in-house real-time PCR (Rotorgene; QIAGEN). DOBV RNA was detected in urine samples by PCR (Table).

Meningococcemia, acute hemorrhagic fever, and Crimean-Congo hemorrhagic fever were considered in the differential diagnosis for the patient. Other diseases were excluded by biochemical, serologic, and microbiologic test results. Hantavirus infection was diagnosed in this patient on the basis of criteria recommended by the European Network for Diagnostics of Imported Viral Diseases (*5*). On the second day of treatment, the patient died of cardiopulmonary arrest.

The patient had worked as a security guard in a new prison located in an area that had contained oak and hornbeam forests. DOBV is carried by rodents (*Apodemus flavicollis*), and the habitat of this rodent in Europe is open oak or beech forest. In a field study performed in rural areas of Turkey near the Black Sea and Aegean Sea, hantavirus was detected in *Microtus* spp. voles (*6*). In another study performed in regions near the Aegean Sea, DOBV was detected in 7 (3.5%) of 200 patients with acute or chronic renal failure (*7*). However, information about specific regions in Turkey in which hantavirus is endemic is limited.

Hantavirus infections, which were first identified in northwestern Turkey in 2009 and subsequently in Istanbul, should be considered in the diagnosis of patients who have fever and bleeding. Because of recent emergence of hantavirus in Turkey, areas to which this virus is endemic and where risk for infection is highest have not been identified. Therefore, all inhabitants at high risk for infection (forest workers, military personnel, farmers, persons living in or near a forest, persons handling wood) should be informed about this risk.

Table. Detection of Dobrava virus in 22-year-old patient, Turkey*

	Hantavirus immunoblot		Real-time PCR			
			Serum		Urine	
Test	IgM	IgG	DOBV	PUUV	DOBV	PUUV
Result	Pos	Neg	Neg	Neg	Pos	Neg

*Ig, immunoglobulin; DOBV, Dobrava virus; PUUV, Puumala virus; pos, positive; neg; negative.

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Maternal–Fetal Transmission of *Cryptococcus gattii* in Harbor Porpoise

To the Editor: We report maternal–fetal transmission of *Cryptococcus gattii* and death in a wild porpoise. *Cryptococcus neoformans* and *C. gattii* are 2 environmental, encapsulated yeasts that cause invasive, potentially life-threatening infections in humans and animals (*1*). *C. neoformans* causes disease in immunocompromised hosts, and *C. gattii* is also pathogenic in immunocompetent hosts (*2*). Since 1999, cryptococcosis caused by *C. gattii* has appeared on southern Vancouver Island (British Columbia, Canada) and nearby surrounding areas (*2*,*3*). Spread beyond Vancouver Island has been documented along the Pacific Northwest Coast, but the mechanism remains undetermined (*4*).

A pregnant, dead, stranded, harbor porpoise (*Phocoena phocoena*) was reported on February 22, 2007, on western Whidbey Island, in Puget Sound, Washington State (48.2833°N, 122.7283°W). The carcass was iced and necropsy was performed on February 24. Sampled tissues from the adult and fetus were divided: half fixed in 10% formalin for histopathologic analysis, and half frozen for ancillary studies.

For histologic analysis, tissues were embedded in paraffin, sectioned to 3–5 μm, and stained with hematoxylin and eosin. Selected sections were stained with mucicarmine. The adult porpoise (length 177 cm, weight ≈57.7 kg) was in poor condition (reduced blubber layer). Both lungs were exposed, extensively scavenged, firm, and nodular; a sectioned surface exuded clear to slightly opaque gelatinous to mucinous discharge. Mediastinal lymph nodes were grossly enlarged, multinodular, and firm with large numbers of yeasts visible by microscopy (online Appendix Figure, panel A, www.cdc.gov/EID/content/17/2/302-appF.htm). The first stomach chamber contained two 3.5 cm × 2.5 cm raised, centrally umbilicated ulcers and several embedded anisakid nematodes. The uterus was gravid in the right horn with a mid-term fetus. No other gross lesions were identified. Microscopically, the lung lesions correlated with granulomatous to pyogranulomatous infiltrates, often with a myriad of yeasts.

The male fetus (length 30 cm, weight 2.4 kg), was examined separately at a different facility than the dam. It appeared grossly normal externally and was at a gestation of ≈5–6 months. Mediastinal lymph nodes had mild granulomatous inflammation and contained numerous yeasts morphologically consistent with *Cryptococcus* spp. (online Appendix Figure, panel B). The lymph nodes were partially replaced with intracellular and extracellular mulilobulated yeast aggregates (length 8–20 μm) with pale eosinophilic central regions and a thin refractile wall peripherally bound by a 5-μm nonstaining capsule. Around the periphery of these aggregates, there were small numbers of macrophages and lymphocytes and fewer neutrophils. Specific staining showed a prominent mucicarminophilic capsule consistent with *Cryptococcus* spp.

Yeasts were found in the amniotic fluid and interspersed within the chorioallantoic villi and submucosal vasculature of the placenta. Mild multifocal nonsuppurative myocarditis was detected. However, no yeasts were seen in inflamed areas. There were no overt lesions in the remaining organs.

Maternal and fetal tissues were cultured for fungi, and diagnosis was based on Gram stain (budding yeast-like cells), India ink stain (positive for encapsulated cells), hydrolysis of urea (positive), and final confirmation by using API 20C Aux V3.0 (bioMérieux, Marcy l'Etoile, France). Canavanine-glycine-bromthymol blue agar was used to differentiate between *C. gattii*

and *C. neoformans* (*5*). Molecular typing by restriction fragment length polymorphism was used to definitively speciate and subtype *C. gattii* (*6*). Fungal culture showed heavy growth of *Cryptococcus* spp. from the dam (lungs, mediastinal lymph nodes, and placenta) and fetus (mediastinal lymph nodes). Genotyping of primary isolates identified VGIIa *C. gattii* in both animals. Test results for enteric pathogens, intestinal nematodes, morbillivirus, and *Brucella* spp. were negative.

Fetal infection was most likely hematogenous, disseminated from a primary maternal pulmonary source to the uterus and subsequently to placental vasculature and internal fetal tissues. Infection by aspiration or ingestion of contaminated amniotic fluid was also possible. Although close evaluation of the lung did not show any discernible yeasts, the organism may have been present in an area other than that sectioned.

During 1998–2007, ≈450 harbor and Dall's porpoises (*Phocoenoides dalli*) and Pacific white-sided dolphins (*Lagenorhynchus obliquidens*) along the Pacific Northwest Coast were recovered and subjected to necropsy. Disseminated cryptococcosis caused by *C. gattii* since 2000 was diagnosed in 15 harbor porpoises, 10 Dall's porpoises, 2 adult Pacific white-sided dolphins, and 3 unrecorded species (10 females, 15 males, and 5 unknown sex; 24 adults, 4 juveniles, 1 fetus, and 1 undocumented age; S. Raverty, unpub. data).

Wild porpoises in the Pacific Northwest Region, being near shore inhabitants in waters surrounding Vancouver Island, may come into contact with air containing *C. gattii* at the air–water interface or ingest seawater containing yeasts while feeding (*7*). Their proximity to a habitat containing Coastal Douglas fir (*Pseudotsuga menziesii*) and Western hemlock (*Tsuga heterophylla*) may play a role in the epidemiology of *C. gattii* because these trees have been associated with cases of *C. gattii* (*8*). Cryptococcal infection during pregnancy has been reported in humans and horses (*9*,*10*).

This fetal case of cryptococcosis may have major human and animal health implications. Further studies should be undertaken to assess possible fetal involvement, identify infections in pregnant females, and provide information on risk reduction and improving diagnosis and treatment.

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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.

New Delhi Metallo-β-Lactamase, Ontario, Canada

To the Editor: The New Delhi metallo-β-lactamase (NDM-1) was first characterized in 2009 from *Klebsiella pneumoniae* and *Escherichia coli* isolated from a patient in Sweden who had received medical care in New Delhi, India (*1*). Further studies have shown broad dissemination of this β-lactamase gene (bla_{NDM-1}) in India, Pakistan, Bangladesh, and the United Kingdom (*2*). Additional isolates have been detected in other countries, and many of the patients with NDM-1–producing *Enterobacteriaceae* reported receiving medical care in the Indian subcontinent (*1*–*7*). We describe detection and characterization of an NDM-1–producing *K. pneumoniae* isolated in Ontario, Canada.

In August 2010, a urinary tract infection was diagnosed in a 36-year-old woman in a hospital in Brampton, Ontario. An *E. coli* strain sensitive to multiple antibacterial drugs (including carbapenems) was isolated from a midstream urine sample; the patient was successfully treated with ciprofloxacin. One week after treatment, when the patient did not have a fever or other clinical signs, a urine culture was repeated, and a carbapenem-resistant *K. pneumoniae* isolate (GN529) was recovered. Travel history indicated that the patient had recently returned from India, where in mid-July she had had a miscarriage and had been hospitalized in Mumbai for 2 days. At that time, no antimicrobial drug treatment was prescribed.

Susceptibility profiles of *K. pneumoniae* GN529 and its *E. coli* transconjugant were obtained by using Etest (bioMérieux, Marcy l'Etoile, France) and the agar dilution method based on the Clinical and Laboratory Standards Institute guidelines (*8*). Multilocus sequence typing (MLST) of isolate GN529 was performed as described (*9*). The Pasteur Institute online database (www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) was used to assign the allelic numbers and sequence type (ST).

To screen for the most commonly known β-lactamase genes in enterobacteria, we performed multiplex PCRs (*10*). Primers were designed (NDM-F, 5′-AATGGAATTGCCCAATATTATGC-3′; NDM-R, 5′-CGAAAGTCAGGCTGTGTTG C-3′) for the specific detection of bla_{NDM-1} and included in 1 of the multiplex PCRs (multiplex V). Primers NDM-F and NDM-R2 (5′-TCAGCGCAGCTTGTCGGC-3′) were used to amplify and sequence the entire bla_{NDM-1} gene. The samples were screened for the presence of six 16S methylase genes (*armA*, *rmtA–D*, and *npmA*) by PCR. *E. coli* J53 transconjugants were selected on Luria-Bertani plates containing sodium azide and meropenem (100 μg/mL and 1 μg/mL, respectively). The plasmid harboring bla_{NDM-1} was identified by Southern blot analysis by using a specific digoxigenin-labeled bla_{NDM-1} probe (Roche Diagnostics, Indianapolis, IN, USA).

K. pneumoniae GN529 was highly resistant to all β-lactams, aminoglycosides, quinolones, tetracycline, nitrofurantoin, and co-trimoxazole. MICs of 0.5 μg/mL for colistin (European Committee on Antimicrobial Susceptibility Testing colistin breakpoint for *Enterobacteriaceae*: susceptibility ≤2 μg/mL) and 1 μg/mL for tigecycline (European Committee on Antimicrobial Susceptibility Testing and US Food and Drug Administration tigecycline breakpoint for *Enterobacteriaceae*: susceptibility ≤1 and ≤2 μg/mL, respectively) were also obtained (Table).

Considering the travel history of the patient and the high level re-

Table. Antibacterial drug susceptibility profiles and resistance genes of *Klebsiella pneumoniae* GN529 clinical isolate and its *Escherichia coli* transconjugant, Ontario, Canada, 2010*

Antibacterial drug or gene	MIC, μg/mL		
	Kpn GN529	Eco J529	Eco J53
Ampicillin	≥256	≥256	6
Cefoxitin	≥256	≥256	8
Ceftazidime	≥256	≥256	0.19
Cefotaxime	≥256	≥256	0.094
Cefepime	48	48	0.064
Ertapenem	32	12	0.008
Meropenem	≥32	4	0.023
Imipenem	≥32	32	0.38
Amikacin	≥256	≥256	1.5
Gentamicin	≥256	≥256	1.5
Tobramycin	≥256	≥256	1
Ciprofloxacin	≥32	0.012	0.012
Tetracycline	≥16	0.78	1
Tigecycline	1	ND	ND
Nitrofurantoin	≥512	ND	ND
Colistin	0.5	ND	ND
Co-trimoxazole	4/76	ND	ND
PCR† and sequencing			
bla_{NDM-1}	+	+	ND
$bla_{CTX-M-15}$	+	–	ND
bla_{SHV-12}	+	+	ND
bla_{SHV-11}	+	–	ND
bla_{OXA-1}	+	–	ND
bla_{TEM-1}	+	–	ND
armA	+	+	ND

*Kpn, *Klebsiella pneumoniae*; Eco J529, *Escherichia coli* transconjugant strain; Eco J53, recipient *E. coli* J53; ND, not determined.
†PCR screening included bla_{TEM}, bla_{SHV}, bla_{OXA-1}-like, bla_{CTX-M} groups 1, 2, and 9, bla_{VEB}, bla_{PER}, bla_{GES}, bla_{OXA-48}-like, bla_{IMP}, bla_{VIM}, bla_{KPC}, bla_{NDM-1}, and 6 groups of bla_{AmpC} genes.

sistance to all β-lactams, molecular screening of β-lactamases in strain GN529 was initiated to identify possible carbapenemases (e.g., bla_{NDM-1}) in that isolate. Five β-lactamases genes (bla_{NDM}, bla_{SHV}, bla_{TEM}, group 1 bla_{CTX-M} , and bla_{OXA}) and one 16S rRNA methylase (*armA*) were detected. By using primers for amplification of complete genes, we obtained sequences of bla_{NDM-1}, 2 extended-spectrum β-lactamases ($bla_{CTX-M-15}$ and bla_{SHV-12}), 3 broad-spectrum β-lactamases (bla_{SHV-11}, bla_{TEM-1} and bla_{OXA-1}), and methyltransferase *armA*. No AmpC β-lactamases were linked to this isolate. Southern blotting identified a plasmid of ≈150 kb harboring bla_{NDM-1} (data not shown). A transconjugant *E. coli* positive for bla_{NDM-1} (*E. coli* J529, Table) was resistant to all β-lactams and aminoglycosides tested. In addition, bla_{SHV-12} and *armA* were detected in strain J529 (Table), indicating the potential for the horizontal spread of these resistance genes.

K. pneumoniae GN529 was typed by MLST as ST147, the same type as a clinical NDM-1–producing strain isolated in Australia (*6*) but distinct from ST14 and ST16 strains described (*1*,*7*). There are insufficient MLST data to confirm polyclonal dissemination of NDM-1, but previous pulsed-field gel electrophoresis results support that hypothesis (*2*).

K. pneumoniae GN529 was isolated from a patient who had recently received emergency medical care in India, suggesting importation of this clinical strain. In the United Kingdom, where *Enterobacteriaceae* containing bla_{NDM-1} are increasingly common, carriage of these organisms has been closely linked to receipt of medical care in the Indian subcontinent (*2*). Similar association as a risk factor was observed in other regions, including bla_{NDM-1}-positive clinical strains isolated in North America, Australia, and Africa (*3*–*6*,*10*).

The NDM-1–producing enterobacteria described in this study previously had low MICs only for colistin and tigecycline (*1*,*2*,*5*,*6*). However, an NDM-1 isolate resistant to these antimicrobial drugs has also been described (*2*). Early detection and implementation of infection control interventions is essential for preventing the spread of multidrug-resistant organisms such as these. It may be prudent to consider medical exposure in the Indian subcontinent as a risk factor for possible infection, colonization, or both with multidrug-resistant, NDM-1–producing *Enterobacteriaceae*.

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Genetic Detection of Dobrava/ Belgrade Virus, Bulgaria

To the Editor: Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) cause 2 clinical syndromes in humans: hemorrhagic fever with renal syndrome (HFRS) in the Old World and hantavirus pulmonary syndrome in the New World (*1*). Humans are infected by inhaling the excreta of infected rodents. Persons at increased risk for infection are farmers, loggers/ forest workers, and soldiers.

Bulgaria is a country in southeastern Europe with 8 million inhabitants. Two types of hemorrhagic fevers are endemic to Bulgaria: Crimean-Congo hemorrhagic fever and HFRS. Both diseases have been subject to mandatory reporting since 1953. During the past decade, 36 cases of HFRS have been reported, mainly in the areas of the Balkan and Rila-Pirin-Rodopa mountain ranges in southwestern Bulgaria. Diagnosis was based on clinical symptoms and serologic test results. We report 3 HFRS cases, 2 of which were fatal. Apart from serologic diagnosis, genetic detection of hantaviruses was also achieved, resulting in gaining insight into the genetic relationships of hantavirus sequences from Bulgaria with respective sequences retrieved in neighboring countries.

On September 2, 2009, high fever, chills, headache, and myalgia developed in a 21-year-old man who lived in Simitli town (Blagoevgrad Province, southwestern Bulgaria). Five days after symptom onset, he was admitted to the regional hospital of Blagoevgrad. His condition rapidly deteriorated. Clinical signs were pharyngeal hyperemia, oliguria, and febrile toxic syndrome. The patient became hypotensive, reporting abdominal pain in the liver and spleen. Laboratory findings showed the following: leukocyte count 11.8 × 10^9 cells/L, hematocrit 51%, blood hemoglobin 161 g/L, platelet count 10 × 10^9 cells/L, aspartate aminotransferase (AST) 118 U/L, alanine aminotransferase (ALT) 89 U/L, urea 26.4 mmol/L, and creatinine 501 μmol/L. An echograph showed enlarged kidneys, liver, spleen, and pancreas, and abdominal and bilateral pleural effusions. Urine analysis disclosed proteinuria and microscopic hematuria. The patient was admitted with acute renal failure and multiple organ insufficiency. Despite multiple blood transfusions and hemodialysis, he died 14 days after hospitalization.

On April 9, 2010, a 54-year-old man, a resident of Kirkovo village (Kardjali Province, southern Bulgaria), was admitted to the regional hospital in Kardjali City with a 7-day history of fever, weakness, and myalgia in the lower extremities and a 4-day history of abdominal pain and diarrhea. At admission, physical examination showed skin petechiae, subconjunctival and gingival hemorrhages, and oliguria. Laboratory findings showed the following: leukocyte count of 23 × 10^9 cells/L, platelet count of 50 × 10^9 cells/L, AST 96 U/L, ALT 167 U/L, urea 58.7 mmol/L, and creatinine 1,033 μmol/L. Urea and creatinine levels continued to rise. Proteinurua and hematuria were present. After 3 sessions of hemodyalisis, the patient gradually improved, and he was discharged without sequelae.

On May 7, 2010, a 28-year-old man, a resident of Smilyan village, (Smolyan Province, southern Bulgaria) was admitted to the Infectious Diseases Clinic in Smolyan Regional Hospital with a 4-day history of fever, vomiting, and diarrhea. Physical examination on admission showed skin petechiae and gingival hemorrhages. Laboratory findings showed the following: leukocyte count of 6 × 10^9 cells/L, platelet count of 50 × 10^9 cells/L, urea 10.5 mmol/L, creatinine 230 mmol/L, AST 1697 U/L, and ALT 1,119 U/L. Proteinuria and hematuria were present. The patient became anuric and underwent hemodialysis. On May 9, the patient died.

Serum samples from these 3 patients were tested for immunoglobulin (Ig) G and IgM against Hantaan virus (HTNV) and Puumala virus by ELISA (Progen, Biotechnik GmbH, Heidelberg, Germany). High titers of HTNV IgM were detected in all 3 patients; in 1 patient HTNV IgG was also detected; antibodies against Puumala virus were not detected. Thus, a HTNV-like infection was suggested.

Viral RNA was extracted from the earliest available serum sample, and a 1-step SYBR Green real time reverse transcription–PCR (RT-PCR) (Bio-Rad, Hercules, CA, USA) (*2*) and 2 nested RT-PCRs amplifying partial small (S) and medium (M) RNA segments were applied (*3*,*4*). Dobrava/Belgrade virus (DOBV) RNA was detected by RT-PCR. Sequencing and phylogenetic analysis of the nested RT-PCR products showed that the causative agent in all 3 cases was DOBV (Figure).

Sequences were submitted to GenBank under accession nos. HQ174468–HQ174473. Bulgarian sequences cluster with respective sequences retrieved from *Apodemus flavicollis* mouse tissues or from HFRS cases from central and southeastern Europe. Briefly, the closest genetic strains in S and M RNA segments are strains isolated from *A. flavicollis* mice in northeastern Greece, near the border with Bulgaria (*5*). The genetic difference at nucleotide level among the Bulgarian strains is 1.2%–2.1% and 2.2%–7.4% in the S and M segments, respectively.

HFRS is endemic to the Balkan Peninsula. Severe HFRS cases caused by DOBV have been reported in Greece (*4*–*6*), Slovenia (*7*,*8*), Serbia and Montenegro (*9*), the Czech Republic (*3*), and Hungary (*10*). Our results confirm that DOBV also circulates in Bulgaria and causes severe HFRS cases; thus, clinicians have to include HFRS in differential diagnosis

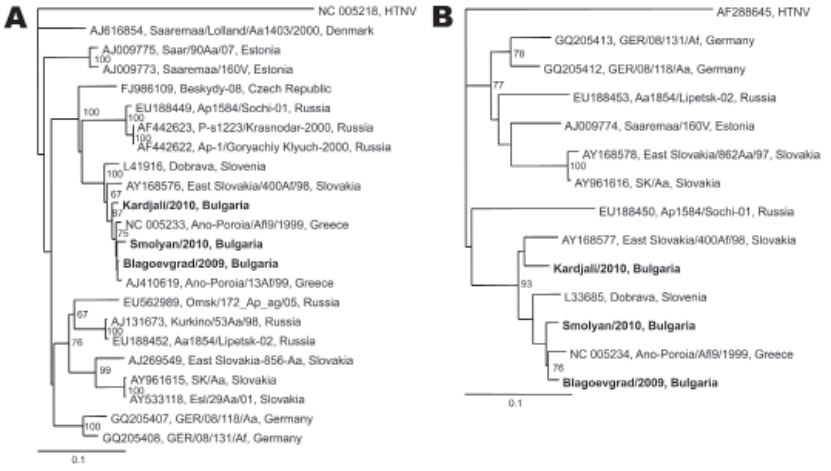

Figure. Phylogenetic trees based on a 560-bp fragment of the small RNA segment (A) and a 224-bp fragment of the medium RNA segment (B) of hantaviruses. Hantaan virus (HTNV) was used as the outgroup. The numbers at the nodes indicate percentage bootstrap replicates of 100; values <60% are not shown. Horizontal distances are proportional to the nucleotide differences. Sequences in the tree are indicated as GenBank accession number, strain name, country. Strains from this study are shown in **boldface**. Scale bars indicate 10% nucleotide sequence divergence.

of febrile cases accompanied by acute nephropathy. Further studies on patients and small mammals in Bulgaria will elucidate the hantavirus epidemiology in this Balkan region.

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A226V Strains of Chikungunya Virus, Réunion Island, 2010

To the Editor: Chikungunya virus (CHIKV) first emerged in Indian Ocean islands off the eastern coast of Africa in 2005 and was responsible for large-scale epidemics on the islands of Réunion, Comoros, Mayotte, Mauritius, Madagascar, and Seychelles (*1–4*). On Réunion Island, a French overseas territory of 810,000 inhabitants, herd immunity reached 38% in October 2006 (*5*). Molecular epidemiology of the strain responsible for these outbreaks indicated that it had originated in Kenya (*6*). The epidemic on Réunion Island was associated with a mutation in the envelope protein gene (E1-A226V) that improves replication and transmission efficiency in *Aedes albopictus* mosquitoes (*7*).

Since 2006, the Regional Office of the French Institute for Public Health Surveillance in the Indian Ocean has conducted epidemiologic and biological surveillance for CHIKV infection. Case definitions have been described (*8*). During December 2006–July 2009, no confirmed case was detected

on Réunion Island and Mayotte, but new outbreaks were reported in Madagascar (*9*). In August 2009, a cluster of cases was identified on the western coast of Réunion Island (*8*).

We report an outbreak of CHIKV infection that occurred on Réunion Island in 2010. The first case was detected on March 17, 2010. As of July 6, a total of 100 confirmed and 32 probable cases had been identified (online Appendix Figure, www.cdc.gov/EID/content/17/2/307-appF.htm). Median age of case-patients was 39 years (range 6 months–80 years), and the ratio of male to female case-patients was 0.81:1. In addition to fever (95%), case-patients had arthralgia (95%), headache (78%), and myalgia (75%). Seven (5%) were admitted to hospitals. No severe illness or death was reported. The outbreak remained largely restricted to residents of Saint Paul (75%) on the western coast. Sporadic cases in other cities also were detected.

Sequence comparison based on partial envelope gene or complete genome showed a high level (>99.6%) of nucleotide and amino acid identity of 2010 isolates from Réunion Island with the strains of the 2009 sporadic cases on Réunion Island, as well as with the Malagasy strains circulating since 2006. All isolates sequenced bore the A226V substitution within the E1 protein. Altogether, these results support the hypothesis of a continuous circulation of A226V strains in the southwestern Indian Ocean since 2006 and the possible reintroduction of CHIKV on Réunion Island, most probably from Madagascar. Once again, human travel may have contributed to the rapid spread of the virus between islands because imported and autochthonous cases on Réunion Island occurred after a holiday period for residents on Réunion Island who often traveled to Madagascar. Migration and birth rate on Réunion Island might have contributed to a decrease in the immunity of the population. Furthermore, seroprevalence in 2007 was not homogenous throughout the territory. A hypothesis would be that a lower immunity of the population in the Saint Paul area and environmental and vectorial characteristics contributed to the emergence of this CHIKV disease cluster.

On Réunion Island, *Ae. albopictus* mosquitoes have been described as the main vector responsible for transmitting CHIKV (*10*). The austral winter may contribute to moderate vector activity and transmission. We cannot exclude a continuous transmission until next austral summer, followed by an increase of cases and an extension to the whole island, as occurred in 2005 (*1*). Epidemiologic and entomologic surveillance has been reorganized to prevent this risk. Medical staff, the general population, and travelers have been informed about the situation through the news media and meetings organized by health authorities, and recommendations have been issued about destroying mosquito breeding sites and preventing mosquito bites.

In recent years, the area of circulation and the epidemic potential of CHIKV have increased, and CHIKV has emerged as a major public health problem. This outbreak could be a new warning to Réunion Island health authorities about the need for preparation not only for CHIKV but also for dengue virus (DENV). With the extent of human travel to and from areas with active CHIKV and DENV circulation, viremic returning travelers constitute an ongoing risk for introduction of such viruses on Réunion Island. In May 2010, two locally acquired DENV-3 cases were also detected, illustrating this threat. These cases occurred during an outbreak of DENV-3 in Comoros Island. Public health efforts to control *Ae. albopictus* mosquitoes have not been completely effective.

This outbreak of CHIKV infection, the detection of autochthonous cases of DENV infection, and the influenza season on Réunion Island emphasize the difficulty of making the appropriate clinical diagnosis. Clinicians and biologists should be aware of the cocirculation of CHIKV, DENV, and influenza viruses. The reemergence of CHIKV on Réunion Island illustrates the permanent threat of circulation of exotic pathogens in the Indian Ocean and the need for strong epidemiologic and laboratory surveillance. Human travel and the geographic expansion of *Ae. albopictus* mosquitoes raise concern for the spread of CHIKV in Europe and North America.

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Segniliparus rugosus–associated Bronchiolitis in California Sea Lion

To the Editor: Until now, *Segniliparus rugosus* has not been isolated from nonhuman animals or the environment (*1*). On April 14, 2010, a rescue team from Pacific Marine Mammal Center impounded an emaciated and unresponsive subadult female California sea lion (*Zalophus californicus*) stranded on the beach at San Onofre, California, USA. Physical examination showed the animal to be obtunded and emaciated (third-stage malnutrition), with moderate bradycardia, hypoventilation, and hypothermia. Euthanasia was elected because of a poor prognosis. Immediately before euthanasia, a blood sample was taken for a complete blood count and serum chemistry evaluation. A postmortem examination was conducted immediately after euthanasia.

The postmortem examination showed marked subcutaneous and visceral adipose tissue depletion, as well as moderate skeletal muscle loss, especially in the axial skeleton. In the lungs, a frothy, greenish, mucoid material exuded from several dozen bronchioles. Samples of the exudate were submitted for cytologic examination and bacterial culturing (IDEXX Laboratories, Irvine, CA, USA). Selected tissues were sampled and fixed in 10% neutral buffered formalin for histopathologic examination.

Complete blood count and serum chemistry analysis showed moderate anemia; relative neutrophilia and monocytosis; mild to moderate relative lymphopenia; moderate to markedly reduced albumin, globulin, and total protein levels; and elevated creatine kinase and alkaline phosphatase levels. Such values are common in California sea lions with severe malnutrition (starvation).

Cytologic examination of the bronchiolar exudate indicated large amounts of mucin with erythrocytes; occasional epithelial cells; and small to moderate numbers of eosinophils, neutrophils, monocytes, and lymphoid cells, characteristic of a mild to moderate, subacute, mixed bronchiolitis. Histologic examinations of 3 sections of lung showed 33 bronchioalveolar foci containing varying numbers of adult *Parafilaroides decorus* nematodes, without associated inflammation. Eleven other foci showed moderate to marked chronic inflammation, with nematodes in only 2 foci. Gram stain did not show bacteria in any of these foci. Lesions were not found in sections of liver, kidney, bladder, spleen, and heart.

A commercial veterinary laboratory (IDEXX Laboratories) isolated an acid-fast organism from the lung swab. This organism was referred to National Jewish Medical and Research Center (Denver, CO, USA) for species identification and sensitivity analysis. By 16S rDNA sequencing, the organism was identified as *S. rugosus*. Sensitivity testing showed that it was susceptible to rifabutin, cycloserine, clofazimine, moxifloxacin, ciprofloxacin, and clarithromycin and resistant to rifampin, streptomycin, amikacin, kanamycin, capreomycin, ethambutol, and ethionamide.

As in humans, this isolation of *S. rugosus* was associated with pathologic changes in the respiratory tract. Whether the relationship was causal or simply a fortuitous isolation of a previously unrecognized part of the normal respiratory flora is uncertain. However, a recent report by Sikorski et al. stated that "Environmental screens and metagenomic surveys did not detect a single phylotype… of the members of the genus *Segniliparus*" (*2*). In contrast, this case report begs the question of whether *S. rugosus* could be free-living in the oceans or part of the flora of any number of ocean-dwelling vertebrates or invertebrates.

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Orbiviruses in Rusa Deer, Mauritius, 2007

To the Editor: Bluetongue and epizootic hemorrhagic disease are caused by orbiviruses transmitted by *Culicoides* spp. biting midges (Diptera: Ceratopogonidae). These diseases are restricted to regions where their vectors exist (*1*) and seem to be expanding to previously unaffected areas (*2*). Infection of wild and domestic ruminants is common. Bluetongue virus (BTV) causes severe clinical disease in certain breeds of sheep; BTV and epizootic hemorrhagic disease virus (EHDV) cause clinical disease in some species of deer (*3*,*4*).

Rusa deer (*Cervus timorensis rusa*), originally from Indonesia, are found in diverse countries in the Pacific region (Papua New Guinea, New Caledonia, New Zealand, and Australia). Introduced to the island of Mauritius in 1639, they are commonly raised in high numbers (≈60,000) for meat production (*5*). Mauritius is considered free from major livestock diseases; its animal health surveillance is based mainly on clinical monitoring and inspection of carcasses at slaughter. To our knowledge, circulation of orbiviruses in Rusa deer has not been reported in detail in any country where this deer is present.

Our study was an initial screening survey of the deer population on the island. A total of 369 deer, representing 28 private farms, were chosen from a list of 42,959 deer. Blood was collected at slaughter, and serum samples were sent to Onderstepoort Veterinary Institute, South Africa, to be tested for antibodies against orbiviruses with a homemade indirect ELISA. To distinguish between BTV and EHDV, samples positive by indirect ELISA were tested for BTV antibodies with the competitive ELISA produced by the Institute for Animal Health (Pirbright, UK). Of the samples positive by competitive ELISA, 3 were tested by serum neutralization against the 24 BTV serotypes (cutoff value >16).

Simultaneously, *Culicoides* spp. midges were trapped in Onderstepoort-type blacklight traps at 3 deer farms in coastal areas (Figure). Trapping was conducted 1 night at each farm, during optimal weather conditions. Midges were kept at room temperature in 95% ethanol until sent to Strasbourg University, France, for identification.

Of the 369 deer serum samples tested, 15 were positive for BTV and 5 for EHDV; seroprevalence was 4.1% for BTV (95% confidence interval 2.0%–6.1%) and 1.3% for EHDV (95% confidence interval 2.0%–6.1%). No significant differences were observed for sex ($\chi^2 = 0.05$,

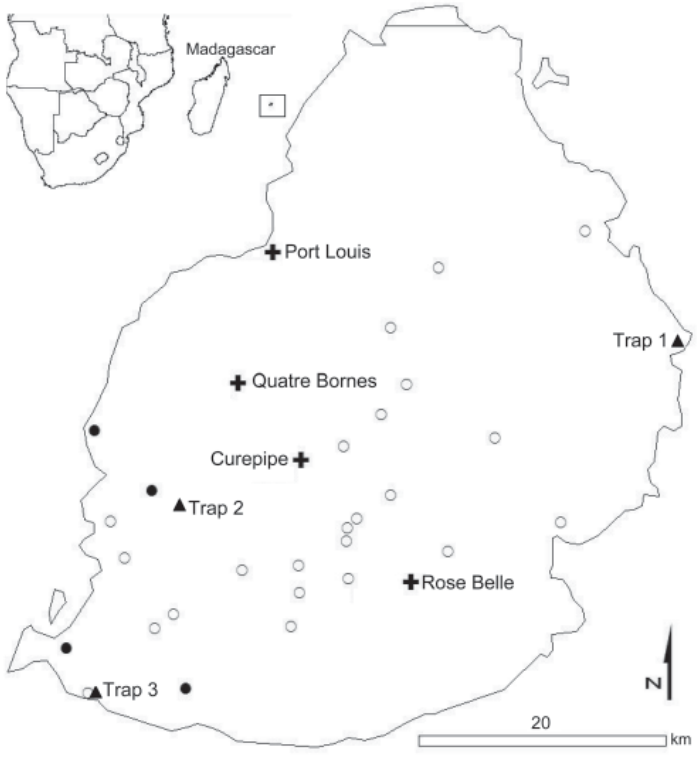

Figure. Location of farms where Rusa deer were sampled (open circles), herds with orbivirus-seropositive deer (closed circles), biting midge collection sites (triangles), and main cities (crosses) in Mauritius. Most (99%) *Culicoides* spp. midges were trapped at sites 1 and 3. Inset show location of Mauritius (in square) in relation to Africa and Madagascar.

p = 0.82). Antibodies (indirect ELISA) against orbiviruses were more prevalent among adults ($\chi^2 = 4.56$, p = 0.03). The 3 samples tested by serum neutralization had high titers against BTV-2 (256, 256, and 128) and lower but positive titers against BTV-17 (32 in each), BTV-10 (16 in each), and BTV 21 (32 in 1). Despite reports of clinical signs in other deer species infected with both viruses (*3*,*4*), no signs of bluetongue or epizootic hemorrhagic disease were reported for the Rusa deer population in our survey. This absence of clinical disease might be the result of natural resistance of this species to orbiviruses or to the fact that the circulating serotypes are endemic to the area.

A total of 13,356 *Culicoides* spp. midges were obtained; 12% were identified as C. *imicola* (1,459 females, 138 males) and 88% as *C. enderleini* (8,800 females, 2,878 males). The former species has been reported in Mauritius (*6*). In our study, the positive serum came from deer on 4 farms located in the coastal area. This finding could indicate a higher abundance and activity of *C. imicola* midges in coastal areas, where climate and altitude are more favorable for the vector.

Serum neutralization results suggested that at least 4 serotypes could have been circulating in deer from Mauritius. During outbreaks of both viruses in neighboring Réunion Island, several serotypes of BTV were isolated from sheep (*7*,*8*) and of EHDV from cattle (*9*). However, BTV serotypes 17 and 21 have never been isolated from Indian Ocean countries, and serotype 21 has been detected only in Australia (*2*). Equally, diverse BTV serotypes circulate at different locations on the Indian Ocean and the east coast of Africa (*2*,*5*,*6*).

Our results provide serologic indication that EHDV and BTV circulate in Rusa deer in Mauritius. The large population of Rusa deer can represent a potential reservoir host for those viruses and a risk for transmission to other ruminants in Mauritius and neighboring countries. However, Rusa deer could be used as a sentinel population to regularly monitor the circulation of orbiviruses and the introduction of new serotypes to Mauritius. To detect and isolate circulating serotypes and genotypes of these viruses in ruminant species and in potential vectors in Mauritius, further research is needed. In addition, the extent of both viruses and the distribution of *Culicoides* midges over the island should be investigated in more detail.

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No Xenotropic Murine Leukemia Virus–related Virus Detected in Fibromyalgia Patients

To the Editor: Xenotropic murine leukemia virus–related virus (XMRV) is a recently described human retrovirus that has been associated with prostate cancer and chronic fatigue syndrome (CFS) (*1*,*2*). XMRV is similar to a classic murine endogenous leukemia retrovirus, murine leukemia virus (MLV), which infects strains of mice that do not express the specific viral receptor. XMRV is genetically close to, although differentiable from, MLV. The first evidence of its presence in humans was obtained by Urisman et al. in prostate cancer tissue (*1*). In 2009, Lombardi et al. (*2*) found XMRV sequences and specific antibody responses in 67% of a large group of patients with CFS in North America. This association was notable because XMRV sequences were found in only 4% of healthy controls. These results have generated controversy because several independent studies, mainly in Europe (*3*–*5*) but also in North America (*6*), have been unable to detect XMRV sequences in patients with CFS. Furthermore, a recent report from North America (*7*) appears to confirm the initial results by Lombardi et al. (*2*) in patients with CFS and expands the viral association to a wider variety of XMLV-related viruses that seem closer to polytropic mouse endogenous retroviruses.

Fibromyalgia is a multifactor condition characterized by widespread pain and diffuse tenderness. Although trauma and stress can worsen or even precipitate development of the syndrome, infections with certain viruses, including hepatitis C virus and HIV, have been associated with development of fibromyalgia (*8*). Nevertheless, fibromyalgia remains a disease of unknown etiology. Although CFS is a distinct entity, features shared by both diseases suggest that CFS and fibromyalgia represent the same underlying condition (*9*). Additionally, because they are often accompanied by a noticeable mental health effect (*9*), the presence of a potential neurotropic retroviral agent in both diseases could explain these similarities. Therefore, we studied the presence of XMRV and polytropic MLV–related retroviruses in a group of patients with fibromyalgia.

During January 2010, blood samples were collected from 15 patients in whom fibromyalgia had been previously diagnosed according to American College of Rheumatology criteria (www.rheumatology.org/practice/clinical/classification/fibromyalgia/1990_Criteria_for_Classification_Fibro.pdf). Ten healthy blood donors served as controls. For XMRV screening, we used DNA extracted from 400 μL of whole blood collected in EDTA tubes by the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Nested PCR was done by using 5 sets of primers corresponding to the *gag* (*3*) and *env* (*2*) regions of XMRV as described (*2*,*3*,*7*). The first round of PCR was conducted by using 500 ng of genomic DNA, equivalent to 7.5 × 10^4 nucleated blood cells, in a final volume of 50 μL, by using the Expand High Fidelity PCR System (Roche Applied Science, Basel, Switzerland). A second round of PCR was conducted under the same conditions by using 5 μL of the first reaction product. Details of the nested-PCR strategy were as follows: *gag* region was amplified by outer primers 419F and 1154R (*2*) and 3 sets of inner primers: XMRV-FI-441/XMRV-RI-566 (*3*), MLV-GAG-I-F/MLV-GAG-I-R, and MLV-NP116/MLV-NP117 (*7*). Nested PCR for *env* was performed by using outer primers 5922F and 6273R (*2*) and 2 sets of inner primers: 5922F/6173R and 5942F/6159R (*7*). Primers for human β-globin were used as positive controls of human DNA amplification (*3*). The full-length molecular viral clone VP62 (obtained through the National Institutes of Health AIDS Research and Reference Reagent Program [Rockville, MD, USA] from R.H. Silverman and B. Dong) (*10*) was used as a positive XMRV control. All samples were examined on a 2% agarose gel stained with ethidium bromide (Figure). The overall sensitivity of the nested PCR procedure, estimated by spiking VP62 into negative samples, was 1–10 copies per sample.

Using highly sensitive PCR tools and a multiple set of primers to detect xenotropic and polytropic MLV–related sequences, we found no evidence of MLV-related sequences in blood cells from fibromyalgia patients or controls. Our results agree with those from studies of CFS cohorts in Europe and North America that also failed to confirm XMRV in blood samples (*3*–*6*). Technical issues or geographic specificities probably could not account for such a difference; therefore, these negative results raise concerns about the role of XMRV in these syndromes. Nevertheless, with this relatively small population we cannot absolutely exclude an association of XMRV or polytropic MLV–related viruses with fibromyalgia. However, a proportion of fibromyalgia cases with XMRV >22% would be unlikely (3/15 cases, 95% confidence interval 0–3), which is clearly insufficient to support a significant association between XMRV and fibromyalgia.

Fibromyalgia does not appear to be associated with XMRV or polytropic MLV–related viruses. The role of these new agents in human disease, and specifically in CFS, remains to be clearly confirmed in multicenter and standardized studies.

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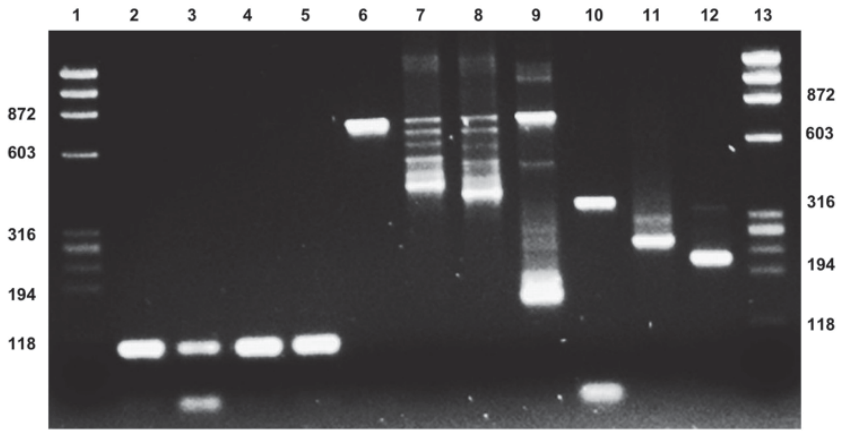

Figure. Testing for xenotropic murine leukemia virus–related virus (XMRV) in patients with fibromyalgia. Lanes 1 and 13, molecular weight marker ΦX174RF *Hae*III; lanes 2–5, hBG for patients 1–4 (primers: hBG-FI-170/hBG-RI-273 (103 bp); lanes 6–12, positive control (pcDNA3.1-XMRV-Vp62) 1,000 copies (lanes 6 and 10) and 100 copies (lanes 7–9 and 11–12); lane 6, primers *gag* 419F/1154R (735 bp); lane 7, primers *gag* MLV-GAG-I-F/MLV-GAG-I-R (413 bp); lane 8, primers *gag* MLV-NP116/MLV-NP117 (380 bp); lane 9, primers *gag* XMRV-FI-441/XMRV-RI-566 (125 bp); lane 10, primers *env* 5922F/6273R (351 bp); lane 11, primers *env* 5922F/6173R (252 bp); lane 12, primers *env* 5942F/6159R (218 bp).

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Clonal Spread of *Streptococcus pyogenes emm*44 among Homeless Persons, Rennes, France

To the Editor: *Streptococcus pyogenes,* or group A streptococci (GAS), are human pathogens responsible for pharyngitis as well as skin and soft tissue infections. Invasive GAS diseases, including bacteremia, cellulitis, and necrotizing fasciitis, are life-threatening, especially when associated with toxic shock syndrome. Several risk factors for GAS infections are known, such as diabetes, immunosuppression, drug use, and skin lesions (*1*,*2*).

In France in 2008, 12% of GAS strains were reported resistant to tetracycline by the national reference center. Unexpected recognition of 8 tetracycline-resistant GAS isolates in January and February 2009 at the 1,950-bed

University Hospital of Rennes in western France led to further investigation. We report results of characterization of tetracycline-resistant GAS isolates collected during 2009 from hospitalized and outclinic patients.

Isolates were identified as GAS on the basis of β-hemolysis, Gram staining, negative catalase test result, positive pyrrolidonyl arylamidase test result, and agglutination with Lancefield group A antiserum. Antimicrobial drug susceptibility to penicillin G, amoxicillin, erythromycin, lincomycin, tetracycline, rifampin, streptomycin, kanamycin, gentamicin, and vancomycin was tested by using the disk diffusion method according to the criteria of the French Society for Microbiology (www.sfm.asso.fr). Of 72 nonduplicate GAS isolates collected, 25 (17 from inpatients, 8 from outpatients) were identified as tetracycline resistant; they were further characterized as described (*3*).

The *emm* types of these 25 tetracycline-resistant strains were determined by sequencing the variable 5′ end of the *emm* gene and comparing sequences with the Centers for Disease Control and Prevention database (www.cdc.gov/ncidod/biotech/strep/doc.htm). Twenty-three strains were *emm*44 type, 1 was *emm*105, and 1 *emm*83. Pulsed-field gel electrophoresis (PFGE) patterns obtained after DNA digestion by *Sma*I restriction enzyme were compared according to Tenover criteria (*4*). The epidemic clone including 22 strains was characterized by an identical PFGE pattern 44-A1, whereas PFGE pattern 44-A5 of the remaining *emm*44 strain differed by 4 DNA bands (Figure). Epidemic strains also shared the same biotype 3 obtained on rapid ID 32 Strep strips (bioMérieux, Marcy l'Etoile, France). T types were determined on trypsinated bacteria by slide agglutination with type-specific antisera. Eleven strains were type T11, 4 type T11/12, 1 type T11/3/13/B3264, and 6 non–T-typeable.

All epidemic *emm*44 strains were susceptible to all antibacterial agents tested except tetracycline. MICs of tetracycline, determined with Etest method (AB Biodisk, Solna, Sweden), were 24–48 mg/L. Screening of strains by PCR for *tet*(*M*), *tet*(*O*), *tet*(*K*), and *tet*(*L*) genes showed tetracycline resistance was related to *tet*(*M*) gene. A multiplex PCR for detection of *speA*, *speB*, *speC*, *smeZ*, and *ssa* toxin genes showed that epidemic strain possessed only *speB* gene.

Investigation conducted by local health authorities showed that the first 5 patients with *emm*44 strain were drug users sharing a squat (illegally occupied housing). Although this place was shut down at the end of February after an outbreak of scabies, additional cases of infections caused by *emm*44 strain occurred. Medical care is difficult to implement for homeless persons, thus, we limited our action to swabbing symptomatic persons to treat them and to limit spread of the epidemic strain. Following recommendations from the Institute for Public Health Surveillance, in mid-April nurses at the 2 main social centers for homeless persons obtained samples from 17 persons. Eleven persons were infected with GAS, of whom 8 had not been swabbed before. All but 1 isolate was *emm*44.

Among the 22 patients infected with epidemic 44-A1 clone, 4 had several successive isolations of this strain. Most (19) infections were secondary infections of skin injuries; others were abscesses (4), septic arthritis (2), necrotizing fasciitis (1), erysipelas (1), and hygroma (1). Five isolates were from sterile sites (1 surgical sample of necrotizing fasciitis, 1 blood culture, and 3 joint fluids). Most infections had favorable outcomes, with the exception of a 79-year old man who died of erysipelas. Patient median age was 37 years (range 20–79 years); all but 1 were men. Eighty-six percent had risk factors such as alcohol abuse (17, 77%), homelessness (16, 73%), drug use (11, 50%), hepatitis C infection (4, 8%), and HIV infection (1, 4.5%). Two patients had no identified risk factors. Complete characteristics of 50 patients infected with a strain of GAS different from 44-A1 clone were not available. However, this population did differ by its sex ratio (28 men:22 women) and by older median age (47.3 years).

We report clonal spread of an *emm*44 tetracycline-resistant GAS strain in marginal populations (drug users and homeless persons) in

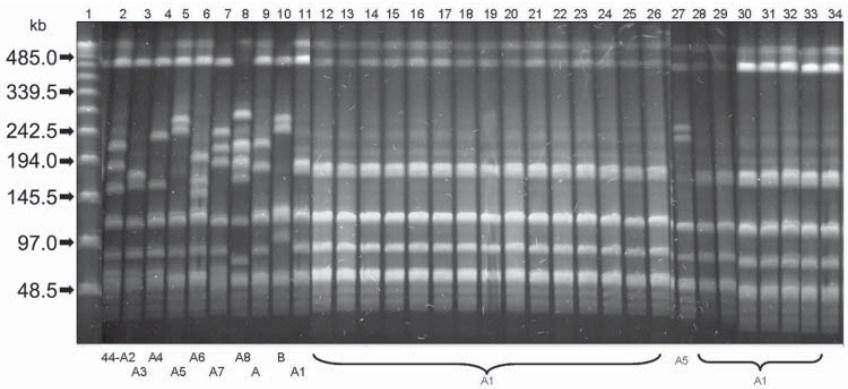

Figure. Pulsed-field gel electrophoresis (PFGE) patterns of *Sma*I-restricted chromosomal DNA of *Streptococcus pyogenes emm*44 strains. Lane 1, Bacteriophage Lambda ladder PFGE Marker (New England Biolabs Inc., Beverly, MA, USA); lanes 2–11, PFGE patterns 44-A2, 44-A3, 44-A4, 44-A5, 44-A6, 44-A7, 44-A8, 44-A, 44-B, and 44-A1 of *emm*44 unrelated control strains; lanes 12–26 and 28–34, 22 identical 44-A1 PFGE patterns shared by the tetracycline-resistant outbreak isolates; lane 27, PFGE pattern 44-A5 of the nonclonal *emm*44 strain isolated during the same outbreak, which differs by 4 bands from the pattern 44-A1.

Rennes. This strain, characterized by PFGE pattern 44-A1, represented 22/25 tetracycline resistant GAS isolates and 30% of the 72 GAS isolates identified at the hospital in Pontchaillou in 2009. Locally, emergence of the 44-A1 clone led to the dramatic increase of GAS tetracycline resistance, from 17% in 2008 to 35% in 2009. *emm*44 GAS strains, which share identical 5′*emm* sequences with previously designated M/ *emm*61 strains (*5*), have mainly been isolated in Asia from throat and skin specimens (*6*,*7*). They were rarely reported as responsible for invasive infections in France or other parts of the world (*5*,*8*). Polyclonal and *emm*25 and *emm*83 monoclonal GAS outbreaks have been recently described among drug users in Switzerland, the United Kingdom, and Spain (*9*,*10*) without robust evidence of enhanced virulence of the causative GAS strains. In the outbreak we report, skin infections might be a leading cause of bacterial transmission between people living in poor hygienic conditions and overcrowded spaces.

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Surface Layer Protein A Variant of *Clostridium difficile* PCR-Ribotype 027

To the Editor: Rates and severity of *Clostridium difficile* infection (CDI) have recently increased worldwide and correlate with dissemination of hypervirulent epidemic strains designated PCR-ribotype 027. CDI caused by this PCR-ribotype is characterized by strong toxin A and B production, presence of binary toxin genes, and, usually, a high level of resistance to fluoroquinolones (*1*).

The mechanisms by which *C. difficile* colonizes the gut during infection are poorly understood. In addition to the toxins, surface protein components are undoubtedly involved. In particular, the surface layer (S-layer) mediates adhesion to enteric cells (*2*), but other functions have been proposed for this S-layer structure: it may act as a molecular sieve, protect against parasitic attack, or be a mechanism to evade the host immune system (*3*). Furthermore, the *C. difficile* S-layer is the predominant surface antigen and is

among the main potential candidates for multicomponent vaccines against CDI (*4*,*5*). Composed of 2 major components, the *C. difficile* S-layer has high and low molecular weight proteins (HMW and LMW, respectively), which are formed from the posttranslational cleavage of a single precursor, surface layer protein A (slpA) (*6*). Different variants of the *slp*A gene have been identified in *C. difficile* (*7*).

The complete genome sequences of 2 *C. difficile* PCR-ribotype 027 strains (CD196, a nonepidemic strain isolated in France in 1985, and R20291, isolated from an outbreak in Stoke Mandeville, UK, in 2006) have been recently deposited in GenBank (accession nos. FN538970 and FN545816, respectively) (*8*). We analyzed the *slp*A gene of these strains by using the National Center for Biotechnology Information BLAST server (www.ncbi.nlm.nih.gov/blast) and the European Bioinformatics Institute ClustalW server (www.ebi.ac.uk/clustalw). Both strains showed a new and identical *slpA* nucleotide sequence. To determine if the new variant was conserved among PCR-ribotype 027 strains, we characterized 8 additional epidemic strains belonging to this PCR-ribotype that were isolated in different geographic regions and years and showed different patterns of resistance to erythromycin and moxifloxacin. Three strains, AI13, AII6, and AIII8, were isolated in 3 hospitals in Belgium during a European prospective study conducted in 2005 (*9*). *C. difficile* DI12 was isolated in Ireland during the same study. *C. difficile* GII7 and LUMC46 were isolated in the Netherlands in 2005 and 2008, respectively. *C. difficile* M43 and A422 were isolated in Calgary (Canada) in 2001 from 2 outbreaks.

Six strains were resistant to erythromycin (MICs ≥256 mg/L) and moxifloxacin (MICs 12–256 mg/L). AIII8 was resistant to erythromycin (MIC ≥256 mg/L) and intermediately resistant moxifloxacin (MIC = 6 mg/L), whereas CD196, LUMC46, and A422 were susceptible to both drugs.

The *slp*A genes of all strains were amplified by PCR mapping. Nine primers were designed on the *slpA* region to obtain 10 overlapping PCR products. The positions of the primers on the reference sequence FN545816 were 3161991–31612012, 3162346–3162365,3162728–3162746, 3162746–3162728,3163514–3163495, 3164222–3164205,3163264–3163284, 3163284–3163264, and 3164518–3164499. Target amplification was performed by an initial denaturation at 94°C for 5 min, then 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Sequence assembly was performed by using DNAStar Lasergene version 8.0 software (DNAStar, Madison, WI, USA). The protein analysis was performed by using the SignalP 3.0 server (www.cbs.dtu.dk/services/SignalP/) and the ExPASy Proteomics server (www.expasy.ch/tools/pi_tool.html). Amino acid comparisons were accomplished by using ClustalW (www.ebi.ac.uk/clustalw), and the output was used for construction of the phylogenetic tree by TreeView version 1.6.6 (http://en.bio-soft.net/tree/TreeView.html). All PCR-ribotype 027 strains showed the same *slp*A gene nucleotide sequence. The slpA precursor encoded by this gene contained a signal peptide, and its cleavage site was located between aa 24 and aa 25. The cleavage of the slpA precursor into LMW and HMW proteins was predicted between aa 342 and aa 343 (N terminal to an Ala amino acid residue and C-terminal to a consensus motif Thr-Lys-Ser). The molecular masses of the LMW and HMW proteins were 33.871 kDa and 44.174 kDa, respectively. These protein sizes were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, after a low pH glycine extraction (data not shown). The phylogenetic tree (Figure), obtained by comparison with the amino acid sequences of other PCR ribotypes (*6*), showed that *C. difficile* strain 027 slpA was strongly related (identity 89%) to that of strains belonging to

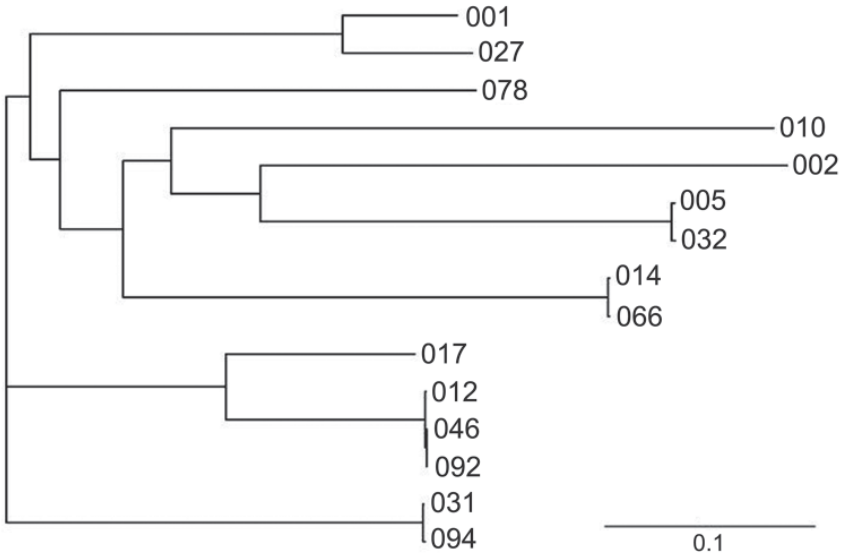

Figure. Phylogenetic tree based on the alignment of the surface layer protein A amino acid sequence of *Clostridium difficile* 027 (GenBank accession no. CBE06198) with those of PCR-ribotypes 001, 002, 005, 010, 012, 014, 017, 031, 046, 054, 066, 078, 092, and 094 (GenBank accession nos. AAZ05957, AAZ05964, AAZ05968, AAZ05974, AAZ05975, AAZ05984, AAZ05988, AAZ05989, AAZ05980, AAZ05972, AAZ05986, AAZ05994, AAZ05982, and AAZ05991, respectively). The phylogram was generated by using TreeView version 1.6.6 (http://en.bio-soft.net/tree/TreeView.html). The branch lengths are scaled in proportion to the extent of the change per position, as indicated by the scale bar.

the epidemic PCR-ribotype 001. In particular, the identity between the 2 PCR ribotypes was 100% for the HMW proteins and 77% for the LMW proteins.

This study provides convincing evidence that the S-layer is well conserved in *C. difficile* PCR-ribotype 027 strains and has high identity with the slpA of the epidemic PCR-ribotype 001. Because *C. difficile* PCR-ribotypes 027 and 001 are the most frequently isolated strains from severe CDIs across both North America and Europe (*9*,*10*), the result obtained suggests that the S-layer of these virulent strains presents peculiar and common characteristics that could be an advantage for these bacteria during the infection process.

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Introduction of Japanese Encephalitis Virus Genotype I, India

To the Editor: Seasonal outbreaks of fatal acute encephalitis syndrome (AES) occur regularly in several parts of India. Japanese encephalitis virus (JEV) has been the major and consistent cause of these outbreaks in the Gorakhpur region of Uttar Pradesh State, accounting for ≈10%–15% of total AES cases annually (*1*–*3*). In India, vaccinations against Japanese encephalitis (JE) are administered in areas where the disease is hyperendemic, including Gorakhpur, and AES cases are regularly investigated to clarify the effects of vaccination. Currently, >2,000 patients with AES are admitted each year to Baba Raghav Das Medical College, Gorakhpur.

JEV is classified into 5 genotypes. Genotype III (GIII) is widely distributed in Asian countries, including Japan, South Korea, the People's Republic of China, Taiwan, Vietnam, the Philippines, India, Nepal, and Sri Lanka (*4*). However, during the past decade, JEV GI has been introduced into South Korea, Thailand, and China and has replaced the GIII strains that had been circulating in Japan and Vietnam during the mid-1990s (*5*). Until 2007, all known JEV strains isolated in India belonged to GIII (*2*–*4*,*6*).

The JE-endemic Gorakhpur region recorded a sudden increase in AES cases during September–November 2009. Clinical specimens collected from 694 hospitalized patients were examined for JEV infection by JEV-specific immunoglobulin M capture ELISA (*7*). Clinical specimens comprising 115 (16.6%) cerebrospinal fluid (CSF) specimens and 114 (16.4%) serum specimens showed recent JE infection among 158 (22.7%) of the case-patients.

All CSF specimens were processed for JEV genome detection by diagnostic reverse transcription–PCR

(RT-PCR), which amplified the nucleocapsid-premembrane genes (*7*). Additionally, envelope (E) gene–specific primers designed from Indian JEV isolate GP78 (GenBank accession no. AF075723) were used for E gene amplification.

The diagnostic RT-PCR detected JEV in 66 (9.5%) of 694 CSF specimens (GenBank accession nos. HM156543–HM156569, HM156573–HM156611). Among them, 27 sequences differed from the remaining 39, with only 86.2%–88.7% nt identity. The group of 27 sequences showed 99.2%–100% nt identity with each other and a high of 95.0% nt identity with Japanese GI swine JEV isolate (AB241119). The 39 sequences showed 94.2%–100.0% nt identity with each other and with other Indian GIII JEV strains. These findings indicate that both GI and GIII JEV strains circulate in the Gorakhpur region.

The E gene sequence was amplified from 4/66 JEV-positive CSF samples (GenBank accession nos. HM156570–HM156572, HM156612). A comparison of E gene nucleotide sequences those of other JEV isolates from the region showed that 1 E gene sequence belonged to GIII and the other 3 to GI (Figure). The 3 GI E gene sequences were most similar (98.6%) with Japanese isolate 95–167/1995/swine (AY377579), followed by 98.5% similarity with Korean isolate K96A07/1996 (FJ938219). The single GIII E gene sequence showed 95.6%–99.8% nt identity with other Indian GIII isolates with the highest similarity (99.8% nt) with the 014178 (EF623987) JEV isolate from the 2001 Uttar Pradesh outbreak. Analysis of the E gene sequence amplified from 2 JEV isolates, obtained by injecting 29 CSF samples, positive by RT-PCR, into baby hamster kidney cells, showed 100.0% nt identity with sequences directly amplified from respective CSF specimens (*3*). Phylogenetic analysis of these E gene sequences, along with other 55 GenBank sequences, confirmed that 3 sequences belonged to JEV GI, and 1 belonged to GIII (Figure).

The first JE outbreak in the Gorakhpur region was documented during 1978. Since then, JE epidemics have occurred regularly (*8*). This study demonstrated simultaneous detection and isolation of GI and GIII JEV strains from AES case-patients. Documented clinical symptoms among patients infected with the 2 strains were indistinguishable.

Figure. Phylogenetic tree constructed by using a 1,381-nt Japanese encephalitis virus (JEV) envelope sequence directly amplified from cerebrospinal fluid specimens collected during the acute phase of illness from hospitalized acute encephalitis syndrome patients, India, September–November 2009. Multiple sequence alignment and phylogenetic analysis were conducted by using ClustalW software (www.ebi.ac.uk/Tools/clustalw2/index.html) and MEGA version 4 (www.megasoftware.net). The phylogenetic tree was constructed by the neighbor-joining method and the maximum composite likelihood model. The robustness of branching patterns was tested by 1,000 bootstrap pseudo replications. Sequences obtained in this study are indicated in **boldface**. Genotypes are indicated on the right. Viruses were identified by using the nomenclature of accession number–strain name–country of origin/year of isolation. Bootstrap values are indicated above the major branch. The tree was rooted within the Japanese encephalitis serogoup by using Murray Valley encephalitis virus (AF161266) and 55 JEV sequences from GenBank were used in the analysis. Scale bar indicates nucleotide substitutions per site.

GI JEV isolates from India share close genetic relationship with GI strains from Japan and Korea. In India, JEV neutralizing antibodies have been detected in 179 (34.8%) of 514 birds, including pond herons and cattle egrets, indicating a possible role in virus maintenance (*9*). Large perennial lakes, swamps, and rice fields provide a wintering and staging ground for several migratory waterfowl; such areas also favor breeding and survival of mosquitoes (*10*). Considering these conditions, GI JEV may have been introduced into India through migratory birds, as it has in other Asian countries (*5*). However, the exact mode of introduction of GI JEV into India is not known, and further studies are needed to determine the role of migratory birds in JE transmission.

This study suggests the recent introduction of JEV GI strain in India. Simultaneous detection of GI and GIII strains indicates their co-circulation and association with human infections in Gorakhpur region. Because the live attenuated JE vaccine used in India is derived from GIII strain SA14–14–2, the efficacy of the vaccine to protect against GI JEV must be carefully evaluated. Thus, the genetic and antigenic variation among JEV strains circulating in India should be monitored to determine effects on JE epidemiology and ongoing vaccination efforts. Additionally, the expansion of GI JEV into other parts of India should be continuously tracked.

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Dengue Virus Serotype 3 Subtype III, Zhejiang Province, China

To the Editor: Beginning in July 2009, physicians in the city of Yiwu, Zhejiang Province, People's Republic of China, noted an outbreak of illness characterized by rash, headache, subjective fever, itching, anorexia, and arthritis. We present the results of the investigation of this outbreak, which was caused by dengue virus (DENV) serotype 3 (DENV-3) subtype III.

DENV-3 subtype III has been continuously circulating in the Indian subcontinent since the 1960s. The virus was first isolated from East Africa in 1985 in Mozambique and subsequently in Kenya (1991) and Somalia (1993) (*1*,*2*). Although dengue has occurred frequently in southern China, including Guangdong, Guangxi, Hainan, Fujian, and Zhejiang Provinces and in Taiwan (*3*–*6*), to our knowledge, DENV-3 subtype III has not been reported in China.

Yiwu is in the center of Zhejiang Province, southeastern China. This investigation included the entire town of Yiwu and towns that are part of the larger town of Yiting where the outbreak took place. We reviewed medical records and conducted prospective surveillance at all hospitals, health centers, and outpatient clinics in Yiwu to identify patients with suspected dengue fever (DF) during July 1 through October 31, 2009. According to the diagnostic criteria for DF (WS216–2008) enacted by the Chinese Ministry of Health, a patient with suspected disease had at least 2 of the following symptoms: acute onset of rash, headache, subjective fever, itching, anorexia, or arthralgia. Patients with suspected disease were asked to provide blood specimens during the acute phase (within 7 days after symptom onset).

Serum samples were tested by ELISA for immunoglobulin (Ig) M against DENV by using the E-DEN01M kit (Panbio, Sinnamon Park, Queensland, Australia). Acute-phase serum samples were tested by real-time PCR for DENV RNA, according to the diagnostic criteria for dengue fever enacted by the Chinese Ministry of Health. Samples that were positive for DENV-3 by real-time PCR were inoculated into *Aedes albopictus* mosquito clone C6/36. Primers for reverse transcription–PCR and sequencing of the envelope gene of DENV isolates were used to identify DENV (*4*).

We considered a patient to have a confirmed case if DENV RNA was detected in the serum by real-time PCR or if IgM against DENV was present. A patient was considered to have a clinically diagnosed case if he or she had acute onset of rash, headache, subjective fever, itching, anorexia, and leukopenia, and lived in Qingsu, Fantianzhu, Xitian, or Shangzhai (4 adjoining villages in the area of the first confirmed case).

The sequences of isolates from case-patients were compared with published sequences by using the BLAST program (www.ncbi.nlm.nih.gov/BLAST/), and phylogenic analysis was calculated with PAUP 4.0 β 10 (*7*), which ran an unrooted tree with 1,000 bootstrap replicates.

We identified 196 cases of DF; 279 suspected cases were excluded, and no cases of dengue hemorrhagic fever or dengue shock syndrome were found. Of DF cases, 71 (36.2%) were confirmed and 125 (63.8%) were clinically diagnosed. Acute-phase serum samples were collected within 7 days after the onset of illness from 350 patients with suspected DF, and dengue virus RNA was detected in samples from 65 patients (18.6%). Six samples had IgM against DENV.

Twenty-six samples positive for DENV RNA by real-time PCR were randomly selected to isolate viruses; 23 isolates were cultured. All isolates were amplified by reverse transcription–PCR, and amplicons were sequenced. The envelope gene sequences of all isolates were identical and 1,479 nt in length. All sequences had 99% similarity to 1 DENV serotype 3 partial envelope gene (GenBank accession no. AM746229), which had been detected in Jeddah, Saudi Arabia, in 2004. According to evolutionary analysis (Figure), sequences of our study were also most closely related to the isolate from Saudi Arabia, which suggests that the outbreak may have been initiated by imported cases from the Indian subcontinent or western Asia.

The date of symptom onset among patients with confirmed or clinically

Figure. Evolutionary analysis of dengue virus isolates from this study (**boldface**) compared with established dengue virus serotype 3 subtypes, Zhejiang Province, People's Republic of China, 2009.

diagnosed cases ranged from July 20 to October 4, 2009. Cases peaked in early September and subsided in early October. The median age of patients with confirmed or probable disease was 47.3 years (range 3–96 years). Infections occurred in all age groups, but most infections occurred among persons 41 to 65 years of age; 118 (60.2%) were women, and 172 were farm workers.

Confirmed and clinically diagnosed cases occurred in 18 villages, which were part of 7 towns. Most cases (182) were reported in Yiting, where the first case was confirmed, and in particular, were in persons who lived in the villages of Qingsu, 100 cases; Fantianzhu, 49 cases; Xitian, 19 cases; Shangzhai, 4 cases; and Xiateng, 4 cases.

The outbreak shows that DENV-3 subtype III is easily transmitted among humans and mosquitoes and can adapt efficiently to a new area. Other countries where the climate is similar to that of Zhejiang Province (subtropical monsoon) should be aware of the risk for expansion of DENV-3 subtype III transmission. Clinical vigilance and strong epidemiologic and laboratory surveillance are essential**.**

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European Subtype Tick-borne Encephalitis Virus in *Ixodes persulcatus* Ticks

To the Editor: The northernmost tick-borne encephalitis (TBE) focus is in Simo, Finnish Lapland. Four TBE cases were confirmed during 2008–2009. Tick-borne encephalitis virus (TBEV) is transmitted by *Ixodes* spp. ticks and is endemic to Eurasia from central Europe to the Far East. The virus has 3 subtypes: European (TBEV-Eur), Siberian (TBEV-Sib), and Far Eastern (TBEV-FE). TBEV-Eur is mainly transmitted by *I. ricinus* ticks (sheep ticks) and the 2 other subtypes by *I. persulcatus* ticks (taiga ticks). The range of *I. ricinus* ticks covers most of continental Europe and the British Isles; *I. persulcatus* ticks are distributed throughout eastern Europe and Asia to the People's Republic of China and Japan.

The transmission cycle of at least TBEV-Eur in nature is fragile and depends on microclimatic conditions. Thus, within the *I. ricinus* distribution area, TBE is endemic merely focally (*1*,*2*)**.** In Finland, TBE foci are located by the sea or large lakes (online Appendix Figure, www.cdc.gov/EID/content/17/2/321-appF.htm). Both vector tick species are found: *I. ricinus* ticks in the southern and central parts of the country, but *I. persulcatus* ticks are in scattered foci along the western coast, including the Kokkola archipelago and Närpiö municipality, where they carry TBEV-Sib (*3*,*4*) (online Appendix Figure).

The first human TBE cases from Simo in Lapland (65°40′N, 24°54′E; online Appendix Figure) were reported during 2008 (n = 2) and 2009 (n = 2). On the basis of interviews with the 2 patients from 2008, we collected 97 ticks and 17 bank voles from the 2 probable sites of infection during June 2009. From the rodents, we extracted

blood from the heart and performed TBEV-antibody tests by immunofluorescence assay. The ticks were placed in 51 pools (1–3 ticks/pool). We isolated RNA from tick pools and rodent lungs and brains by TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA) and performed real-time reverse transcription–PCR (*5*) to detect TBEV RNA. For the positive tick pools, we confirmed the identification species by *Ixodes* mtDNA sequencing (*6*).

Six of 51 tick pools (with a total of 97 *I. persulcatus* ticks) were positive for TBEV in real-time reverse transcription–PCR, resulting in 6% TBEV RNA prevalence. At least 1 organ was positive for TBEV RNA in as many as 15/17 bank voles, in line with our finding that TBEV RNA persists in rodents for months (*7*); 4 rodents had antibodies to TBEV. The TBEV RNA prevalence among ticks and rodents was relatively high, as is the incidence among humans (0.57 cases/year/1,000 inhabitants) in Simo, indicating a focus with high activity.

We isolated 6 TBEV strains from suckling mice (experimental animal permit ESLH-2008–06558/Ym-23): 2 from *I. persulcatus* tick pools (Simo-38 and Simo-48; pools of 2 and 3 ticks, respectively), and 4 from TBEV antibody– and RNA-positive rodent lung–brain suspensions (Simo-2, -5, -7 and -9). Partial envelope (E) and nonstructural protein 3 genes (*4*) of the isolated TBEV strains were sequenced (accession nos. HQ228014–HQ228024, GenBank) and subjected to phylogenetic analysis (online Appendix Figure). Within the 1208 nt from the E gene, Simo-38 and Simo-48 from ticks and Simo-9 from a bank vole were identical. Other sequences differed for 1 nt and Simo-2 for 1 aa compared with the others. All strains were monophyletic and belonged to the TBEV-Eur subtype. The partial nonstructural protein 3 gene sequences were identical, and the phylogenetic tree showed similar topography as for the E gene (not shown).

The only tick species found in Simo was *I. persulcatus,* further widening its known distribution along the western coast of Finland (online Appendix Figure). However, the virus subtype found in Simo was TBEV-Eur strain, the main vector of which is the *I. ricinus* tick.

TBEV-Eur strains are commonly very closely related to each other and do not form clear geographic clusters (*4*). Thus, it is difficult to deduce the origin of the virus. The nearest TBE-endemic focus is the Kokkola archipelago, ≈200 km south (online Appendix Figure), but there *I. persulcatus* ticks carry the TBEV-Sib strain (*3*). The nearest areas to which the TBEV-Eur strain is endemic are in southern Finland where only *I. ricinus* ticks have been found.

Cattle serum samples were negative for antibodies to TBEV in the Simo area in the 1960s (*8*). The first human TBE cases from Simo were identified during 2008 and 2009. We isolated TBEV strains from ticks and rodents in 2009. Simo appears to be a recently established, and the northernmost, TBE focus known. TBEV may have been introduced to Simo from a geographically distinct location recently, likely within the past 50 years.

TBE seems to be moving northward in Europe (*9*) and shifting upward to higher elevations in the mountains (*10*), apparently influenced by climate change. An altered microclimate favoring TBE circulation (*1*), in addition to introduction of the virus, could also explain the recent emergence of TBE in Simo. In conclusion, Simo in Finnish Lapland is a new TBE-endemic focus demonstrating northward movement of foci and an unusual combination of the TBEV-Eur strain and *I. persulcatus* ticks in an area with no evidence of cocirculation of tick species or TBEV subtypes.

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Rickettsia aeschlimannii in *Hyalomma marginatum* Ticks, Germany

To the Editor: *Rickettsia* spp. of the spotted fever group cause worldwide emerging human infections known as tick-borne rickettsioses (*1*). Data on the occurrence and prevalence of *Rickettsia* in Germany are still limited (*2*). Six *Rickettsia* species have been reported to date (*2*). *R. helvetica, R. felis, R. massiliae*, and *R. monacensis* were detected with a relatively low prevalence in *Ixodes ricinus* ticks collected in southern Germany (*2*); *R. raoultii* was identified with high prevalence in the rapidly expanding area where *D. reticulatus* ticks are found (*2*). *R. raoultii* was recently recognized as an agent of tick-borne lymphadenopathy/*Dermacentor*-borne necrosis and erythema lymphadenopathy (*3*). Low prevalence of another tick-borne lymphadenopathy agent, *R. slovaca,* in *Dermacentor marginatus* ticks collected in southern Germany was recently reported (*4*).

We report the detection in Germany of the pathogenic SFG species *R. aeshlimannii* (*1*), which is phylogenetically close to *R. raoultii* and causes an infection with clinical signs similar to those of Mediterranean spotted fever (*1*). To determine the prevalence of *R. raoultii* in the ticks in Berlin/Brandenburg and neighboring regions, we collected 294 ticks; 288 had been collected either from vegetation or domestic animals and morphologically identified as adult *D. reticulatus* ticks. The remaining 6 ticks were delivered by an ornithologist who had removed them from a bird (belonging to the *Acrocephalus scirpaceus* spp.) that he had captured in the reeds near Pakendorf and Zerbst, Saxony-Anhalt, in May 2007. These 6 ticks were reported as *D. reticulatus*–like adults but were damaged in the collection process, making an exact morphologic evaluation impossible.

DNA was isolated from the complete tick body by homogenization in the SpeedMill (Analytik Jena Biosolutions, Jena, Germany) followed by purification by RapideStripe tick DNA/RNA Extraction Kit (Analytik Jena Biosolutions). Multispacer typing (*5*) was used for molecular detection and determination of *Rickettsia* spp. (Figure). DNA sequencing and analysis were performed as described (Figure).

In 51.3% of the intact tick isolates, *R. raoultii* was detected. In each of the 3 damaged isolates, the multispacer type pattern was detected, which was 100% identical to that of *R. aeschlimannii* (*5*) (Figure). Moreover, PCR, which we routinely use as a positive control for molecular identification of *D. reticulatus*, yielded no product in the damaged isolates (Figure).

To determine the species of the damaged ticks, we used 3 tick-specific PCRs. One amplified a 16S rRNA fragment used for phylogenetic studies of ticks (*6*). Use of the other 2 PCRs was

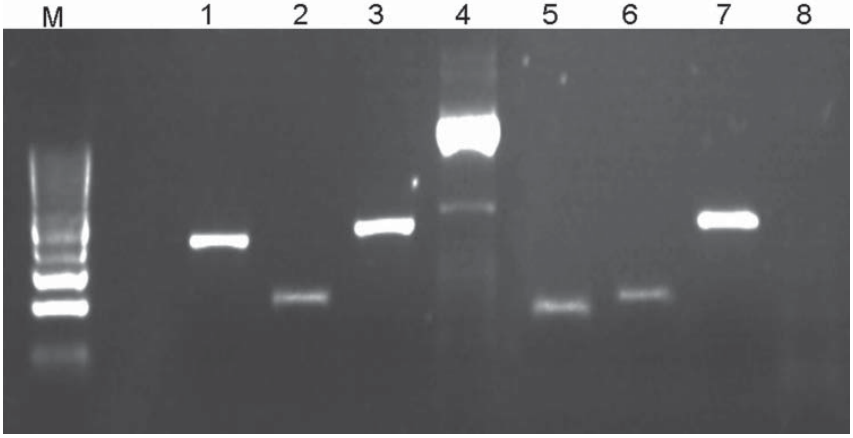

Figure. Illustration of multispacer typing. Amplicons 1–4 result from PCRs on DNA obtained from 1 *Rickettsia raoultii*–infected *Dermacentor reticulatus* tick isolate; lanes 5–8 result from PCRs on 1 damaged isolate. PCRs amplifying dksA-xerC (lanes 1 and 5), mppA-purC (lanes 2 and 6), and rpmE-tRNA (lanes 3 and 7) intergenic spacers were performed as described (*5*). PCR amplifying the entire internal transcribed factor 2 (ITS2) locus of *D. reticulatus* tick (lanes 4 and 8) was involved in each PCR run as a positive control and for validation of *D. reticulatus* tick identity (the primers will be described elsewhere).The negative result of ITS2 PCR with the damaged isolates (lane 8) indicated that they are not *D. reticulatus* ticks. Lane M, DNA size marker (100-bp ladder). PCR products were directly sequenced in both directions with respective primers by an ABI PRISM DNA Sequencer (Applied Biosystems, Foster City, CA, USA). DNA Star package (DNA Star, Madison, WI, USA) and the tools offered by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) were used for DNA search and analysis.

based on the consideration that *R. aeschlimannii* is usually found in ticks of the genus *Hyalomma,* primarily in *H. marginatum* (*1*). Therefore, 1 of the PCRs amplified a fragment of the *Hyalomma* tick mitochondrial cytochrome oxidase I gene and the other a fragment of the internal transcribed spacer 2 (*7*).

The ITS2 fragment displayed the highest (99%) similarity with the respective fragment of *H. marginatum*, *H. dromedarii*, *H. truncatum,* and *H. lusitanicum.* Cytochrome oxidase subunit I fragment was 99% identical to *H. marginatum*, *H. dromedarii*, and *H. truncatum*. The 16S RNA fragment was 98% identical to *H. marginatum*; its identity to the second closest sequence belonging to *H. lusitanicum* was 96%.

Earlier, *R. aeschlimannii* had been detected in sub-Saharan and North Africa, southern Europe, and southwestern Russia (*8*). Therefore, the area of Zerbst, the middle of Germany, marks the northernmost point of *R. aeschlimannii* detection.

Hyalomma spp. ticks are distributed in Africa, the Mediterranean climatic zone of southern Europe, and in Asia. The only documented *Hyalomma* spp. tick in Germany was found on a human in the southern part of the country (Lake Constance area) in May 2006, but the possibility of tick transportation from Spain was not ruled out (*9*).

Acrocephalus scirpaceus birds are migratory birds and live in central Europe from April to October and winter in sub-Saharan Africa in the region inhabited by *Hyalomma* spp. ticks. Therefore, it is reasonable to suggest that the *Hyalomma* spp. ticks that we examined had been transported by the birds from Africa. The fact that a randomly caught bird was infested with *R. aeshlimannii*–infected ticks is suggestive of the intensive stream of new pathogens transported through Europe by migrating birds. The first possible implication of a bird as a vector of infected ticks was proposed for *Hyalomma* spp. ticks infected by *R. sibirica mongolitimonae* (*10*). Until now, the role of migrating birds in distribution of tick-borne pathogens has been poorly understood (*9*). The changing climate and environment in central Europe may facilitate the establishment of pathogen-carrying tick species transported by birds. These new pathogens can be directly transmitted from infected birds to the species of the local fauna.

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Dogs as Reservoirs for *Leishmania braziliensis*

To the Editor: I have read the review by Sousa and Pearson (*1*), which provides a fascinating historical account of the Great Drought and the smallpox epidemic of the 1870s and their association with the emergence of cutaneous leishmaniasis in Ceará, Brazil. In their review, the authors went back to the 19th century, remembering the hard years experienced by those who faced the Great Drought,

which prompted the immigration of thousands of persons from Ceará to the Amazon region, and a devastating smallpox epidemic, which resulted in the death of >100,000 persons. Later, they returned to the present situation of cutaneous leishmaniasis in Brazil.

I would like to address the role of dogs as reservoirs of *Leishmania* (*Viannia*) *braziliensis*. Sousa and Pearson stated that "no animal reservoir other than dogs has been identified in Ceará" and that "a sylvatic reservoir has not been identified for *L.* (*V.*) *braziliensis* in Ceará and other areas," concluding that "dogs appear to be the most important reservoir in domestic and peridomestic transmission."

Conversely, recent studies have indicated that rodents and other small mammals are the primary reservoirs for *L.* (*V.*) *braziliensis* (*2*) and that, so far, no strong evidence indicates that dogs could act as reservoirs for this parasite (*3*,*4*). The finding of dogs infected by *L.* (*V.*) *braziliensis* in leishmaniasis-endemic areas is expected because they are susceptible to this parasite and are often exposed to phlebotomine sandflies. However, this finding does not imply that dogs are important reservoirs. Indeed, they represent a poor source of *L.* (*V.*) *braziliensis* (*3*). For these reasons, dogs cannot be incriminated as the most important reservoirs in the domestic and peridomestic transmission cycles of *L.* (*V.*) *braziliensis*.

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In Response: We agree with most points raised by Dantas-Torres (*1*). However, so far our statement related to the reservoir(s) of *Leishmania* (*Viannia*) *braziliensis* (*2*) is correct for Ceará. Oliviera-Lima (*3*) conducted a case–control study that definitively incriminated dogs as a reservoir host of *L.* (*V.*) *braziliensis* in Baturite, Ceará. He showed that infected dogs in households increased the risk for infection with *L.* (*V.*) *braziliensis* by 3.2-fold among resident children. The risk increased to 6-fold when infected dogs had leishmaniasis-like skin lesions. Additionally, his observations suggested that animals other than dogs might be involved. In another study, Santana (*4*) examined 272 small mammals (213 rodents and 59 marsupials) in the same area; tissue culture and hamster inoculation showed none to be infected with *L.* (*V.*) *braziliensis*, although some cultures were contaminated by fungi and bacteria. On the basis of these findings, a sylvatic reservoir for *L.* (*V.*) *braziliensis* has not been identified in Baturite, Ceará. However, Brandão-Filho et al. found that rodents in Pernambuco State, Brazil, were infected with *L.* (*V.*) *braziliensis* and stated that they were the primary reservoir (*5*). We concur with Lainson and Shaw (*6*) and conclude that information about mammalian reservoirs of *L.* (*V.*) *braziliensis* is incomplete.

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Pandemic (H1N1) 2009 and HIV Co-infection

To The Editor: Barchi et al. report a case of simultaneous pandemic (H1N1) 2009 influenza and HIV infection (*1*). We agree with the authors' conclusion that during influenza epidemics, consideration of alternative diagnoses, such as acute HIV infection, remains essential for patients who seek treatment for severe influenza-like illnesses. However, from our perspective, several points from this letter need additional clarification.

First, we recommend that the authors clarify whether the positive HIV test results reported were for the hospitalized patient (as we suspect) or for the nurse who was exposed to the patient's urine. Occupationally acquired HIV infection in a health care provider after an ocular splash with urine has, to our knowledge, never been reported and, if these test results are for the worker, would represent a novel source of transmission. Precision with respect to the source of these samples and results is critical to reader understanding.

Additionally, the reported negative Western blot results demonstrated p24 and p41 bands; this test result would be considered positive by Centers for Disease Control and Prevention–endorsed interpretive criteria (i.e., Western blot positivity equates to presence of any 2 of the following 3 bands: p24, p41, and gp120/160) (*2*). Thus, the negative Western blot result interpretation, even if caused by different local interpretive criteria, deserves further explanation.

Finally, diagnosing acute HIV infection can be challenging. Although the elevated initial CD4 lymphocyte percentage and viral load are suggestive of recent HIV infection (*3*), the ELISA result was positive. Do the authors have access to a prior HIV test result that may shed further light on the chronicity of HIV infection? The hepatitis C infection in this patient was also diagnosed relatively recently. Co-infection with HIV and hepatitis C virus may alter the course of both infections and may have contributed to the severity of this patient's illness (*4*).

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In Response: In reply to Kuhar and Henderson (*1*), the positive HIV test result reported was for the patient. The nurse who was exposed to the patient's urine is currently HIV negative 12 months after exposure. With regard to the negative result for the Western blot, part of the original sentence was accidentally changed in the published letter (*2*); the correct information is "confirmatory Western blot test results were negative on days 5 and 15, and showed the p24 and p41 bands on day 23." Finally, a prior HIV test, which had been performed for the patient 8 months earlier, showed a negative result.

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Avian Influenza: Science, Policy and Politics

Ian Scoones, editor

Earthscan Publications Ltd., London, UK, 2010
IBSN: 978-1-84971-095-4 (hardcover)
IBSN: 978-1-84971-096-1 (paperback)
Pages: 261; Price: US $39.95

Pandemic (H1N1) 2009 reiterated the lesson that emerging diseases will continue to challenge us. This pandemic once again brought emerging infectious diseases to the world's attention. Pandemics have occurred for centuries and will continue to occur. The question remains as to what we can learn from the past as we address future pandemics and infectious disease crises. Globalization is inexorable, and global spread of disease occurs faster than ever. Shifting wealth and geopolitical power and balance must affect our response as the world's population becomes increasingly urbanized. As populations change, there will be higher expectations for health, higher anxiety in populations as their health is threatened, and a growing distrust in governmental response.

Severe acute respiratory syndrome and avian influenza (H5N1) confirmed that globalization has altered the developing world's relationship with the industrialized world. Indonesia, the epicenter of the avian influenza (H5N1) epidemic, illustrates the geopolitical debates about equity, public goods, and global responsibilities, as demonstrated in the controversy surrounding virus sharing.

Avian Influenza: Science, Policy and Politics offers insight from the avian influenza (H5N1) response into the wider dilemmas regarding animal health, production and trade, public health, emergency response, and long-term development. Disease threats have transnational implications operating in a complex multilateral system. A one-size-fits-all response is not the answer. Although we think globally, our actions are local: livelihoods affected are local, ecology impacted is local, and disease dynamics are local. Surveillance and disease response systems must be congruent with local social, political, and cultural realities.

The authors provide an in-depth analysis of the political and economic structure of Cambodia, Vietnam, Indonesia, and Thailand and illustrate the many actors and networks involved in policy and response to avian influenza (H5N1) and to intersections of science and politics. These examples uncover key insights into how policy was formulated, and it is this often disguised arena, in which some of the most important indicators of future actions and options are found, that can open up the experience to a wider, more nuanced debate. The authors note, "taking seriously the politics of policy, and not just focusing on the technical or economic dimensions, is vital for a more complete understanding of what works and what doesn't as well as ensuring that the trade-offs between different disease control pathways are made clear."

This book provides a well-documented approach to laying out issues involved in features of an effective, equitable, accountable, and resilient response infrastructure at international, national, and local levels for influenza and other emerging infectious diseases. The ultimate challenge will be to interpret and implement such recommendations locally, regionally, and globally. The chapters are well written and complete with multiple examples of science, policy, politics, actors, and networks involved in such efforts. This book would interest public health practitioners, those involved in policy and emergency response, and anyone interested in intricacies of policy development. It is essential reading for those involved in infectious disease detection and control and is highly recommended for epidemiologists; human and veterinary medicine practitioners; experts in international health and policy formulation; and persons involved in nongovernmental organizations, political science, business, and industry.

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Bacterial Population Genetics in Infectious Disease

D. Ashley Robinson, Edward J. Feil, and Daniel Falush, editors

John Wiley and Sons, Inc., Hoboken, New Jersey, USA, 2010
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Pages: 420; Price: US $130.00

Bacterial Population Genetics in Infectious Disease addresses a set of issues that are extremely provocative in the current climate in which progress is rapidly changing the microbiological landscape. From this perspective, the volume appears at a key moment because of 2 major challenges that have arisen since the beginning of the genomic era: 1) defining what constitutes a species and how to determine this, and 2) determining the structure of the population of the most common bacterial pathogens.

The book opens with a review of the concepts and methods that make it possible to analyze bacterial popu-

lations genetically. This presentation immediately brings to the forefront the question of how to define what constitutes a bacterial species (if there is conceptually even such a thing) and the variable evolutionary nature of bacteria. The differences among complexes within species, species, and clones are shown with clear diagrams useful for teaching. In addition, the text also evaluates the lateral transfer of genes, one of the elements that constitute the bacterial genome repertoire, including its effects on the attempts at classification of bacteria. The evolutionary demography of bacterial populations is then examined. These concepts are essential for comprehending and teaching modern microbiology.

In addition, the text describes techniques for evaluating bacterial diversity, such as the search for single-nucleotide polymorphisms or repeats by using sequences encoding proteins or not. These techniques can play a key part in the identification of the clones.

The second part of the book is specifically relevant to clinical microbiology, particularly to emerging bacterial pathogens. The genetic populations of *Bacillus anthracis*, *Campylobacter* spp., *Enterococcus* spp., *Borrelia burdorferi*, *Neisseria meningitidis*, *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp., and *Streptococcus* spp. are analyzed. The importance of genomic recombinations in microbial evolution is particularly stressed for *Streptococcus* spp., *Neisseria* spp., and *E. coli,* for which the number of recombinations is considered to be higher than that of the number of changes; this conclusion likely alters our perception of the evolution of bacteria. Lastly, a general hypothesis is proposed for *Salmonella* spp.: that their antigens are selected by passage through specific phagocytic protists from their animal hosts with which they have cospeciation. This assumption is bold and intriguing.

On the whole, this book is of general interest for teachers of microbiology, who need to explain bacteriology in the genomic age, and it may also help clinical microbiologists choose tools for identifying circulating clones. Finally, it can be useful to specialists in the field of emerging bacteria for whom the need to synthesize genomic data is part of their professional routine. I recommend this book most strongly for all of these bacteriology specialists.

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Caspar David Friedrich (1774–1840) ***The Polar Sea*** **(1824) Oil on canvas (97.8 cm × 128.3 cm)** Hamburger Kunsthalle, Hamburg, Germany/The Bridgeman Art Library

The Icy Realm of the Rime

Polyxeni Potter

The "taciturn man from the North" is how his contemporaries described Caspar David Friedrich, referring to his melancholy, or in his own words, his "dreadful weariness," especially in later years. Loneliness pervaded his work as well as his life, which was marred by early deaths in the family—of his mother when he was seven and several siblings, among them, a young brother, who drowned in a frozen lake, according to some, trying to rescue him.

Friedrich was born in Greifswald, then Swedish Pomerania, on the Baltic coast of Germany, the son of a candle maker and soap boiler in a family of 10 children. As a youth he studied with architect and painter Johann Gottfried Quistorp but later moved to Copenhagen to attend the Academy, one of the leading centers of art in Europe, and eventually settled in Dresden. His training in the neoclassical tradition relied on extensive preliminary studies, drawings, and sketches to depict the physical world and is reflected in the disciplined quality of all his works. But while his landscapes were always actual studies of nature, they were more than a representation of nature.

This man, who according to his contemporaries discovered "the tragedy of landscape" and gained by it fame in his own time, soon embraced an untested individual approach to painting, despite a lingering attachment to the systematic techniques of his training. "The artist should paint not only what he sees before him, but also what he sees within him," he wrote. This belief was rooted in his view of nature as a subject itself worthy of study, imbued with spiritual qualities and portrayed entirely without human presence, not as backdrop but as protagonist. His interest was not in the beauty of nature alone but in what the romantics called the sublime—powerful natural phenomena: snowstorms, impenetrable fog, impassable mountains—generating conflicted feelings of wonder and helplessness, which he could sense and capture with symbols and allegorical elements.

Viewing and presenting the landscape in an entirely new way was Friedrich's main innovation. He turned the mountains, forests, and vistas of northern German countryside in the times of Beethoven, Schubert, and Goethe into romantic icons, painting them at all times of night and day, around Dresden and the River Elbe, especially in the moonlight and sunlight or covered with mist. "Close your bodily eye so that you may see your picture first with the spiritual eye. Then bring to the light of day that which you have seen in the darkness so that it may react upon others from the outside inwards," he wrote in his notes on aesthetics in 1830. Therefore, his winter landscapes were not about life in the winter but

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about winter itself, stark, still, desolate, where "no man has yet set his foot."

Despite early fame and a prolific career, Friedrich lost ground in his mature years and fell into poverty, becoming the "most solitary of the solitary." Bare trees and stumps populated with ravens and owls near graveyards and ruins filled his works, expressing the passage of time and his own state of mind. But these late paintings also explored a mystical approach, one abandoning the self to reach an intuitive understanding of physical phenomena. This period's frisson of the sublime was later adopted by Hollywood directors to show horror, trepidation, and other emotions caused by human inadequacy against the overpowering forces of nature.

The Polar Sea, on this month's cover, expresses Friedrich's mature vision, which, far ahead of his times, was not well received. The painting was inspired by William Parry's arctic expedition of 1819–20, a venture filled with opportunities for symbolic interpretation. The artist seized these to build a monument to nature's triumph over human efforts to conquer it. The tiny image of the ship, inscribed HMS Griber, against a mount of ice, signals the insignificance of human enterprise. Frightful shards jut into the steel gray sky atop solid slabs of ice that form a frigid grave over what human presence might have existed before the wreck and builds a wall between the viewer and the ship.

Another leading romantic, Samuel Taylor Coleridge wrote prolifically about imagery deep with symbolism. In "The Rime of the Ancient Mariner," he offered his version of beautiful and ominous nature, set in a metaphysical world. Among the many influences on this poem, were vivid accounts by arctic explorers. Like Friedrich, Coleridge was fascinated by their travails, which he immortalized. Here is the Mariner's ship in the grip of polar ice: "And now there came both mist and snow, / And it grew wondrous cold: / The ice, mast high, came floating by / As green as emerald…. / The ice, was here, the ice was there, / the ice was all around: / It cracked and growled, and roared and howled, / Like noises in a swound!/"

The "rime" in the world of both Friedrich and Coleridge is symbolic of the sublime world of nature. At once fascinating and terrifying, it changes forms: water, ice, mist—taxing visual awareness, toying with the artist, challenging the scientist, tempting the poet. When European mariners were searching for the Northwest Passage, formidable polar ice lay between them and navigation. The routing was lined with myth and uncertainty, hunger, and scurvy. How times have changed! Now instead of the powerful solidity of ice, we fear instead its fragility as the polar ice cap threatens to melt into the sea, exposing among other puzzles, the dynamic evolutionary interface between human viruses and the ice that can preserve and protect them for thousands of years. What remains constant is nature's upper hand.

In 1918, as explorers were plowing their way into the Arctic, other events were also making history. World War I was coming to a close, yet weary humanity already had a new serious concern, one that was to cause more deaths around the globe than this and future wars combined. The public health emergency spread widely in the fall of the year. Only 3 days after taking sail, the *Forsete* arrived at Longyearbyen, a tiny village in Spitsbergen Island, Svalbard, Norway, north of the Arctic Circle. An outbreak of flu had broken out on the ship caused as it turned out by an extraordinarily potent strain that would become known as Spanish Flu. Many passengers, young miners, were hospitalized and over the next few weeks, seven of them died. Their bodies, containing the deadliest flu virus the world has ever known, were buried in the local cemetery, 800 miles from the North Pole.

Almost 8 decades later, a similar grave in Alaska permafrost held valuable clues about the Spanish Flu pandemic. Unlike the one concocted in Friedrich's imagination, this grave was not a monument to human failure. Its contents enabled RNA sequencing of much of the 1918 virus. As the ice melts, more secrets of the great pandemic may see the light of day, guiding present flu prevention activities. Moreover, other illnesses become endemic in new areas as a result of changes in climate. Tick-borne encephalitis seems to be moving northwards in Europe and shifting upwards on 84 mountains apparently influenced by such changes. Frozen solid or melting fast, sublime nature rules.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Characteristics of Nontuberculous Mycobacteria and Respiratory Infections, Eastern Asia

Q Fever in France, 1985–2009

Epidemiology of *Staphylococcus aureus* Infections in Veterans, United States, 1999–2008

Fonsecaea spp. and Human Chromoblastomycosis

Emerging RNA Viruses and Bat Maternity Roost

Epidemiology of *Mycobacterium bovis* disease, the Netherlands, 1993–2007

Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus, Japan, 2009–2010

Identification of Novel Picornavirus in Turkey Poults with Hepatitis

Tuberculosis Incidence among Health Care Workers

Swine Influenza Virus Antibodies in Humans, Western Europe, 2009

Active Tuberculosis among Homeless Persons, Toronto, Ontario, Canada, 1998–2007

Tuberculosis Outbreak at Elephant Refuge, Tennessee, 2009

Integrated Approach to Identifying International Foodborne Norovirus Outbreaks

Nontuberculous Mycobacteria from Household Plumbing

Mycobacterium tuberculosis Cluster with Developing Drug Resistance, New York, New York, 2003–2009

Reduction of *Coxiella burnetti* Prevalence by Vaccination of Goats and Sheep, the Netherlands

Serologic Surveillance for Anthrax, Tanzania, 1996–2009

Sporadic Human Cryptosporidiosis caused by *Cryptosporidium cuniculus*, United Kingdom

Surveillance for Invasive Meningococcal Disease in Children, US–Mexico Border

Complete list of articles in the March issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

February 4–7, 2011
International Meeting on Emerging Diseases and Surveillance (IMED 2011)
Hotel Hilton, Vienna, Austria
http://imed.isid.org

February 27–March 2, 2011
CROI 2011: 18th Conference on Retroviruses and Opportunistic Infections
Boston, Massachusetts, USA
http://www.retroconference.org/2011

April 1–4, 2011
Annual Scientific Meeting of The Society for Healthcare Epidemiology of America
(SHEA) 2011
Dallas, Texas, USA
http://www.shea2011.com

July 8–10, 2011
International Society for Infectious Diseases Neglected Tropical Diseases Meeting (ISID-NTD)
Boston, MA, USA
http://ntd.isid.org

November 16–19, 2011
7th World Congress of the World Society for Pediatric Infectious Diseases (WSPID 2011)
Melbourne, Australia
http://www.kenes.com/wspid2011/mailshot/ms3.htm

2012

June 13–16, 2012
15th International Congress on Infectious Diseases (ICID)
Bangkok, Thailand
http://www.isid.org/15th_icid

Announcements

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Announcements may be posted on the journal Web page only, depending on the event date.

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Article Title

Zoonoses in the Bedroom

CME Questions

1. Based on the above review by Drs. Chomel and Sun, which of the following statements about transmission of plague by pets living in very close contact to their owners is most likely correct?

A. Transmission of bubonic plague by pet cats has not been reported
B. Transmission of bubonic plague by pet dogs has not been reported
C. Pets infested with infected fleas may transmit plague to their owners
D. Plague cannot be transmitted from infected pets to their owners without biting

2. Your patient is a 6-month-old boy admitted with irritability and fever. Lumbar puncture reveals cerebrospinal fluid profile consistent with meningitis. Upon questioning, the mother admits that while the infant was in a bassinet, she ran to answer the phone, and when she returned to her son, her pet dog was licking his nose and mouth. Based on the above review, which of the following statements is most likely correct?

A. *Pasteurella multocida* has not been linked to meningitis
B. Being licked by pets is a common source of human infection by *P. multocida*
C. Adults in the household may safely kiss the dog's face without risk of contracting *Pasteurella* spp.
D. Sharing the bed with pets has not been reported to cause *Pasteurella* spp. infections

3. Based on the above review, which of the following statements about parasitic zoonoses associated with pets is most likely correct?

A. In the United States, the most common parasitic zoonoses associated with dogs are due to tapeworms
B. Transmission of toxocariasis to humans may occur from contact with embryonated eggs on a dog's hair coat
C. Aging dogs are most likely to be infected with *Toxocara* spp.
D. No measures are currently available to prevent human toxocariasis

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

Hepatitis E Virus and Neurologic Disorders

CME Questions

1. Based on the above study by Dr. Kamar and colleagues, which of the following statements about the overall spectrum of neurological manifestations of HEV infection is correct?

A. Approximately 15% of patients with HEV infection in this series had neurological manifestations
B. The pattern of neurological involvement was exclusively polyradiculoneuropathy in all cases
C. One patient presented with central and peripheral manifestations
D. There was marked cerebrospinal fluid (CSF) lymphocytic meningitis in most cases with central involvement

2. You are asked to consult on a 49-year-old man suspected to have neurological manifestations of HEV infection. He presented with a history of severe low back pain, followed by tingling in the legs and then in the arms, and then followed by limb weakness. Examination showed global arm weakness, proximal leg weakness, patchy loss to pinprick sensation in a radicular pattern, stocking distribution of sensory loss, and globally decreased or absent reflexes. Routine blood chemistries showed normal bilirubin but markedly elevated alanine aminotransferase (ALT). Based on the above study, which of the following statements would be most likely to be correct regarding his diagnostic workup?

A. HEV serology is sufficient to confirm the diagnosis
B. Diagnosis should be confirmed using molecular techniques to document HEV RNA in the serum and/or CSF
C. MRI of the lumbar spine is likely to be abnormal
D. Nerve conduction velocity testing is likely to be completely normal

3. The patient in Question 2 has HEV RNA in the serum and CSF and is diagnosed with acute inflammatory polyradiculoneuropathy secondary to HEV genotype 3 infection. Based on the above study, which of the following statements about his condition is most likely correct?

A. Proximal peripheral nerve involvement does not resemble that seen in most immune disorders
B. HEV infection may elicit an immune response that cross-reacts with axolemmal or Schwann cell antigens, thereby damaging peripheral nerves
C. The patient's condition is likely to rapidly deteriorate, causing death
D. Steroid treatment is recommended

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.				
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4. The activity was presented objectively and free of commercial bias.				
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1	2	3	4	5

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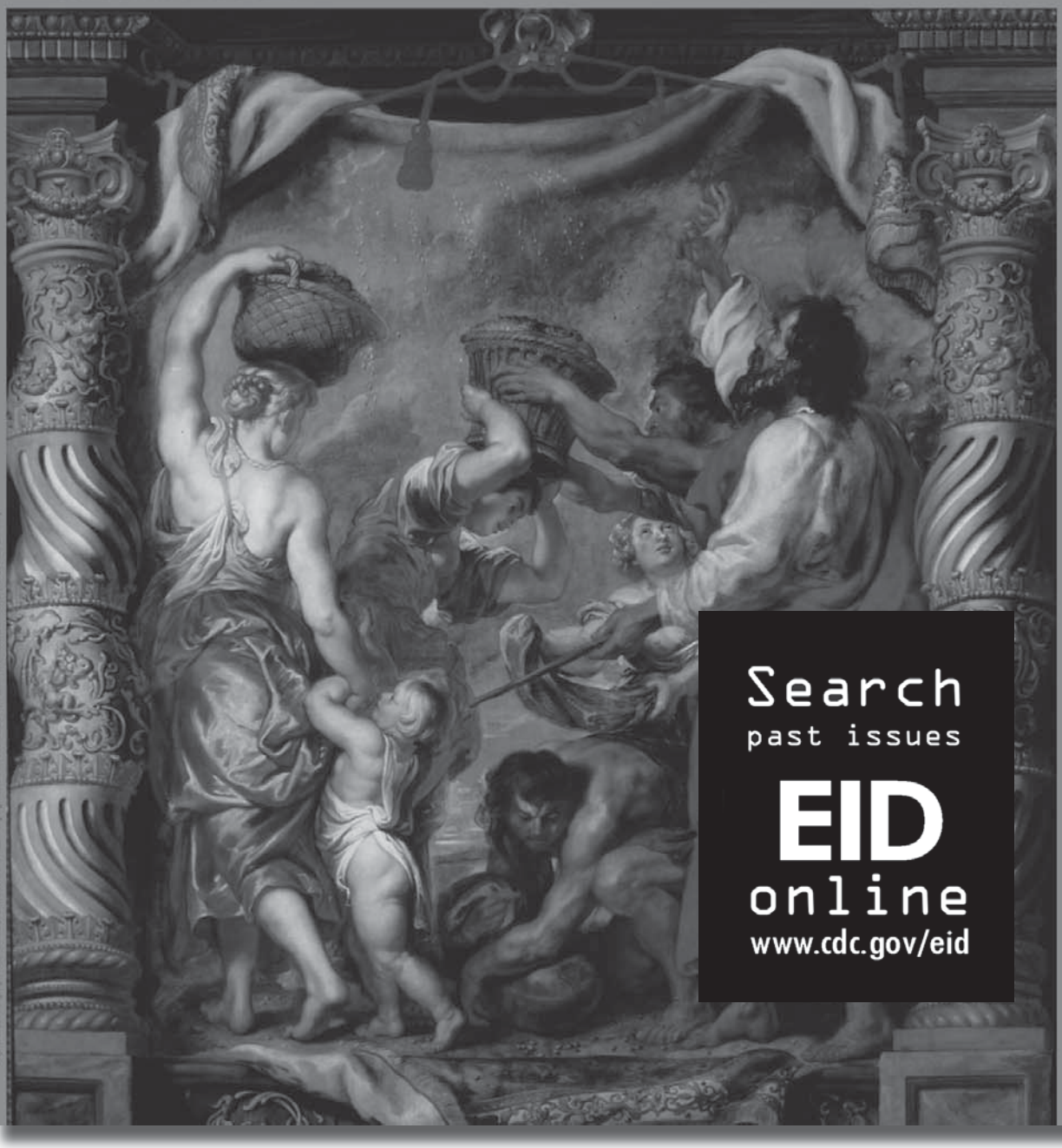

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