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September 2014

Emerging Pathogens

Pornchai Jaima (b. 1970) Integrated Farming (2013) Acrylic on canvas (39¼ x 31½ inches/100 cm x 80 cm) Courtesy of the Artist

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On the Cover

Pornchai Jaima (b. 1970)
*Integrated Farming (*2013)
Acrylic on canvas
(39¼ x 31½ inches/
100 cm x 80 cm)

Courtesy of the Artist

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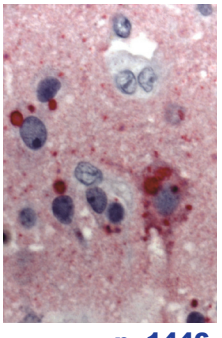

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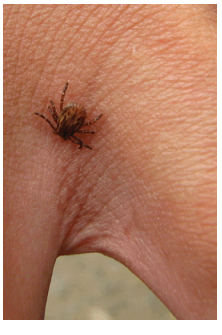

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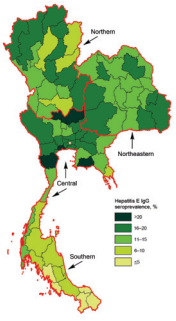

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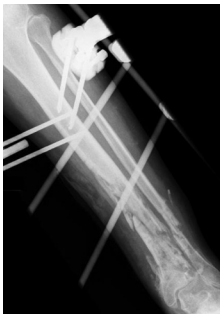

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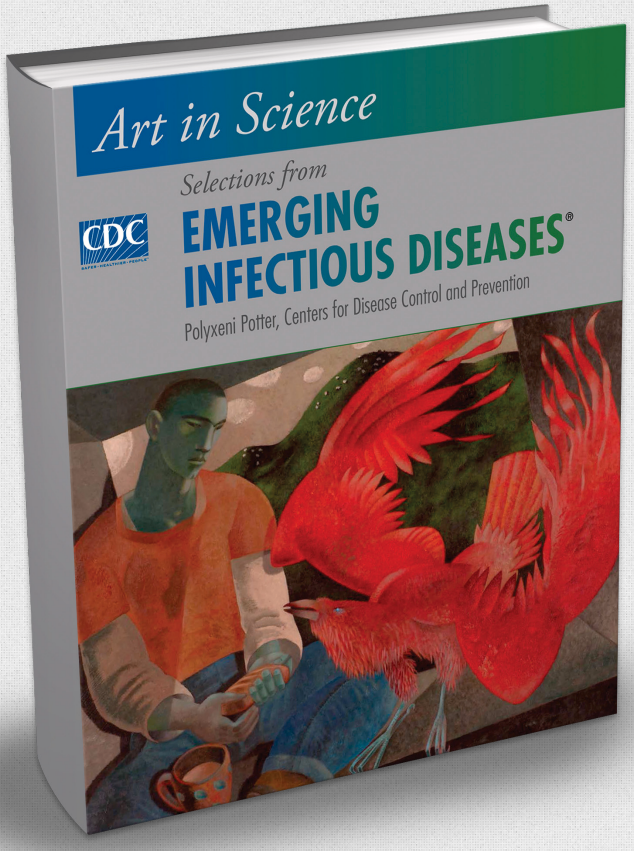

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This collection of 92 excerpts and covers from **Emerging Infectious Diseases** will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.

Molecular Epidemiology of Reemergent Rabies in Yunnan Province, Southwestern China

Hai-Lin Zhang,[1] Yu-Zhen Zhang,[1] Wei-Hong Yang,[1] Xiao-Yan Tao, Hao Li, Ji-Chao Ding, Yun Feng, Du-Juan Yang, Juan Zhang, Jiang He, Xin-Xin Shen, Li-Hua Wang, Yun-Zhi Zhang, Miao Song, and Qing Tang

Yunnan Province in China borders 3 countries (Vietnam, Laos, and Myanmar) in Southeast Asia. In the 1980s, a large-scale rabies epidemic occurred in this province, which subsided by the late 1990s. However, 3 human cases of rabies in 2000 indicated reemergence of the disease in 1 county. In 2012, rabies was detected in 77 counties; 663 persons died of rabies during this new epidemic. Fifty two rabies virus strains obtained during 2008–2012 were identified and analyzed phylogenetically by sequencing the nucleoprotein gene. Of the 4 clades identified, clades YN-A and YN-C were closely related to strains from neighboring provinces, and clade YN-B was closely related to strains from Southeast Asia, but formed a distinct branch. Rabies virus diversity might be attributed to dog movements among counties, provinces, and neighboring countries. These findings suggest that Yunnan Province is a focal point for spread of rabies between Southeast Asia and China.

Although rabies is distributed globally, it is especially prevalent in developing countries in Asia and Africa. China has the second highest number of deaths caused by rabies, exceeded only by India (*1–3*). A massive rabies epidemic occurred in China in the 1980s (*4–6*), which subsided by the mid-1990s. However, rabies has once again become a serious public health concern in China. A sharply increasing dog population and a lack of efficient management and vaccination of dogs, especially in rural areas, has led to a dramatic increase in human rabies cases in many provinces in China (*6–9*). Yunnan Province in China shows the same temporal pattern of rabies outbreaks as the rest of China; rabies was first reported in this province in 1956 (*10,11*). However, during the present reemergence of rabies, the threat to public health has intensified because the disease-endemic area has increased (Figure 1).

Yunnan Province, which comprises 16 prefectures and 129 counties, is located in southwestern China. It is adjacent to Guangxi, Guizhou, and Sichunag Provinces, which have the highest incidences of human rabies in China (*9*). Yunnan Province also has a 4,060 km border with Vietnam, Laos and Myanmar, which are countries to which rabies is endemic (Figure 1). This province has an area of ≈394,000 km^2, and mountains account for >84% of the terrain; the highest elevation (6,740 m) is in the northwestern region and the lowest elevation (76.4 m) is in the southeastern region. According to 2010 census data, Yunnan Province has a population of 45.6 million persons; most persons live in the eastern part of the province. In western areas, the difference in altitude between mountain peaks and river valleys can be as much as 3,000 m.

The primary objective of this study was to clarify the epidemiology of rabies in Yunnan Province. During 2008–2012, we obtained brain tissue specimens from patients who had died of rabies and from dogs, and other animals with suspected rabies, and cerebrospinal fluid (CSF) and saliva specimens from surviving patients. Antigens of rabies viruses (RABVs) were tested, and nucleoprotein genes from 52 rabies-positive specimens were sequenced. Results were used to characterize patterns of rabies transmission and to evaluate the factors that may influence spread of rabies. Our secondary objective was to evaluate effects of rabies control and preventive measures adjusted to local conditions.

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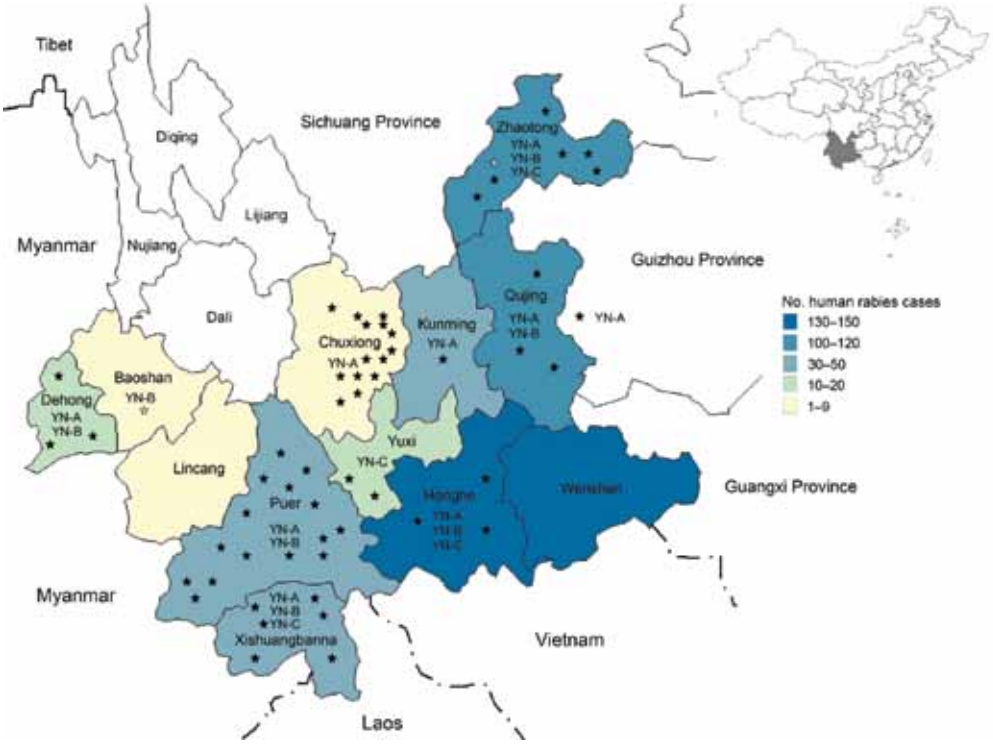

Figure 1. Distribution of rabies cases, 2000–2012, and clades of rabies virus isolates, 2008–2012, Yunnan Province, China. Shown are the 16 prefectures in Yunnan Province. Black stars indicate 52 specimens collected in the present study. White stars indicate specimens obtained before the present study. YN-A, YN-B, and YN-C indicate clades identified in different prefectures.

Human Rabies Case Data

A human case of rabies was defined according to diagnostic criteria (WS281–2008) of the Health Department of the People's Republic of China. A person with rabies had to have the following features: a history of being bitten or scratched by a dog, cat, or a wild animal, or had a wound that was licked by these animals; and clinical manifestations of itching, pain, numbness, and formication around the healed wound, followed by hyperactivity, hydrophobia, aerophobia, spasms of the pharyngeal muscle, and sympathetic excitability. The prodromal stage of paralytic rabies shows hyperpyrexia, headache, emesis, and pain at the site of the wound. Muscles of patients gradually become paralyzed, and patients with rabies die of cardiorespiratory arrest within a few days.

A case-patient with laboratory-diagnosed rabies had RABV antigen, antibody, or nucleic acid was detected in specimens. In China, rabies is a reportable disease; all human cases are reported to the Chinese Center for Disease Control and Prevention (CDC), and nearly all cases are confirmed by clinical features, rather than laboratory diagnosis. A person who satisfied these criteria was confirmed as having a clinical case of rabies.

Human rabies case data were obtained from an infectious disease database report that was officially compiled by the Yunnan Center for Disease Control and Prevention, and the Chinese CDC. Detailed information was obtained by epidemiologic investigations. These data were sorted and analyzed by using Excel (Microsoft, Redmond, WA, USA) and a descriptive epidemiologic method.

Sample Collection

During 2008–2012, brain tissues from 1 cow and 86 sick dogs suspected of having rabies, including dogs that had bitten humans or animals (during abnormally aggressive incidents), were obtained in Baoshan, Dehong, Honghe, Xishuangbanna, Qujing, Zhaotong, Yuxi, Puer, and Chuxiong Prefectures of Yunnan Province. In addition, brain tissues from 1,069 apparently healthy dogs were collected during depopulation of dogs in 11 villages in which a rabies outbreak suddenly occurred and threatened local inhabitants. Brain tissues from 300 dogs used for meat were also obtained from local restaurants. Human brain tissues were obtained from 3 patients within 24 h of death. In addition, 14 saliva samples and 1 CSF sample were obtained from surviving patients. All specimens were kept in airtight screw-cap tubes, transported in liquid nitrogen, and stored at –70°C until tested.

Detection of RABV Antigen

All brain tissues were analyzed by using a direct immunofluorescence assay (DFA) (*12*) and fluorescent-labeled monoclonal antibody against RABV nucleoprotein (Rabies DFA Reagent; Chemicon, Temecula, CA, USA). Fluid specimens were also screened for specific gene fragments of the nucleoprotein gene by using a nested PCR.

Reverse Transcription PCR

Viral RNA was extracted by using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and used as template for synthesis of cDNA with Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Piscataway, NJ, USA). Specific regions of nucleoprotein genes were amplified by using nested PCR.

DNA Sequencing

Complete nucleoprotein gene sequences were obtained by using primers specific for this gene as described (*12,13*). PCR products were purified by using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced.

Phylogenetic Analysis

Complete nucleoprotein gene sequences were analyzed by using BioEdit software (http://bioedit.software.informer.com/) and ClustalX version 1.8 (http://www.clustal.org/) software. Nucleotide homologies were analyzed using the MegAlign software version 5 (DNAStar, Madison, WI, USA). Phylogenetic trees were generated by the neighbor-joining algorithm in MEGA version 5 (http://www.megasoftware.net/).

Distribution of Human Cases

Temporal Distribution

On the basis of published reports, during 1956–2012, a total of 1,841 human rabies cases were reported officially in Yunnan Province (annual average incidence 0.115 cases/100,000 population; range 0.00–0.69 cases/100,000 population). However, only 33 cases occurred during 1956–1979. In the 1980s, the number of human cases increased and accounted for 57.9% of the total number since official records were initiated in 1956. The highest numbers of human rabies cases were reported in the late 1980s. From 1990 onwards, the number of reported cases fell from 73 cases in 1991 to 5 cases in 1994; during 1995–1999, human deaths caused by rabies were rare. However, this decreasing trend in the incidence of rabies then reversed; 3 cases were reported in 2000, and the number of cases increased to 130 by 2010. During 2000–2012, a total of 663 human cases were reported.

Regional Distribution

During the past 13 years, rabies-endemic areas in Yunnan Province showed an increase in the number of rabies cases. In 2000, a total of 3 human deaths caused by rabies were reported in 1 county in Wenshan Prefecture. By the end of 2012, human cases were detected in 12 prefectures (77 counties). However, no cases have been reported in 4 prefectures in the northwestern Yunnan Province since 2000 (Figures 1–3).

In Yunnan Province, most cases occurred in eastern and central areas (Figures 1, 3). Zhaotong, Qujing, Honghe, and Wenshan were the most affected prefectures in rabies-endemic areas of Yunnan Province, which borders Guizhou, Guangxi, and Sichuang Provinces. Human cases were reported in these 3 provinces (maximum no. cases: 664 in Guizhou Province in 2006; 518 in Gaungxi Province in 2006; and 372 in Sichuang Province in 2007; Center for Public Health Surveillance and Information Service and China CDC). This proximity might have been responsible for introduction of RABV into Yunnan Province. Prefectures with a comparatively low incidence of human rabies are located in southern and

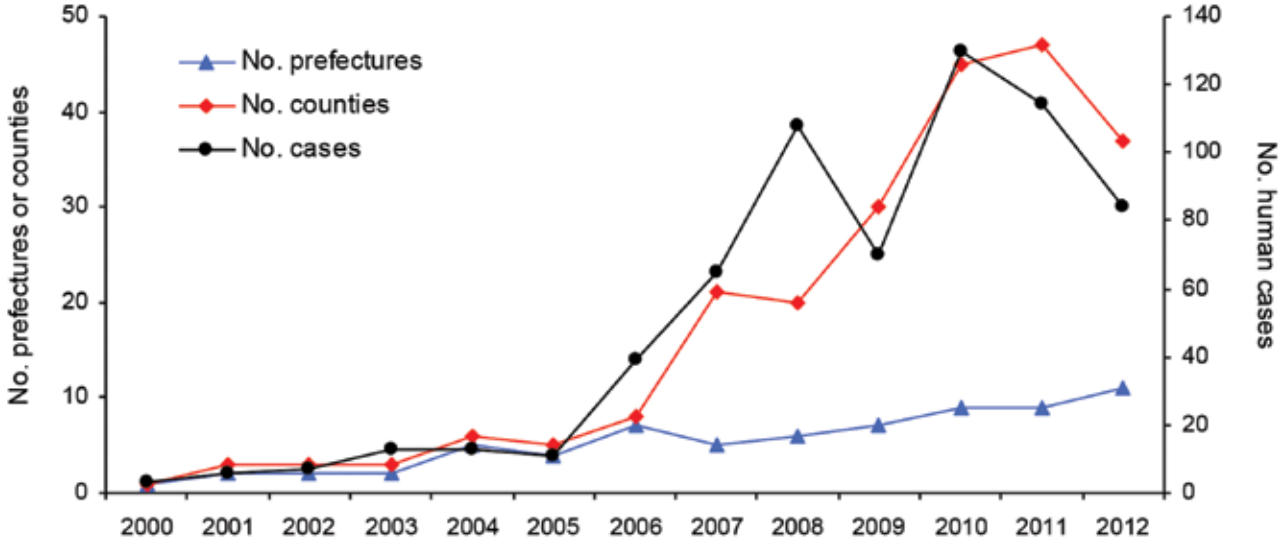

Figure 2. Temporal trends of rabies-affected prefectures, counties, and human cases, Yunnan Province, China, 2000–2012. Yunnan Province is divided into 16 prefectures and 129 counties.

southwestern regions of Yunnan Province, which borders Myanmar and Laos.

Since 2000, Wenshan Prefecture has been the most affected prefecture in Yunnan Province; a high incidence of human rabies was reported during 2007–2011 (Figure 2). Furthermore, rapid increases in numbers of human cases were reported in Qujing, Zhaotong, Honghe and Kunming Prefectures during 2006, 2007, 2008, and 2010, respectively; the number of cases in Yuxi, Puer, and Xishuangbanna Prefectures also began to show an increasing trend. This distribution pattern suggests that the disease might have spread from east to west (Figure 3). In Baoshan and Dehong Prefectures in western Yunnan Province, human rabies cases were documented every year during 2004–2007; there was a decrease in cases during 2008–2010 and an increase in cases during 2011–2012. However, in 2012, four human

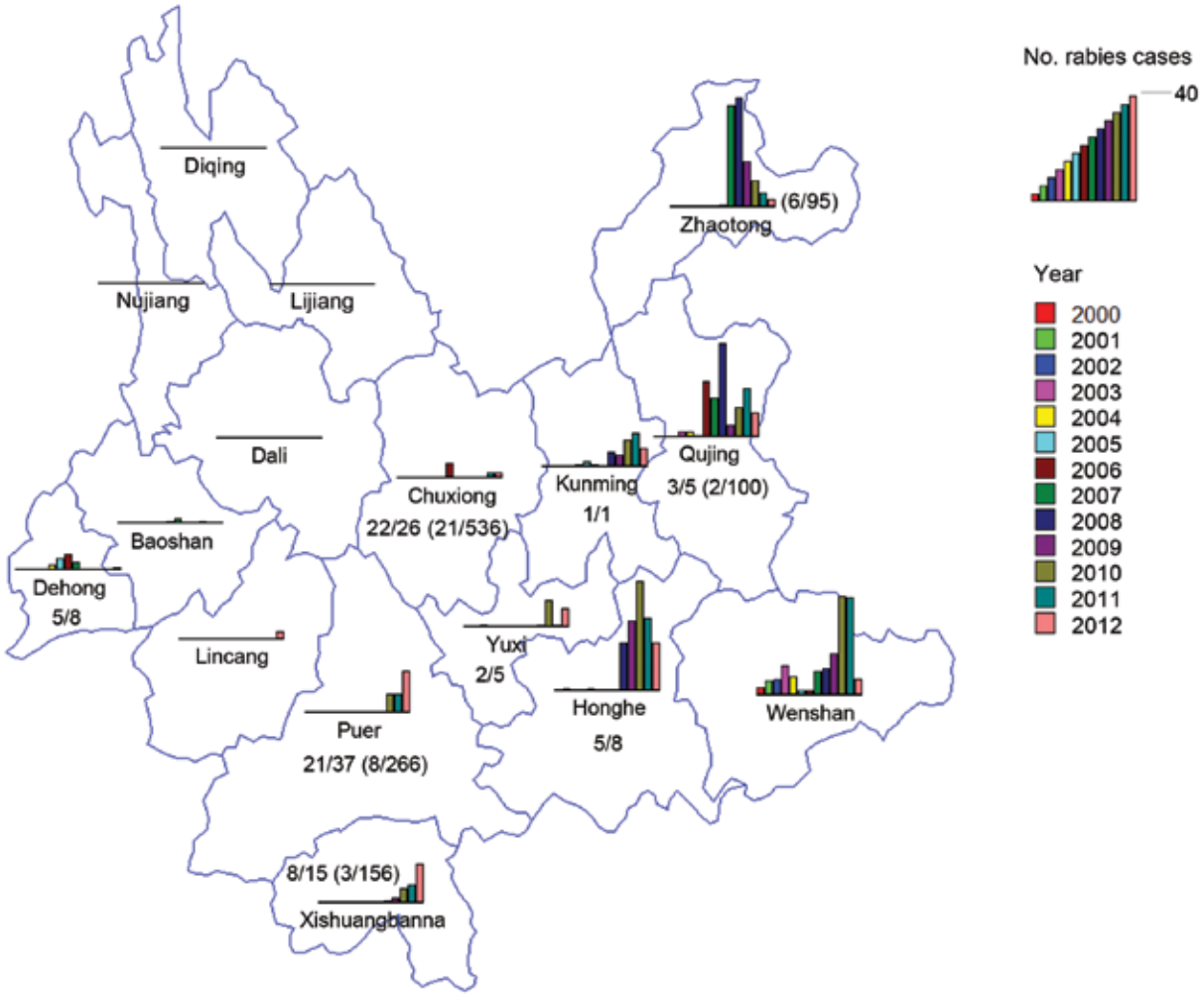

Figure 3. Distribution of rabies cases in 16 prefectures in Yunnan Province, China, 2000–2012, and data analysis of human and animal specimens. Except for Dali, Lijiang, Nujiang, and Diqing Prefectures, 12 prefectures had reported human cases. The color key is a scale in which each color bar indicates the year and its length indicates the number of human cases in that year. The longest bar indicates 40 human cases. Values indicate number of rabies-positive samples/number of samples submitted for testing from sick dogs, dogs with suspected rabies, and patients. Values in parentheses indicate number of rabies-positive samples/number of samples submitted for testing from apparently healthy dogs in areas to which rabies is epidemic. During 2008–2012, a total of 1,267 specimens from 9 prefectures were submitted for testing, including 95 brains of apparently healthy dogs from Zhaotong where rabies is endemic (6/95); five brains of sick dogs (3/5) and 100 brains of apparently healthy dogs (2/100) from the rabies-endemic area of Qujing; 1 human brain (positive) and 7 saliva samples of patients with rabies (4/7) from Honghe; 5 saliva samples (2/5) from Yuxi; 1 brain of a sick dog (positive) from Kunming; 26 brains of sick dogs or dogs with suspected rabies (22/26) and 536 brains of apparently healthy dogs (21/536) from rabies-endemic areas in Chuxiong; 36 brains of sick dogs or dogs with suspected rabies (20/36), 1 brain of 1 sick cow, and 266 brains of apparently dogs (8/266) from rabies-endemic areas in Puer; 12 brains of sick dogs or dogs with suspected rabies (5/12), 165 brain samples of apparently healthy dogs (3/165), 1 human cerebrospinal fluid sample (positive), and 2 saliva samples (positive) of patients with rabies from the rabies-endemic area of Xishuangbanna; 7 brains of sick dogs or dogs with suspected rabies (4/7) and 1 human brain sample (positive) from Dehong; and 1 human brain sample (positive) from Pan County in Guizhou Province, a county bordering Qujing Prefecture in Yunnan Province. Samples were not obtained from Wenshan, Lincang, Dali, Baoshan, Lijiang, Nujiang, and Diqing Prefectures.

cases were reported in Lincang Prefecture in Yunnan Province, where no rabies cases had been reported before 2011.

Seasonal and Demographic Distribution

Human rabies cases were reported year-round. However, reports of human cases usually peaked during May–October. Adult farmers were the most common case-patients (64%), followed by school-age children living in the countryside. Frequency of cases in male patients (73%) greatly exceeded that in female patients (27%).

RABV Detection in Specimens

All brain tissues were examined by DFA for RABV antigen. Three human brains were confirmed to be positive for RABV. Of 86 brains from sick (rabid) dogs and dogs suspected of having rabies, 54 were positive for RABV, and 45/47 dogs that bit humans were positive for RABV. In addition, 1 bovine brain was positive for RABV. The frequency of rabies-positive dogs in rabies-endemic villages was 3.44% (40/1,162) (Figure 3), and 300 specimens obtained from dogs used as food in restaurants were negative. Eight of the 14 saliva samples and 1 CSF sample were confirmed as positive for RABV RNA.

Nucleoprotein Gene Sequencing

Complete gene sequences of the nucleoprotein gene were obtained from 52 RABV-positive specimens: brain specimens from 42 dogs, brain specimens from 3 persons who died, 1 specimen from a cow that died, 5 human saliva specimens, and 1 human CSF specimen. All 52 sequences were submitted to GenBank under accession nos. JF819603–JF819612, JF819614-JF819624, JQ040591–JQ040600, and JX276405–JX276425.

Molecular Diversity and Phylogenetic Analysis

Phylogenetic analysis showed that the 52 RABV s isolated belonged to group I, clustered in 3 clades (designated YN-A, YN-B, and YN-C) (Figure 4), and had distinct distribution characteristics (*14–17*). YN-A, which contained 35 viruses, circulated primarily in eastern and central Yunnan Province; 1 virus (CYN1025H) was obtained from a patient who had been exposed to RABV in Pangxian County in Guizhou Province, which is adjacent to eastern Yunnan. A virus in this clade was identified in 2012 in a patient who lived in Yunnan Province near Myanmar. Nine YN-B viruses were obtained in western and southern Yunnan Province near the border with Myanmar and in northeastern Yunnan Province. YN-C, which contained 8 viruses, was found usually in the central and southern regions of Yunnan Province.

In general, RABVs obtained from the same prefecture or county within a similar time frame clustered in the same clade, as shown by a canine rabies outbreak in Chuxiong Prefecture in 2012. However, with the spread of RABV over time, viruses in the same clade, such as YN-A and YN-B, were found in the eastern and western regions of Yunnan. This finding suggests that long-distance transmission of RABV is closely connected with the movement of dogs.

A nucleoprotein gene sequence of RABV previously isolated in Yunnan Province belongs to another clade (YN-D) (Figure 4). Thus, 4 phylogenetic clades are present in Yunnan Province YN-A, a distinct branch of the China group of RABVs (Figure 4), showed a close relationship with RABVs from most neighboring provinces. Thus, it is highly likely that these viruses had been introduced into Yunnan Province and then spread to other areas. YN-C belongs to China clade II, which circulates primarily in provinces in southern China, such as Guangdong, Guangxi, Fujian, Jiangxi, and Zhejiang (Figure 4). In Yunnan Province, YN-D (which belongs to China clade III) appeared to be present at a low prevalence because no other specimens of this type were documented.

YN-B is phylogenetically similar to RABVs in Thailand, Myanmar, Laos, Vietnam, and Cambodia, but formed a distinct branch within China clade IV. Results show that RABVs from Guangxi Province, which borders Vietnam, remained within the same branch as RABVs from other countries in Southeast Asia (Figures 4, 5).

Conclusions

In China, dogs are a major reservoir of RABVs (*14*), although wildlife rabies has been reported occasionally in southeastern China (*15,18*). In Yunnan Province, domestic dogs are the predominant source of human rabies (*10,11,19*), and no wildlife rabies cases have been found.

Rabies was previously reported in only 1 county in Yunnan Province in 2000, but has now been reported in 77 counties in this province. Despite the obvious increase in the dog population, canine rabies has remained a neglected disease in Yunnan over recent decades. Currently, rural families in many villages commonly own 2–3 dogs. However, there is no national or provincial law or related department to ensure compulsory annual routine vaccinations in dogs. Because of this large reservoir of susceptible animals, dog-to-dog transmission of RABV has been difficult to interrupt.

Yunnan Province is affected not only by neighboring provinces, but also by other countries in Southeast Asia because of the extensive border. Two investigations aggregately documented 5 rabid dogs from Myanmar that entered villages in China (Dehong Prefecture); attacked humans, pigs, horses, and other domestic animals; and caused turmoil in the affected rural communities (*20,21*). In the present study, RABV from all 15 brain specimens of dogs obtained from 5 counties in Chuxiong Prefecture within a

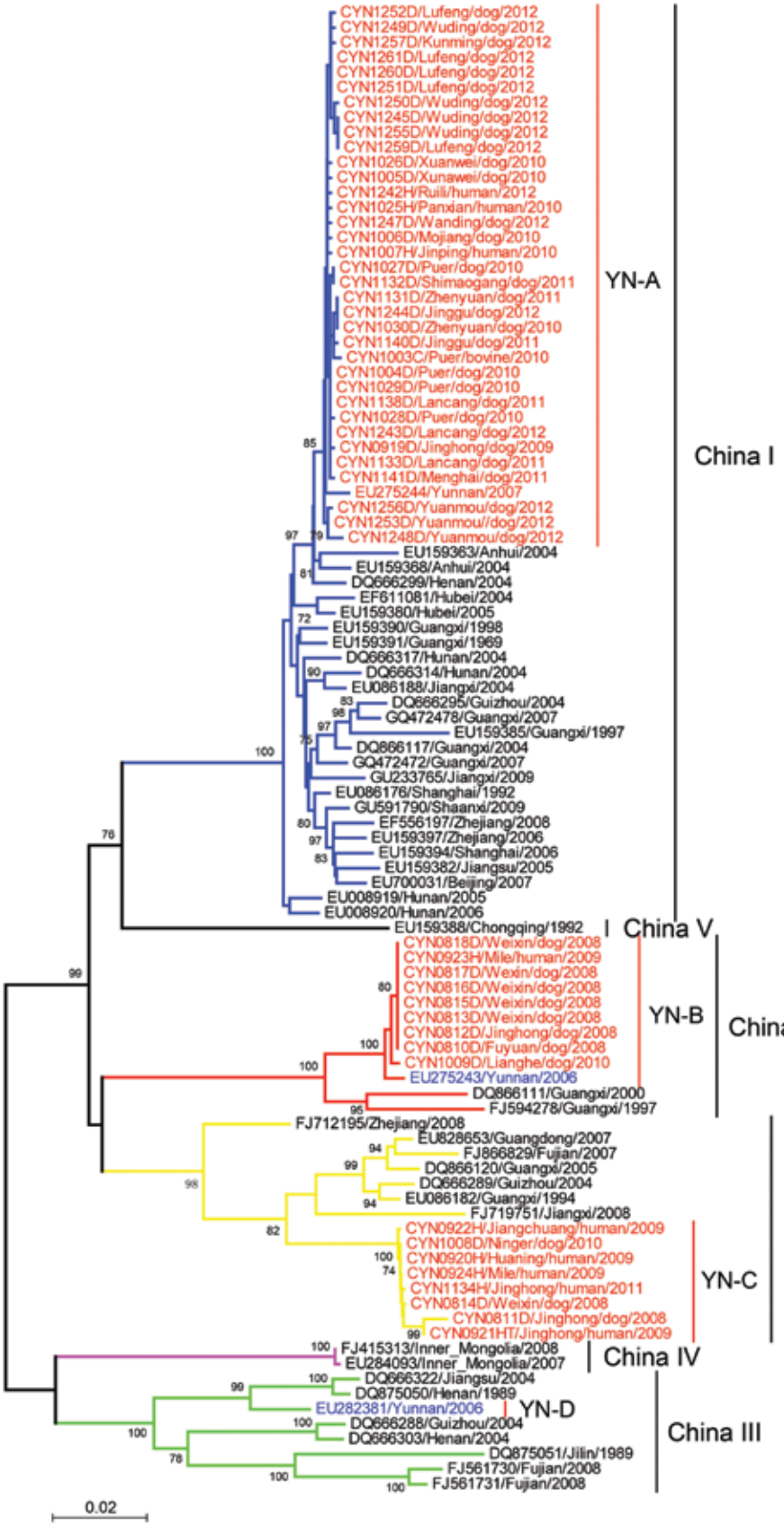

Figure 4. Phylogenetic relationship of nucleoprotein gene sequences of rabies virus isolates from Yunnan Province, China, 2008–2012, with isolates from neighboring provinces in China. Numbers at each node indicate degree of bootstrap support (only values >70% are indicated). Red indicates taxa sequenced in this study; blue indicates taxa from Yunnan Province; black indicates taxa from other provinces in China. Blue branches indicate China I clade; yellow branches indicate China II clade; green branches indicate China III clade; purple branch indicates China IV clade; black branches indicate China V clade; red branches indicate China IV clade. Scale bar indicates nucleotide substitutions per site.

6-month period (47 abnormally aggressive incidents) were grouped into clade YN-A. This grouping suggested a dramatic rabies outbreak in dogs associated with the traditional laissez-faire dog-keeping practice.

At least 2 mechanisms of canine translocation due to human activities that could play a major role in RABV transmission exist. First, unrestricted by law, dogs can be traded over short distances at local fairs and delivered as gifts by friends or relatives who live in neighboring villages that are not subject to a quarantine process. These dogs might be kept and valued principally as watch dogs that guard and protect the properties of their owners. Second, long-distance translocation can occur through restaurants that sell cooked dog meat. Although no specimens from dogs used as meat were positive for RABV in this study, some managers of local restaurants reported that their dogs were obtained from neighboring provinces or even Myanmar, and not all were sold for meat. Among dogs in Guizhou, Guangxi, and Hunan Provinces, some were confirmed as positive for rabies by Li et al. (*22*).

On the basis of studies conducted by Wu et al (*23*), which demonstrated that phylogenetic analyses of 1 gene of lyssavirus can show similar results as analysis of complete genomes, we sequenced and analyzed the nucleoprotein

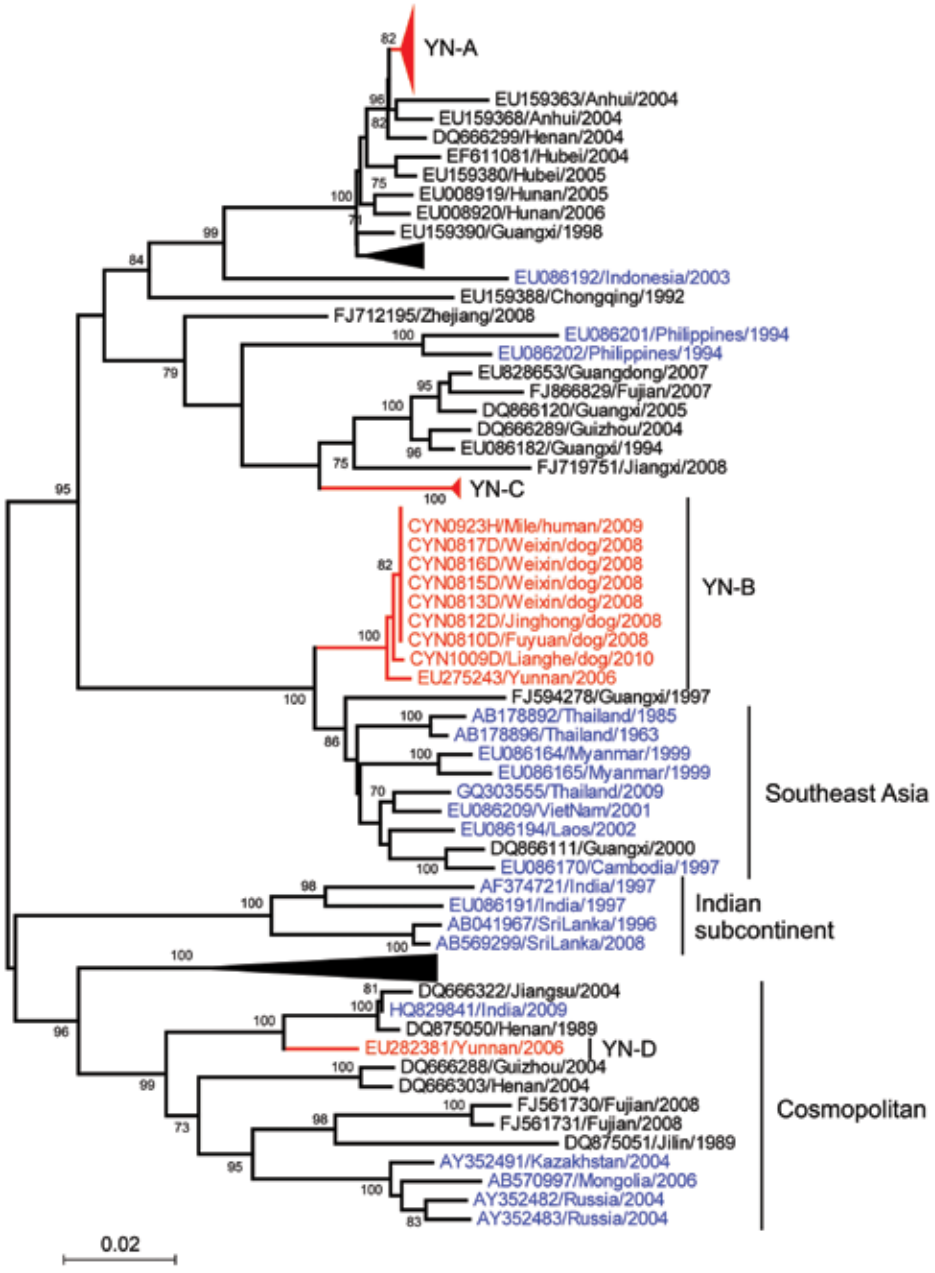

Figure 5. Phylogenetic relationship of nucleoprotein gene sequences of rabies virus isolates from Yunnan Province, China, 2008–2012, with those from neighboring countries in Asia. Numbers at each node indicate degree of bootstrap support (only values >70% are indicated). Red indicates taxa from Yunnan Province; blue indicates taxa from Asia; and black indicates taxa from other provinces in China. Black triangles indicate virus isolates shown in Figure 4. Scale bar indicates nucleotide substitutions per site.

gene of RABVs. Our results were supported by molecular epidemiologic analysis of nucleoprotein gene sequences of RABVs. Nucleoprotein gene sequences are the most conserved of the 5 RABV genes and have been extensively analyzed (*24–28*). Zhang et al. (*29*) grouped 37 RABV strains from China into 2 phylogenetic clades. Four other studies (*16,17,30,31*) reported grouping of RABVs from China into 6 clades. In our study, we found 4 clades in Yunnan, which suggests genetic diversity of RABVs in this province.

Despite the paucity of data for Myanmar, Laos, and Vietnam before 2008, we elucidated the pattern of RABV spread. Clade YN-A, which was initially introduced into eastern Yunnan Province from neighboring provinces, probably spread westward to adjacent prefectures and might have spread into neighboring countries from which it returned to Yunnan Province (Figure 1). Early in 2006–2007 we verified the presence of clade YN-B in a human brain specimen from Longyang County and a domestic dog brain specimen (EU275243) from Tengchong County in Baoshan Prefecture, near Myanmar (*32)*. This clade was also detected in other prefectures in this study. The phylogenetic relationship of RABVs in other countries in Southeast Asia and their independent branch (Figure 5) indicate that RABV was introduced from neighboring countries, became established locally as an enzootic virus, and then spread northward into eastern and other areas of Yunnan.

We cannot exclude the possibility that clade YN-B will spread further in China. In addition, more specimens should be obtained from Wenshan Prefecture, other prefectures, and neighboring countries to clarify the pattern of spread of YN-C. We speculate that rabies epidemics in Yunnan Province will remain complicated because of the unique location of this province, which also plays a major role in rabies dispersal among developing countries in Asia.

In eastern Yunnan Province, the high population densities of humans and dogs and the relatively advanced transportation network are advantageous for regional spread of RABV. In contrast, mountains and valleys in the northwestern province act as barriers to slow the spread of the virus. However, the major factor associated with RABV circulation in dogs is dog population density, which correlates with the human population density. In the most affected prefectures (Wenshan, Zhaotong, Qujing, Honghe, and Kunming), population densities were 109.25–299.58 persons/km^2 and 10.76–19.81 dogs/km^2. In Diqing, Nujiang, and Lijiang Prefectures, where no human or dog rabies cases have been reported, the population densities were 16.76–60.44 persons/km^2 and 3.44–8.74 dogs/km^2 (2012 census and 2012 dog population statistics derived from the Yunnan Animal Diseases Control Center). In contrast, the human:dog ratio (an indicator of the average number of persons who own a dog) was lower in Diqing, Nujiang, and Lijiang Prefectures (4.87–9.08) than in Wenshan, Zhaotong, Qujing, Honghe, and Kunming Prefectures (10.61–15.41).

Because the recommended preventive strategy (annual vaccination) has not been implemented in the dog population in Yunnan Province, the following temporary interventions and remedies are suggested for a village affected by rabies. First, persuade villagers to confine their dogs, regardless of any negative response. Second, euthanize unsupervised dogs and domestic dogs within the affected villages. Third, vaccinate all dogs in neighboring villages as an emergency and temporary measure. Fourth, vaccinate all susceptible persons, educate inhabitants regarding the risk of rabies, and subsidize poor persons for expenses relating to these issues.

Previous studies showed that in rabies-endemic areas, some apparently healthy dogs are infected with RABV (*22,33*). In the present study, of 1,162 brain samples from apparently healthy dogs in rabies-endemic areas, 40 (3.44%) were confirmed to be infected with RABV, which indicates that the virus will be disseminated by local dogs in any village containing even 1 rabid dog (Figure 3). This finding supports the rationality of culling dogs indiscriminately, given the absence of conclusive means of distinguishing apparently healthy dogs (i.e., no clinical symptoms although infected) from healthy animals. However, culling is a costly practice that usually forces local authorities into a financial dilemma. Moreover, its effectiveness is deficient because of virus transmission among dogs beyond culled areas. In contrast, vaccination of all exposed persons has been largely unrealized because of cost of rabies vaccine and human rabies immunoglobulin, as well as the ignorance of the risk of rabies in humans. Thus, human/canine rabies has continued to spread into more counties in Yunnan Province. In the near future, Dali and Lijiang Prefectures will have human cases of rabies because these areas have dog population densities of 11.02 dogs/km^2 and 8.74 dogs/km^2, respectively.

We did not obtain specimens from dogs in all prefectures of Yunnan Province (Figure 3). A canine rabies surveillance laboratory has not yet been established, but as described by Banyard and others (*34*), such a laboratory would play a major role in controlling spread of rabies. In addition, some regions of Yunnan Province have established canine entry–exit inspections and quarantine measures. However, Yunnan Province and other countries in Southeast Asia have extensive borders (4,060 km) with each other, and it would be difficult to restrict the movement of dogs, especially stray dogs, across these borders. Therefore, we recommend increased surveillance programs in the border areas of adjacent countries to control the spread of RABV.

The greatest obstacle to removing the threat of rabies is the low level of political commitment because dogs are not

regarded as economically useful animals in Yunnan Province. After the goal of eradicating rabies in China before 2020, as proposed by the World Health Organization, was set, the principal concern undoubtedly was to stop RABV transmission among dogs (*34–37*). To achieve this goal, the following programs should be deployed. First, rabies vaccination of dogs should be made compulsory by passing of a national or provincial law. Second, for political and medical reasons, a database of adequate surveillance and laboratory data should be established to document dog rabies cases throughout Yunnan Province. Third, an inexpensive, safe, and highly effective dog rabies vaccine should be developed and the problematic vaccines currently used should be phased out. Fourth, a cost-efficiency analysis of rabies eradication should be performed because of political/economic considerations.

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Encephalitis Caused by Pathogens Transmitted through Organ Transplants, United States, 2002–2013

Sridhar V. Basavaraju, Matthew J. Kuehnert, Sherif R. Zaki, and James J. Sejvar

The cause of encephalitis among solid organ transplant recipients may be multifactorial; the disease can result from infectious or noninfectious etiologies. During 2002–2013, the US Centers for Disease Control and Prevention investigated several encephalitis clusters among transplant recipients. Cases were caused by infections from transplant-transmitted pathogens: West Nile virus, rabies virus, lymphocytic choriomeningitis virus, and *Balamuthia mandrillaris* amebae. In many of the clusters, identification of the cause was complicated by delayed diagnosis due to the rarity of the disease, geographic distance separating transplant recipients, and lack of prompt recognition and reporting systems. Establishment of surveillance systems to detect illness among organ recipients, including communication among transplant center physicians, organ procurement organizations, and public health authorities, may enable the rapid discovery and investigation of infectious encephalitis clusters. These transplant-transmitted pathogen clusters highlight the need for greater awareness among clinicians, pathologists, and public health workers, of emerging infectious agents causing encephalitis among organ recipients.

More than 500,000 solid organ transplants have been performed worldwide, and >28,000 are performed annually in the United States (*1*). Improvements in immune-modulating therapy, critical care medicine, and surgical techniques have led to the increased success of organ transplantations, and more patients are now eligible for these procedures. In the United States, >100,000 patients are currently on organ transplant waiting lists (*1*).

The risk for infections caused by pathogens transmitted through solid organ or tissue transplants (hereafter referred to as donor-derived or transplant-transmitted infections) has been recognized for decades and remains a worldwide public health problem (*2*). The gravity of these infections took on greater focus after the HIV epidemic emerged in the 1980s (*3*). Infections caused by other donor-derived pathogens in transplant recipients are often asymptomatic or may result in nonspecific signs and symptoms, including unexplained fever or end-organ injury (*4*). Because of immunosuppression and underlying co-existing conditions in transplant recipients, these infections can be severe and fatal. The recognition of this risk led to the screening of donors for some infectious agents (e.g., HIV and hepatitis B and C), which made the organ supply substantially safer. However, a residual transmission risk persists, which might be further reduced by the use of new technologies, such as nucleic acid testing (NAT) (*5*).

Since 2002, several types of emerging donor-derived infections have been reported with increasing frequency among solid organ transplant recipients seeking medical care for encephalitis. These cases can present a diagnostic challenge for clinicians and highlight the need to increase awareness among transplant clinicians regarding the necessity for prompt recognition and treatment of transplant-transmitted infections.

The signs and symptoms of encephalitis vary, depending on the region of the brain involved, but most cases are characterized by global cerebral dysfunction or focal neurologic deficits (*6*). Diagnosing the cause of encephalitis in transplant recipients may be particularly difficult because the cardinal sign of encephalitis (alteration of mental status) can be attributed to numerous other systemic processes. In addition, there are other noninfectious causes of encephalitis, including toxic, metabolic, neoplastic, and autoimmune processes. The signs and symptoms of donor-derived infections can be obscured by co-existing conditions in the transplant recipient, or they can appear more abruptly than

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in natural infection because of a higher inoculum of organisms and immunosuppression in the transplant recipient. Thus, transplant-transmitted pathogens may be an underrecognized cause of encephalitis.

Since 2002, the US Centers for Disease Control and Prevention (CDC) has investigated clusters of encephalitis among transplant recipients. Cases have been caused by emerging pathogens, including West Nile virus (WNV) (*7*,*8*), rabies virus (*9*), lymphocytic choriomeningitis virus (LCMV) (*10*), and *Balamuthia mandrillaris* amebae (*11*). The cases highlight the difficulties in diagnosing or recognizing clusters of infectious encephalitis among transplant recipients. We review the emerging infectious agents known to cause transplant-transmitted encephalitis, as described from several recent outbreak clusters reported to and investigated by CDC, and suggest methods for better identifying possible donor-derived infections.

West Nile Virus

WNV, an enveloped, positive, single-stranded RNA flavivirus within the Japanese encephalitis serologic complex, was historically associated with infrequent epidemics of relatively mild febrile illness in parts of Africa, Asia, and Europe (*12*). In 1999, WNV virus was first identified in North America, where it caused an outbreak of encephalitic illness in New York City. Within 5 years, WNV caused the largest epidemic of arboviral encephalitis and became the most common etiologic agent of arboviral encephalitis in the Western Hemisphere. By the end of 2011, >30,000 human cases of WNV encephalitis throughout the United States had been reported to CDC, including >13,000 cases of neuroinvasive disease (*13*).

The natural mode for WNV transmission is through the bite of infected mosquitoes, primarily *Culex* spp. mosquitoes; birds serve as amplifying hosts (*12*). In 2002, several cases of serologically confirmed WNV infection occurred in persons with little or no known exposure to mosquitoes, and epidemiologic evidence suggested transmission of the virus through blood transfusions. Later that year, the first recognized US cases of organ transplant–transmitted WNV were described (*8*). For these infections, the initial link to the transplanted organ was made by histopathologic evaluation and immunohistochemical testing of tissue from an organ recipient who died 4 weeks after undergoing transplantation (Figure 1). However, a link between the infections and the 2 mechanisms of pathogen transmission (i.e., blood transfusion and solid organ transplant) had been suspected early in the epidemic, when WNV infection was diagnosed in 1 of the organ donors who had received a transfusion of WNV-infected blood. In addition, concern was raised over the possibility of a transplant-transmitted infectious agent when illness occurred in 4 recipients of organs from a common donor: febrile illness developed in the liver recipient, and WNV encephalitis developed in 2 kidney recipients and the heart recipient (*8*). Laboratory and epidemiologic data substantiated this mode of virus transmission and documented that the organ donor had likely acquired WNV through a blood transfusion. Subsequent investigation by CDC verified 23 cases in 2002 of WNV acquired through blood or blood components (*7*).

Since blood screening was put into place through pooled donation NAT, there have been 12 published cases of transfusion-transmitted WNV. Of these 12 cases, 5 occurred after blood collection agencies voluntarily implemented processes to trigger more sensitive individual NAT based on local WNV activity (*14*–*16*). There have been no recognized cases of transfusion-transmitted WNV when individual NAT has been used. In 4 of the 5 cases that

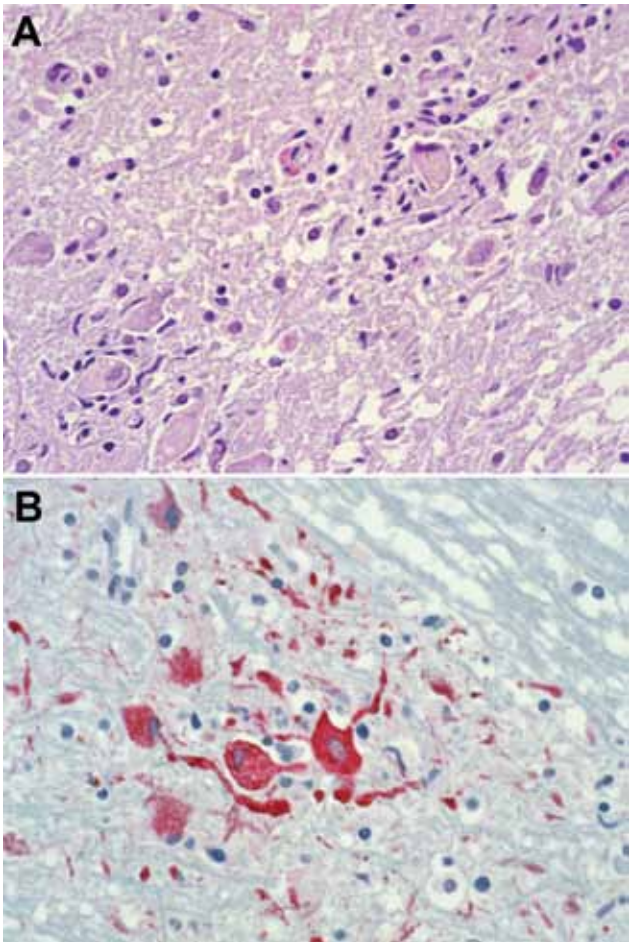

Figure 1. Photomicrographs showing histopathologic features and immunolocalization of West Nile virus antigens in central nervous system tissue from a kidney transplant recipient with transplant-transmitted West Nile virus infection. A) Mononuclear inflammation, gliosis, and neuronophagia. Hematoxylin and eosin staining. Original magnification ×125. B) West Nile virus antigens within neurons and neuronal processes. Immunoalkaline phosphate staining, naphthol fast red substrate with light hematoxylin counterstain. Original magnification ×125.

occurred after testing of individual blood samples was begun, individual testing was not triggered by local events, resulting in breakthrough infection that was not detected by pooled NAT. In another case, individual screening was triggered, and NAT was done, but before the screening results were received, a granulocyte unit was released for transfusion use; testing results later showed that the WNV load was sufficient for detection by NAT of pooled and individual blood samples (*16*).

The 2002 transplant-transmitted WNV cluster heightened awareness of the potential increased severity of WNV infection, particularly neuroinvasive disease, in transplant recipients and immunocompromised persons. It is believed that WNV transmission through blood transfusion has become rare since NAT of donated blood was implemented; however, this may not be the case in the solid organ–transplant setting because organ donor screening has not been mandated.

Six clusters of organ transplant–transmitted WNV were reported to CDC during 2002–2013. In those clusters, WNV infection developed in 12 of 16 transplant recipients; encephalitis developed in 9 of the 12 infected persons, and 4 of those 9 patients died (*17*). It is likely that signs and symptoms of encephalitis among transplant recipients during a WNV outbreak led to the recognition that WNV had been transmitted through organ transplants (*17*). In 2005, another cluster of donor-derived infection was observed among 3 of 4 recipients of organs from a common donor who had naturally acquired WNV infection (*18*).

In the blood transfusion setting, experience has suggested that it is unusual for WNV to be transmitted though the blood of a WNV IgM–positive donor. It has been assumed that a WNV-specific neutralizing antibody response results in clearing of viremia, rendering the blood no longer infectious and probably safe for transfusion use (*19*). However, this may not be the case in the solid organ–transplant setting. The organ donor in the 2005 transplant-transmitted WNV cluster had detectable WNV-specific IgM and IgG but no detectable WNV RNA, suggesting that WNV may be transmitted through transplanted organs from infected persons who have already mounted an antibody response. It may be that transmission is possible because of viral persistence in donated organs after peripheral viremia has cleared or because of intermittent viremia from a reservoir organ, such as a kidney (*20*). Thus, it may prove challenging to implement WNV screening of potential organ donors.

Rabies Virus

Rabies virus (family *Rhabdoviridae*, genus *Lyssavirus*) is an enveloped, negative, single-stranded RNA virus (*21*). After the virus is inoculated into humans, typically through the saliva or bites or scratches from infected animals, it is taken up through peripheral nerves. Then, through retrograde transport, the virus infects the central nervous system and causes encephalitis; this process may take weeks or months (*21*). Clinical manifestations of rabies virus infection include a nonspecific prodrome (malaise, fever, headache) that lasts days to weeks, followed by confusion and paresthesias, which progress to paresis, hydrophobia, coma, and death (*22*). Rabies is nearly always fatal once neurologic signs develop. In industrialized countries, the disease occurs infrequently because of good veterinary public health practices and postexposure prophylaxis of person exposed or potentially exposed to rabies virus. However, in developing countries, rabies is a common cause of encephalitis (*22*).

The transmission of rabies virus through cornea transplantation has been described, but transmission through solid organ transplantation was not recognized before 2004 (*23*). In July 2004, CDC was notified that 3 recipients of solid organs and 1 recipient of an iliac artery segment from a common donor had died from encephalitis, which was eventually found to be caused by rabies virus infection (*9*). The initial link to rabies virus was made by histopathologic evaluation and immunohistochemical testing of central nervous system tissue (Figure 2). The common-source outbreak of donor-derived rabies infection highlighted the difficulties inherent in the evaluation of encephalopathy in a potential organ donor. Before dying, the donor associated with this outbreak cluster had been evaluated twice in an emergency department for nausea, vomiting, and dysphagia. He eventually sought care for a subarachnoid hemorrhage and then died. After it was determined that rabies virus had been transmitted to multiple recipients of organs from this donor, it was noted that the donor had had other symptoms consistent with encephalitis and had been bitten by a bat (*9*).

Although it was thought that this type of rabies virus transmission was extremely uncommon, another cluster of donor-derived rabies encephalitis cases was reported from Germany soon after the 2004 cases. Recognition of the second cluster demonstrates the potential ability to improve clinical recognition and diagnosis of transplant-transmitted encephalitis through increased awareness generated from knowledge of previous transmission events (*24*).

In these 2 clusters of solid organ transplant–transmitted rabies cases, infection was attributed to bat and canine virus variants, respectively. Within 6 weeks of undergoing transplantation, all but 1 recipient had rabies symptoms and died; the transplant recipient who survived was from the German cluster and had been previously vaccinated against rabies (*9*,*24*). These observations suggested a high rate of infectivity and incubation periods of ≈6 weeks in unvaccinated recipients of solid organs from donors with rabies.

In 2013, another case of transplant-transmitted rabies was identified in the United States (*25*). A raccoon rabies virus variant was identified in the organ donor and the infected recipient. Signs and symptoms of rabies developed

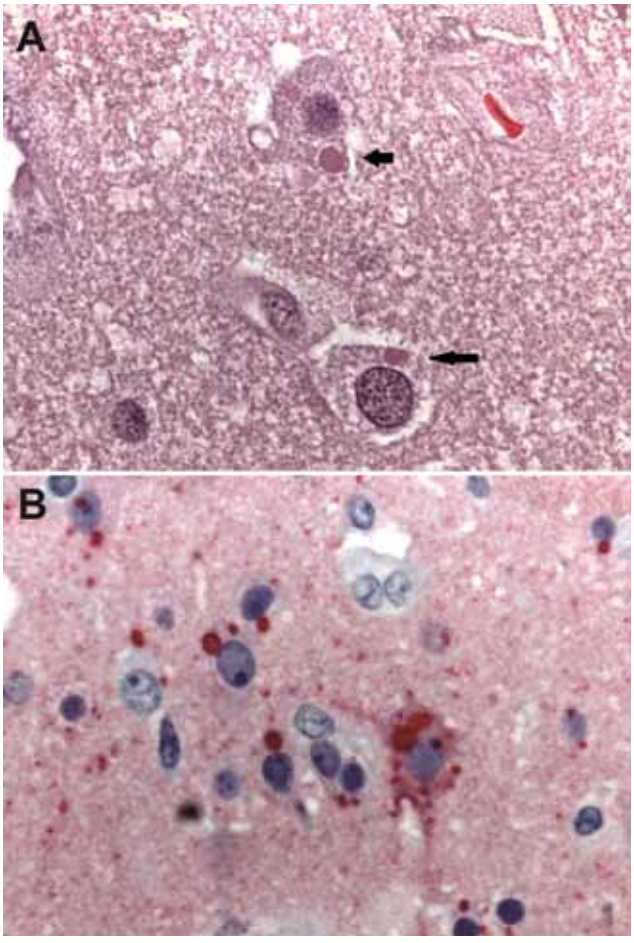

Figure 2. Photomicrographs showing histopathologic features and immunolocalization of rabies virus antigens in central nervous system tissue from a kidney transplant recipient with donor-derived rabies infection. A) Typical intracytoplasmic eosinophilic inclusions (Negri bodies, arrows). Hematoxylin and eosin staining. Original magnification ×158. B) Rabies virus antigens within neurons and neuronal processes. Immunoalkaline phosphate staining, naphthol fast red substrate with light hematoxylin counterstain. Original magnification ×158.

in the organ recipient ≈17 months after the transplantation (Figure 2); 3 unvaccinated recipients of organs from the same donor remained asymptomatic. These findings contrast with those from previously reported clusters of transplant-transmitted rabies cases. Postexposure prophylaxis was initiated in the asymptomatic organ transplant recipients, and protective neutralizing antibody responses developed in all 3, suggesting that raccoon rabies infection can be prevented in organ transplant recipients if timely preventive measures are implemented (*25*).

Lymphocytic Choriomeningitis Virus

LCMV (family *Arenaviridae*) is an enveloped virus with 2 negative-stranded RNA segments (*26*). The disease is an uncommon, primarily rodentborne infection that occurs among persons who have substantial contact with infected small rodents (*27*). Infection in immunocompetent persons is believed to be asymptomatic, and it is generally mild and self-limited in persons in whom clinical disease develops (*27*). Severe meningoencephalitis has been reported among immunocompromised patients (*28*).

In 2003 and 2005, CDC investigated clusters of meningoencephalitis among solid organ transplant recipients in the United States (*29*). The 2003 cluster occurred among recipients of organs from a common donor from Wisconsin who had died from a subdural hematoma (*29*). There was no serologic evidence of LCMV infection in the donor and no evidence of exposure to rodents. LCMV was eventually detected in specimens from the donor by cell culture and electron microscopy evaluation, and the donor was identified as the source of the infections (*29*). The 2005 cluster of cases occurred among 4 recipients of organs from a common donor who died from an ischemic stroke (*30*). Pathologic investigation and immunohistochemical staining of specimens led to the diagnosis of LCMV infection in the transplant recipients (Figure 3), and epidemiologic investigation showed that the donor had a pet hamster (*10*). Although the donor had no evidence of active LCMV infection, the hamster was infected with a strain that was genetically similar to those that infected the transplant recipients.

In 2008, CDC investigated reports of hepatic insufficiency, multi-organ system failure, and death among 2 recipients of kidneys from a common organ donor (*31*). Although the recipients had no signs or symptoms of encephalitis, the donor had aseptic meningitis at death and was retrospectively found to have serum antibodies against LCMV. The donor was homeless and thus likely had multiple opportunities for exposure to rodents (*31*).

Another cluster of infections with a novel LCMV-related virus among 3 organ transplant recipients was reported in Australia; the patients died of encephalitis within 6 weeks of undergoing transplantation (*32*). In that cluster, the novel arenavirus was identified by the use of high-throughput sequencing with digital transcriptome subtraction (*32*). The donor, who died of a hemorrhagic stroke, had serologic evidence of recent LCMV infection and had recently traveled to rural southern Europe, where he may have been exposed to rodents (*32*).

These cases indicate that transplant-transmitted LCMV infection and encephalitis may be an uncommon, but quantifiable, infectious risk in organ transplantation. Immunosuppression in transplant recipients predisposes them to severe disease, even though infected donors may be asymptomatic.

Balamuthia mandrillaris Amebae

B. mandrillaris, a species of small, free-living, aerobic amebae, has been reported as a cause of granulomatous

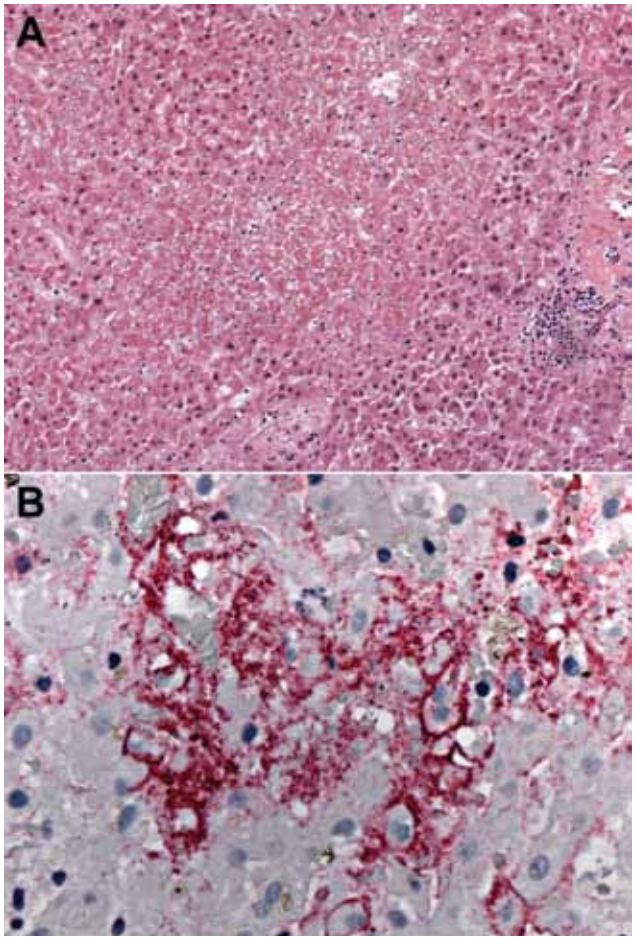

Figure 3. Photomicrographs showing histopathologic features and immunolocalization of lymphocytic choriomeningitis virus (LCMV) antigens in liver tissue from a liver transplant recipient with donor-derived LCMV infection. A) Massive hepatic necrosis, without prominent inflammation. Hematoxylin and eosin staining. Original magnification ×150. B) LCMV antigens within hepatocytes and sinusoidal lining cells. Immunoalkaline phosphate staining, naphthol fast red substrate with light hematoxylin counterstain. Original magnification ×158.

amebic encephalitis (*33*). *B. mandrillaris*–associated skin lesions have been described, and untreated infection can progress to fatal encephalitis. Neurologic disease is characterized by the presence of single or multiple space-occupying intracranial lesions that cause a variety of focal and diffuse neurologic signs and symptoms (*33*). *B. mandrillaris*–associated encephalitis is almost always fatal, even with treatment (*34*). Diagnosis has traditionally required culture of the organism or identification of amebic trophozoites or cysts from biopsy samples of affected tissue; however, a real-time PCR for use with cerebrospinal fluid samples is available (*34*). Unlike other free-living amebae, which are typically found in fresh water, the natural reservoir for *Balamuthia* amebae is believed to be the soil. Clinical infection has been described among immunosuppressed patients, alcoholics, and otherwise debilitated persons (*33*,*34*).

Infection with transplant-transmitted *B. mandrillaris* amebae was first identified by CDC in 2009 following reports by clinicians in Mississippi of encephalitis among 2 recipients of kidneys from a common donor (*11*). The 4-year-old organ donor reportedly had numerous exposures to soil and water before dying of seizures and subarachnoid hemorrhage. An initial diagnosis of immune-mediated encephalitis was made (Figure 4), but subsequent histopathologic evaluation and immunohistochemical testing showed that the child had *B. mandrillaris* infection (Figure 5) (*11*). A second cluster of infections with transplant-transmitted *B. mandrillaris* amebae was reported to CDC in 2010. In this cluster, encephalitis developed in 2 organ transplant recipients in Arizona; cerebral magnetic resonance imaging showed multiple ring-enhancing lesions (*35*). The organ donor was presumed to have died of a stroke. Both transplant recipients died, and a postmortem diagnosis was determined by immunohistochemistry and real-time PCR detection of *B. mandrillaris* amebae DNA in brain biopsy specimens (*35*).

Discussion

The transplant-transmitted cases of encephalitis we described highlight several important diagnostic and clinical challenges related to the recognition and treatment of certain emerging infections (Table). The precise rate of donor-derived transmission of WNV, rabies virus, LCMV, and *B. mandrillaris* amebae that cause encephalitis among transplant recipients is not known, but such cases are rare and may not be immediately recognized by clinicians. Diagnosis is further complicated because of laboratory screening limitations for some of these pathogens. Moreover, few effective treatment options are available once patients exhibit signs or symptoms of infection. However, there is limited evidence that prophylaxis or treatment, even for asymptomatic transplant recipients, may be effective following exposure to these pathogens (*11*,*25*,*29*,*37*,*38*). Identification of possible infectious encephalitis among organ donors and establishment of proactive notification systems for transplant centers is crucial. Furthermore, surveillance systems for possible donor-derived infectious encephalitis could reduce illness and death among organ transplant recipients.

Several difficulties are inherent in the identification and diagnosis of possible transplant-transmitted encephalitis. The hallmark clinical features of encephalitis (i.e., fever and altered mental status) are common in severely ill hospitalized patients, including persons who have undergone organ transplantation. Differentiating between encephalopathy caused by a severe underlying injury or illness and

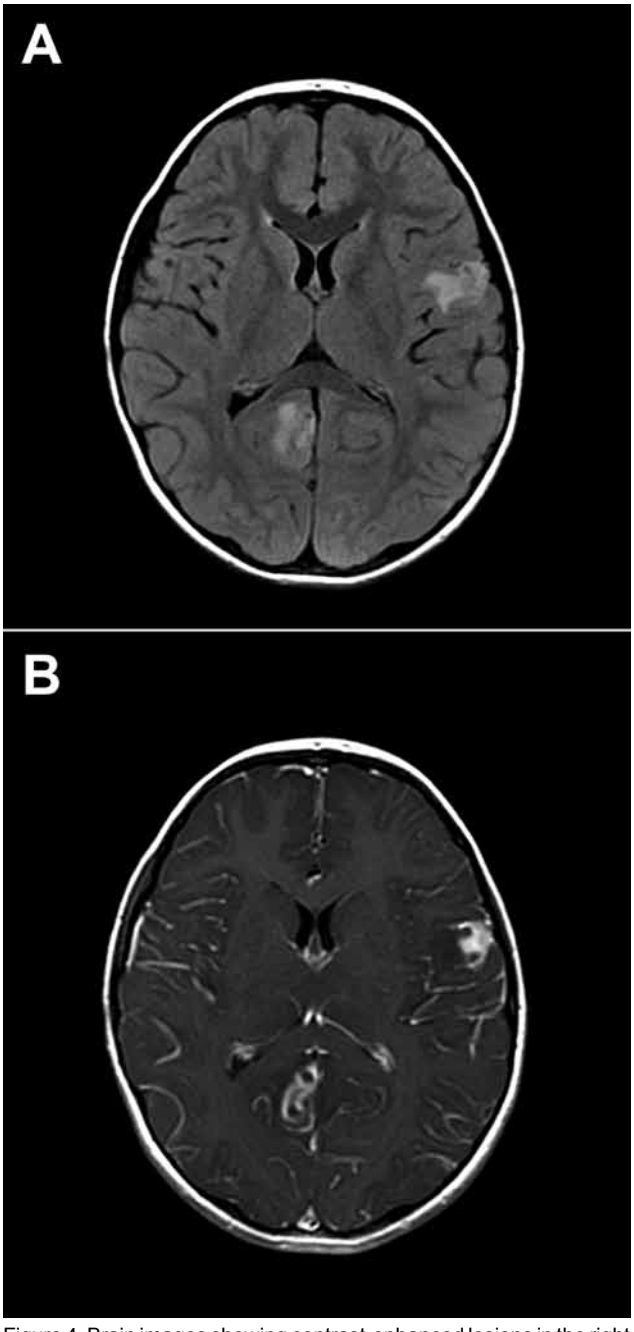

Figure 4. Brain images showing contrast-enhanced lesions in the right occipital and left parietal lobes of a 4-year-old boy with encephalitis caused by infection with *Balamuthia mandrillaris* amebae. A) T2-weighted fluid-attenuated inversion recovery (FLAIR) image. B) T1-weighted contrasted magnetic resonance image.

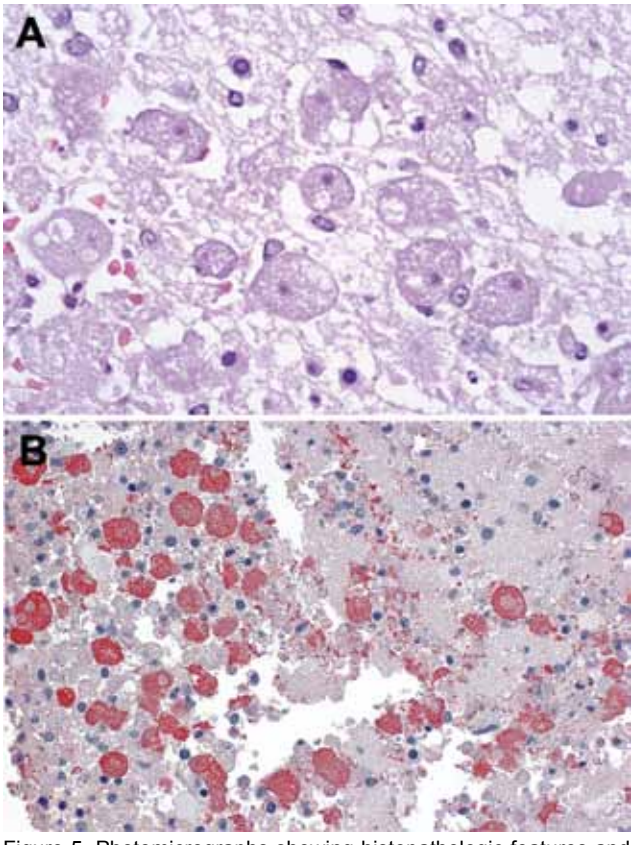

Figure 5. Photomicrographs showing histopathologic features and immunolocalization of *Balamuthia mandrillaris* antigens in central nervous system tissue from a donor with *B. mandrillaris* infection. A) Typical amebic trophozoites with prominent karyosomes in central nervous system. Hematoxylin and eosin staining. Original magnification ×158. B) *B. mandrillaris* antigens in amebic trophozoites. Immunoalkaline phosphate staining, naphthol fast red substrate with light hematoxylin counterstain. Original magnification ×100.

transplant-transmitted infection may be clinically challenging. It is therefore possible, that cases of encephalitis caused by transplant-transmitted pathogens may go unrecognized. Organs from a single donor are transplanted to recipients in multiple centers, frequently in widely separated geographic areas. Linking fever and encephalitis in transplant recipients to an infectious pathogen transmitted from a common donor may be relatively easy if all recipients are in the same hospital. Such a coincidence facilitated recognition of many of the clusters referenced here (*8*,*9*,*11*,*29*). However, if recipients are located in widely dispersed geographic areas, this linkage may not be recognized. Even when transplant-transmitted encephalitis is suspected, establishing a donor or recipient diagnosis may be challenging. Diagnosis of the infections that we described required specialized laboratory techniques, capacity, and training, all of which may not be available in most transplant centers. Furthermore, many laboratory techniques are not standardized. Even where testing is possible, transplant recipient samples may be unavailable or difficult to obtain, particularly if a brain biopsy specimen is required to establish a diagnosis. In some cases, donor specimens may not be available for definitive testing, thus leaving unanswered the possibility of a common etiologic agent.

Table. Infectious agents associated with encephalitis reported among clusters of solid organ transplant recipients in the United States, 2002–2013*

Infectious agent	Classification	Natural transmission route	No. reported clusters	Clinical features of infection	Laboratory detection method	Treatment
WNV	Enveloped, positive, single-stranded RNA virus; *Flavivirus* family	Bites from infected mosquitoes (*Culex* spp.)	6	Febrile illness, meningitis, encephalitis, poliomyelitis-like limb paralysis	Detection of WNV-specific antibodies or WNV nucleic acid in serum or CSF samples	None; several experimental therapies under investigation
Rabies virus	Enveloped, negative, single-stranded RNA virus; *Rhabdoviridae* family, *Lyssavirus* genus	Exposure to secretions, typically saliva, of infected animals (in North America, most commonly bats, raccoons, and skunks)	2	Nonspecific prodrome followed by confusion, paresthesias, insomnia, agitation, paresis, spasm of swallowing muscles, coma, and death	Before death: PCR or virus isolation in saliva, PCR and fluorescent antibody testing of nuchal biopsy samples, antibody testing of serum, and PCR or antibody testing of CSF; after death: fluorescent antibody staining of brain tissue or frozen tissue from nuchal biopsy, and serologic diagnosis by neutralization tests in mice or cell culture	Supportive; treatment with induced coma and antiviral therapy, as reported (*36*); postexposure prophylaxis for asymptomatic recipients of organs from infected donors
LCMV	Enveloped, RNA virus; *Arenaviridae* family	Exposure to infected rodents, presumably to urine	3	Febrile illness in most symptomatic persons; aseptic meningitis, encephalitis	Cell culture, electron microscopy, immunohistochemistry, detection of LCMV antibodies, PCR or high-throughput sequencing	Supportive
Balamuthia mandrillaris	Free-living aerobic amebae	Ubiquitous in soil	2	Skin lesions; single or multiple space-occupying intracranial lesions; granulomatous amebic encephalitis characterized by hemiparesis, aphasia, seizures	Culture or identification of amebic trophozoites or cysts in biopsy sample of affected tissue; real-time PCR of CSF	Multidrug combinations, which may include pentamidine isethionate, 5-flucytosine, fluconazole, clarithromycin or azithromycin, sulfadiazine, miltefosine, thioridazine, or liposomal amphotericin B†

*CSF, cerebrospinal fluid; LCMV, lymphocytic choriomeningitis virus; WNV, West Nile virus.
†http://www.cdc.gov/parasites/balamuthia/treatment.html.

Better recognition of encephalitis among organ donors and prompt notification of transplant centers regarding possible transmission are essential to improve clinical management of recipients, including prophylaxis or treatment. However, because of several logistical issues, it is currently challenging to recognize encephalitis in organ donors. Clinical, demographic, and social histories are often obtained from family members of deceased donors who may not have access to or recall historic details associated with the donor.

Further complicating organ safety in the United States is the regulatory oversight of solid organs. Although some policies are set by the Health Resources and Services Administration through the Organ Procurement and Transplantation Network, and infectious disease guidelines are available, screening of potential organ donors is under the purview of the individual organ procurement organizations, and variability exists in the testing that is performed for many agents (*39*). No organ procurement organization currently screens for all of the diseases discussed in this review. Given that the diseases are rare, laboratory screening of all donors is unlikely to be cost effective. However, introduction of a standardized risk assessment tool to gather medical, demographic, and social risk factors for infectious encephalitis from all organ donors may reduce the risk of transmitting infectious pathogens to transplant recipients. If a donor is perceived as

a high risk for transmitting an infectious pathogen, transplant centers could be informed through a standardized proactive, notification system so that appropriate clinical management of transplant recipients could be considered.

The challenges encountered in investigating the clusters described here highlight the need for establishing surveillance systems for infectious illnesses among transplant recipients. Although infectious disease reporting systems already exist and potential cases of donor-derived infections are reviewed by the Organ Procurement and Transplantation Network Disease Transmission Advisory Committee, there is a need for a national system for rapid communication on disease clusters, particularly infectious encephalitis, involving organ and tissue recipients from a common donor. Given that prevention and treatment options are available, recognition of donor or recipient infection, even after transplantation, could improve clinical outcomes among recipients. In many of the detected transmission clusters, the implicated pathogen could not be identified in the donor, despite intensive examination. In others, the pathogen was identified in procured organs but could not be detected in the archived serum sample, which was readily available for testing. Rapid recognition of disease transmission in transplant recipients could facilitate intervention in recipients of a common donor, particularly if tissue, which may be transplanted in up to 100 recipients, has also been donated.

Availability of appropriate samples to test is also a critical issue. The Retrovirus Epidemiology Donor Study Allogeneic Donor and Recipient (RADAR) repository, which was previously instituted in the blood transfusion community, could serve as an example of potentially effective surveillance linking organ donors and recipients (*40*). During 2000–2003, the RADAR repository contained pre- and posttransfusion specimens from 3,575 surgical patients. The specimens were linked to 13,201 blood donation samples and used to study the transmission of emerging infections (*40*). Such a repository, with voluntary participation, if linked with a surveillance system for transplant-transmitted encephalitis could obviate difficulties related to specimen availability and laboratory testing, facilitate diagnosis, better inform clinical management, and guide policy decisions related to organ donor guidelines. Cost and informed consent considerations may result in challenges to implementation of a specimen repository. All of the emerging infections described in this review were initially recognized by histopathologic evaluation and immunohistochemical testing of both autopsy and biopsy tissues. Increased rates of autopsy among organ donors could enable the identification of new and emerging infections in transplant recipients.

The investigation of infections resulting from organ transplant–transmitted pathogens requires rapid communication among transplant physicians, organ procurement organizations, and public health authorities. The presence of an unusual syndrome of signs and symptoms, including fever and change in mental status, particularly in the first few weeks following transplantation, should alert clinicians to the possibility of encephalitis caused by a transplant-transmitted infectious agent. Prompt notification to public health authorities can enable rapid investigation and discovery of clusters from a common donor. Until active surveillance can be implemented, timely communication and use of traditional and novel diagnostic testing can be crucial in identifying unusual and emerging infections caused by transplant-transmitted pathogens.

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Confirmed *Bacillus anthracis* Infection among Persons Who Inject Drugs, Scotland, 2009–2010

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Release date: August 15, 2014; Expiration date: August 15, 2015

Learning Objectives

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

1. Distinguish patient factors associated with a higher risk for mortality from injection anthrax
2. Evaluate variables at patient presentation associated with a higher risk for death from injection anthrax
3. Assess medical treatment variables associated with a higher risk for death from injection anthrax
4. Analyze hospital events associated with a higher risk for mortality from injection anthrax.

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In Scotland, the 2009 outbreak of *Bacillus anthracis* infection among persons who inject drugs resulted in a 28% death rate. To compare nonsurvivors and survivors, we obtained data on 11 nonsurvivors and 16 survivors. Time from *B. anthracis* exposure to symptoms or hospitalization and skin and limb findings at presentation did not differ between nonsurvivors and survivors. Proportionately more nonsurvivors had histories of excessive alcohol use ($p = 0.05$) and required vasopressors and/or mechanical ventilation ($p \leq 0.01$ for each individually). Nonsurvivors also had higher sequential organ failure assessment scores (mean ± SEM) (7.3 ± 0.9 vs. 1.2 ± 0.4, $p<0.0001$). Antibacterial drug administration, surgery, and anthrax polyclonal immune globulin treatments did not differ between nonsurvivors and survivors. Of the 14 patients who required vasopressors during hospitalization, 11 died. Sequential organ failure assessment score or vasopressor requirement during hospitalization might identify patients with injectional anthrax for whom limited adjunctive therapies might be beneficial.

Bacillus anthracis infection in humans has typically been classified as cutaneous, gastrointestinal, or inhalational on the basis of the bacterium's route of entry (*1*). However, in Scotland, United Kingdom, during 2009–2010, a total of 47 patients had confirmed *B. anthracis* soft tissue infection related to injection of contaminated heroin (*2*,*3*). This form of *B. anthracis* infection appears to be distinct from cutaneous disease and has been termed "injectional" anthrax (*2*–*5*). In addition to confirmed cases, 35 probable and 37 possible cases in Scotland, 5 confirmed cases in England, and 2 confirmed cases in Germany also were identified. This initial outbreak ended in late 2010, but since the summer of 2012, new cases have been reported in the United Kingdom and Europe (*3*,*4*). Although 1 case of injectional anthrax was recognized in Norway in 2001, the patients in 2009–2010 constitute the first large outbreak of this newly recognized and poorly characterized form of anthrax (*5*).

Health Protection Scotland (HPS) has published epidemiologic analyses of the 2009–2010 outbreak (*3*,*6*,*7*). Among other findings, analysis suggested associations between longer injecting histories, opioid substitution therapy, and alcohol use and risk for *B. anthracis* infection in persons who inject drugs (PWID) (*6*). Several case reports from the outbreak also have been published (*8*–*12*), but they did not include systematic examinations of the physical, laboratory, and surgical findings or of therapies administered. Notably, although 13 of the 47 persons from Scotland who had confirmed cases died, no published report has compared findings in survivors and nonsurvivors. Such a comparison is needed for the prognosis and management of future cases. We therefore sent a questionnaire regarding these issues to clinicians who had treated PWIDs in whom *B. anthracis* infection was confirmed in Scotland during the outbreak.

Methods

Approval

We used data collected during routine hospital care of patients. Patient identifiers were removed from data before analysis. Because of the retrospective nature of the study and the anonymity of data, the West of Scotland Research Ethics Service (Glasgow, Scotland, UK) and the Office of Human Subjects Research from the Clinical Center at the National Institutes of Health (Bethesda, MD, USA) exempted the study from formal review.

Data Collection

We developed an electronic questionnaire that requested information in several areas. These were: general information (i.e., age, sex, and medical and drug histories); current illness; data at the time the patient sought care, including skin and limb findings, vital signs, laboratory findings, and diagnosis (i.e., was anthrax infection or sepsis initially suspected?); medical and surgical treatments at the time the patient sought care or later (including antibacterial drugs; need for hemodynamic, respiratory, renal replacement, or blood product support; and use of anthrax immune globulin, a polyclonal antibody produced by Cangene [Winnipeg, MB, Canada] and made available by the Centers for Diseases Control and Prevention [Atlanta, GA, USA]; microbiological data supporting the diagnosis of anthrax infection and the time at which the diagnosis was confirmed; surgical findings; other procedures performed during hospitalization; levels of organ injury based on the sequential organ failure assessment (SOFA) score; and outcomes, including survival, time in the intensive care unit (ICU), and total time in hospital.

During this anthrax outbreak, HPS identified 13 hospitals to which the 47 patients with confirmed anthrax were admitted (*3*). In March 2012, two of the authors (L.D. and M.B.), who were members of the HPS anthrax outbreak control team, asked physicians known to have treated persons with confirmed anthrax to complete the questionnaire. The questionnaire was sent to these physicians in early April 2012. In June 2012, physicians who had not yet returned it were asked to do so. Data from all questionnaires completed by the end of August 2012 were analyzed. We received no additional questionnaires after August 2012. Contributors were subsequently contacted to clarify missing or unclear responses.

Data Analysis

A variable was reported on only if >50% (i.e., ≥14) of questionnaires provided definitive data. SOFA score was calculated as previously described for each patient for whom data were obtained within the initial 24 hours after they sought care (*13*). Measures of time were expressed as medians (interquartile range [IQR]). We used Wilcoxon rank sum test to compare these times between survivors and nonsurvivors. Categorical data (i.e., chief complaints,

types of initial surgery and gross tissue findings, skin and limb findings, and treatments) were analyzed with Fisher exact or χ^2 test, where applicable, and continuous data (i.e., laboratory data, age, vital signs, and SOFA score) were examined with 1-way ANOVA (analysis of variance).

Results

Initial Findings

We received data on 27 confirmed cases from the outbreak: 16 of the 33 survivors and 11 of the 14 nonsurvivors from 10 of 13 hospitals that admitted PWID with outbreak-associated anthrax. The median (IQR) times (days) from exposure to onset of symptoms and from onset of symptoms to hospital admission were 1 (0–4) and 2 (2–4), respectively, and these did not differ significantly between survivors (1 [0–4.5] and 3 [2–5] days) and nonsurvivors (1 (0–2] and 2 [2– 2] days) (p = 0.90 and p = 0.19, respectively) (Figure 1).

Mean (± SEM) age of patients was 34.5 ± 1.7 years. Nonsurvivors tended to be older than survivors (38.2 ± 2.8 vs. 31.9 ± 1.9, p = 0.07). The proportion who were male did not differ significantly (9 [56%] survivors vs. 9 [82%] nonsurvivors, p = 0.23). Although history of excessive alcohol use was higher in nonsurvivors than in survivors (4 [80%] of 5 vs. 3 [23%] of 13, p = 0.05), tobacco use and suspected injection sites (arm, groin, buttock, or leg) and routes

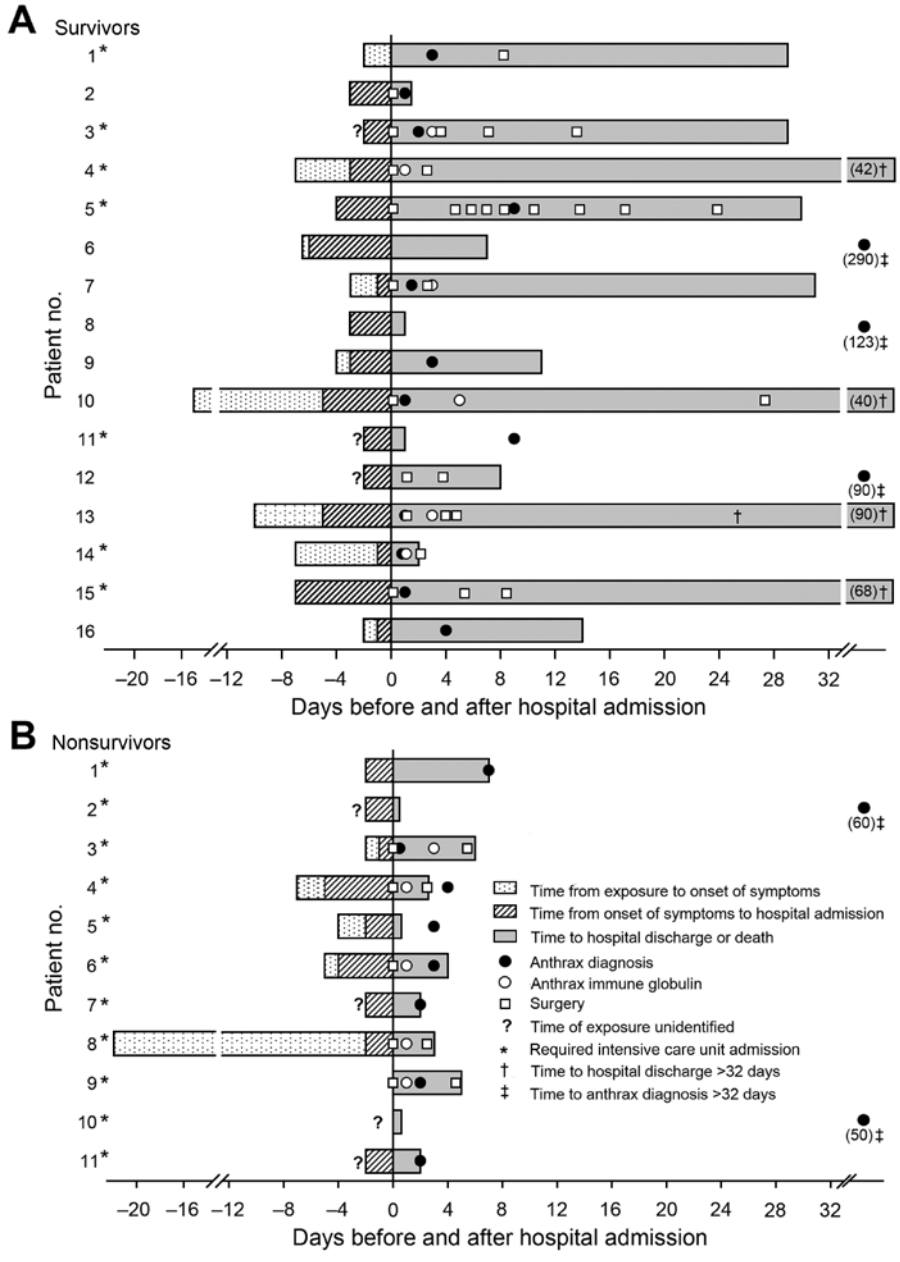

Figure 1. Key events for 16 survivors (A) and 11 nonsurvivors (B) in an outbreak of *Bacillus anthracis* infection in persons who inject drugs, Scotland, UK, 2009–2010. Patients are numbered in the order in which they sought care. Time period is from patients' suspected exposure to contaminated heroin to their discharge from hospital or to death. Day 0 is day of hospital admission. ICU, intensive care unit.

(intravenous or intramuscular) of contaminated drug injection did not differ significantly ($p \geq 0.19$ for all, data not shown). HIV infection status was available for only 10 patients (4 nonsurvivors), and hepatitis C infection status was available for only 12 patients (5 nonsurvivors). One survivor was HIV positive, and 4 survivors and 5 nonsurvivors had histories of hepatitis C virus infection. Although the questionnaire requested other medical history (e.g., hepatitis B virus infection status), these data were not provided in sufficient numbers for analysis.

Eleven (69%) survivors and 2 (18%) nonsurvivors had only localized skin or limb symptoms (i.e., pain, swelling, erythema, exudate); 5 (31%) survivors and 4 (36%) nonsurvivors had both localized and generalized symptoms (i.e., fever, confusion, seizures, abdominal pain, fatigue, malaise, sweating, headache), and 0 survivors and 5 (45%) nonsurvivors had only generalized symptom (Table 1). The proportion of nonsurvivors who had only a localized or only a generalized complaint was less ($p = 0.02$) and greater ($p = 0.006$) than the proportion of survivors.

At the time they sought care, nonsurvivors had lower temperatures and systolic blood pressures and higher respiratory rates than survivors ($p \leq 0.01$) (Figure 2), but other vital signs (mean ± SEM), including diastolic blood pressure (68 ± 4 vs. 61 ± 5 mm Hg), heart rate (111 ± 5 vs. 118 ± 8 beats per minute), and capillary refill (2.6 ± 0.4 vs. 3.6 ± 0.5 seconds) did not differ significantly. Four nonsurvivors, but only 1 survivor, had a temperature <36°C when they sought care, whereas 1 nonsurvivor and 7 survivors had temperatures >38°C. Also, whereas 4 nonsurvivors had systolic blood pressures <90 mm Hg, no survivor did. The Glasgow coma score recorded during the initial 24 hours was lower for nonsurvivors than for survivors ($p = 0.008$) (Figure 2). The proportion of survivors and nonsurvivors for whom specific skin and limb findings were available did not differ significantly ($p \geq 0.19$ for all) (Table 2).

Nonsurvivors had lower serum sodium, corrected calcium, albumin, and platelet levels and higher bilirubin, percentage circulating neutrophils, hemoglobin concentration, international normalized ratio, prothrombin time, partial thromboplastin time, and C-reactive protein levels ($p \leq 0.05$) (all shown in Figure 3 except prothrombin time, which was median [IQR] 12 [11–13] vs.15 [13.5–16.1]). For a greater proportion of nonsurvivors than survivors, arterial blood gases were measured when they sought care (10 [91%] vs. 6 [38%], $p = 0.008$). In patients with for whom the following values were measured, nonsurvivors had lower bicarbonates and greater base deficits ($p = 0.02$). Other laboratory data did not differ significantly between survivors and nonsurvivors (Table 2).

Table 1. Sogns and symptoms prompting hospital visits for persons who inject drugs who had *Bacillus anthracis* Infection, Scotland, UK, 2009–2010

Patient no.*	Signs and symptoms	Degree of complaint
Survivor		
1	Pain, swelling, exudate of left groin, fever, sweating	Local, generalized
2	Pain of left buttock	Local
3	Swelling, erythema, exudate of left forearm	Local
4	Abscess of left thigh, malaise, pallor, fainting	Local, generalized
5	Pain, swelling, exudate of right antecubital fossa	Local
6	Pain, erythema, swelling of right antecubital fossa	Local
7	Pain of left buttock radiating to groin, fever, chills	Local, generalized
8	Swelling of left arm	Local
9	Pain, swelling of right arm	Local
10	Pain, swelling of right arm	Local
11	Pain, swelling of left leg	Local
12	Pain, swelling of left groin, headache, photophobia, fever	Local, generalized
13	Pain, swelling of left testis and scrotum	Local
14	Swelling, erythema of right hand	Local
15	Pain, swelling of right hand to elbow	Local
16	Swelling and exudate of left groin, fever	Local, generalized
Nonsurvivor		
1	Pain, swelling, erythema of left thigh, fever, abdominal pain	Local, generalized
2	Headache followed by delirium	Generalized
3	Pain of right thigh	Local
4	Pain of right buttock, chills, malaise	Local, generalized
5	Swelling of right arm, seizure	Local, generalized
6	Pain, erythema of right groin increasing in area	Local
7	Confusion, lethargy, malaise	Generalized
8	Pain of right hip, abdominal pain, seizures	Local, generalized
9	Abdominal pain, fever	Generalized
10	Fainting	Generalized
11	Fatigue, malaise	Generalized

*Patients are numbered in the order in which they sought care during the outbreak.

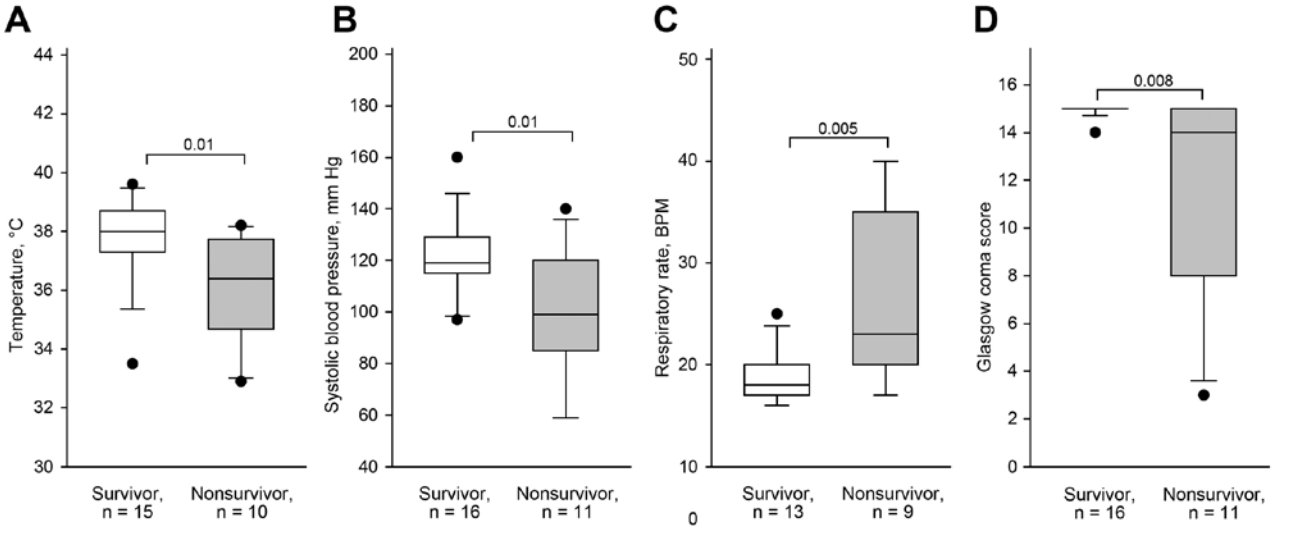

Figure 2. Physical examination findings for persons who inject drugs and were part of an outbreak of *Bacillus anthracis* infection, Scotland, UK, 2009–2010. Included are data from 27 patients for whom data were available. Median (interquartile range) for temperature (A), systolic arterial blood pressure (B), respiratory rate (C), and Glasgow coma scores (D) were recorded during the first 24 hours after patients sought care. Horizontal black lines indicate median values; lower and upper boundaries indicate the 25% and 75% ranges. BPM, breaths per minute.

Clinical Impression, Treatment, and SOFA Score at Presentation

Sepsis was noted in a greater proportion of nonsurvivors than survivors at presentation (7 [70%] vs. 3 [19%], p = 0.02), but *B. anthracis* infection was not (5 [46%] vs. 12 [75%], p = 0.22). All patients were initially treated with at least 1 antibacterial drug (Table 3). Neither type nor number (3.8 ± 0.3 vs. 3.6 ± 0.7, respectively) of antibacterial drugs received differed significantly between survivors and nonsurvivors ($p \geq 0.06$). Among survivors, 75% received ciprofloxacin and 88% received clindamycin; among nonsurvivors, 55% and 64% received these drugs, respectively. More nonsurvivors than survivors received vasopressors, oxygen support, mechanical ventilation, and corticosteroids (all $p \leq 0.002$) (Figure 4) but not undergo surgery (5 [46%] nonsurvivors vs. 11 [69%] survivors, p = 0.26) (Table 4). SOFA score calculated within the first 24 hours after patients sought care was higher for nonsurvivors than for survivors and for only those requiring ICU admission ($p \leq 0.003$) (Figure 4).

Confirmation of *B. anthracis* Infection

The basis for the microbiological diagnosis of *B. anthracis* for the 47 confirmed anthrax cases has been published (*3*). For the 27 cases reported here, the median (IQR) time (days) to confirmation was 3 (1.5–9.0) and did not differ between nonsurvivors and survivors (3.0 [2.0–7.0] vs. 3.0 [1.0–9.0], respectively, p = 0.83) (Figure 1). Results of tests confirming *B. anthracis* infection did not differ significantly between survivors and nonsurvivors ($p \geq 0.05$ for all) (Table 2). We had insufficient data to determine how often bacteria other than *B. anthracis* were found in blood or tissue samples.

Hospital Course

Proportionately more nonsurvivors than survivors received ICU care (11 [100%] vs. 7 [44%], p = 0.003) (Figure 4). The median (IQR) time (days) survivors remained in the ICU and hospital were 2.0 (1.0–13.3) and 21.5 (4.5–35.5), respectively. For nonsurvivors, median time from hospital admission to death was 2.1 (0.63–4) days. During hospitalization, proportionately more nonsurvivors received vasopressors (p = 0.0001) (Figure 4). More nonsurvivors also required mechanical ventilation (p = 0.005). Among all patients, 11 (79%) of the 14 who received vasopressors at any time died. Twelve (44%) of the 27 patients received anthrax immune globulin. We found no significant difference in the proportions of survivors and nonsurvivors who received anthrax immune globulin (p = 0.93, Figure 4) or in the median (IQR) time (days) to treatment (3 [1–3] vs. 1 [1–1]), respectively; p = 0.13).

We found no significant difference in the proportions of survivors and nonsurvivors who required surgery during hospitalization (11 [69%] vs. 5 [46%]; p = 0.26) or who required >1 surgery (7 [64%] vs. 4 (80%); p = 0.52) or in the median (IQR) time (days) to initial surgery (0 [0–1] vs. 0 [0–0.125]; p = 0.53) (Figure 1). Even when we examined data from the first week of hospitalization only, survivors and nonsurvivors did not differ significantly in the proportion having surgery (7 [44%] survivors vs. 4 [36%] nonsurvivors; p = 0.56). However, nonsurvivors more often bled excessively during surgery (4 [80%] nonsurvivors vs.

Table 2. Clinical and laboratory findings, confirmatory laboratory results, and therapies administered during hospitalization of persons who inject drugs and had *Bacillus anthracis* infection, Scotland, UK, 2009–2010*

Finding or test	Overall*	Survivor	Nonsurvivor
Skin/limb findings			
Localized edema	22/24 (92)	14/15 (93)	8/9 (89)
Local pain	19/21 (90)	14/15 (93)	5/6 (83)
Local erythema	20/24 (83)	13/16 (81)	7/8 (88)
Skin lesion	19/26 (73)	11/16 (69)	8/10 (80)
Limb pain	12/22 (55)	10/16 (63)	2/6 (33)
Limb edema	13/25 (52)	10/16 (63)	3/9 (33)
Limb mottling	10/24 (42)	5/15 (33)	5/9 (56)
Exudate	5/22 (23)	4/16 (25)	1/6 (17)
Distant edema	4/21 (19)	3/13 (23)	1/8 (13)
Ulcer	4/23 (17)	3/15 (20)	1/8 (13)
Eschar	4/24 (17)	3/16 (19)	1/8 (13)
Chemistry, hematology, and arterial blood gas results (reference)†			
Potassium, mmol/L (3.5–5.3 mmol/L)	4.2 ± 0.2	3.9 ± 0.1	4.5 ± 0.4
Chloride, mmol/L (96–108 mmol/L)	98 ± 2	101 ± 2	95 ± 3
Creatinine, μmol/L (40–130 μmol/L)	108 ± 14	93.4 ± 16.1	130 ± 24
Blood urea nitrogen, mmol/L (2.5–7.8 mmol/L)	8.3 ± 1.6	7.3 ± 2.5	9.8 ± 1.7
Glucose, mmol/L (3.6–6.0 mmol/L)	8.9 ± 1.0	7.3 ± 1.4	10.4 ± 1.2
Alkaline phosphatase, U/L (30–130 U/L)	113 ± 14	106 ± 15	121 ± 27
Alanine aminotransaminase, U/L (<50 U/L)	44 ± 15	53 ± 33	37 ± 6
Total protein, g/L (60–80 g/L)	61 ± 3	65 ± 3	55 ± 5
Leukocyte × 10^9/L (4–11 × 10^9/L)	16.7 ± 1.5	14.7 ± 1.5	18.3 ± 2.8
Lymphocyte, % (18%–44%)	0.15 ± 0.02	0.16 ± 0.03	0.13 ± 0.02
Fibrinogen, g/L (2–4.10 g/L)	1.8 ± 0.2	2.0 ± 0.4	1.5 ± 0.2
Hydrogen ion concentration, nmol/L (35–50 nmol/L)	43 ± 2	40 ± 3	45 ± 3
PaO_2, kPa (9–20 kPa)	21 ± 3	18 ± 4	23 ± 4
$PaCO_2$, kPa (3.5–6.5 kPa)	5.1 ± 0.5	6.0 ± 0.5	4.6 ± 0.5
Lactate, mmol/L (<2 mmol/L)	3.8 ± 1.0	1.7 ± 0.4	5.2 ± 1.5
Confirmatory laboratory results			
Blood culture	14/26 (54)	7/15 (47)	7/11 (64)
Wound culture	4/12 (33)	4/10 (40)	0/2 (0)
Tissue culture	10/14 (71)	5/8 (63)	5/6 (83)
PCR	12/20 (60)	5/12 (42)	7/8 (88)
Protective antigen antibody test	12/17 (71)	9/11 (82)	3/6 (50)
Lethal factor antibody test	10/18 (56)	8/12 (67)	2/6 (33)
Protective antigen ELISA	10/15 (67)	5/10 (50)	5/5 (100)
Lethal factor ELISA	8/13 (62)	4/9 (44)	4/4 (100)
Tissue Immunohistochemistry	3/3 (100)	2/2 (100)	1/1 (100)
Therapies administered			
Packed erythrocytes	13/22 (59)	7/13 (54)	6/9 (67)
Fresh frozen plasma	13/22 (59)	6/13 (46)	7/9 (78)
Platelets	10/27 (37)	4/16 (25)	6/11 (55)
Cryoprecipitate	3/20 (15)	1/12 (8)	2/8 (25)
Renal replacement therapy	7/26 (27)	3/15 (20)	4/11 (36)
Pleural drainage	4/27 (15)	3/16 (19)	1/11 (9)
Peritoneal drainage	3/25 (12)	0/15 (0)	3/10 (3)

*No. (%) patients noted to have the finding/total no. patients for whom data were provided, except for chemistry, hematology, and arterial blood gas results, for which values are indicated.
†Mean ± SEM.

1 [8%] survivor; p = 0.01) (Table 4). One survivor had an arm amputated above the elbow, and 4 received skin grafts.

Receipt of packed erythrocytes, fresh frozen plasma, cryoglobulin, and platelets; renal replacement therapy; and pleural or peritoneal drainage did not differ significantly between survivors and nonsurvivors (Table 2). For 9 (36%) of 25 patients for whom information was available, cardiac function was assessed: echocardiography for 4 patients, troponin measures for 3, and lithium dilution cardiac output and pulse contour cardiac output for 1 each. Of these, 3 nonsurvivors were noted to have evidence of myocardial dysfunction on the basis of echocardiography, lithium dilution cardiac output, or pulse contour cardiac output; 1 survivor had an elevated troponin level. Causes of death for the 7 patients for whom autopsies were reported were as follows; multiple system organ failure caused by *B. anthracis* sepsis, 2 patients; necrotizing fasciitis related to *B. anthracis*, 1; sepsis and hemorrhagic meningitis with *B. anthracis* infection, 2; subarachnoid hemorrhage, 1; and myocardial infarction, 1.

Discussion

Our review of 27 confirmed cases of *B. anthracis* infection in PWID compares clinical findings in survivors

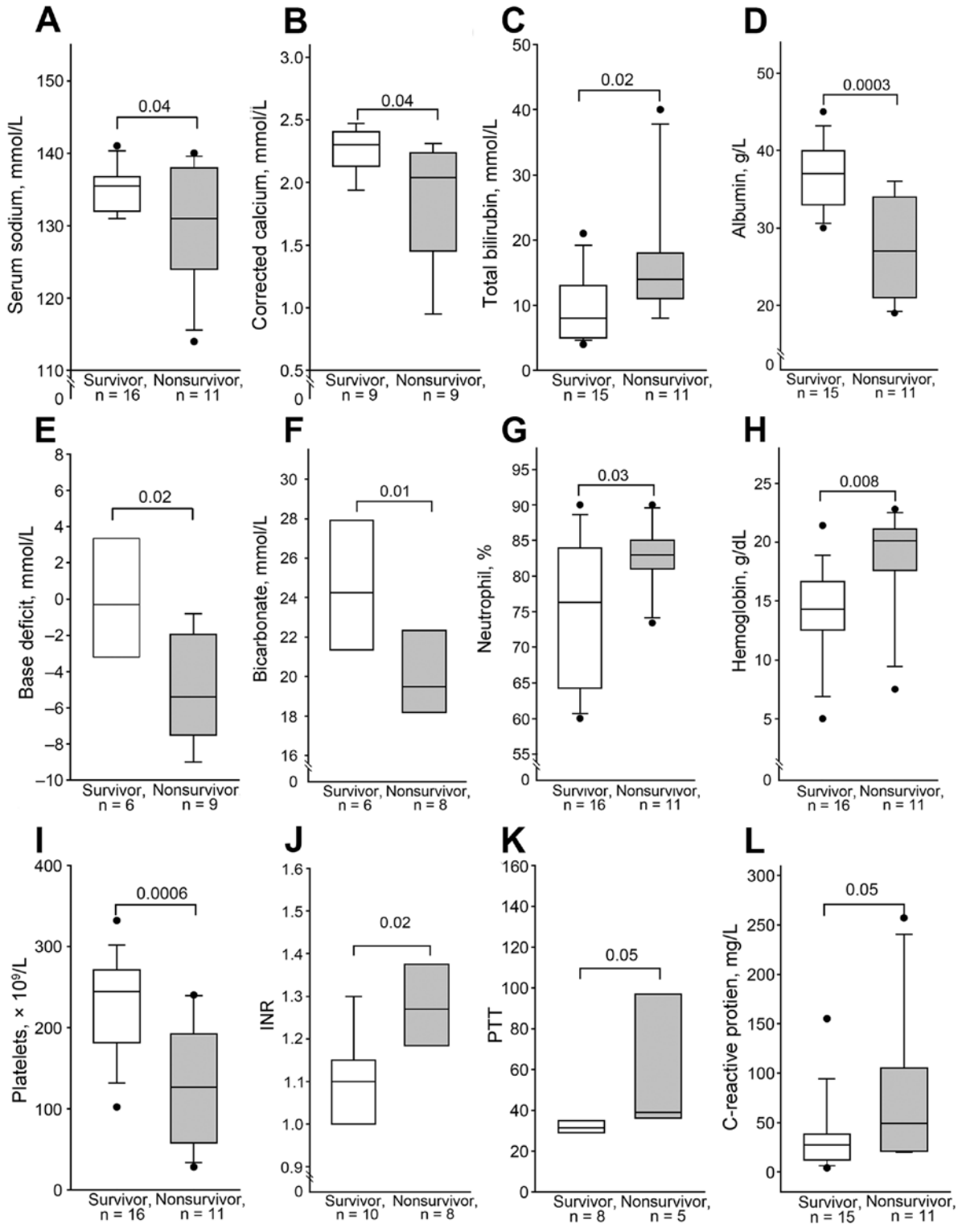

Figure 3. Laboratory findings for persons who inject drugs and were part of an outbreak of *Bacillus anthracis* infection, Scotland, UK, 2009–2010. Included are the 27 patients for whom data were available. Shown are median (interquartile range) for levels of serum sodium (A), corrected calcium (B), total bilirubin (C), albumin (D), base deficit (E), bicarbonate (F), percentage neutrophil (G), hemoglobin (H), and platelets (I); international normalized ratio (INR) (J); partial thromboplastin times (PTT) (K); and C-reactive protein levels (L). Horizontal black lines indicate median values; lower and upper boundaries indicate 25% and 75% ranges.

Table 3. Initial administration of antibacterial drugs to persons who inject drugs and had *Bacillus anthracis* Infection, Scotland, UK, 2009–2010*

Patient no.†	CLI	CIP	BPC	FLUX	MTZ	CRO	GEN	VAN	MEM	TZP	AMC	AMX
Survivor, n = 16‡	14 (88)	12 (75)	11 (69)	11 (69)	8 (50)	1 (6)	0	2 (13)	1 (6)	0	1 (6)	0
1	+	+	–	–	+	–	–	+	–	–	–	–
2	+	–	–	+	+	–	–	–	–	–	–	–
3	–	–	+	+	–	–	–	–	–	–	+	–
4	+	+	+	+	+	–	–	–	–	–	–	–
5	+	+	+	–	+	–	–	–	–	–	–	–
6	+	+	+	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	–	–	–	–	–	–	–
8	+	+	+	+	–	–	–	–	–	–	–	–
9	+	+	+	+	+	–	–	–	–	–	–	–
10	+	+	+	+	–	–	–	–	–	–	–	–
11	–	–	–	+	–	–	–	–	–	–	–	–
12	+	+	+	+	+	–	–	–	–	–	–	–
13	+	–	–	–	–	–	–	–	+	–	–	–
14	+	+	+	+	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	–	–	–	–	–	–
16	+	+	–	–	–	–	–	+	–	–	–	–
Nonsurvivor, n = 11‡	7 (64)	6 (55)	5 (45)	5 (45)	6 (55)	4 (36)	3 (27)	1 (9)	1 (9)	1 (9)	0	1 (9)
1	–	–	–	–	+	+	–	–	–	–	–	–
2	–	–	–	–	–	+	–	–	–	–	–	–
3	+	+	+	+	+	–	+	–	–	–	–	–
4	+	+	+	+	+	–	–	–	–	–	–	–
5	–	–	–	+	–	–	–	–	–	–	–	–
6	+	+	+	+	+	–	–	–	–	–	–	–
7	+	+	+	–	–	–	–	–	–	–	–	–
8	+	–	+	+	–	–	+	–	–	–	–	–
9	+	+	–	–	+	+	+	+	+	+	–	–
10	+	+	–	–	+	+	–	–	–	–	–	–
11	–	–	–	–	–	–	–	–	–	–	–	+
Total, n = 27	21 (78)	18 (67)	16 (59)	16 (59)	14 (52)	5 (19)	3 (11)	3 (11)	2 (7)	1 (4)	1 (4)	1 (4)

*CLI, clindamycin; CIP, ciprofloxacin; BPC, benzylpenicillin (penicillin G); FLUX, flucloxacillin; MTZ, metronidazole; CRO, ceftriaxone; GEN, gentamicin; VAN, vancomycin; MEM, meropenem; TZP, piperacillin/tazobactam; AMC, amoxicillin/clavulanic acid; AMX, amoxicillin; +, antibacterial drug administered; –, antibacterial drug not administered.
†Patients for whom data were available are numbered in the order in which they sought care during the outbreak.
‡No. (%) patients receiving an antibacterial drug.

and nonsurvivors of this newly described form of infection. Although duration of symptoms and time to seeking hospital care did not differ between survivors and nonsurvivors, the severity of illness did. Most survivors reported localized symptoms related to the injection site, and none required vasopressor therapy or mechanical ventilation. In contrast, most nonsurvivors had generalized symptoms and evidence of sepsis, which required both vasopressor support and mechanical ventilation. Nonsurvivors also had lower systolic blood pressures and Glasgow coma scores; higher respiratory rates; worsened base deficits; higher levels of hemoglobin (consistent with hemoconcentration) and C-reactive protein; higher international normalized ratio; and lower sodium and albumin levels and platelet counts. During hospitalization, all nonsurvivors required vasopressor and ICU support, whereas only 3 and 7 survivors, respectively, required these. SOFA scores were substantially higher in nonsurvivors than survivors. Thus, assessing the need for aggressive cardiopulmonary support or determining a score like SOFA for patients with injectional anthrax can help identify those for whom prognosis is particularly poor and more aggressive therapy is needed.

Possibly consistent with prior analysis showing an association between excessive alcohol use and risk for *B. anthracis* infection in PWID, we found a higher incidence of excessive alcohol use among nonsurvivors than survivors (*6*). Increased bilirubin and decreased albumin levels in nonsurvivors might in part have reflected preexisting alcoholic liver disease. Although age did not differ significantly between survivors and nonsurvivors, the latter tended to be older, a finding consistent with analysis of inhalational *B. anthracis* infection (*14*).

Differences in outcome between survivors and nonsurvivors did not appear related to variation in treatment. All patients received antibacterial drugs from the time they sought care, and the types and numbers of antibacterial drugs administered did not differ. Also, the proportion of patients who had ≥1 surgeries and the time from admission to initial surgery did not differ. Finally, similar proportions of survivors and nonsurvivors received anthrax immune globulin, and the median time to treatment for these groups did not differ.

The most common skin and limb findings were localized edema, pain, and erythema. Although these findings are consistent with soft tissue infection, their presence did

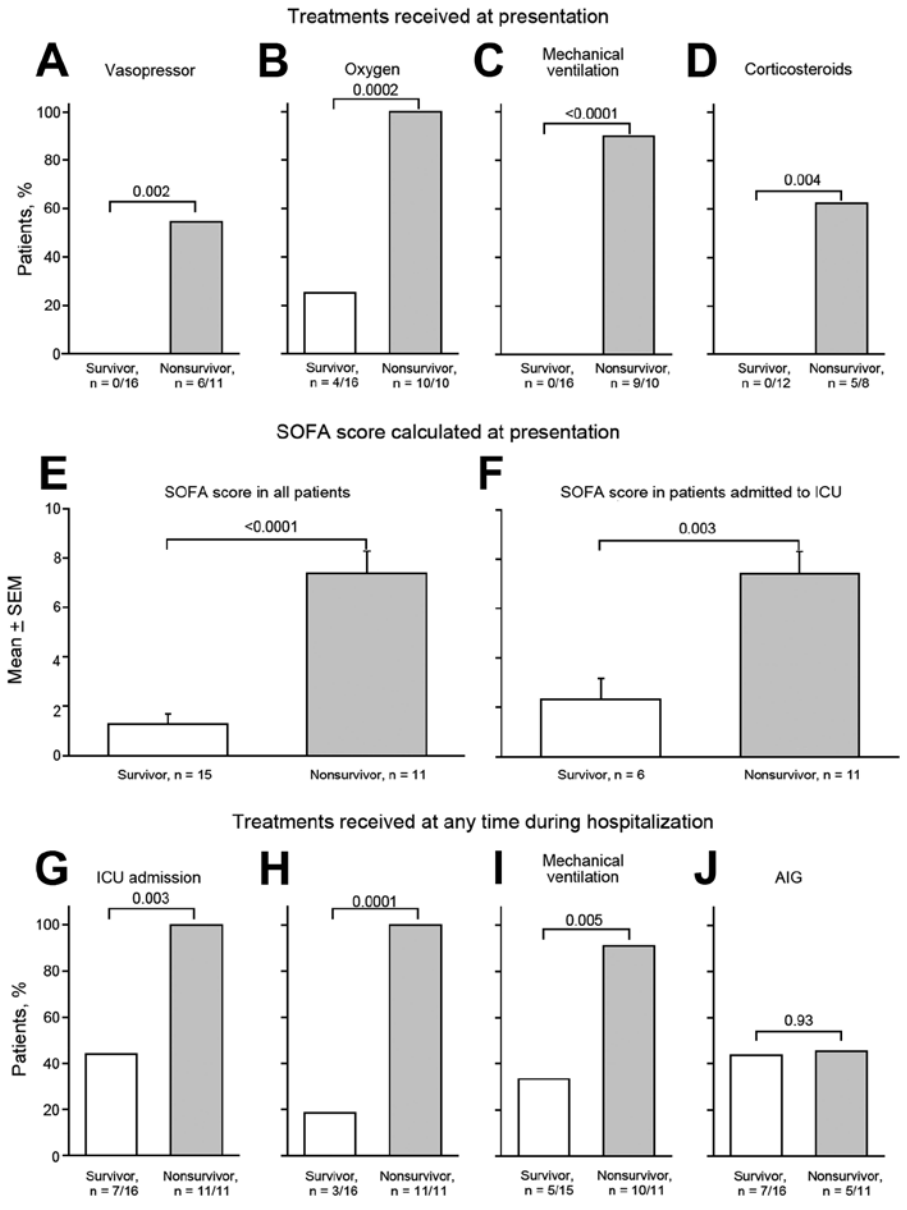

Figure 4. Treatments at time patients sought care, SOFA scores, and treatments anytime during hospitalization of persons who inject drugs and were part of an outbreak of *Bacillus anthracis* infection, Scotland, UK, 2009–2010. Included are the 27 patients for whom data were available on treatment with vasopressors (A), oxygen therapy (B), mechanical ventilation (C), or steroids (D); the mean (± SEM) SOFA score calculated within the first 24 h of hospitalization in all patients (E) and in only those who required ICU admission (F); and the proportion of patients at time they sought care who required ICU admission (G) or were treated with vasopressors (H), mechanical ventilation (I), or anthrax immune globulin (J) at any time during hospitalization. For panels A–D and G–J, n = the number of survivors or nonsurvivors receiving treatment/total number for whom data were available. For panels E and F, n = the number of survivors or nonsurvivors for whom SOFA scores were calculated. SOFA, sequential organ failure assessment; ICU, intensive care unit.

not differ between survivors and nonsurvivors (*15*). Thus, skin and limb findings and even the need for surgery did not appear to predict worsened prognoses. Notably absent in most patients was the eschar formation classically associated with cutaneous *B. anthracis* infection (*16*,*17*). This absence combined, with the frequent need for surgery and the overall high death rate despite receipt of antibacterial drugs, supports the observation that the pathogeneses of injectional and cutaneous anthrax differ (*3*,*4*–*6*).

In general, the severity of soft tissue infection and its requirement for surgery varies from mild to severe on the basis of the depth of tissue involvement and tissue necrosis (*15*). The *B. anthracis* soft tissue infections reviewed here reflect this range of disease. Two survivors had symptoms consistent with cellulitis, were treated only with antibacterial drugs, and were discharged within 1–2 days. However, 10 survivors and 5 nonsurvivors required debridement, fasciotomy, or laparotomy on ≥1 occasion; 10 had evidence of tissue necrosis. Even among the 5 survivors who did not have surgery, 3 required hospitalization for ≥7 days, which suggests severe infection. Without additional data about co-existing conditions, we cannot determine whether outcomes from soft tissue infection in PWID differ between *B. anthracis* and other bacteria.

B. anthracis has a cell wall that elicits a robust host inflammatory response and the endothelial dysfunction, shock, and organ injury with which this response is associated (*1*,*18*–*21*). However, *B. anthracis* also releases lethal and edema toxins that can produce this same dysfunction but through very different mechanisms than the cell wall

Table 4. Types of initial surgery and tissue findings on gross examination for persons who inject drugs and had *Bacillus anthracis* Infection, Scotland, UK, 2009–2010*

	Surgery					Gross tissue findings at surgery				
Patient no.†	Debride	Fasciotomy	Thigh ex	Laparotomy	Incision drainage	Excessive edema	Necrotic tissue	Excessive bleeding	Fasciitis	Liquefied necrosis
Survivor‡	5/11 (45)	4/11 (36)	1/11 (9)	0/11 (0)	1/11 (9)	5/9 (56)	7/11 (64)	1/11 (9)	1/10 (10)	1/10 (10)
1	–	–	+	–	–	+	–	+	–	–
2	–	–	–	–	+	–	+	–	–	+
3	+	–	–	–	–	+	+	–	–	–
4	+	–	–	–	–	–	+	–	–	–
5	–	+	–	–	–	+	–	–	–	–
7	+	–	–	–	–	+	+	–	–	–
10	–	+	–	–	–	–	–	–	–	–
12	+	–	–	–	–	NA	+	–	+	–
13	+	–	–	–	–	+	+	–	–	–
14	–	+	–	–	–	–	–	–	–	–
15	–	+	–	–	–	NA	+	–	NA	NA
Nonsurvivor‡	4/5 (80)	0/5	0/5	1/5 (20)	0/5 (0)	5/5 (100)	3/5 (60)	4/5 (80)§	2/4 (50)	1/4 (25)
3	+	–	–	–	–	+	+	+	+	–
4	+	–	–	–	–	+	+	+	+	–
6	+	–	–	–	–	+	+	+	NA	NA
8	+	–	–	–	–	+	–	–	–	–
9	–	–	–	+	–	+	–	+	–	+
Total	9/16 (56)	4/16 (25)	1/16 (6)	1/16 (6)	1/16 (6)	10/14 (71)	10/16 (63)	5/16 (31)	3/14 (21)	2/14 (14)

*Debride, debridement; thigh ex., thigh exploration; +, type of surgery or tissue finding performed or noted; –, type of surgery or tissue finding not performed or noted; NA, observation not available.
†Patients are numbered in the order in which they sought care.
‡No. (%) patients with the type of surgery or finding/total no. patients for whom data were reported.
§For proportion of positive responses by survivors vs. nonsurvivors for whom data were reported, $p<0.05$.

(*22*,*23*). Whether, as a result of these diverse nontoxin and toxin components, the manifestations of soft tissue infection with *B. anthracis* differ from those of other bacteria is unclear. Several differences between nonsurvivors and survivors, such as reduced systolic blood pressure and sodium and worsened acidosis, are associated with worsened outcome with other types of soft tissue infection (*24*–*26*). However, of patients who required vasopressor treatment, almost 80% died. This death rate is high, even for patients identified with septic shock on the basis of need for vasopressors. However, this finding is consistent with the 2001 US outbreak of inhalational *B. anthracis* in which all patients in whom shock developed died (*27*). Also in the current review, nonsurvivors bled more during surgery, possibly because of an increase in international normalized ratio and a decrease in platelets. Although excessive bleeding is not typically associated with soft tissue infection, it is associated with inhalational and gastrointestinal *B. anthracis* infection (*28*,*29*).

B. anthracis lethal and edema toxins inhibit components in the innate and adaptive immune responses (*22*,*23*). This inhibition might contribute to infection, as well as suppress signs typically associated with an activated host inflammatory response (*3*). However, although temperature was lower in nonsurvivors than in survivors, circulating leukocyte counts, percentage of neutrophils, and C-reactive protein were higher and in ranges approaching or consistent with invasive soft tissue infection caused by other bacteria (*30*,*31*). Whether toxin production interferes with host defense and influences the features and course of injectional *B. anthracis* infection requires further study.

Several lines of evidence suggest that *B. anthracis* lethal toxin produces direct cardiac dysfunction (*22*). Whether such dysfunction contributes to clinical *B. anthracis* infection is unclear because there are few measures of cardiac function in patients. Although 4 of the 9 patients in the current review were described as having evidence of cardiac dysfunction, these data were limited. More comprehensive investigation of cardiac function is necessary during future outbreaks of *B. anthracis*.

This study has limitations. First, data were collected ≥2 years after patients sought care, were not obtainable for some questions on patients we included in the analysis, and were unavailable for 20 of the 47 confirmed cases. However, these 27 patients included 11 of the 14 nonsurvivors from the outbreak and probably were fairly representative of nonsurvivors. Second, data were limited regarding co-morbidities, particularly HIV infection and viral hepatitis status, which might have influenced outcome. Information about other co-morbidities (e.g., diabetes, heart disease, and chronic lung disease) might have been informative as well. Third, although 1 survivor and 1 nonsurvivor described headache, using the Glasgow coma scale to assess neurologic status might not have captured other patients with this symptom, a possible manifestation of underlying

meningeal infection. Fourth, comparisons of therapies administered to survivors and nonsurvivors later than when they initially sought care might have been confounded by patients' length of hospitalization; however, data were insufficient to analyze the influence of this variable. Finally, autopsy findings were available only for 7 patients.

The 2009–2010 outbreak of *B. anthracis* infection among PWID in Scotland was considered over at the end of 2010 (*3*). However, during June 2012–December 2013, thirteen new cases were reported in the United Kingdom and Europe (*4*,*32*). The death rate for these patients has been close to 50%. Findings from the patients in the current review, combined with findings from newer cases, emphasize the need to better understand the pathogenesis and management of this recently identified form of *B. anthracis* infection.

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etymologia

Bacillus anthracis [bə-sil′əs an-thra′sis]

A large, gram-positive, rod (bacillus), *Bacillus anthracis* is the causative agent of anthrax (Greek for "coal"), named for the black lesions of cutaneous anthrax. In 1850, Rayer and Davaine discovered the rods in the blood of anthrax-infected sheep, setting the stage for Koch to link the disease to the bacterium in 1876, after he performed a series of experiments that fulfilled what came to be known as Koch's postulates. This was among the first times a microorganism was conclusively linked with a specific disease.

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Distance from Construction Site and Risk for Coccidioidomycosis, Arizona, USA[1]

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Coccidioides spp. fungi, which are present in soil in the southwestern United States, can become airborne when the soil is disrupted, and humans who inhale the spores can become infected. In 2012, our institution in Maricopa County, Arizona, USA, began a building project requiring extensive excavation of soil. One year after construction began, we compared the acquisition of coccidioidomycosis in employees working adjacent to the construction site (campus A) with that of employees working 13 miles away (campus B). Initial testing indicated prior occult coccidioidal infection in 20 (11.4%) of 176 campus A employees and in 19 (13.6%) of 140 campus B employees (p = 0.55). At the 1-year follow-up, 3 (2.5%) of 120 employees from campus A and 8 (8.9%) of 90 from campus B had flow cytometric evidence of new coccidioidal infection (p = 0.04). The rate of coccidioidal acquisition differed significantly between campuses, but was not higher on the campus with construction.

The fungal infection coccidioidomycosis, which is also called Valley fever, is caused by *Coccidioides* spp. and is acquired through inhalation of airborne spores. Of the estimated 150,000 infections that occur annually, ≈60% occur in Arizona, USA. In Arizona, Maricopa County has been the center of a coccidioidal epidemic for years (*1*). Coccidioidomycosis is the second most commonly reported infectious disease in Arizona (*2*), although reported cases are likely an underestimate of the true number of cases. Respiratory illness develops in persons with symptomatic infection. The severity of illness varies from person to person; some patients require prolonged medical evaluation, time away from work or school, treatment, or hospitalization (*2,3*). In 2007, estimated hospital-related charges for coccidioidomycosis totaled $89 million in Arizona (*3*). It has been estimated that 3% of the nonimmune population residing in *Coccidioides* spp.–endemic areas is infected annually (*4*); thus, even if up to 60% of the infected population is asymptomatic, the potential number of patients who may lose the ability to perform daily activities, work, or go to school because of illness is substantial.

Once a person is infected with coccidioidomycosis, the immune system mounts a complex reaction to control the infection; this reaction eventually results in the presence of cell-mediated and humoral immunity (*5*,*6*). The cell-mediated immunity is measured by using a delayed-type hypersensitivity (DTH) skin test (*5*) or an in vitro assay of cellular immunity to *Coccidioides* spp.

In areas of the US Southwest where *Coccidioides* spp. are endemic, the fungi grow in the top 18 inches of soil. Climate and soil conditions in the area foster growth of the fungi, and after rainfall, the fungi proliferate in the mold form with arthroconidia. As the weather dries, arthroconidia break off and become airborne spores when the soil is disrupted (*7*). Situations and activities that increase exposure to dust increase the risk for coccidioidomycosis in humans (*7*); these situations and activities include, but are not limited to, dust storms, earthquakes, construction work, outdoor occupations or activities, and military maneuvers (*7*). Little data exist to quantify the effects of construction activities on the local epidemiology of coccidioidomycosis. Measures to control construction-associated dust have been codified into law, but no data exist to demonstrate the efficacy of these mandatory, dust-control measures in eliminating airborne arthroconidia or associated coccidioidal infections.

In late 2011, our institution embarked on the construction of a new medical facility at the site of a previously undisturbed native desert area in Maricopa County (hereafter referred to as campus A). This construction project required a year-long process of excavation and hauling of large amounts of desert soil. With the current study, we

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[1]Preliminary findings from this study were presented at the 57th Annual Meeting of the Coccidioidomycosis Study Group, Pasadena, California, USA, April 6, 2013.

sought to quantify and compare the rate of acquisition of coccidioidomycosis among employees working at an existing facility on campus A with that among employees working at another campus 13 miles away (hereafter referred to as campus B).

Methods

After approval was given by the Mayo Clinic Institutional Review Board, all employees at the 2 campuses were invited by email to participate in the study. Employees were included if they were ≥18 years of age, spent ≥95% of their work time on a single campus (A or B), and were self-reported to be immunocompetent. Exclusion criteria included the following: presence of any immunosuppressive illness or medication (including seropositivity for HIV; history of hematologic malignancy; and receipt of cancer chemotherapy, antirejection medication, inhibitors of tumor necrosis factor, or other immunosuppressants); a history of anergy to tests of DTH, unless subsequent skin test reactivity had been demonstrated; a history of coccidioidal illness (diagnosed by a physician or confirmed by skin testing or serologic, microbiologic, or pathologic evidence); a history of positive results for coccidioidal serology or coccidioidal skin test; current use of an oral or intravenous antifungal drug (azole or amphotericin) that could prevent coccidioidomycosis; or current pregnancy (because of a theoretical decrease in cellular immunity).

During January 22–February 13, 2012, employees who provided verbal consent completed a questionnaire to ascertain whether they met inclusion criteria and to provide additional information, such as demographic information (sex, race/ethnicity, duration of residence in the *Coccidioides* spp.–endemic area, and residential zip code); the types of regular outdoor activities they participated in; and any perception they might have that construction was occurring near their area of employment or residence. A 10-mL blood sample was collected from each participant and assayed for cellular immunity to *Coccidioides* spp. All campus A participants were recruited and had a blood sample collected before excavation and construction began. Campus B participants were recruited and tested within 2 weeks of construction onset. Twelve to 13 months later, during January 29–March 27, 2013, we again collected and assayed blood samples from participants and administered a second questionnaire. Data were eliminated from analysis if a participant's employment site changed from 1 campus to the other after enrollment.

We used a whole-blood CD69 lymphocyte-activation assay to determine whether study participants were infected with *Coccidioides* fungi; the assay methods used were similar to previously described methods (*8–10*). In brief, we incubated 0.5 mL of whole peripheral blood with 5 μg of coccidioidin filtrate (provided by Mitch Magee, Arizona State University, Tempe, AZ, USA) for 24 h at 37°C in a humidified incubator containing 5% CO_2. Phytohemagglutinin lectin (5 μg) was used as a positive stimulatory control, and 10 mL of phosphate-buffered saline (PBS) was added to the control tubes. After the 24-h incubation, we lysed the erythrocytes by using BD FACS lysing solution (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. We resuspended the resultant peripheral blood mononuclear cell (PBMC) pellet in 200 mL of PBS and then added 20 mL each of fluorescein–conjugated anti-CD3 and phycoerythrin-conjugated anti-CD69 antibodies (Becton, Dickinson and Company). The antibodies and PBMCs were gently mixed, incubated for 30 min at room temperature, and then washed twice with 3 mL of PBS. The final PBMC pellet was resuspended in 500 mL of PBS and analyzed on a Becton Dickinson CyAn flow cytometer. Before each flow cytometry run, the instrument was calibrated according to the manufacturer's protocol. Isotype controls for fluorescein isothiocyanate–labeled and phycoerythrin-labeled CD3 and CD69 antibodies were used to establish a CD3-positive cell gate. From that CD3-positive population, we quantified CD69-positive cell populations.

For the initial assay in 2012, we classified test results for all participants into 3 groups: definite negative (mean fluorescence intensity of CD69 above control; range 0%–5.9%), possibly negative (intermediate mean fluorescence intensity; range 6.1%–8.1%), and definite positive (mean fluorescence intensity; range 9.4%–33.1%). On the basis of results from healthy controls with known or no known history of definite coccidioidomycosis, we used 6.1% as a cutoff for differentiating between study participants with a positive or a negative test result for coccidioidomycosis. For participants eligible for the second test in 2013, a similar process was undertaken.

The percentage of employees who converted from a negative to a positive test result was calculated for each study site and compared by using the χ^2 test or the Fisher exact test, as applicable. For other employee characteristics, categorical variables were reported in numbers and percentages and compared by using the χ^2 test or the Fisher exact test; for the ages of participants, we reported the medians and compared them by using the Wilcoxon rank sum test.

After reviewing the results of our study, we conducted a logistic regression analysis, using only information collected in the initial questionnaire, to explore possible factors associated with conversion of cellular immunity. The univariate analysis was performed first, and any variables with $p<0.30$ were considered in the model-selection process. We used the backward elimination procedure to identify the variables, and any variable with $p<0.15$ was retained in the model. Since the comparison between employees from different campuses was of interest, campus location was

retained in the model. These liberal criteria were used for the exploratory purposes of our analysis. For the final model, adjusted odds ratios (ORs), 95% CIs, and p values were reported. All analyses were conducted by using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). All tests were 2-sided; $p<0.05$ was considered statistically significant.

With regard to the construction, the medical institution's Construction Safety and Infection Control policy required that the contractor develop a plan for using every appropriate precaution to avoid or limit dust in the air and in adjacent buildings during construction. The plan included precautions compliant with the Maricopa County Air Quality Department specifications to limit dust pollution (*11*), including a dust control permit (*11*). Trained county inspectors made periodic unannounced inspections of the construction site.

Construction commenced in late January 2012, and most of the excavation and movement of dirt was completed within 1 year. During that time, 15 unannounced inspections were conducted, and no violations of dust control regulations were documented. In total, 154,600 cubic yards of soil was excavated to a depth of 33 feet; pockets for concrete and steel caissons were excavated another 40–90 feet. The top 18 inches of soil was removed in the first 4 months. All excavated soil was initially moved to on-site stockpiles, but from the fourth month onward, 80% of the stockpiled soil was hauled off site; the balance of soil was re-used on the construction site for backfill or for building up new parking lots or an electric substation. It is not known when the movement of topsoil was completed or how much of the original top 18 inches of soil was in the soil that was re-used.

Results

In January 2012, campus A employees were recruited and enrolled in the study during the 2 weeks before onset of the construction site excavation; campus B employees were enrolled 2 weeks later. A total of 316 employees met inclusion criteria: 176 from campus A and 140 from campus B. Of these employees, 20 (11.4%, 95% CI 6.7%–12.1%) from campus A and 19 (13.6%, 95% CI 7.9%–19.2%) from campus B were excluded because test results for the CD69 lymphocyte–activation assay were positive, indicating previous coccidioidal infection and current immunity ($p = 0.55$).

The flow of study participation, from the beginning to the end of the study, is summarized in Figure 1. After an initial positive test result, making participants ineligible for the second test a year later, the most common reasons for exclusion were employee attrition, change of employment campus, or medical leave ($n = 16$ for campus A; $n = 14$ for campus B). A year after the study was initiated, campus A had 140 eligible employees available for participation, of whom 120 (85.7%) continued in the study, and campus B had 107, of whom 90 (84.1%) continued.

At the 1-year follow-up, 3 (2.5%) of 120 participants from campus A who had previously negative test results had lymphocyte proliferation evidence of newly acquired

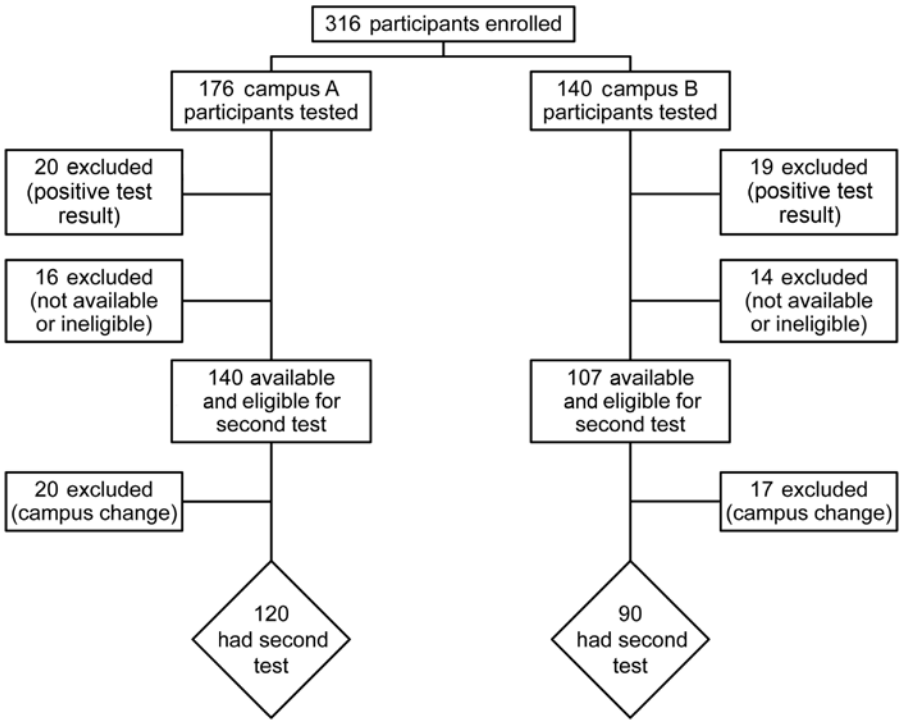

Figure 1. Flowchart of study participants in a study of the acquisition of immunity to *Coccidioides* spp. among persons working adjacent to and 13 miles away from a construction project requiring extensive excavation of soil, Arizona, USA, 2012–2013.

coccidioidal infection, compared with 8 (8.9%) of 90 participants from campus B (p = 0.04). Figure 2 shows test results for representative study participants from each campus who showed immunologic conversion from negative for coccidioidal infection in 2012 to positive in 2013.

Table 1 summarizes the demographics, perceptions of risk for coccidioidomycosis, and outdoor activities of the study population. Campus B employees were older and more likely to regularly walk outdoors than were campus A employees. At the 1-year follow-up, there was a disproportionate drop in male participants on campus B and an increase in the proportion of participants on campus B who reported construction activity near their homes. Table 2 summarizes the comparison of demographic characteristics and risk factors for coccidioidomycosis among participants who did and those who did not show immunologic conversion after 1 year. Campus location and walking outdoors for recreation were associated with conversion of cellular immunity. Participant variables, including age, participation in other (or any) outdoor activities, and residential zip code, were assessed by logistic regression, and did not correlate with conversion of cellular immunity (data not shown).

The final model, which evaluated factors associated with the conversion of cellular immunity, showed that participants on campus A, compared with those on campus B, had a lower odds of acquiring coccidioidal infection (adjusted OR 0.42, 95% CI 0.15–1.19; p = 0.10). Regularly taking walks outdoors was associated with increased odds of acquisition (adjusted OR 3.39, 95% CI 0.74–15.49; p = 0.11).

During the 1-year study period, 1 participant had a clinical episode consistent with new coccidioidal infection. This otherwise healthy 54-year-old woman experienced an insidious onset of heart palpitations, dyspnea, cough, profound fatigue, and new back pain; chest imaging showed

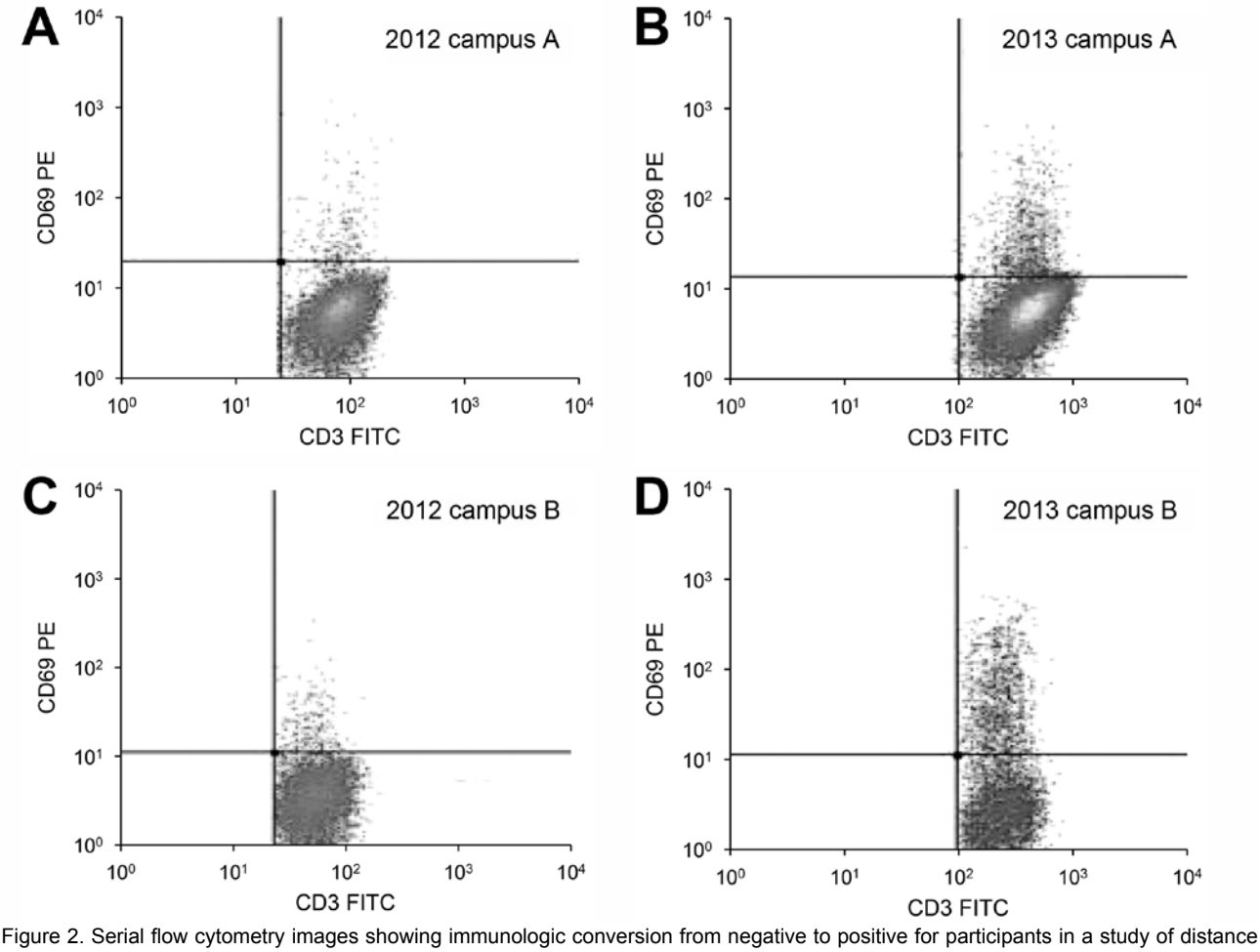

Figure 2. Serial flow cytometry images showing immunologic conversion from negative to positive for participants in a study of distance from a construction site as a risk factor for coccidioidomycosis, Arizona, USA, 2012–2013.Conversion was measured by using the CD69 lymphocyte-activation assay. A, B) Images for a representative participant from campus A, which was adjacent to the construction site. C, D) Images for a representative participant from campus B, which was 13 miles from the construction site. A, C) Images were done in 2012, before construction began. B, D) Images were done in 2013, a year after construction began. The participants' CD3-positive T-cell populations are shown in the lower right quadrant of each image. The percentage of CD3/CD69-positive T cells changed from 1.9% to 6.4% in the campus A participant and from 2.9% to 17.7% in the campus B participant. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Table 1. Characteristics of participants, at enrollment and 1 year later, in a study of distance from a construction site as a risk factor for coccidioidomycosis, Arizona, USA, 2012–2013*

Characteristic	At enrollment, n = 316			At 1-year follow-up, n = 210		
	Campus A†	Campus B†	p value	Campus A†	Campus B†	p value
Sex						0.03‡
M	28/176 (15.9)	20/140 (14.3)	0.69‡	22/120 (18.3)	7/90 (7.8)	
F	148/176 (84.1)	120/140 (85.7)		98/120 (81.7)	83/90 (92.2)	
Median age, y (range)	47 (21–75)	53 (18–76)	0.04§	49 (23–72)	53 (25–76)	0.04§
Race/ethnicity						0.75‡
White	144/176 (81.8)	119/140 (85.0)	0.66‡	101/120 (84.2)	75/89 (84.3)	
Hispanic	13/176 (7.4)	10/140 (7.1)		7/120 (5.8)	7/89 (7.9)	
Other	19/176 (10.8)	11/140 (7.9)		12/120 (10.0)	7/89 (7.9)	
Indoor work location	168/176 (95.5)	137/140 (97.9)	0.35‡	117/119 (98.3)	89/90 (98.9)	0.67‡
Work near a construction site	77/168 (45.8)	2/140 (1.4)	<0.001‡	86/114 (75.4)	4/89 (4.5)	<0.001‡
Live near a construction site	18/170 (10.6)	6/135 (4.4)	0.048‡	17/120 (14.2)	8/88 (9.1)	0.27‡
New home construction, remodeling, landscaping in home or neighborhood since enrollment¶	NA	NA	NA	28/116 (24.1)	19/88 (21.6)	0.67‡
Regular weekly participation in outdoor activities#						
Running	26/176 (14.8)	23/140 (16.4)	0.69‡	18/120 (15.0)	9/90 (10.0)	0.28‡
Hiking	52/176 (29.5)	39/140 (27.9)	0.74‡	34/120 (28.3)	20/90 (22.2)	0.32‡
Walking	107/176 (60.8)	110/140 (78.6)	0.007‡	72/120 (60.0)	71/90 (78.9)	0.004‡
Yard work	53/176 (30.1)	53/140 (37.9)	0.15‡	37/120 (30.8)	33/90 (36.7)	0.37‡
Received diagnosis of coccidioidomycosis during study period	NA	NA	NA	1/117 (0.9)	0	>0.99**
Initiated antifungal therapy after enrollment	NA	NA	NA	1/120 (0.9)	1/90 (1.1)	>0.99**

*Campus A was adjacent to and campus B was 13 miles away from the construction site, which required extensive excavation of soil inhabited by *Coccidioides* fungi. NA, not available.
†Values are no. with characteristic/no. total (%), except as noted for age.
‡By χ^2 test.
§By Wilcoxon rank sum test.
¶Work that took place within the past year.
#Other activities that were evaluated but did not differ significantly between campuses were jogging, gardening, landscaping, golfing, playing team sports, swimming, and biking.
**By Fisher exact test.

a 12-mm solid pulmonary nodule with satellite lesions that were not present in a radiograph from 4 years earlier. The results of coccidioidal serologic testing were positive by enzyme immunoassay for IgG and indeterminate for IgM; immunodiffusion was indeterminate for IgM. This participant received a clinical diagnosis of probable coccidioidomycosis; she recovered clinically and had negative coccidioidal serology results within 6 months, without antifungal treatment. Results of her enrollment and follow-up lymphocyte activation studies were negative. During the study period, short-term (<2 weeks' duration) antifungal treatments were administered to 2 other study participants (1 from each campus) for noncoccidioidal illnesses.

Discussion

Coccidioidomycosis is a respiratory illness with a variety of clinical manifestations. Approximately two thirds of infected persons are asymptomatic; the remainder show signs and symptoms of systemic and respiratory illness that range from mild to severe and life threatening.

Once a person is infected with *Coccidioides* fungi, the immune system mounts a complex reaction to control the infection; this reaction eventually results in the presence of cell-mediated and humoral immunity (*5*,*6*). Persons who recover uneventfully from coccidioidomycosis become immune and are unlikely to have subsequent coccidioidal infections. Such immunity can be assessed (regardless of the presence or absence of symptomatic illness) with a DTH skin test (*5*) or an in vitro assay of cellular immunity to *Coccidioides* spp., such as the assay described in this report. Because the DTH skin test is not commercially available, we elected to use a lymphocyte activation assay to identify any study participants with such immunity (*10*). As reported by Ampel et al. (*10*,*12*), this assay detects the activation marker CD69 on the surface of CD3+ T cells, correlates well with skin test reactivity, and indicates previous (or current) exposure to *Coccidioides* spp. Although Johnson et al. (*13*) later used T27K, a coccidioidal antigen preparation, we elected to use coccidioidin filtrate, which has historically been shown to be a good coccidioidal preparation for DTH testing and to be an even better preparation for determining cellular immunity in vitro (*14*). We chose to use the lymphocyte activation assay rather than standard serologic testing to measure cellular immunity for 3 reasons: 1) serology, while often adequately sensitive for evaluation of clinical illness, is not 100% sensitive (*15*); 2) serologic sensitivity depends on the time from onset of symptoms and may be undetectable in early or resolved

Table 2. Characteristics of participants, by cellular immunity conversion status at 1-year follow up, in a study of distance from a construction site as a risk factor for coccidioidomycosis, Arizona, USA, 2012–2013*

Characteristic	Cellular immunity status†		p value‡
	Negative, n = 199	Positive, n = 11	
Sex			0.65
M	27/199 (13.6)	2/11 (18.2)	
F	172/199 (86.4)	9/11 (81.8)	
Median age, y (range)	50.0 (23.0–76.0)	52.0 (26.0–71.0)	0.88
Race/ethnicity			0.66
White	165/198 (83.3)	11/11 (100)	
Hispanic	14/198 (7.1)	0	
Other	19/198 (9.6)	0	
Work near a construction site	87/192 (45.3)	3/11 (27.3)	0.35
Live near a construction site	23/198 (11.6)	2/10 (20.0)	0.34
New home construction, remodeling, landscaping in home or neighborhood since enrollment§	40/198 (20.2)	1/9 (11.1)	0.69
Regular weekly participation in outdoor activities¶			
Running	26/199 (13.1)	1/11 (9.1)	>0.99
Hiking	51/199 (25.6)	3/11 (27.3)	>0.99
Walking	133/199 (66.8)	10/11 (90.9)	0.18
Yard work	64/199 (32.2)	6 /11 (54.6)	0.19
Employment site			0.06
Campus A	117/199 (58.8)	3/11 (27.3)	
Campus B	82/199 (41.2)	8/11 (72.7)	

*A CD69 lymphocyte-activation test was used to determine if the cellular immunity status of participants had converted from negative to positive.
†Values are no. with characteristic/no. total (%), except as noted for age.
‡For age, the Wilcoxon rank sum test was used; for other variables, the Fisher exact test was used.
§Work that took place within the past year.
¶Other activities that were evaluated but that did not differ significantly between participants who did and did not convert from negative to positive cellular immunity status were jogging, gardening, landscaping, golfing, playing team sports, swimming, and biking.

illness (*15*,*16*); and 3) in the absence of clinical illness, it may be difficult to distinguish true-positive from false-positive serologic testing results (*17*). Up to 60% of infections may be asymptomatic, so we wanted an assay that would measure asymptomatic infection.

Coccidioides fungi naturally reside in the top 18 inches of soil in areas where *Coccidioides* spp. are endemic. However, even within such areas, soil sampling studies aimed at isolating the fungus by culture or by molecular amplification have shown the distribution of *Coccidioides* fungi to be spotty and erratic, even where the fungi are highly prevalent (*18*,*19*). We did not undertake soil sampling studies before construction began, and it is certainly possible that, unbeknownst to us, the soil of the 2 campuses assessed in this study had different concentrations of *Coccidioides* fungi, and, more specifically, that the soil at the campus A construction site did not have a high level of fungal organisms.

Where present, *Coccidioides* fungi naturally reside in the top layers of soil; thus, activities that disrupt the soil and create dust, increasing the airborne dissemination of *Coccidioides* spores, are recognized as risk factors for an increased likelihood of coccidioidal acquisition. Persons engaged in construction, agriculture, archeological digs, and other soil-disrupting activities within areas where *Coccidioides* fungi are endemic have experienced increased dust exposure and subsequent coccidioidal infection (*20*–*23*). In addition, 2 reports have implicated construction as a risk for development of coccidioidomycosis among persons in the surrounding community (*24*,*25*).

In 2002, the onset of construction of a mental hospital adjacent to the Pleasant Valley State Prison in California was temporally associated with 127 new cases of coccidioidomycosis among prisoners over the subsequent 15 months; these 127 new cases compared with only 7 cases from the same institution in the preceding year (*24*). In other, more limited observations, dust control at military bases by natural means (i.e., rainfall) or by artificial measures (e.g., planting lawns or oiling down unpaved roads and airstrips) has been associated with a temporary reduction of airborne dust and with the subsequent rate of coccidioidal infection (*25*). However, the observations in both of these reports took place over 2 sequential years, and neither study controlled for year-to-year variations in weather (e.g., rainfall, temperature, or wind) or for background cases of coccidioidomycosis within the same area.

In planning this study, we hypothesized that the dust generated from the construction on campus A would result in an increase in the acquisition of coccidioidomycosis among employees at campus A, compared with the acquisition of coccidioidomycosis among employees on campus B, 13 miles away. Knowing that dust-suppression measures would be used at the construction site, we were uncertain about what magnitude of difference to expect in infection rates. However, our findings did not show that the rate of newly acquired coccidioidomycosis was higher among study participants from campus A than among participants from campus B. In fact, the 2.5% rate of newly acquired coccidioidomycosis cases on campus A is essentially the

same as the 3% rate of infection previously estimated for residents of *Coccidioides* spp.–endemic areas (*4*). Whether the construction and/or the concurrent dust control measures had any effect on the acquisition of infection is not known.

Our findings showed an overall 1-year risk of coccidioidal acquisition of 5.2% (11/210 persons; 95% CI 2.2%–8.3%) among the study participants; this rate is similar to a previous acquisition estimate of 3% per year (*4*). Rather than finding an increase of coccidioidomycosis among participants on campus A, we instead observed a statistically significantly higher rate of acquisition at the control site, campus B, which is not in an area of known higher risk for coccidioidomycosis and which had no construction being conducted on or in the vicinity of its grounds.

Several factors can affect any person's risk for contact with arthroconidia and subsequent coccidioidal infection (e.g., recreational and other outdoor activities, exposure to dust storms, home or work location close to construction, or prevalent wind patterns). Thus, we examined demographic information provided by study participants to ascertain any risk factors among those with newly identified coccidioidomycosis. We observed an increased risk for coccidioidal acquisition not only among study participants who worked on campus B, but also a trend to significance in risk for participants at both campuses who regularly walked outdoors; no other risk factors emerged. Although walking is a common form of exercise, whether regularly walking outdoors represents a unique risk factor is not clear. In addition, this variable trended to statistical significance by virtue of a larger cohort participating in the activity; it is possible that this activity is merely an indirect marker of time spent outdoors. We also observed that the participants on each campus tended to reside in separate groups of zip codes, with only some overlap, but no particular residential zip codes were associated with a higher likelihood of infection (data not shown).

This study has several limitations. The study participants were predominantly female and white, reflecting the employee population on the 2 campuses, a factor that may limit the generalizability of our findings. In addition, the CD69 lymphocyte-activation assay has been shown to correlate with helper T cell, subtype 1 (T_H1) cytokines, but not with T_H2 cytokines. Therefore, if any participants had coccidioidomycosis that did not resolve because of a T_H2 immune response, we may not have been able to detect the infection because of inadequate lymphocyte activation (i.e., a false-negative test result) (*9*). This scenario may explain the situation of the employee from campus A who had protracted, probable, symptomatic coccidioidal infection and an atypical serologic pattern, but who had a negative test result on the second CD69 lymphocyte-activation assay. Alternatively, since CD69 is a nonspecific marker of lymphocyte activation, an immune response to another infectious agent would have elevated a participant's baseline CD69 level, making it difficult to determine whether their PBMCs were responding to coccidioidin or another infection or both.

In summary, by using the CD69 lymphocyte-activation assay, we determined that employees working adjacent to a large construction project involving the excavation of previously undisturbed native desert soil and the use of active dust-control measures, compared with co-workers at another site 13 miles away, did not have an increased risk for acquisition of coccidioidomycosis. That the control group of employees on the second campus had a statistically higher rate of negative to positive assay conversion at 1 year is a finding that merits further study.

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Dr Blair is a consultant in infectious diseases at Mayo Clinic, Scottsdale, Arizona, and a professor of medicine in the Mayo Clinic College of Medicine. Her research interests include the study of coccidioidomycosis in healthy and immunosuppressed hosts.

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Swine-to-Human Transmission of Influenza A(H3N2) Virus at Agricultural Fairs, Ohio, USA, 2012

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Agricultural fairs provide an opportunity for bidirectional transmission of influenza A viruses. We sought to determine influenza A virus activity among swine at fairs in the United States. As part of an ongoing active influenza A virus surveillance project, nasal swab samples were collected from exhibition swine at 40 selected Ohio agricultural fairs during 2012. Influenza A(H3N2) virus was isolated from swine at 10 of the fairs. According to a concurrent public health investigation, 7 of the 10 fairs were epidemiologically linked to confirmed human infections with influenza A(H3N2) variant virus. Comparison of genome sequences of the subtype H3N2 isolates recovered from humans and swine from each fair revealed nucleotide identities of >99.7%, confirming zoonotic transmission between swine and humans. All influenza A(H3N2) viruses isolated in this study, regardless of host species or fair, were >99.5% identical, indicating that 1 virus strain was widely circulating among exhibition swine in Ohio during 2012.

In the United States during 2012, approximately 300 cases of human infection with influenza A(H3N2) variant (H3N2v) virus were reported; they resulted in 16 hospitalizations and 1 death (*1*). The variant designation (swine-origin influenza A virus infecting humans) of these cases must be acknowledged because interspecies transmission of influenza A virus plays a substantial role in the evolution of influenza A viruses that infect swine and humans (*2*,*3*). Genomic reassortment resulting in novel influenza A viruses can occur in swine because they are susceptible hosts for avian and human strains as well as strains endemic among swine (*4*,*5*). Thus, swine play a critical role in the ecology and emergence of influenza A viruses that affect human health, as illustrated by the emergence of the 2009 pandemic influenza virus (influenza A[H1N1]pdm09 virus), a reassortant virus with origins that have been traced to influenza A viruses circulating among swine in North America and Eurasia (*6*–*8*).

Bidirectional transmission of influenza A viruses between swine and humans is facilitated by unique swine–human interfaces such as agricultural fairs, where swine from multiple sources commingle with human exhibitors and visitors (*9*). In 2007, novel influenza A viruses, including those of nonhuman origin, became part of the National Notifiable Diseases Surveillance System, and before 2012, outbreaks of variant influenza A virus were reported only occasionally in the medical literature (*10*–*13*); these cases were frequently linked to human exposure to swine at agricultural fairs (*14*–*16*). Epidemiologic investigations by public health officials into human cases of influenza virus subtype H3N2v infection that occurred during 2012 concluded that swine exposure at agricultural fairs was the primary source of the viruses (*17*–*19*).

During the investigation of the 2012 outbreak of influenza A(H3N2v) virus infection, public health officials in Ohio documented 107 confirmed human cases, second only to the number of cases reported from Indiana. In late July 2012, interspecies transmission (from swine to humans) of swine-origin influenza A(H3N2) viruses containing the matrix gene from the influenza A(H1N1) pdm09 virus (H3N2pM virus) was initially confirmed at 1 Ohio agricultural fair (*20*). Retrospective epidemiologic investigations of all subtype H3N2v virus cases determined that human-to-human transmission of subtype H3N2v virus

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was limited and that most human patients had been directly or indirectly exposed to swine at a total of 14 agricultural fairs across the state (*19*). We investigated influenza A virus activity among swine (the swine side of the swine–human interface) and describe the results of active influenza A virus surveillance among swine at Ohio agricultural fairs during the entire 2012 fair season. When combined with the results of the public health epidemiologic investigation, our data provide molecular corroboration that swine-to-human transmission of influenza A(H3N2v) virus occurred at multiple agricultural fairs.

Materials and Methods

During 2012, a total of 40 agricultural fairs geographically distributed across Ohio were enrolled in the study. They represented a base of 22 fairs sampled in 2011 that were selected with predetermined criteria and supplemented with 18 fairs randomly sampled in 2012 (*9*). At the end of each fair, study team members visually examined the swine for signs of respiratory disease and collected nasal swab samples from at least 20 swine that were selected without regard to visually determined health status (healthy or ill). Nasal swab samples were placed in individual vials containing viral transport medium and frozen at –70°C until the time of testing. The Ohio State University Institutional Animal Care and Use Committee approved the use of animals in this study under protocol no. 2009A0134.

Detection and characterization of influenza A virus from nasal swab samples were performed as previously described (*21*,*22*). Briefly, samples were screened by real-time reverse transcription PCR (rRT-PCR) for influenza A virus (VetMAX-Gold SIV Detection Kit; Applied Biosystems, Austin, TX, USA). If ≥1 sample from a fair was positive for influenza A virus, then viral transport medium for all nasal swab samples from that fair was inoculated individually into serum-free medium-adapted MDCK cells for virus isolation (*21*). Cells were observed daily for 72 hours, at which time cell culture supernatant was tested for hemagglutination activity. Hemagglutinin and neuraminidase subtype determination, along with matrix gene lineage characterization, were performed on virus isolates with rRT-PCR by using a commercially available swine influenza viral subtyping kit (Applied Biosystems).

Previously described procedures were used to generate full-length nucleotide sequences for 2 representative swine-origin H3N2pM virus isolates from each fair (*20*). Two isolates from Fair D had previously been sequenced and reported (*20*). All gene segments of the 18 remaining swine-origin isolates underwent amplification by PCR, followed by purification of the cDNA and preparation of cDNA libraries. Quantitated libraries were diluted and pooled for library amplification. After enrichment, DNA was sequenced and the sequences were assembled by using standard procedures. Sequences from the swine-origin influenza A virus isolates reported here have been deposited in GenBank (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/9/13-1082-Techapp1.pdf).

Of the 14 Ohio fairs that Jhung et al. epidemiologically linked to cases of subtype H3N2v virus infection in 2012 (*19*), 7 (Fairs D–J) had participated in our active influenza A virus surveillance among exhibition swine during the same year (Table 1). As part of the public health investigation into the outbreak of subtype H3N2v virus infections, specimens collected from humans with suspected cases of subtype H3N2v virus infection were submitted to the Ohio Department of Health laboratory for influenza testing. Representative samples with preliminary test results consistent with H3N2v virus infection were forwarded to the Centers for Disease Control and Prevention for confirmatory testing and sequencing. Sequences of the human-origin subtype H3N2v virus isolates were

Table 1. Influenza A virus–specific results, 40 agricultural fairs, Ohio, USA, 2012*

Fair	Week of fair season	Length of swine exhibition, d	ILI among swine reported	No. swine sampled	Positive by rRT-PCR, no. (%)	Positive by VI, no. (%)	Virus subtypes recovered	Associated with H3N2v in humans
A	1	5	Yes	20	14 (70)	5 (25)	H1N1pM and H3N2pM	No
B	4	5	No	20	13 (65)	9 (45)	H3N2pM	No
C	6	4	No	20	6 (30)	6 (30)	H3N2pM	No
D	7	7	No	34	31 (91)	29 (85)	H3N2pM	Yes
E	7	5	Yes	40	39 (98)	28 (70)	H3N2pM	Yes
F	8	5	No	20	20 (100)	18 (90)	H3N2pM	Yes
G	8	7	Yes	20	14 (70)	16 (80)	H3N2pM	Yes
H	8	6	No	20	20 (100)	18 (90)	H3N2pM	Yes
I	9	4	No	20	17 (85)	15 (75)	H3N2pM	Yes
J	10	4	Yes	20	20 (100)	17 (85)	H3N2pM	Yes
30 other fairs	NA		NA	600	29 (5)	0	NA	No
Totals	NA		NA	834	223 (27)	161 (19)	NA	NA

*Real-time reverse transcription PCR (rRT-PCR) and virus isolation (VI) assays performed on nasal swab samples collected from swine at the end of the fairs. Additional details (week of the fair season, clinical signs of influenza-like illness [ILI], and influenza A virus subtypes) are shown for 10 agricultural fairs from which influenza A virus was isolated from ≥1 pig. Pigs at the other 30 fairs were negative for influenza A virus by VI. NA, not applicable.

retrieved from the EpiFlu database (http://www.gisaid.org). The human-origin influenza A virus isolates with full-length complete-genome sequences were then categorized by the fair with which the case had been associated during the epidemiologic investigation. Isolates from cases that were associated with >1 fair were removed from the study. This process identified at least 1 human-origin subtype H3N2v virus isolate per fair; if multiple isolates were identified, 1 isolate per fair was randomly selected for further analysis.

Comparative genome analysis was conducted by using the full-length sequences of 1 human-origin and 2 swine-origin subtype H3N2 virus isolates per fair (total of 27 influenza A virus isolates) (online Technical Appendix). These sequences were combined with all unique complete influenza A virus gene segment sequences from swine from North America that were available in the Influenza Resource Database (*23*). The nucleotide sequences of each segment were aligned individually by using Geneious version 6.0.5 (Biomatters Limited, Auckland, New Zealand); phylogenetic trees were generated by using maximum-likelihood methods (*24*), and the resulting trees were edited with MEGA version 5.2.2 (*25*). Full-length sequences for each isolate were concatenated to form a 13,133-nt sequence for each isolate; these sequences were then used to examine the genetic distance (number of nucleotide differences) between each isolate. The estimates of genetic distance were calculated by using MEGA version 5.2.2.

Results

Influenza A virus was isolated from ≥1 pig at 10 (25%) of the 40 agricultural fairs. Influenza A(H3N2pM) virus was recovered from swine at all 7 fairs that had been epidemiologically linked to cases of human infection with subtype H3N2v virus. The 30 fairs at which influenza A virus was not isolated from swine were not linked to any cases of human infection with subtype H3N2v virus. The 7 fairs in this study that were associated with cases of influenza A(H3N2v) virus infection in humans occurred during 4 consecutive weeks (weeks 7–10) of Ohio's 18-week agricultural fair season (June–October) (Figure 1). The 3 fairs at which swine were shedding influenza A(H3N2pM) virus not associated with any cases of human influenza A(H3N2v) infection (Fairs A, B, and C) were held during weeks 1, 4, and 6, respectively. As shown in Table 1, a total of 834 swine were sampled, and influenza A virus was recovered from 161 (19.3%). Although influenza A(H1N1) and A(H3N2) viruses were recovered from exhibition swine during 2012, most (158 [98.1%] of 161) of the isolates were subtype H3N2pM viruses. All isolates, including the 3 subtype H1N1 virus isolates, contained a matrix gene derived from the A(H1N1)pdm09 virus.

The overall nucleotide identity of all 27 isolates included in the investigation was >99.50%. The mean number of differences between each human-origin influenza A(H3N2v) isolate and its 2 matched swine-origin isolates was 9.7 nt (95% CI 2.7–16.7). Conversely, the mean number of differences between each human-origin influenza A(H3N2v)

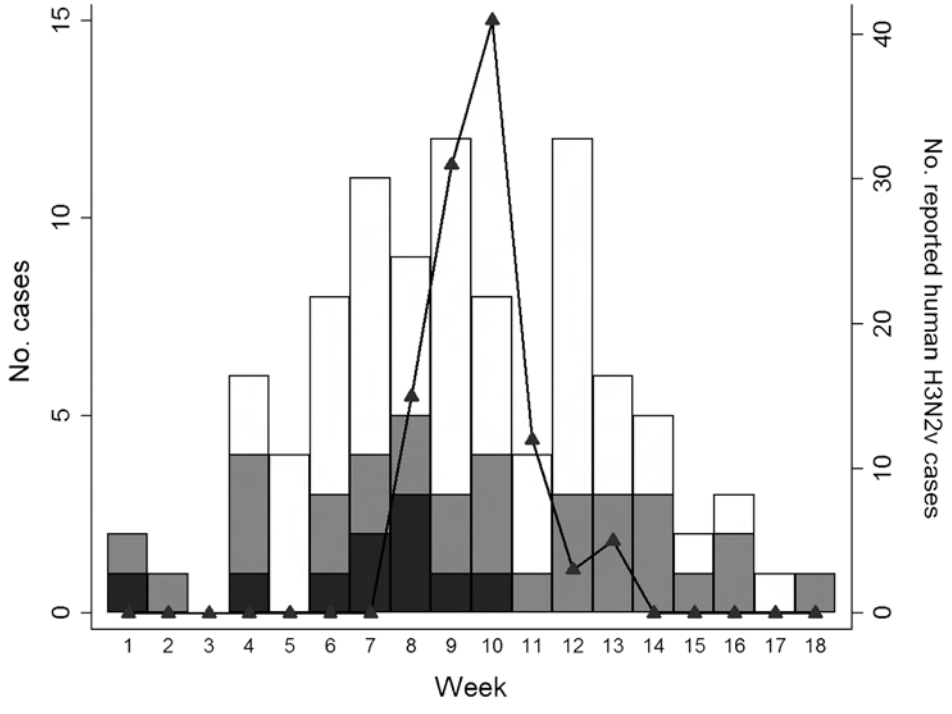

Figure 1. Distribution of agricultural fairs and human infection with influenza A variant virus (H3N2v), by week of the Ohio fair season, June–October 2012. Black bar sections, fairs with swine positive for influenza A virus; gray bar sections, fairs with no swine positive for influenza A virus; white bar sections, fairs not enrolled in this study. Black triangles, reported human cases of H3N2v virus infection.

isolate and the 18 swine-origin influenza A(H3N2pM) isolates to which it was not linked was 36.9 nt (95% CI 28.94–44.80). At 5 of the 7 fairs from which human cases of subtype H3N2v infection were reported, the nucleotides of the matched subtype H3N2 isolates from humans and swine were >99.90% identical. Nucleotide identities of 99.74% and 99.87% were detected between the matched subtype H3N2 isolates from humans and swine at Fairs E and F, respectively. Pairwise comparisons of nucleotide differences within and between fairs are shown in Table 2.

Phylogenetic analysis of each gene segment of the influenza A(H3N2v) isolates from humans and influenza A(H3N2pM) isolates from swine demonstrated tight clustering with each other (Figures 2, 3, and online Technical Appendix). The hemagglutinin segments are typical of cluster IV H3 influenza A viruses circulating among swine in North America (Figure 2, panel A). The neuraminidase segments of the subtype H3N2pM and H3N2v isolates described here clustered tightly together as a sublineage of the 2002 lineage of swine in North America (N2) (Figure 3, panel A). The only other virus included in this neuraminidase sublineage recovered before 2012 was the neuraminidase segment from isolates of subtype H3N2v that caused infection in West Virginia in 2011 (*11*), represented by the A/West Virginia/06/2011(H3N2) isolate (Figure 3, panel B).

Discussion

The results reported here demonstrate that influenza A(H3N2pM) viruses were commonly circulating among exhibition swine in Ohio during the 2012 agricultural fair season, and genome analysis confirmed zoonotic transmission that resulted in human infection with subtype H3N2v virus. Concurrent infection of swine and humans with swine-origin influenza A(H3N2) virus at Fair D has been reported (*20*), but our molecular investigation demonstrates that the result of that previous study was not an isolated event. Rather, the subtype H3N2pM isolates recovered from swine at 6 additional agricultural fairs in Ohio are molecularly linked to human infections with subtype H3N2v virus contracted at each of those respective fairs. These data provide molecular confirmation of the epidemiologic linkage between cases of subtype H3N2v virus infection and exposure to swine at agricultural fairs.

The fact that all influenza A(H3N2) virus isolates from humans and swine included in this investigation had >99.5% nt identity provides convincing evidence that the same strain of influenza A(H3N2pM) virus was at the 10 Ohio fairs with influenza A virus–positive swine detected in this study. Even within that high degree of similarity among isolates during the 2012 fair season, influenza A(H3N2v) isolates from humans differed from their fair-matched influenza A(H3N2pM) isolates from swine by no more than 16 nt (range 0–16) at 6 of the 7 fairs; the exception was Fair E, for which the closest match between isolates from humans and swine was 34 nt. The 2 sequenced swine-origin subtype H3N2 isolates from Fair E differed from each other by 32 nt, indicating increased genetic diversity among the influenza A viruses circulating among the swine at Fair E compared with that at the other fairs from which isolates from swine differed by ≤7 bases (data not shown). This diversity among isolates from Fair E can be seen in the phylogenetic trees (Figures 2, panel B; Figure 3, panel B; and online Technical Appendix). The genetic diversity among the swine-origin influenza A virus isolates from Fair E is probably a result of multiple independent influenza A virus introductions into the swine population; Fair E hosts a larger number of swine (>800), representing a larger geographic area than the other fairs in the study. Additionally, the swine at Fair E were sampled as part of 2 separate load-outs (shipments out of the fair), which resulted in a total of 40 samples from the fair. Because only 2 isolates from swine were sequenced per fair, it is possible that a closer genetic counterpart for the subtype H3N2v isolate from humans exists within the 26 unsequenced isolates from swine at Fair E.

Table 2. Genetic distances between influenza A(H3N2) viruses isolated from swine and humans at 10 agricultural fairs, Ohio, USA, 2012*

Fair	Fair A	Fair B	Fair C	Fair D	Fair E	Fair F	Fair G	Fair H	Fair I	Fair J
Fair A	4.00	3.38	3.51	6.74	5.59	5.30	7.76	5.00	7.10	7.42
Fair B	19.00	2.00	1.68	5.75	5.15	4.68	7.09	4.12	5.90	7.02
Fair C	17.50	6.00	1.00	6.03	4.48	4.09	6.81	3.84	5.50	6.63
Fair D	48.17	42.17	44.67	2.67	3.53	5.22	5.17	4.83	5.11	5.24
Fair E	48.17	42.17	44.67	31.33	35.33	3.13	3.11	3.47	3.17	3.43
Fair F	34.33	34.17	34.50	32.67	31.67	10.67	4.96	4.56	3.90	5.01
Fair G	53.83	47.83	50.33	33.00	25.00	38.33	2.67	5.43	5.22	1.46
Fair H	31.50	15.50	14.00	32.67	32.67	38.00	38.33	0.00	4.33	5.44
Fair I	47.83	41.83	44.33	27.00	26.00	14.33	32.67	32.33	2.67	5.35
Fair J	55.50	49.50	52.00	34.67	26.67	40.00	4.33	40.00	34.33	6.00

*Full-length concatenated genomes (13,133 nt positions) were used for comparisons. Mean numbers of base differences between isolates within each fair are shown on the highlighted diagonal; mean numbers of nucleotide differences between groups of isolates recovered from each fair are shown in the area below the diagonal. SE estimates, which were obtained by a bootstrap procedure using 1,000 replicates, for the between-group mean number of nucleotide differences are shown above the diagonal. Evolutionary analyses were conducted by using MEGA5.2.2 (http://www.megasoftware.net). Gray shading of mean numbers of nucleotide differences indicates higher percentage identity with darker shaded cells. Influenza A virus isolate names have been shortened to conserve space (OSU, indicates swine isolates; human isolates are identified by their unique number). Fair A, OSU50, OSU52; Fair B, OSU129, OSU138; Fair C, OSU175, OSU176; Fair D, OSU268, OSU293, 17; Fair E, OSU307, OSU420; 57; Fair F, OSU363, OSU370; 38; Fair G, OSU447, OSU450, 56; Fair H, OSU464, OSU467, 62; Fair I, OSU483, OSU484, 71; Fair J, OSU522, OSU527, 80.

The odds of having cases of influenza A(H3N2v) virus infection in humans were much higher for fairs at which ≥1 pig was infected with influenza A virus because cases of subtype H3N2v infection in humans were linked only to fairs with swine infected with influenza A virus. Of note is the lack of human H3N2v cases associated with Fairs A, B, and C (Table 1), although the influenza A(H3N2pM) viruses circulating among the swine at those fairs were highly similar to the viruses recovered from swine and humans later in the fair season (Figure 2, panel B). The frequency of virus isolation from swine was ≤45% at Fairs A, B, and C, whereas frequency at the 7 fairs associated with human cases of subtype H3N2v virus infection (Table 1) was ≥70%. This finding provides a basis for optimism that efforts to decrease the proportion of influenza A virus–infected swine at fairs will decrease the risk to public health. Another possible explanation for the lack of human cases of H3N2v infection during Fairs A, B, and C is that increased awareness and surveillance led to a surveillance artifact caused by previous underdiagnosis and/or underreporting of cases in humans. No human cases of subtype H3N2v infection were reported in Ohio until after the initial reports of such cases in Indiana during July 2012 became public (*26*). After publication of these cases, the Ohio Department of Health began enhanced influenza surveillance, and almost immediately local Ohio public health jurisdictions began alerting the Ohio Department of Health that persons who had been exposed to swine at agricultural fairs were seeking medical care for influenza-like illness.

Influenza A virus–infected exhibition swine threaten public health, and recently, increased emphasis has been placed on educating fair organizers and exhibitors about implementing appropriate precautions when exhibition swine become ill (*27*). Although swine showing clinical signs of influenza-like illness at agricultural fairs are typically removed from public display and/or excused from the exhibition, a previously reported high prevalence of subclinical infection in swine at agricultural fairs (*9*) suggests that many exhibitors and visitors are unknowingly being exposed to swine infected with influenza A virus. In this 2012 investigation, 6 (60%) of 10 agricultural fairs with influenza A–infected swine did not report any influenza-like illness among the exhibition swine (Table 1), further demonstrating the public health risk posed by swine with subclinical influenza infections.

Swine-to-human transmission and human-to-swine transmission of influenza A virus are known to occur at fairs (*28*), highlighting the fact that swine in this setting are potentially exposed to multiple lineages of influenza A viruses simultaneously, making fairs ideal locations for genomic reassortment and novel virus formation. The swine exhibited at agricultural fairs and livestock exhibitions account for a small but distinct subset of the US swine herd, frequently reared in very small herds as part of youth educational programs (*29*) and generally segregated from swine reared for commercial pork production. Influenza A(H3N2pM) viruses are not unique to fairs, and viruses similar to those described in the study reported here have also been detected in commercial swine populations (*30*), indicating that influenza A virus genes and/or whole viruses are shared between commercial and exhibition swine populations. The frequent movement of exhibition swine could serve as a potential pathway for the spread of influenza A virus, which could cause further dissemination of emergent stains in the larger commercial swine population. The rapid dissemination of highly similar subtype H3N2pM viruses among swine at 10 fairs across the state highlights the need to study the transmission dynamics of influenza A viruses within exhibition swine populations. However, although there is certainly fair-to-fair movement of exhibition swine, the role of infected humans spreading these variant viruses fair-to-fair, farm-to-fair, and fair-to-farm needs to be considered and investigated.

The results of this study support previous calls for enhanced surveillance of influenza A viruses among swine, especially at high-risk swine–human interfaces (*31*). In 2012, across the United States, 309 human cases of influenza A(H3N2v) virus infection were reported (http://www.cdc.gov/flu/swineflu/h3n2v-case-count.htm); 306 of these cases occurred during the summer (*19*). Investigations seeking to identify infected swine were frequently hampered by the limitations of retrospectively tracing suspected swine after exhibition. These limitations include not finding and testing the swine until after peak virus shedding and the unavailability of swine for testing after terminal (mandatory harvest) swine exhibitions. The data presented here from active influenza A virus surveillance among exhibition swine, initiated before recognition of cases of subtype H3N2v virus infection, made Ohio uniquely able to investigate the subtype H3N2v virus outbreaks of 2012. Risk factor analyses of fair characteristics and fair-level management practices used during 2012 showed that Ohio fairs with a larger swine inventory were more likely to have had influenza A virus–infected swine during that year (*32*).

Our findings with regard to the swine side of the swine–human interface at fairs seem to be similar to those from previous years in which no human cases of variant influenza virus infection were reported in Ohio. We detected influenza A virus–infected swine at 25% of the fairs tested during 2012, which corresponds with our previous work showing infection at 22.6% of Ohio fairs during 2009–2011 (*9*). The temporal pattern was also similar to that from previous years; fairs with influenza A virus–positive swine were detected sporadically during the early summer, the number peaked in the middle of the season, and none were detected during autumn. Although the seasonal pattern of subtype

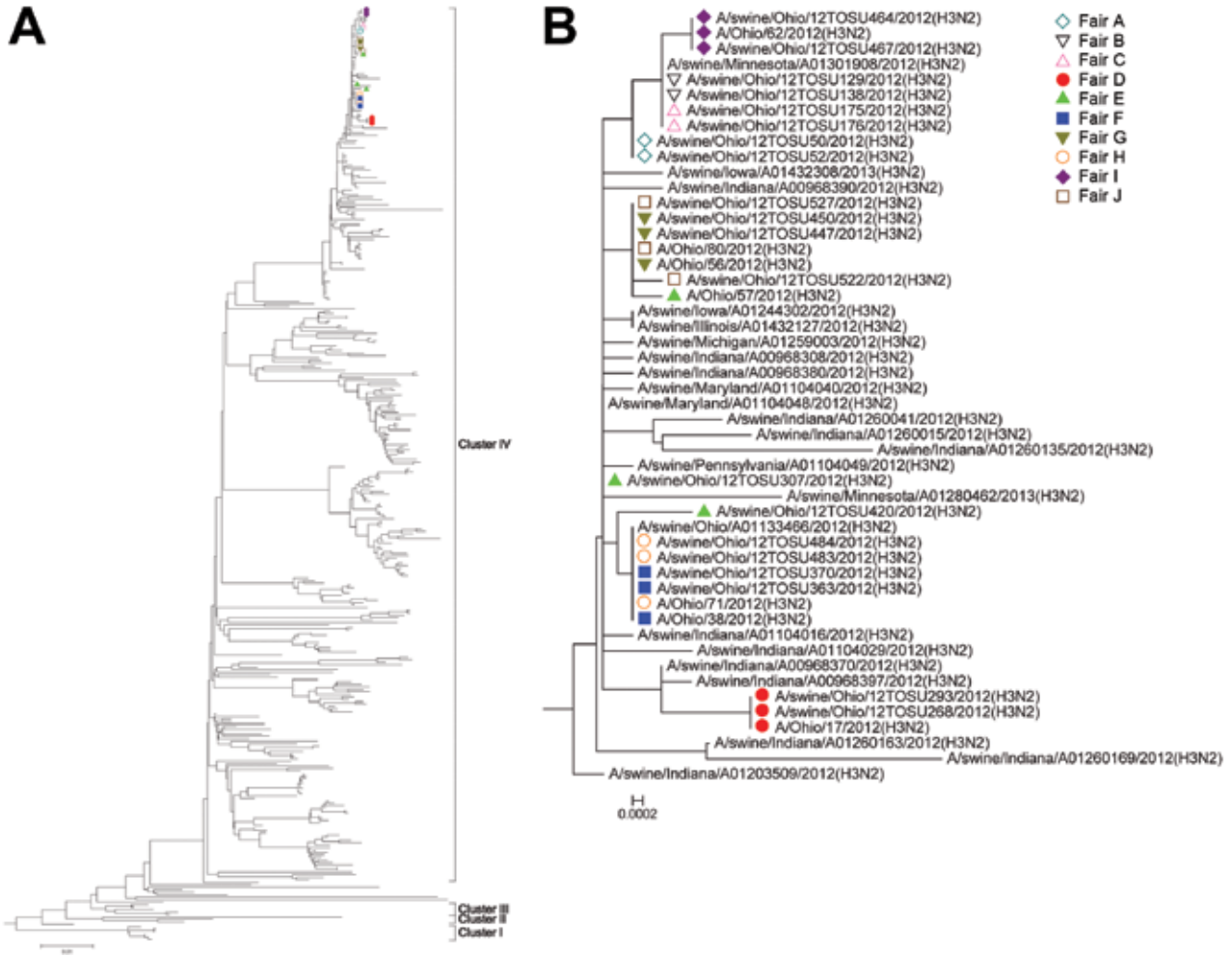

Figure 2. Hemagglutinin phylogeny. A) Phylogenetic relationships of the hemagglutinin sequences of swine-origin subtype H3 influenza A viruses from agricultural fairs, Ohio, USA, 2012. B) Expanded view of isolates. Isolates recovered from swine and humans at the same fair are identified with the same color and symbol. Scale bars indicate nucleotide substitutions per site.

H3N2v infections in humans in other states was similar to that in Ohio, extrapolation of the findings beyond Ohio will require more robust influenza A virus surveillance in swine at agricultural exhibitions in other states. The sublineage of N2 virus associated with the cases of variant influenza virus infection that occurred during 2012 is clearly within the larger previously described 2002 lineage of N2 virus circulating among swine in North America (Figure 3, panel A) (*30*). The fact that before 2012 this sublineage had only been detected in humans and never in swine further illustrates the need for better influenza A virus surveillance in swine populations.

The sporadic nature in which human infections with variant influenza virus had been reported before 2012 (*10*,*13*,*14*,*33*) highlights an unprecedented frequency of interspecies influenza A virus transmission that occurred during the 2012 outbreak of subtype H3N2v virus infections. Although 306 cases of subtype H3N2v infection were identified during the summer of 2012, thousands more probably went undocumented during the same period (*34*,*35*). Possible explanations for the increased number of variant influenza A virus infections during 2012 include increased transmissibility provided by reassorted gene segments from influenza (H1N1)pdm09 virus (*36*,*37*), limited immunity to swine-origin H3N2 among children <12 years of age (*38–40*), and/or increased awareness because of the 2011 cases of subtype H3N2v infection (*15*). During 2013, only 19 cases of infection with subtype H3N2v were reported (http://www.cdc.gov/flu/swineflu/h3n2v-case-count.htm); during that year, public health departments were on the lookout for cases of variant influenza A virus infection. Further evaluation of viral, host, and environmental factors will be needed for elucidation of the difference between the 2012 and 2013 fair seasons.

Whatever the reason for the increased incidence of cases of influenza A(H3N2v) virus infection during 2012,

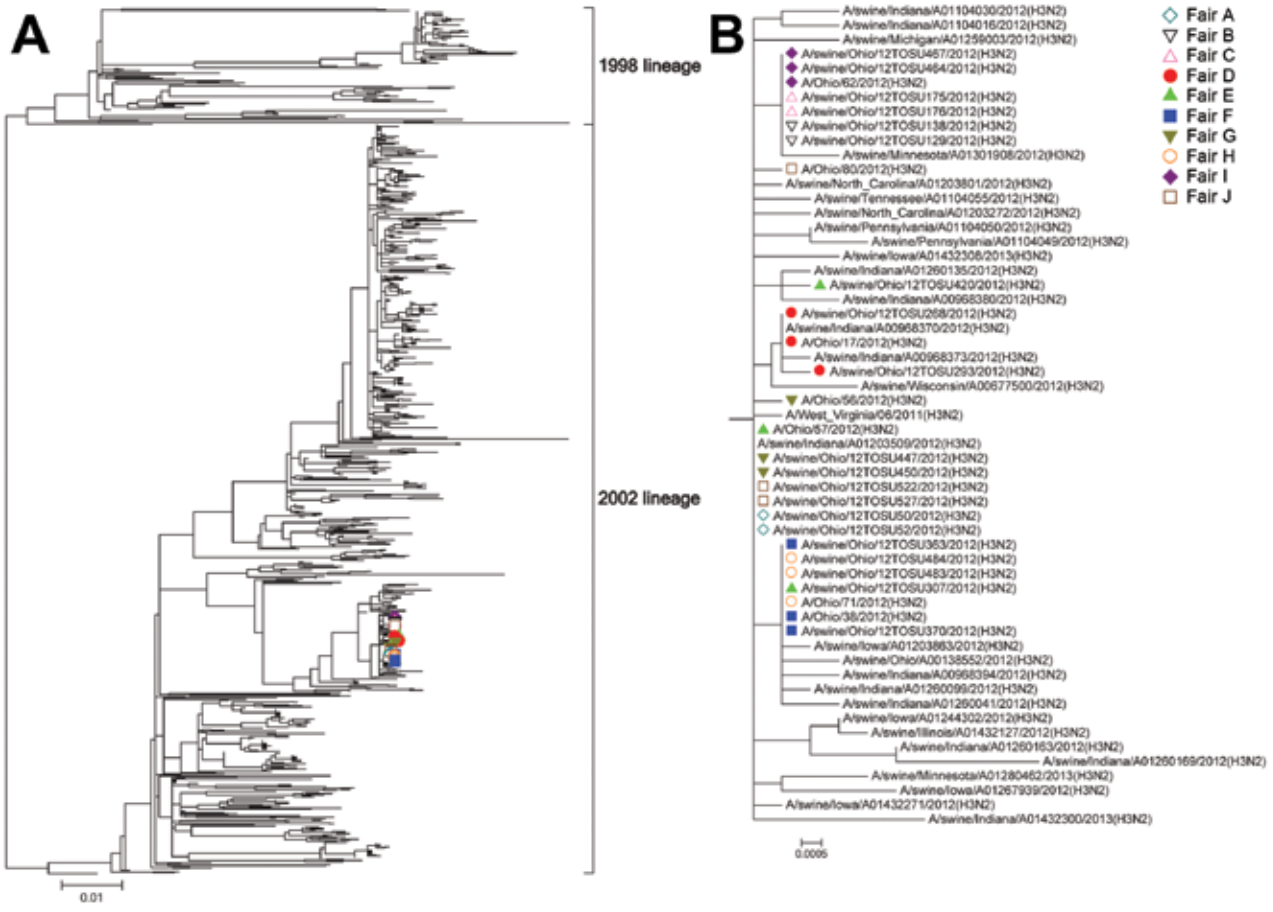

Figure 3. Neuraminidase phylogeny. A) Phylogenetic relationships of the neuraminidase sequences of swine-origin subtype N2 influenza A viruses from agricultural fairs, Ohio, USA, 2012. B) Expanded view of isolates. Isolates recovered from swine and humans at the same fair are identified with the same color and symbol. Scale bars indicate nucleotide substitutions per site.

mitigation strategies must be undertaken to decrease the risk for influenza A virus transmission across the swine–human interface at fairs, animal markets, abattoirs, and commercial swine production units. Influenza A virus infections in exhibition swine represent an unquantified public health risk. Rigorous efficacy evaluations and expanded risk assessments of adopted mitigation strategies to protect public and animal health are needed to help animal and public health experts make evidence-based recommendations for reducing intraspecies and interspecies transmission of influenza A virus in this setting. Fair organizers, animal health officials, and public health agencies should take additional steps to decrease the threat to human and animal health. Recently, the National Assembly of State Animal Health Officials and the National Association of State Public Health Veterinarians jointly released some potential measures for fair organizers and exhibitors to consider when hosting and participating in swine exhibitions (*27*). These measures include, but are not limited to, shortening the length of exhibitions, vaccinating swine and humans against influenza A virus infection, promoting awareness at exhibitions, continuous monitoring of swine for signs of influenza-like illness, posting risk-communication signage for visitors to the swine barns, and decreasing movement of swine between fairs. Active communication and partnerships between human and animal health agencies are needed for development and implementation of appropriate prevention and control plans. Local health care providers should be alerted to the possibility that patients with influenza-like illness might have variant influenza A virus infection, especially when agricultural fairs or exhibitions are being held in the community.

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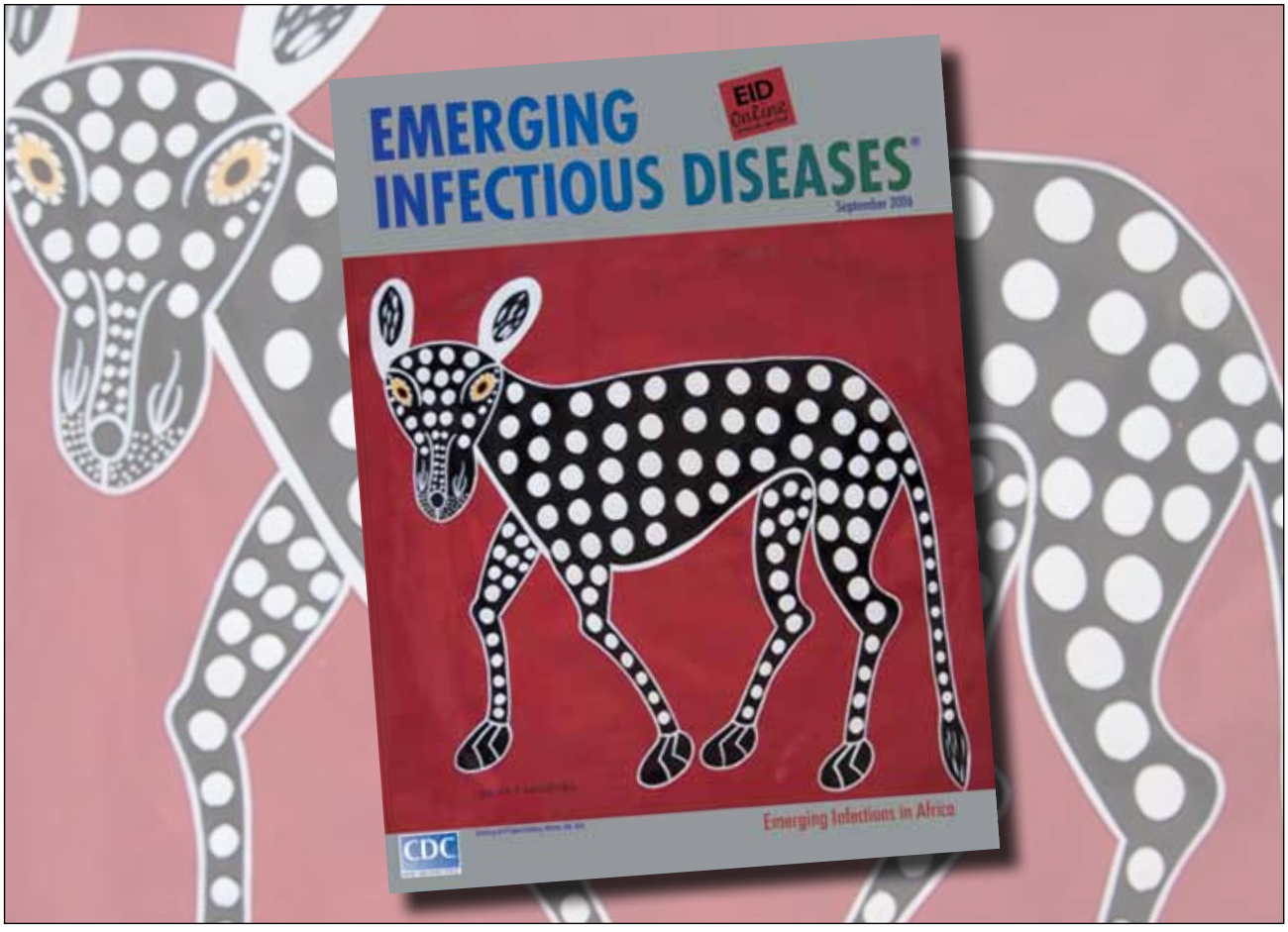

Genomic Epidemiology of *Salmonella enterica* Serotype Enteritidis based on Population Structure of Prevalent Lineages

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Salmonella enterica serotype Enteritidis is one of the most commonly reported causes of human salmonellosis. Its low genetic diversity, measured by fingerprinting methods, has made subtyping a challenge. We used whole-genome sequencing to characterize 125 *S*. enterica Enteritidis and 3 *S. enterica* serotype Nitra strains. Single-nucleotide polymorphisms were filtered to identify 4,887 reliable loci that distinguished all isolates from each other. Our whole-genome single-nucleotide polymorphism typing approach was robust for *S. enterica* Enteritidis subtyping with combined data for different strains from 2 different sequencing platforms. Five major genetic lineages were recognized, which revealed possible patterns of geographic and epidemiologic distribution. Analyses on the population dynamics and evolutionary history estimated that major lineages emerged during the 17th–18th centuries and diversified during the 1920s and 1950s.

Salmonella enterica causes ≈1 million illnesses and >350 deaths annually in the United States (*1*). Among >2,500 known serotypes, *S. enterica* serotype Enteritidis is one of the most commonly reported causes of human salmonellosis in most industrialized countries (*2*). From the 1970s through the mid-1990s, the incidence of serotype Enteritidis infection increased dramatically; shelled eggs were a major vehicle for transmission. Despite a decrease in serotype Enteritidis infection since 1996 in the United States, outbreaks resulting from contaminated eggs continue to occur (*3*), and Enteritidis remains among the most common serotypes isolated from humans worldwide (*2*). Epidemiologic surveillance and outbreak investigation of microbial pathogens require subtyping that provides sufficient resolution to discriminate closely related isolates. Differentiation of *S. enterica* Enteritidis challenges traditional subtyping methods, such as pulsed-field gel electrophoresis (PFGE), because isolates of serotype Enteritidis are more genetically homogeneous than are isolates of many other serotypes (*4*,*5*). Among the serotype Enteritidis isolates reported to PulseNet, ≈45% display a single PFGE *Xba*I pattern (JEGX01.0004), which renders PFGE ineffective in some investigations (*5*). Of the second-generation methods evaluated for *S. enterica* Enteritidis subtyping, multilocus variable number–tandem repeat analysis offers slightly better discrimination, but differentiating common patterns remains a substantial problem (*6*). Therefore, new methods are needed to better subtype and differentiate this serotype. Recent applications of whole-genome sequencing (WGS) have demonstrated exceptional resolution that enables fine delineation of infectious disease outbreaks (*7*–*10*).

In addition to sufficient subtyping resolution, accurately ascribing isolates to epidemiologically meaningful clusters, i.e., grouping isolates associated with an outbreak while discriminating unrelated strains, is critical for pathogen subtyping. Outbreak and epidemiologically unrelated isolates might not be differentiated by using current methods. Despite the high incidence of *S. enterica* Enteritidis

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infection in humans, genome sequencing of this serotype has lagged behind sequencing of other major foodborne pathogens. To our knowledge, only 1 finished *S. enterica* Enteritidis genome is publicly available (*11*). Recent sequencing of *S. enterica* Enteritidis genomes of the common PFGE *Xba*I pattern JEGX01.0004 has provided a valuable resource on the *S. enterica* Enteritidis genome (*12*). Here we present a broad sampling of WGS to include diversity of other major lineages.

We expanded the genomic population structure of *S. enterica* Enteritidis by sequencing a collection of 81 *S. enterica* Enteritidis genomes and 3 *S. enterica* serotype Nitra genomes selected to capture epidemiologic and phylogenetic diversity in current domestic and international serotype Enteritidis populations. We included serotype Nitra in the study because it is thought to be a variant of serotype Enteritidis with its O antigen (serogroup O2) being a minor genetic variant of serogroup O9 found in serotype Enteritidis (*13*). These genomes, along with 44 draft genomes of *S. enterica* Enteritidis (14 historical strains and 30 isolates selected from the 2010 egg outbreak investigation [http://www.cdc.gov/salmonella/enteritidis/]), provided a phylogenetic framework of diverse circulating serotype Enteritidis lineages. Model-based Bayesian estimation of age and effective population size of major *S. enterica* Enteritidis lineages showed that the spreading of *S. enterica* Enteritidis coincided with 2 periods: the 18th century period of colonial trade and the 20th century period of agricultural industrialization. A single-nucleotide polymorphism (SNP) pipeline was developed for high-throughput whole-genome SNP typing and was robust for combining data from different sequencing platforms in the same analysis. This enabled retrospective investigation of recent clinical cases in Thailand and the shelled eggs outbreak in the United States. The ability of whole-genome SNP typing to infer the polyclonal genomic nature of at least some *S. enterica* Enteritidis strains causing outbreaks, despite high genetic homogeneity among *S. enterica* Enteritidis genomes, demonstrates the utility and sensitivity of whole-genome SNP typing in epidemiologic surveillance and outbreak investigations. Potential challenges of whole-genome SNP typing, such as ways to accurately define individual outbreaks, were discussed.

Methods

Isolates

We obtained 125 serotype Enteritidis and 3 serotype Nitra isolates from Centers for Disease Control and Prevention, US Department of Agriculture, and University of California Davis (online Technical Appendix Tables 1, 2, http://wwwnc.cdc.gov/EID/article/20/9/13-1095-Techapp1.pdf). *S. enterica* Enteritidis isolates of diverse PFGE subtypes (18 *Xba*I patterns accounting for >90% of all *S. enterica* Enteritidis isolates reported to PulseNet [online Technical Appendix Figure 1]), spatiotemporal origins, and sources were sampled to span a broad epidemiologic and phylogenetic diversity of prevalent lineages of which we were aware.

WGS

Bacterial strains were grown in Luria broth at 37°C to stationary phase. Genomic DNA was prepared by using the GenElute Genomic DNA isolation kit (Sigma-Aldrich, St. Louis, MO, USA). Eighty-one isolates were sequenced by using Illumina (San Diego, CA, USA) technology (100-bp paired-end reads) at Washington University (St. Louis, MO, USA). Another 44 isolates were sequenced by using Roche (Indianapolis, IN, USA) 454 technology (single-end reads) as described previously (*12*).

SNP Detection

We developed a bioinformatics pipeline to detect high-quality SNPs from raw sequencing reads. The design of the workflow was geared toward a customizable and robust solution for whole-genome SNP typing of many isolates. It enables user-defined parameters for SNP quality filters and provides additional functions, such as assembly of unmapped reads and functional annotation of SNPs (online Technical Appendix Figure 2). The program snp-sites was then used to code missing data and SNP sites from ambiguous sites within the consensus sequences and create an alignment containing variable sites (https://github.com/andrewjpage/snp_sites).

Phylogenetic Analyses

We used BratNextGen (*14*) to detect recombination events in the genomes. The consensus sequences were used as input with 100 replicates (10 iterations each) to infer the significance of detected recombination events. Regions with a significant signal of recombination were excluded, as were highly homoplastic sites (as inferred in PAUP 4.0b10 [Sinauer Associates, Inc., Sunderland, MA, USA]; rescaled consistency index <1) indicative of non-neutral evolution, recombination, or ambiguous SNP calls. The remaining SNP sites were used only for further analysis when unambiguously called for at least 95% of the isolates. We performed maximum-likelihood (ML) analyses in MEGA5.1 (*15*). The resulting ML trees were used to test for a temporal signal by using Path-O-Gen v1.3 (http://tree.bio.ed.ac.uk/software/pathogen/). Bayesian phylogenetic analyses were performed by using BEAST v. 1.7.5 (*16*). The isolation year of each isolate was used to establish a temporal framework for constructing phylogenetic relationship among the isolates and estimating parameters to describe the evolutionary dynamics of the population (*17*).

Comparisons of different molecular clock models and tree priors were performed similarly to a method of Bakker et al. (*18*), except that we used the path sampling method (*19*) to estimate the marginal likelihood (online Technical Appendix Table 4).

Results

Divergent Isolates and Serotypes

Comparison of 84 newly sequenced genomes to the reference genome showed that all but 4 were closely related to the reference strain, differing by no more than 950 SNPs. The 4 divergent genomes (77–0915, 07–0056, SARB17, and SARB19) contained 19,800–43,544 SNPs, comparable to the number of SNPs between phylogenetically distinct serotypes. They also lacked *sdf*, a characteristic marker of commonly circulating serotype Enteritidis organisms, and were phylogenetically apart from the main serotype Enteritidis lineage (online Technical Appendix Figure 3). We did not include these divergent genomovars (genetic lineages) in subsequent SNP and phylogenetic analyses. The 3 *S. enterica* Nitra genomes were highly similar to the reference genome, with numbers of SNPs comparable to those of other *S. enterica* Enteritidis strains.

High-Quality Core Genome SNPs and Phylogeny

We observed 6,542 SNP loci in the remaining strains after we excluded other genomovars. The pairwise homoplasy index test (*20*) found no evidence of recombination for the SNP data of both the 81-isolate set (Illumina sequenced isolates and the reference) and the 125-isolate set (Illumina and 454 sequenced isolates plus the reference). However, putative regions (14 in total, online Technical Appendix Table 3) involved in homologous recombination were detected by BratNextGen (*14*) in the 125-isolate set, comprising 1,519 SNPs. After exclusion of these regions encompassing recombination, and homoplastic sites (136 SNPs, identified by using PAUP 4.0), 4,887 core genome SNP loci were left to be included in the analysis.

The general time-reversible model of nucleotide substitution was the best fit model for the dataset and was subsequently used in phylogenetic analyses. ML analysis based on high-quality core genome SNPs yielded highly congruent phylogenies between the 81-isolate (Illumina data only) and the 125-isolate (Illumina and 454 data combined) datasets (Figure 1). All 454 sequenced isolates clustered in 1 lineage, including the 30 selected for the shelled eggs outbreak investigation. These isolates represented 8 of the 9 clades defined by Allard et al. (*12*). For the Illumina-sequenced isolates in both datasets, the inferred phylogenies were highly congruent (Figure 1). Five major genetic lineages were identified (Figure 1): LI, LII, LIII, LIV, and LV. Isolates from clinical cases in Thailand and associated with a shell egg outbreak in the United States were found predominately in LIII and LV, respectively.

Population Dynamics

The 125-isolate set displayed a temporal signal, as demonstrated by a positive correlation between distances to the most recent common ancestor (MRCA) and dates of sampling. Although this correlation was weak when measured for the whole dataset (correlation coefficient 0.3, $R^2 = 0.09$, $p<0.001$), exclusion of LII from the dataset led to an increased correlation (correlation coefficient 0.6, $R^2 = 0.40$, $p<0.0001$). Both the Path-O-Gen analysis and molecular clock–based analyses indicate that LII evolved at a higher mutation rate than other clades.

We compared 4 population genetics models through Bayes factors (BF) (*21*). For this analysis, we excluded redundant isolates that were derived from the same outbreak and differed by only 1 or 2 SNPs. The final dataset comprised 99 isolates. A relaxed log-normal molecular clock was strongly favored over a strict clock rate ($\log_{10}$ BF>100), suggesting that mutation rates vary significantly among branches (online Technical Appendix Table 4). We found strong evidence ($\log_{10}$ BF>100) in favor of a constant effective population size model over an effective population size model that enables fluctuations of effective population size through time (Gaussian Markov random field skyride model [*22*]). The results of the analyses assuming a constant effective population size model are thus discussed here.

The mean mutation rate across all lineages was inferred to be 2.2×10^{-7} substitutions per site per year or 1.01 SNPs per genome per year. The MRCA of the whole population (Table) was estimated to date to 1549 CE (95% highest posterior probability density, from 1351 to 1704) (*23*). Although the inferred ages of the MRCA differ because of the model of choice, the estimates for the younger nodes appeared to converge between models, with overlapping highest posterior probability densities. We constructed a lineage through time plot (Figure 2) to show the change of inferred number of lineages over time on the basis of a constant effective population size model using BEAST (http://tree.bio.ed.ac.uk/).

Discussion

WGS of the 125 *S. enterica* isolates of serotypes Enteritidis and Nitra enabled us to probe the population dynamics and evolutionary history of prevalent serotype Enteritidis lineages. The inferred mean mutation rates of serotype Enteritidis are comparable to those of short-term evolution in several other pathogens (*24–26*). The prediction that the MRCA possibly emerged between 1351 and 1704 CE is in line with the historical fact that serotype Enteritidis was one of the first recognized *Salmonella* serotypes in 1888 CE

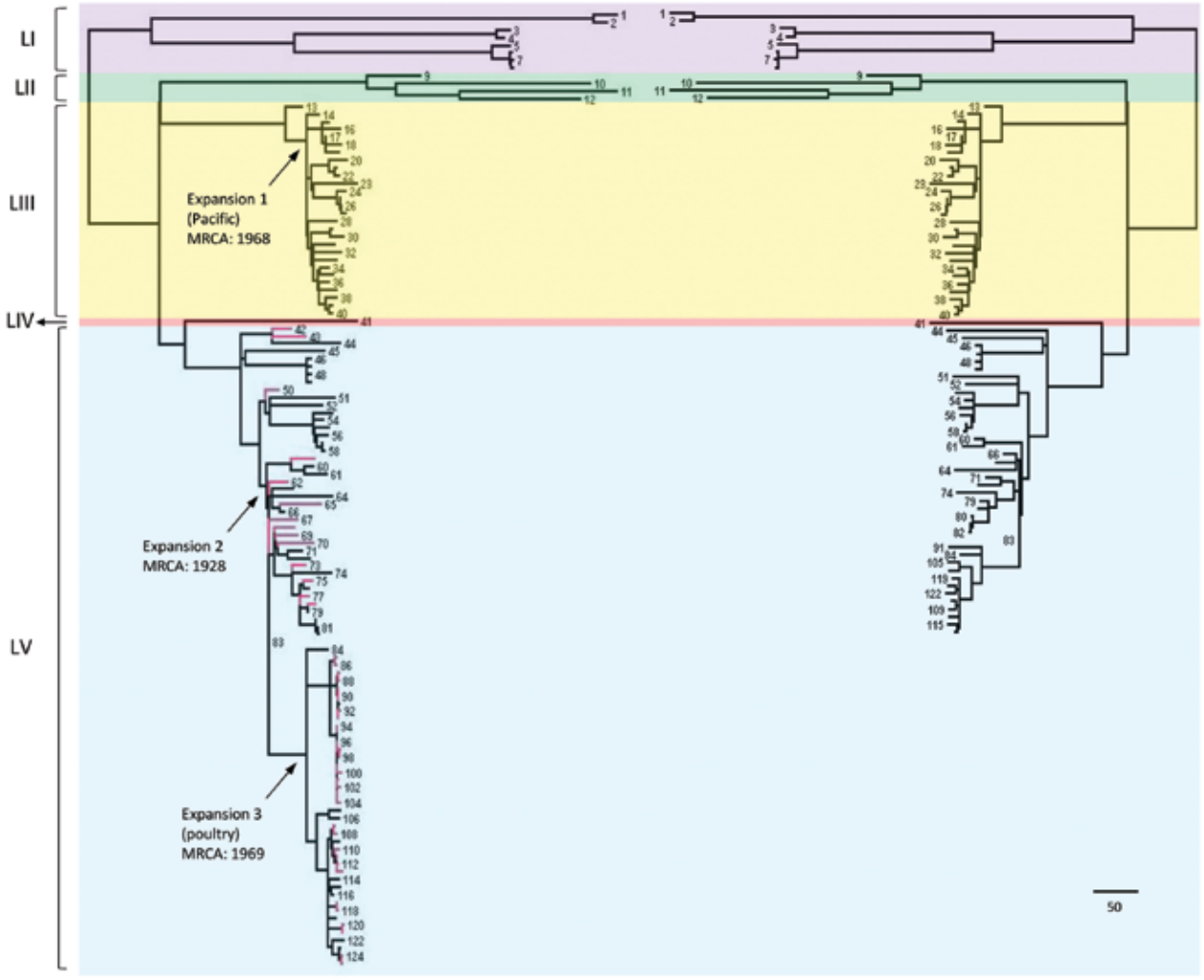

Figure 1. Comparison of *Salmonella enterica* serotype Enteritidis phylogenies inferred from Illumina data and combined data of Illumina (San Diego, CA, USA) and Roche 454 (Indianapolis, IN, USA). The tree on the right incudes 80 Illumina sequenced isolates and the reference genome (PT4). The tree on the left includes both the 80 Illumina and the 44 454 sequenced isolates in addition to the reference. Isolates were numbered (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/20/9/13-1095-Techapp1.pdf). Lineages I, II, III, IV, and V are highlighted in purple, green, yellow, red, and blue, respectively. Branches representing 454 sequenced isolates are labeled in red. Arrows on the left tree indicate the 3 serotype Nitra isolates. MRCA, most recent common ancestor. Scale bar indicates single-nucleotide polymorphisms.

(*27*). According to the same model, the 5 lineages identified in this study originated during 1608–1788 CE (Table). This initial diversification event could possibly be related to the emergence of colonial trade around that time, facilitating effective global dispersal of serotype Enteritidis, as in the case of the intercontinental transmission of the yellow fever virus (*28*).

On the basis of the molecular clock and other assumptions made in the Bayesian analysis, the number of lineages of *S. enterica* Enteritidis is estimated to have increased sharply during 1925–1950 CE, which indicated rapid diversification of serotype Enteritidis (Figure 2). This period coincides with the Green Revolution, a period of increased global agricultural production. We speculate that the start of better poultry farming practices in the United Kingdom and United States might have created poultry farms as a niche for *S. enterica* Enteritidis by reducing closely related traditional avian *Salmonella* serotypes, such as Gallinarum (*29*).

The genes responsible for serotype (primarily *rfb* region, *fliC*, and *fljB*) are commonly subject to horizontal gene transfer, resulting in very similar/the same alleles present in distinct genetic backgrounds. This phenomenon has contributed to the huge diversity of *Salmonella* serotypes

Table. Dating of nodes in the maximum-likelihood tree of *Salmonella enterica* serotype Enteritidis using BEAST and strict and relaxed mutation rates*

	Median MRCA (95% CI)		
	Relaxed clock		
Node, cluster	Constant population size	GMRF	Strict clock, constant population size
MRCA, node N01	1549 (1351–1704)	1896 (1858–1933)	1520 (1455–1576)
MRCA II, III, IV, V	1709 (1608–1788)	NA	1680 (1636–1716)
MRCA II	1831 (1760–1897)	1937 (1903–1966)	1824 (1799–1846)
MRCA III	1941 (1919–1957)	1950 (1933–1962)	1934 (1924–1944)
MRCA V	1888 (1858–1911)	1917 (1900–1936)	1883 (1869–1897)
Expansion 1, Pacific	1968 (1958–1976)	1975 (1964–1982)	1964 (1958–1970)
Expansion 2	1928 (1916–1937)	1934 (1926–1943)	1926 (1919–1933)
Expansion 3, poultry	1969 (1956–1981)	1984 (1974–1995)	1970 (1964–1976)

*BEAST (*16*). MRCA, most recent common ancestor; GMRF, Gaussian Markov random field; NA, not available.

and dictates that the serotyping system sometimes poorly reflects true phylogeny. Multiple genomovars have been noted for some serotypes (*30*) and most likely arose from horizontal gene transfer of the same assortment of serotype antigen genes into distinct genetic lineages by coincidence. Four isolates serotyped as *S. enterica* Enteritidis but lacking *sdf*, a characteristic marker of commonly circulating *S. enterica* Enteritidis, were divergent by WGS; they putatively represent different genomovars. The divergent *S. enterica* Enteritidis lineages are rarely encountered in the United States.

In contrast to the observation of different genomovars within a single serotype, *S. enterica* Nitra represents a separate serotype that is embedded within serotype Enteritidis lineages. Three serotype Nitra isolates clustered with LIII (CDC-STK-1280 and 96–0186) and LIV (2010K-0860), indicating that they are members of these *S. enterica* Enteritidis lineages. This finding is consistent with the finding that serogroup O2 is a minor genetic variant of serogroup O9. The 3 *S. enterica* Nitra isolates originated from different geographic locations and decades apart, suggesting that rare, independent, and distinct mutational events caused the switch of serotype from Enteritidis to Nitra. In this instance, it seems appropriate to consider *S. enterica* Nitra as part of the *S. enterica* Enteritidis lineage. As scientists move toward genetic determination of serotype, *S. enterica* Nitra is likely to be identified as *S. enterica* Enteritidis (*13*).

As shown in this and previous studies, whole-genome SNP typing provides both superior subtyping resolution and phylogenetic accuracy without compromising either, which is rarely possible with traditional molecular subtyping methods. Whole-genome SNP typing achieves this resolution by surveying point mutations across entire genomes rather than by targeting relatively few polymorphic sites characteristic of either high mutation rates for sufficient discriminatory power (e.g., variable number tandem repeats [*31*] and clustered regularly interspaced short palindromic repeats [*32*]) or reliable phylogenetic signals for accurate phylogeny (e.g., housekeeping genes). We found

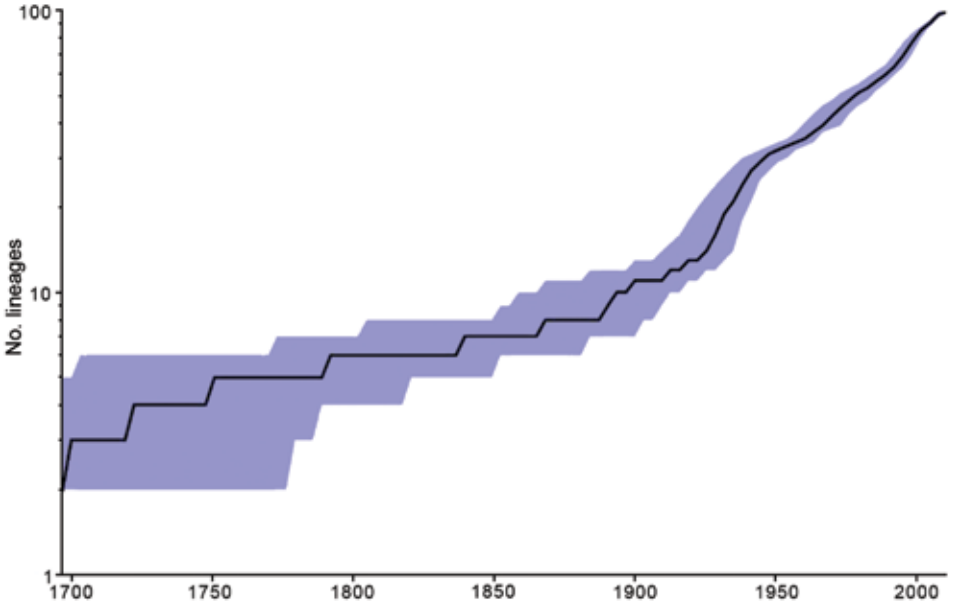

Figure 2. Inferred number of *Salmonella enterica* serotype Enteritidis lineages over time based on a constant effective population size model using BEAST (*16*). Blue shading indicates 95% CIs.

that the 4,887 SNPs resulting from combining data from 2 platforms were sufficient to resolve every serotype Enteritidis isolate in a phylogeny highly congruent with the tree based only on 1 platform (Figure 1). Such robustness facilitates the use of different sequencing platforms in actual surveillance and investigations to achieve repeatable results in subtype differentiation. Specifically, the same analytical approach and bioinformatics pipeline was applied to sequencing data with drastically different error patterns by employing strict criteria to search for high-quality SNPs. This strategy was effective and efficient for phylogenetic inference, subtyping, and routine investigation of serotype Enteritidis, especially when multiple instruments, library preparation, and bioinformatics methods are available for whole-genome SNP typing applications in public health laboratories.

WGS of *S. enterica* Enteritidis isolates with a wide range of genetic, spatiotemporal, and source attributes broadened the understanding of phylogenetic diversity of this genetically homogeneous pathogen. WGS enabled us to build a phylogenetic framework of prevalent serotype Enteritidis lineages that will be highly valuable for ongoing and future surveillance and investigation.

We recognized lineages and clades with geographic and epidemiologic characteristics. Of the 3 seemingly rare or potentially undersampled lineages (LI, LII, and LIV), LI and LII appeared to be associated with specific geographic locations and have diverged earlier than other lineages. LI was further divided into 2 clades, of which one had 2 isolates from Africa (isolates 1 and 2 in Figure 1) and the other had 6 isolates from the western United States and predominately associated with wildlife and environmental sources (isolates 3–8). LII consisted of 3 isolates associated with marine mammals in California and 1 isolate linked to a human in the same state (isolates 9–12). The 3 marine mammal isolates formed a highly supported clade and came from 2 different host species in a 10-year span, suggesting a stable *S. enterica* Enteritidis lineage circulating among those animals. The fact that a human case was linked to this clade suggests possible transmission between marine animals and humans. The marine animal community on and around the Channel Islands off the coast of southern California is extremely rich and diverse and displays the highest known prevalence of *S. enterica* in the world (*33*). Free-ranging and migratory animals and birds from this natural reservoir of *S. enterica* could play a role in long distance dispersal of this pathogen. LIII and LV appeared to be the more prevalent lineages on the international and US domestic scales, respectively. LIII contained isolates from 4 continents; its major clade (isolates 14–40) originated primarily from the Pacific region, including Thailand and California. LIV represented a common lineage in the United States, including a clade predominately associated with poultry products (isolates 84–125).

Our retrospective investigation of clinical cases from Thailand and a shelled eggs outbreak in the United States demonstrated the utility of our method on the basis of the robust bioinformatics pipeline and the broad phylogenetic framework. Fourteen of the 15 isolates from clinical cases in Thailand clustered in LIII and fell into 4 different clusters with high bootstrapping support (Figure 3, clusters A–D), suggesting multiple genetic origins consistent with a previous study (*34*). Isolates from blood and fecal samples clustered with other isolates from the United States and Europe, including the reference strain P125109 from United Kingdom. Previous observation of a disproportionately high percentage of bloodstream infections of *S. enterica* Enteritidis in Thailand (*35*) may be due to host factors (e.g., underlying health conditions, concurrent infections), underreported or unreported gastrointestinal infections less detectable than invasive ones, and/or random invasive infections (e.g., high-dosage exposure). Similarly, compromised immunity from the high percentage of HIV cases in sub-Saharan Africa was proposed as a contributing factor to the perceived high invasiveness of serotype Enteritidis infections in the region (*36*). We recommend caution when interpreting extraintestinal infections of *Salmonella* as evidence for high virulence and that newly proposed

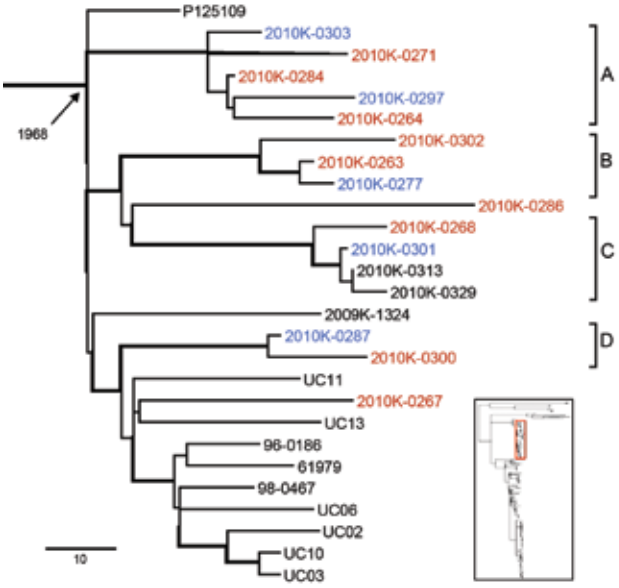

Figure 3. Salmonella enterica serotype Enteritidis genetic lineage III. Isolates from human blood and stool samples are indicated by red and blue, respectively. Four clades are highlighted and designated A–D, representing individual outbreak clades identified from clinical cases in Thailand in 2008. Branches with bootstrap value >0.9 are indicated by thickened lines. Age of the ancestral node (median most recent common ancestor) is labeled. Inset indicates the location of the highlighted lineage on the global phylogenetic tree.

hypervirulent lineages (*37*) be evaluated within a global phylogenetic context for their evolutionary identity and origin.

Concerning the shelled eggs outbreak, 2 definite (Figure 4, clusters A and B, both including isolates traced to the implicated facility) and 3 putative outbreak clades (clusters C–E, none of which had a direct epidemiologic link to the outbreak), i.e., individual clusters each containing isolates originated from a single source of contamination, were identified with phylogenetic and/or epidemiologic support on the basis of 2 criteria: 1) the clade is phylogenetically highly supported and 2) the isolation dates of all the isolates in the clade correspond to the outbreak period. Three isolates from sporadic cases in 2009 and 2010 might be attributed to the outbreak because they fell into the outbreak clades A and E (Figure 4, underlined), suggesting that the outbreak strains might have been circulating before the outbreak. Although A corresponded to a major clade defined by Allard et al. (*12*), B, C, and D clustered and thus were designated as a single clade in that study, possibly because of the absence of reference strains to separate them. Among the 4 isolates associated with poultry (Figure 4, blue highlighted), 60277 and 85366 were respectively isolated in 2002 and 2007 and therefore unlikely to be associated with each other and with the outbreak in 2010. These and other isolates unrelated to the outbreak helped delineate the individual outbreak clades, corroborating the likely polyclonal nature of the outbreak, which also was recognized by Allard et al. (*12*), and emphasizing the importance of incorporating multiple proper background references into outbreak investigations.

During outbreak investigations, putative outbreak isolates are analyzed with epidemiologically unrelated strains to determine whether they are related and thus likely to be associated with the same source. The availability and selection of background references can be critical (online Technical Appendix Figure 4). To maximize the availability of suitable background reference datasets, researchers desire phylogenetic frameworks with sufficient coverage of the genetic diversity in major pathogens, which is an aim of the ongoing 100K pathogen genome project (http://100kgenome.vetmed.ucdavis.edu/index.cfm).

Phylogenetic data alone are insufficient for defining an outbreak. Determining the polyclonal nature and scope of an outbreak remains a challenge, and no definitive criteria yet exist. For example, investigations of a recent *S. enterica* serotype Montevideo outbreak associated with red and black peppers reached discrepant conclusions. A proposed domestic source isolate from the implicated food processing facility (*38*) was excluded from the

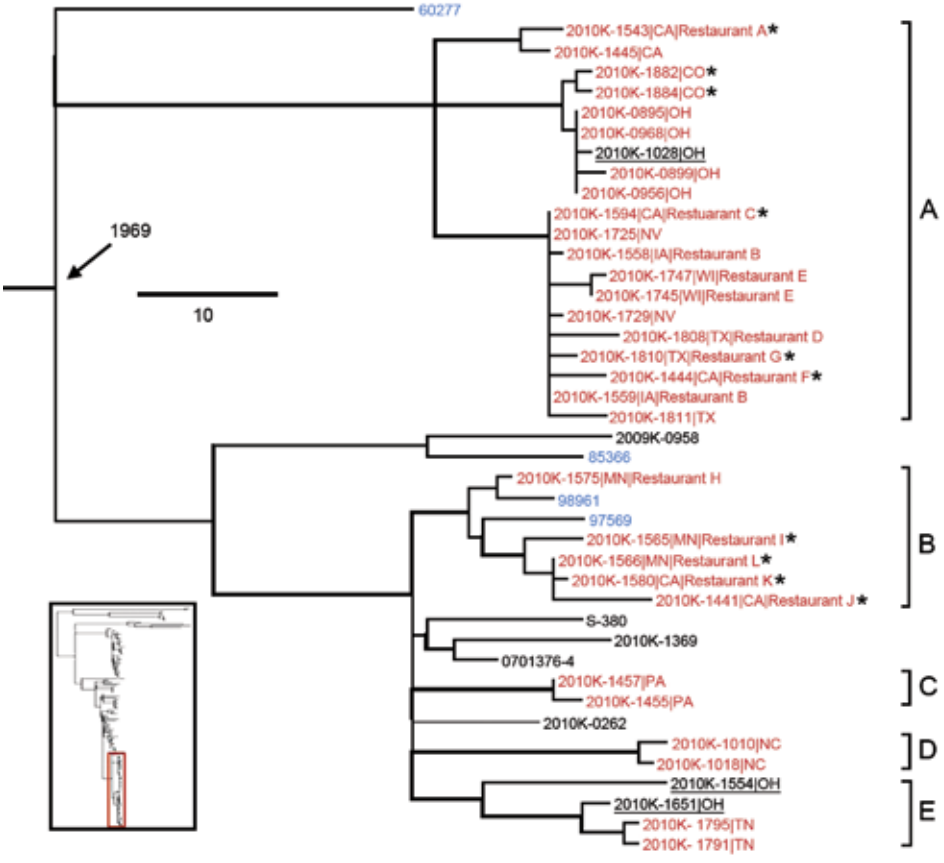

Figure 4. Salmonella enterica serotype Enteritidis clades associated with the 2010 US shelled eggs outbreak. Red indicates isolates sequenced as part of the outbreak investigation using Roche 454 technology (Indianapolis, IN, USA); blue indicates isolates associated with chicken or chicken products; asterisk (*) indicates the isolate was traced back to the implicated egg production facility. Five outbreak clades are highlighted and designated as A–E, of which A and B are definite and C, D, and E are putative. Isolates ascribed to the egg outbreak in this study are underlined. Branches with bootstrap values >0.9 are shown by thickened lines. Age of the ancestral node (median most recent common ancestor) is labeled. Scale bar indicates single-nucleotide polymorphisms. Inset indicates the location of the highlighted lineage on the global phylogenetic tree..

outbreak clade defined in another study (*18*), presumably because of differences in the definition of the scope of the outbreak between the 2 studies. Although a polyclonal outbreak is among the explanations for this discrepancy, the possibility of other scenarios cannot be rejected by available phylogenomic and epidemiologic evidence. For instance, in a case of continuous or intermittent outbreak, a single persistent founder strain can shed divergent descendants that contaminate food and cause disease over an extended time, as reported by Orsi et al. (*39*). Such microevolution events give rise to clones that might or might not be considered as distinct genotypes or separate outbreak clades depending on levels of mutation, epidemiologic information, or even subjective interpretation. Therefore, case-by-case understanding of evolutionary dynamics and population structure of major foodborne pathogens (*40*), which might vary among different species and be affected by particular food-processing environments and outbreak vehicles, is necessary for elucidating population genetics and transmission dynamics of foodborne pathogens and lays the groundwork for the increasing application of genomic epidemiology in pathogen surveillance and outbreak investigation. Ultimately, if some strains in an outbreak have continued to evolve quickly, then the ability to cluster strains and identify outbreaks will rely even more on a suitable set of outgroups.

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Pneumocystis jirovecii Pneumonia in Patients with or without AIDS, France

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Learning Objectives

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

1. Describe presentations of *Pneumocystis jirovecii* pneumonia, based on a prospective, multicenter observational study in France
2. Assess outcomes of *Pneumocystis jirovecii* pneumonia
3. Identify risk factors for mortality in *Pneumocystis jirovecii* pneumonia.

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Pneumocystis jirovecii pneumonia (PCP) in patients without AIDS is increasingly common. We conducted a prospective cohort study of consecutive patients with proven PCP; of 544 patients, 223 (41%) had AIDS (AIDS patients) and 321 (59%) had other immunosuppressive disorders (non-AIDS patients). Fewer AIDS than non-AIDS patients required intensive care or ventilation, and the rate of hospital deaths—17.4% overall—was significantly lower for AIDS versus non-AIDS patients (4% vs. 27%; p<0.0001). Multivariable analysis showed the odds of hospital death increased with older age, receipt of allogeneic bone marrow transplant, immediate use of oxygen, need for mechanical ventilation, and longer time to treatment; HIV-positive status or receipt of a solid organ transplant decreased odds for death. PCP is more often fatal in non-AIDS patients, but time to diagnosis affects survival and is longer for non-AIDS patients. Clinicians must maintain a high index of suspicion for PCP in immunocompromised patients who do not have AIDS.

Pneumocystis jirovecii pneumonia (PCP), caused by the fungus *P. jirovecii* (formerly *P. carinii*), is a life-threatening, opportunistic infection that is often the AIDS-defining illness in patients with HIV infection. Consequently, PCP has been extensively studied as a manifestation of the AIDS epidemic. However, in high-resource countries, the decrease in the prevalence of AIDS and the use of highly active antiretroviral therapy and routine primary PCP prophylaxis have diminished the number of patients with HIV-related PCP (*1*,*2*). At the same time, PCP has emerged as a concern in patients with non–HIV-related immune deficiencies. PCP has become more common among these patients as a result of treatment changes such as increasing use of immunosuppressive agents to treat malignancies, autoimmune diseases, and inflammatory diseases, as well as an increase in the number of solid organ transplants (SOTs) (*3*,*4*). Thus, patients who have hematologic or solid-organ malignancies or autoimmune or chronic inflammatory diseases, or those who have received an SOT or hematopoietic stem cell transplant (HSCT), are at high risk for PCP (*5*–*8*).

Whether these changes in PCP epidemiology have affected the clinical manifestation and outcome of the disease remains unclear. As early as 1989, a biological study that compared PCP in patients with and without AIDS found significant differences in fungi counts and lung inflammation (*9*). Another study evaluated clinical manifestations and outcomes of PCP in patients without AIDS seen during 1980–1993 (*10*) but did not include a comparison with AIDS patients. In a study comparing PCP in AIDS and non-AIDS patients in Basel, Switzerland, during 1982–1998, non-AIDS patients more often required intensive care and mechanical ventilation, although rates of death were not significantly different for the 2 groups (*11*). The Switzerland study and another comparison of AIDS and non-AIDS patients with PCP published in 1984 (*12*) found that symptom duration was longer and oxygen tension needs higher for AIDS patients.

These studies suggest that differences in PCP pathogenesis and influences of the underlying disease or treatment may affect the expression of PCP. However, recent data are lacking on the differences between clinical features and outcomes of PCP in AIDS and non-AIDS patients. Clinicians need this information to help identify patients who require prophylaxis and to enable early diagnosis of PCP at a stage when treatment is most likely to be effective. To obtain data on manifestations and outcomes of PCP in recent years and to identify risk factors for death, we performed a prospective, multicenter, observational study of consecutive patients with confirmed PCP admitted to 17 hospitals in France during 2007–2010.

Materials and Methods

Patients and Management

The appropriate ethics committee approved this study; informed consent was not required because of the observational design. For the study, the head mycologist at each of 17 university-affiliated hospitals in France prospectively included consecutive patients with confirmed PCP who were admitted during January 1, 2007–December 31, 2010. We defined confirmed PCP as a positive result for *Pneumocystis jirovecii* by Gomori-Grocott or toluidine blue stain or positive immunofluorescence test results (*4*) for a bronchoalveolar lavage (BAL) fluid or induced sputum specimen. Induced sputum testing or bronchoscopy with BAL was performed at the discretion of the clinicians by using previously described procedures (*13*,*14*). We did not include patients for whom only PCR results were positive.

For all included patients, chest radiographs were obtained; computed tomography (CT) scans were performed

when deemed necessary by clinicians. Bilateral interstitial or alveolointerstitial opacities on chest radiographs and diffuse ground-glass opacities on CT images were considered typical findings for PCP. Septal lines and centrolobular nodules were also interpreted to support a diagnosis of PCP. Focal consolidation, pleural effusion, subpleural nodules, and cavitation were considered atypical findings. Criteria for microbiologically documented pneumonia were as follows: clinical symptoms of pneumonia; pulmonary infiltrates; and ≥1 positive noncolonizing microbiological sample (i.e., blood culture, tracheal aspirate, sputa examination, BAL, protected sample, or pleural fluid). Urine antigens (*Streptococcus pneumoniae* and *Legionella pneumophila*) were included in routine testing for the microbiological documentation of pneumonia. When the microbiological data were negative but the patient had symptoms of pneumonia and pulmonary infiltrates, the case was classified as clinically documented pneumonia (*13*).

Data Collection

Data were collected prospectively. Steroid treatment was either high dose (>1 mg/kg for >1 mo) or long term (>3 mo at any dose) (*3*,*14*). Mycologists and study investigators obtained missing and follow-up data by reviewing patients' medical charts and by interviewing the specialists who provided usual care to the patients. Bacterial, viral, fungal, and parasitic infections were diagnosed on the basis of criteria reported elsewhere (*13*). Information on PCP prophylaxis was recorded; trimethoprim-sulfamethoxazole (TMP-SMX), aerosolized pentamidine (1×/mo), dapsone, and atovaquone were classified as effective prophylaxis options (*3*,*4*). Information on treatments used for PCP and the time from admission to treatment initiation were recorded; TMP-SMX, pentamidine, atovaquone, dapsone, and clindamycin-primaquine were considered acceptable options for PCP treatment (*4*). Shock was defined as persistent hypotension despite appropriate fluid load, requiring treatment with a vasopressive drug.

Steroids as adjunctive therapy were used on the basis of standard protocol recommendations for patients with AIDS at a dose that depended on patient location (e.g., medical unit). In deeply hypoxemic, critically ill patients, steroids were implemented at the dosage of 240 mg/day for 3 days, then at 1 mg/kg/day for 7 days, followed by tapering doses to be stopped before day 21 (*15*,*16*). In patients who were less critically ill, the dose was 1 mg/kg/day followed by a tapering dose after day 7 to be stopped before day 21. Similar protocols were used for AIDS and non-AIDS patients.

Statistical Analyses

The variables in the dataset are described or summarized by using either median and interquartile range or number and proportion of the total (%). Categorical variables were compared by using the Fisher exact test and continuous variables by using the nonparametric Wilcoxon test or Mann-Whitney test for pairwise comparisons. All tests were 2-sided, and $p<0.05$ was considered statistically significant. Kaplan-Meier curves and log-rank tests were used to compare hospital death rates between groups of patients. Logistic regression analysis was used to identify variables significantly associated with hospital deaths by estimating the odds ratios (OR) with 95% CIs. Variables yielding p values <0.20 in the univariable analyses were entered into a multivariable logistic regression model with stepwise variable selection using an automatic procedure based on the Akaike Information Criterion, with hospital deaths as the variable of interest. The co-variates were entered into the model with a p value cutoff for removal of 0.1. Co-linearity and interactions were tested. For the multivariable analysis, missing data were handled by using multiple imputation with chained equations. The imputed missing data focused on 3 variables: oxygen saturation on admission (35 patients), time to treatment (17 patients), and time from respiratory symptom onset to diagnosis (11 patients). The Hosmer-Lemeshow test was used to check goodness-of-fit of the logistic regression model.

Results

Causes of Immunodeficiency

We included 544 patients in the study. Median age was 51 (interquartile range 40–62) years, and 370 (68%) were men A total of 223 (41%) patients had AIDS and 321 (59%) had other immunosuppressive conditions (Figure 1). Among patients with AIDS, PCP was the first manifestation of AIDS for 105 (44.8%); only 4 (5.0%) had CD4 lymphocyte counts >200 cells/mm^3. As shown in Table 1, the main causes of immunodeficiency in the non-AIDS patients were SOT (n = 99, 30.8%), chiefly of a kidney (80/99); and hematologic malignancies (n = 84, 26.2%), chiefly lymphoproliferative diseases (72/84). Twenty-seven (8.4%) patients were HSCT recipients (14 allogeneic, 13 autologous); 65 (20.2%) had autoimmune or chronic inflammatory disease; and 46 (14.3%) had solid-organ malignancies.

At the time of PCP diagnosis, high-dose or long-term steroid therapy was the most common immunosuppressive treatment for non-AIDS patients: 88% (n = 63) for those with SOTs, 85% (n = 71) for those with hematologic malignancies, 100% (n = 13) for those with HSCTs, 91% (n= 41) for those with solid-organ malignancies, and 89% (n = 52) for those with autoimmune or chronic inflammatory disease. SOT recipients were also receiving anticalcineurin agents (n = 59, 82%), purine inhibitors (n = 56, 77%), rituximab (n = 1, 6%), mechanistic target of rapamycin inhibitors (n = 6, 8%), tumor necrosis factor–α antagonists (n = 3,

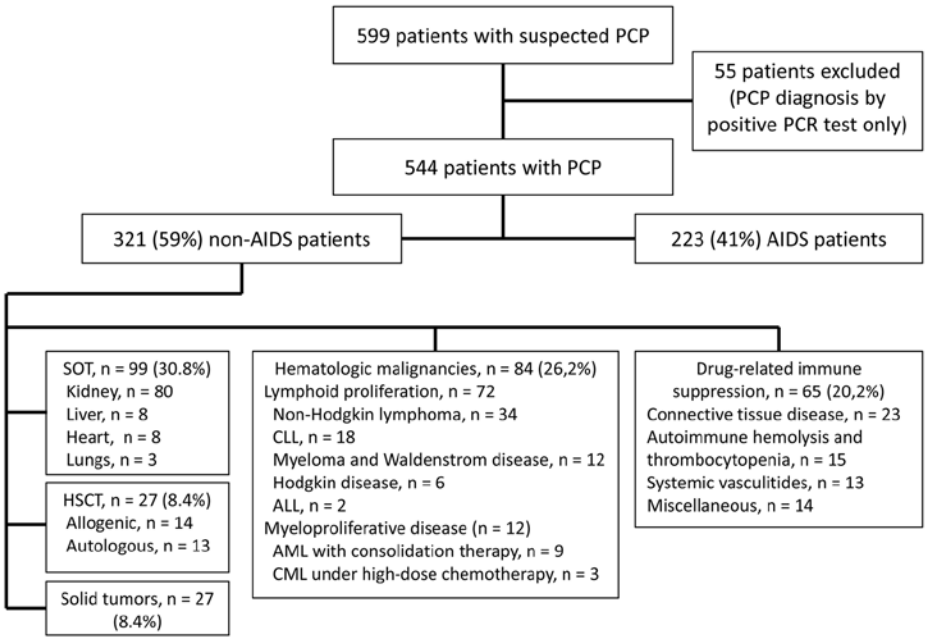

Figure 1. Flowchart of selection of patients with *Pneumocystis jirovecii* pneumonia (PCP) for study and underlying conditions among non-AIDS patients, France, January 1, 2007–December 31, 2010. Miscellaneous conditions: inflammatory diseases or automimmune (n = 4); common variable immunodeficiency (n = 2); focal segmental glomerulosclerosis (n = 2); sarcoidosis (n = 1); steroid-dependent asthma (n = 1); idiopathic pulmonary fibrosis (n = 1); acute alcoholic hepatitis (n = 3). ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphoid leukemia; CML, chronic myeloid leukemia; HSCT, hematopoietic stem cell transplant; SOT, solid organ transplant.

4%), or intravenous immunoglobulins (n = 1, 1%). Patients with hematological malignancies also had received rituximab (n = 29, 35%) and fludarabin (n = 8, 9.5%). All HSCT patients were receiving anticalcineurin agents. Patients with autoimmune or chronic inflammatory diseases were receiving methotrexate (n = 14, 21.5%), rituximab (n = 6, 9.2%), tumor necrosis factor–α antagonists (n = 6, 9.2%), anticalcineurin agents (n = 5, 7.7%), or purine inhibitors (n = 4, 6.1%). (Note that values are approximate; data are missing among each subpopulation.)

Clinical Manifestations of PCP

The clinical manifestations of PCP in AIDS and non-AIDS patients are shown in Table 2 and Figure 1. Overall,

Table 1. Clinical characteristics of 544 patients with and without AIDS at diagnosis with PCP, France, January 1, 2007–December 31, 2010*

Characteristic	AIDS patients, n = 223	Non-AIDS patients, n = 321	p value
Clinical features			
Prophylaxis prescribed†	3 (1)	12 (4)	0.06
Temperature >38°C	165 (74)	263 (82)	0.05
Days from constitutional symptom onset to diagnosis, median (IQR)	30 (14–60)	7 (2–15)	<0.0001
Shock	5 (2.2)	23 (7)	0.01
Respiratory symptoms			
Cough	170 (76.2)	173 (54)	<0.0001
Dyspnea	176 (79)	234 (73)	0.10
Days from respiratory symptom onset to diagnosis, median (IQR)	21 (7–30)	5 (1–15)	<0.0001
Laboratory test results			
SpO_2, median (IQR)	95 (90–97)	91 (86–96)	0.003
Lymphocyte count, cells/mm^3, median (IQR)	802 (499–1,200)	500 (278–880)	0.0004
CD4+ T-cell count, cells/mm^3, median (IQR)	167 (89–342)	32 (12–75)	<0.0001
C-reactive protein	48 (17–128)	120 (59–210)	<0.0001
Radiologic findings			
Chest radiograph results typical for PCP	183 (82)	247 (77)	0.23
Chest radiograph results atypical for PCP‡	31 (14)	48 (15)	0.66
Pneumothorax	7 (3.1)	7 (2.2)	0.50
Chest radiograph results unremarkable	9 (4)	26 (8)	0.34
Atypical computed tomography scan pattern§	15 (14)	22 (14)	0.47

*Values are no. (%) patients except as indicated. Data were missing for CD4+ T-cell count (372 patients), C-reactive protein (274 patients), and CT scan pattern (265 patients). PCP, *Pneumocystis jirovecii* pneumonia; IQR, interquartile range.
†Adherence to prescribed prophylaxis was unknown. Among already known HIV+ patients without prophylaxis and with available CD4+ T cell count (n = 74), median CD4+ T cell count was 33 cells/mm^3 (range 12–85).
‡Defined as focal interstitial or alveolar consolidation.
§Defined as subpleural nodules, focal condensation, cavitation, or marked pleural effusion.

Table 2. Clinical management of 544 AIDS and non-AIDS patients after diagnosis with PCP, France, January 1, 2007–December 31, 2010*

Characteristic	AIDS patients, n = 223	Non-AIDS patients, n = 321	p value
Days from admission to treatment initiation, median (IQR)	1 (0–2)	2 (0–6)	<0.0001
Intensive care admission	65 (35)	134 (50)	0.0015
Immediate oxygen needed	87 (49)	160 (69)	<0.0001
Oxygen flow rate, L/min, mean (95% CI)	2 (1.3–2.8)	3.8 (2.8–4.8)	0.015
Mechanical ventilation			
Noninvasive needed	17 (8)	50 (16)	0.0053
Noninvasive failed	16 (8)	46 (15)	0.013
Invasive needed	25 (11.0)	98 (30.5)	<0.0001
Hospital deaths	8 (4)	75 (27)	<0.0001

*Values are no. (%) patients except as indicated. PCP, *Pneumocystis jirovecii* pneumonia; IQR, interquartile range.

96% of patients were not receiving PCP prophylaxis. The median time from the onset of respiratory symptoms to PCP diagnosis was significantly shorter for non-AIDS patients (5 [range 1–15] days) than for AIDS patients (21 [7–30] days) (p<0.0001). However, as shown in Tables 1 and 2, hypoxemia was more severe in non-AIDS patients, who required higher oxygen flow rates (4 [range 3–5] L/min) than did AIDS patients (2 [1.3–3] L/min). Non-AIDS patients also more often required intensive care and noninvasive or invasive ventilation. Shock was more common in non-AIDS patients and was significantly associated with pulmonary microbial co-infection (OR 3.09 [95% CI 1.44–6.68]; p = 0.004), in agreement with the presence of microbiologically documented pneumonia (*13*) in half of the patients with shock. CT scans were obtained for 279 patients; typical findings were seen in 37 patients, of whom 29 were believed to have pulmonary bacterial microbial co-infection and 8 microbial co-infections with a second opportunistic microorganism.

Diagnosis and Treatment of PCP

A BAL sample was diagnostic for 87% and 97% of AIDS and non-AIDS patients, respectively (p = 0.0003). Overall, microbial co-infection as previously defined (*13*) was suspected or confirmed for 169 (31%) patients, including 92 (16.9%) with bacterial infection, 65 (6.6%) with viral infection, and 38 (6.9%) with fungal infection. For 32 (5.8%) patients, ≥2 microbial co-infections were identified; the most frequent site of microbial co-infection was the lung (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/20/9/13-1668-Techapp1.pdf). Univariable analysis showed an association between microbial co-infection and death (OR 2.29, 95% CI 1.41–3.71) (online Technical Appendix Figure), but multivariable analysis did not confirm this finding (OR 1.99, 95% CI 0.91–4.33; p = 0.09).

Time from hospital admission to the start of treatment for PCP was longer for non-AIDS patients than for AIDS patients (2 [range 0–6]) days vs. 1 [0–2] days; p<0.0001). TMP-SMX was the first-line anti-PCP agent used for 97% of patients. However, patients with AIDS more often required a switch to second-line therapy than did non-AIDS patients (25.5% vs. 7%; p<0.0001); this change was most often made because of allergic reactions or hepatic or renal toxicity. Adjunctive steroid therapy was used in 40.4% of patients overall and in a significantly higher proportion of AIDS than in non-AIDS patients (55% vs. 43%; p = 0.01).

Risk Factors for Death

Hospital death data were available for 478 (88%) patients, and the percentage of hospital deaths was significantly higher for non-AIDS than AIDS patients (27% vs. 4%; p<0.0001). For non-AIDS patients, death rates varied by cause of immunodeficiency, from a low of 3.75% for kidney transplant recipients to a high of 43% for allogeneic HSCT recipients. Invasive mechanical ventilation was used in 28% and 43% of patients in these 2 groups, respectively (p<0.0001).

Of 7 variables independently associated with hospital death by multivariable analysis, 2 were associated with lower death rates: AIDS diagnosis (OR 0.33, 95% CI 0.12–0.92) and receipt of a SOT (OR 0.08, 95% CI 0.02–0.31) (Table 3). Five variables were associated with increased death rates: older age (OR 1.04/additional year, 95% CI 1.02–1.06); receipt of a HSCT (OR 8.6, 95% CI 1.40–53.02); need for oxygen on admission (OR 4.06, 95% CI 1.44–11.5); need for invasive mechanical ventilation (OR 16.70, 95% CI 7.25–38.47); and longer time from

Table 3. Multivariate analysis of independent predictors of hospital death for AIDS and non-AIDS patients with PCP, France, January 1, 2007–December 31, 2010*

Variable	Odds ratio (95% CI)
HIV infection	0.33 (0.12–0.92)
Solid organ transplant	0.08 (0.02–0.31)
Age, per additional year	1.04 (1.02–1.06)
Allogeneic HSCT	8.6 (1.40–53.02)
Need for immediate oxygen therapy	4.06 (1.44–11.5)
Need for intubation and mechanical ventilation	16.70 (7.25–38.47)
Time to PCP treatment, per additional day	1.11 (1.04–1.18)

*Variables from Tables 1 and 2 were introduced into the multivariate model based on their association with hospital mortality by bivariate analysis with p values <0.20. Higher numbers indicate increased odds, with 1 as the cutoff point (i.e., values <1.00 indicate decreased risk, >1.00 increased risk). Goodness of fit (Hosmer-Lemeshow test) was 0.61. PCP, *Pneumocystis jirovecii* pneumonia; HSCT, hematopoetic stem cell transplant.

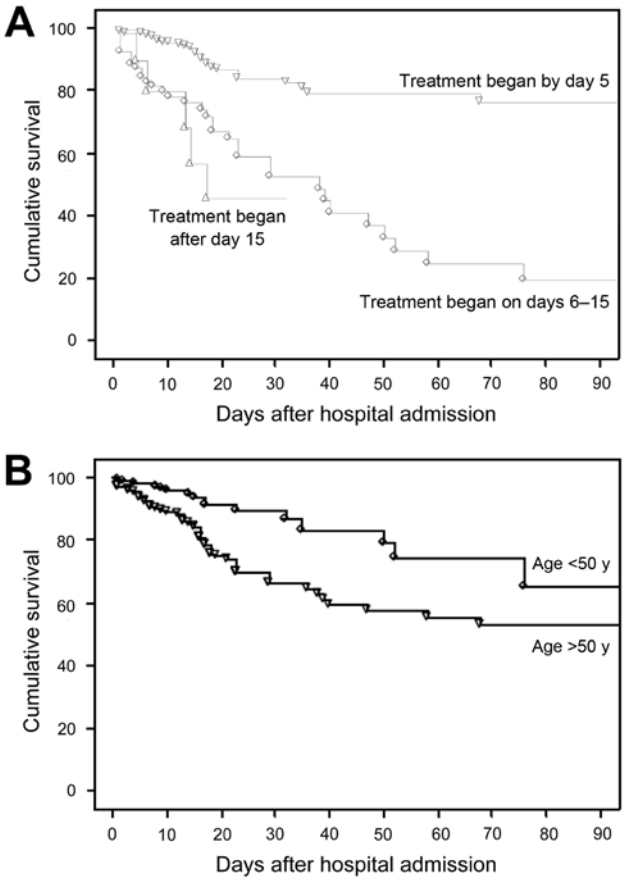

Figure 2. Survival in 544 patients with *Pneumocystis jirovecii* pneumonia by A) number of days from admission to treatment initiation and B) underlying disease (AIDS versus non-AIDS), France, January 1, 2007–December 31, 2010. p<0.0001 by log-rank test for both comparisons.

admission to initiation of PCP treatment (OR 1.11/additional day, 95% CI 1.04–1.18).

Improved cumulative survival was significantly associated with underlying condition (p<0.0001 for AIDS vs. non-AIDS comparison; Figure 2). Shorter time from admission to treatment initiation was also associated with improved cumulative survival (Figure 2).

Discussion

This multicenter, prospective study describes the current picture of PCP in immunocompromised patients with or without AIDS in a high-resource country. In this cohort, AIDS-related PCP was less common than was PCP associated with other types of immunosuppression. Our findings confirm several differences between AIDS and non-AIDS patients in clinical presentation and outcomes related to PCP, as described by Kovacs et al. (*12*). The progression of PCP was faster for non-AIDS patients; these patients had a significantly shorter time from onset of symptoms to diagnosis but still experienced a faster progression of illness, including more severe hypoxemia, greater need for intensive care and invasive mechanical ventilation, higher prevalence of shock, and a longer time to PCP treatment initiation. Death rates were also significantly higher for non-AIDS patients, and one of the variables independently associated with death was longer time to PCP treatment initiation for non-AIDS patients.

This study offers several contributions toward the development of PCP prophylaxis guidelines for specific at-risk groups. One of the reasons for the lower proportion of AIDS patients than of non-AIDS patients in this study population could have been the widespread use of highly active antiretroviral therapy and PCP prophylaxis among AIDS patients (*1*,*2*). However, among patients with AIDS in our study, only 3 (2.7%) were receiving PCP prophylaxis; for 100 (44.8%) patients, the PCP diagnosis was the reason for the AIDS diagnosis.

In our study, PCP treatment was started later after admission in non-AIDS patients than in AIDS patients, and longer time to treatment independently predicted odds for death, which is in agreement with findings of an earlier study (*12*). Longer time to treatment was the only predictor of death in our study that could be mitigated. Treatment initiation differed by only 1 day for AIDS versus non-AIDS patients, yet this difference was associated with reduced death rates for AIDS patients. Therefore, because routine implementation of PCP treatment on admission may be associated with higher survival, clinicians should implement treatment as soon as the diagnosis is suspected, without waiting the 2 days required to confirm the diagnosis.

Our findings indicate that PCP prophylaxis could improve outcomes for high-risk patients without AIDS. Among non-AIDS patients in this study, 99 (30.8%) were SOT recipients, a population for which recent guidelines recommend PCP prophylaxis for 6–12 months, a period that might be extended on the basis of level of immunosuppression and immunosuppressive drug requirements (*18*,*19*). Despite this recommendation, however, recent studies suggest that 1 month of prophylaxis would be sufficient for kidney transplant patients (*20*). Given the high rate of death in our cohort, this conclusion should be challenged. Maintaining a high index of suspicion for PCP in immunocompromised patients appears to be of the utmost importance. In addition, as with AIDS patients, every effort should be made to ensure compliance with PCP prophylaxis in non-AIDS patients. Non-AIDS patients may be less aware than AIDS patients that they are at risk for PCP. This point may be of particular relevance, as the course of PCP was significantly more acute in non-AIDS patients, with a median symptom duration of 5 (range 1–15) days compared with 21 (7–30) days for AIDS patients (p<0.0001). Educating non-AIDS patients about

PCP might result in earlier medical evaluation and hospital admission and, consequently, in shorter lengths of time to PCP diagnosis and treatment. In addition, development of rapid and minimally invasive diagnostic tests could improve the early diagnosis and treatment of PCP (*21*).

We found marked differences in death rates across patient groups. Deaths were lowest for AIDS patients and highest for HSCT recipients, and rates in our study were consistent with earlier data (*6*,*12*). Microbial co-infection rates in our study were also in agreement with earlier data (*22*). More than one fourth of our patients overall had microbial co-infection, which indicates a need for comprehensive diagnostic investigations in patients with PCP and for routine broad-spectrum antimicrobial drug therapy when findings are atypical for PCP.

Adjunctive steroid therapy has been proven to increase survival in AIDS patients with severe PCP (*23*,*24*), but 2 small retrospective studies found that adjunctive steroids had no effect on survival for non-AIDS patients (*17*,*25*). In our study, although non-AIDS patients had more severe hypoxemia and more often required invasive mechanical ventilation, they received adjunctive steroid therapy significantly less often than did AIDS patients.

Our study has several limitations. First, the patients were recruited at university hospitals, which may have influenced the distribution of risk factors for PCP. However, most of these risk factors are associated with diseases that require management in university hospitals. Our data obtained for consecutive patients from 17 centers are probably representative of PCP in other countries where optimal AIDS treatment and critical care are widely available. In addition, the diagnosis and therapeutic management of these patients was not standardized, and variations in testing and treatment strategies may have affected outcomes and determinants of death. Moreover, the diagnostic strategy may have been different for AIDS versus non-AIDS patients, as well as for critically ill patients versus non–critically ill patients; these differences could have resulted in different proportions of patients with documented microbial superinfections. However, our objective was to describe all PCP patients seen during the past few years, and we found no effect of the center at which a patient was treated on mortality rates (data not shown). This study also included only episodes of PCP for which a patient was hospitalized. AIDS patients with mild PCP episodes could be treated as outpatients, and the prognosis and characteristics for these patients may vary substantially from those of our study population. Last, we included only PCP cases proven by tinctorial or immunofluorescence staining. PCP may be present in some patients who have positive PCR test results for *P. jirovecii* but for whom stain results are negative or unavailable (*26*). However, because isolated PCR test positivity can indicate colonization and not infection (*27*), we confined our study to cases of confirmed infection to maximize the validity of our data.

In summary, PCP occurs in patients with a range of conditions associated with immunosuppression. For AIDS patients, efforts should focus on improving the early detection of HIV infection and adherence to PCP prophylaxis. For non-AIDS patients, guidelines regarding PCP risk evaluation and prophylaxis are needed. We found higher death rates and longer time from hospital admission to initiation of PCP treatment for non-AIDS patients. Clinicians must maintain a high index of suspicion for PCP in immunocompromised patients who do not have AIDS, and these patients should be educated about the early symptoms that can indicate PCP. Treatment should be implemented early in high-risk patients, even before appropriate diagnostic tests are completed.

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Passive Surveillance for Azole-Resistant *Aspergillus fumigatus*, United States, 2011–2013

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Emergence of *Aspergillus fumigatus* strains containing mutations that lead to azole resistance has become a serious public health threat in many countries. Nucleotide polymorphisms leading to amino acid substitutions in the lanosterol demethylase gene (*cyp51A*) are associated with reduced susceptibility to azole drugs. The most widely recognized mutation is a lysine to histidine substitution at aa 98 (L98H) and a duplication of the untranscribed promoter region, together known as TR_{34}/L98H. This mechanism of resistance has been reported in Europe, Asia, and the Middle East, and is associated with resistance to all azole drugs and subsequent treatment failures. To determine whether isolates with this mutation are spreading into the United States, we conducted a passive surveillance–based study of 1,026 clinical isolates of *A. fumigatus* from 22 US states during 2011–2013. No isolates harboring the TR_{34}/L98H mutation were detected, and MICs of itraconazole were generally low.

Azole antifungal drugs are the first line of therapy against *Aspergillus fumigatus*, a common etiologic agent of aspergillosis. These drugs are used as empirical prophylaxis and as targeted therapy for invasive aspergillosis. Resistance to azole drugs has been associated with treatment failure and deaths in patients with aspergillosis. In the past 2 decades, azole resistance in *A. fumigatus* has been documented in many regions (*1–7*). The high prevalence of azole resistance in *A. fumigatus* has prompted the European Centre for Disease Prevention and Control to increase the risk level of this organism to a public health risk (*8*).

A common mechanism that confers resistance to azole drugs is a mutation in the lanosterol 14 α-demethylase gene that encodes the CYP51A protein. This protein is the primary target of azole drugs and sterol demethylation inhibitor (DMI) fungicides (*9–11*). However, not all *cyp51A* mutations contribute to azole resistance (some are benign), and not all azole resistance is caused by mutations in the *cyp51A* gene (*12*).

In the past decade, novel *cyp51A* promoter duplication mutations, especially the cooperative pair of *cyp51A* mutations known as TR_{34}/L98H, have emerged as a predominant azole-resistance mechanism in *A. fumigatus* (*1,4,6,10,11,13–22*). Isolates harboring the TR_{34}/L98H mutation are cross-resistant to multiple azoles (*4,15,17*). A second *cyp51A*-promoter duplication genotype, the recently discovered TR_{46}/Y121F+T289A, also displays high tolerance for voriconazole (*13,14*). Both genotypes are associated with in vitro MICs that exceed by multiple dilutions the established epidemiologic cutoff value (ECV) of ≤1 μg/mL published by the European Committee on Antimicrobial Susceptibility Testing and Clinical and Laboratory Standards Institute members for medical azole agents (*9,13*). Moreover, these mutations often contribute to failure of azole therapy in aspergillosis patients (*13–23*). Their presence rules out the use of voriconazole, the treatment recommended by the Infectious Diseases Society of America for aspergillosis, and leaves amphotericin B, an antifungal drug with toxic side effects, as the primary therapy choice.

Since the discovery of the TR_{34}/L98H mutation in the Netherlands by Verweij et al. in 2007 (*15*), other countries in Europe, including Austria, Belgium, Germany, Spain, France, the United Kingdom, Denmark, and Norway, have also reported isolates harboring TR_{34}/L98H (*5,7,16–20,24*). *A. fumigatus* isolates with the TR_{34}/L98H mutation have also been reported in China, India, and Iran (*1,4,6,21*). Because of rapid increase in detection of the TR_{34}/L98H mutation in many regions and its adverse effect on patient management, isolates harboring this mutation pose a serious public health threat. Isolates bearing the TR_{34}/L98H mutation have not been documented in United States, but

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given the number of aspergillosis cases in this country, its presence could pose a serious public health threat, as it does in Europe.

The TR_{34}/L98H mutation is not associated with use of long-term medical azole therapy in patients. Instead, prolonged exposure of the fungus to sterol DMI fungicides in the environment, in conjunction with sexual reproduction, has probably led to dissemination of this mutation (*19,25*). As in many countries in which this genotype has been reported, DMI fungicides are used in various agricultural practices across the United States. According to the US Department of Agriculture data for 2010–2013, DMI fungicides, such as tebuconazole, propiconazole, prothioconazole, tetraconazole, metconazole, and epoxiconazole, have been used by several agricultural producers (*26*). This shared agriculture practice indicates that the TR_{34}/L98H mutation could potentially arise in the United States.

Thus, the Centers for Disease Control and Prevention initiated passive surveillance for *A. fumigatus* isolates in 2011 to identify resistance to the representative azole itraconazole. More than 1,000 clinical isolates from 22 states were screened by using an itraconazole antifungal plate assay and the Etest to measure itraconazole MICs. For isolates that require an MIC greater than the ECV, DNA sequence analysis of the *cyp51A* gene did not yield any isolates with the TR_{34}/L98H genotype.

Materials and Methods

Fungal Isolates

A. fumigatus isolates were obtained through the American Society for Microbiology listservs (DivC) and (ClinMicroNet) (http://www.asm.org/index.php/online-community-groups/listservs) and through the US Association of Public Health Laboratories. The request for isolates read as follows: "To determine the US level of *A. fumigatus* azole resistance, we are requesting submission of: 1) All new or stored isolates of *Aspergillus fumigatus* from US collections; 2) Any new isolates of *A. fumigatus* regardless of clinical relevance." Isolates were sent to the Centers for Disease Control and Prevention where the species was confirmed by colony color and morphologic features observed by microscopy. Cryptic species that required increased MICs were subsequently ruled out by DNA sequencing of the *cyp51A* gene as described below.

Itraconazole Susceptibility Assay

All isolates were screened by using 2 methods: antifungal plate culture and itraconazole Etest (bioMérieux, Marcy l'Etoile, France). For plate culture, a 24-well culture plate containing 1 mL of Sabouraud agar supplemented with 4 μg/mL of itraconazole was used. Twenty microliters of a suspension containing $\approx 2 \times 10^4$ conidia were transferred to each well and allowed to dry at ambient temperature for 30 min before incubation. Etest determination of itraconazole MICs was performed as described by Pfaller et al. (*27*) with minor modifications. Isolates of *A. fumigatus* were allowed to sporulate on potato dextrose agar slants for 5–7 days. Conidial suspensions were prepared in sterile distilled water containing 1% Tween-20. The concentration was adjusted to an optical density of 0.09–0.13 or $\approx 10^6$ conidia/mL equivalent at 530 nm. An RPMI 1640 agar plate was inoculated by streaking a conidia-laden cotton swab bidirectionally across the surface of the plate. An itraconazole Etest strip was placed across the central surface of the freshly inoculated RPMI plate. An azole-resistant and an azole-susceptible isolate for which broth microdilution azole MICs were known were used as controls (*1*). The Etest and itraconazole plates were incubated at 37°C, and results were recorded 48 hours postinoculation.

Genetic Profiling of *cyp51A*

DNA sequence analysis of the *cyp51A* gene and its promoter region was performed for all isolates that required an MIC above the ECV (1 μg/mL) by using primers described (*1*). A subset of the first 561 isolates was also examined by using a mixed-format real-time PCR to detect nucleotide polymorphisms that cause single amino acid substitutions at Gly54, Leu98, Gly138, and Met220, and the recently discovered TR_{46}/Y121F/T289A in the CYP51A protein as described by Klaassen et al. (*28*).

A. fumigatus genomic DNA was purified by using the DNeasy blood and tissue kit (QIAGEN, Valencia, CA, USA) with some modifications. Hyphae were transferred to a 1.5-mL microcentrifuge tube containing 360 mL of Z-buffer (*29*) (60 mmol/L Na_2HPO_4, 40 mmol/L NaH_2PO_4, 10 mmol/L KCl, 1 mmol/L $MgSO_4$, 38 mmol/L b-mercaptoethanol, pH 7) and 40 mL of proteinase K (QIAGEN). A total of 250 mg of 0.5-mm zirconia beads (BioSpec, Bartlesville, OK, USA) was added to the cell suspension, and cells were homogenized for 1 min at maximum speed by using a mini-beadbeater (BioSpec). Four hundred microliters of AL buffer (QIAGEN) was added to the cell lysate and mixed by vortexing for 10 s. The lysate was then centrifuged at 13,200 rpm for 5 min. The supernatant was transferred to a clean 1.5-mL microcentrifuge tube, and an equal volume of absolute ethanol was added to the supernatant. After briefly undergoing vortexing, the DNA suspension was transferred to a spin column (QIAGEN). DNA binding, washes, and elution steps were followed as described by the manufacturer.

Results

Characteristics of *A. fumigatus* Isolates

A request for *A. fumigatus* clinical isolates collected beginning in 2011 was initiated in June 2011. Because the

TR_{34}/L98H mutation was believed to be environmentally induced, all *A. fumigatus* clinical isolates were accepted, not just those causing infection. A total of 1,026 clinical isolates were obtained during September 2011–September 2013 from hospitals, clinics, and state public health laboratories across 22 states (Figure 1), including Arizona (n = 17), California (n = 139), Connecticut (n = 140), Florida (n = 78), Georgia (n = 133), Iowa (n = 56), Illinois (n = 111), Indiana (n = 13), Kansas (n = 3), Massachusetts (n = 2), Maine (n = 52), Michigan (n = 105), Minnesota (n = 84), Missouri (n = 7), Montana (n = 6), North Carolina (n = 9), New York (n = 15), Oregon (n = 35), Tennessee (n = 5), Texas (n = 8), Virginia (n = 1), Wyoming (n = 1), and unknown (n = 7). The isolates were form respiratory tracts (≈57%), ears (≈4%), other tissues (≈5%), and unknown sources (≈34%). Of respiratory tract isolates, 65% were collected from sputum and 25% from bronchoalveolar lavage specimens.

Most *A. fumigatus* isolates were susceptible to itraconazole. Historically, the TR_{34}/L98H mutation has been detected by itraconazole plate assay. We also used the Etest validate its utility for detecting high MICs. The same 3 isolates were identified by their growth on the itraconazole plate and an itraconazole Etest MIC >4 μg/mL, which indicated congruence for the 2 tests. Overall MICs ranged from 0.05 μg/mL to 32 μg/mL, and 95% of the isolates required an MIC ≤1 μg/mL) (Figure 2). The 50% MIC (MIC_{50},), MIC_{90}, and MIC mode for isolates were 0.5 μg/mL, 1.0 μg/mL, and 0.5 μg/mL, respectively. Of 51 isolates that required an increased MIC for itraconazole, >94% (n = 48) required an MIC <4 μg/mL and were negative by agar screening, and 3 isolates required MICs of 6 μg/mL (n = 1), 16 μg/mL (n = 1), or ≥32 μg/mL (n = 1), the maximum value on the Etest strip.

Genetic Analysis of the *cyp51A* Gene

DNA sequence analysis of the *cyp51A* gene was performed for 51 isolates that required an itraconazole MIC >1 μg/mL and for 36 isolates that required an itraconazole MIC ≤1 μg/mL. The duplication mutations (TR_{34} and TR_{46}) in the promoter region of *cyp51A* and the L98H or Y121F/T289A substitutions in *cyp*51A were not detected. Eighteen (35%) of the 51 isolates that required an increased MIC had a *cyp51A* mutation. The most common mutation in this group was a novel valine→isoleucine substitution at residue 242 (I242V), which was found in 13 of the isolates that required increased MICs and 2 of the control isolates; all required MICs ≤4 μg/mL. There was also a set of 4 linked mutations in 2 of the control isolates that are commonly seen in susceptible isolates (*1,3*). The only mutation found that has been associated with azole treatment failure was an M220I mutation in the isolate that required an MIC ≥32 μg/mL. A mixed-format real-time PCR was also used to screen the first 561 isolates collected for other known resistance-inducing *cyp51A* single nucleotide polymorphisms. Except for the M220I mutation, no other polymorphisms were identified by using this method.

Discussion

Azoles, specifically voriconazole and (in some instances) itraconazole, are the recommended treatment for aspergillosis (*30*). These drugs have a high potency against the most common causes of aspergillosis (i.e., *A. fumigatus*, *A. niger*, *A. flavus*, *and A. terreus*). However, reduced

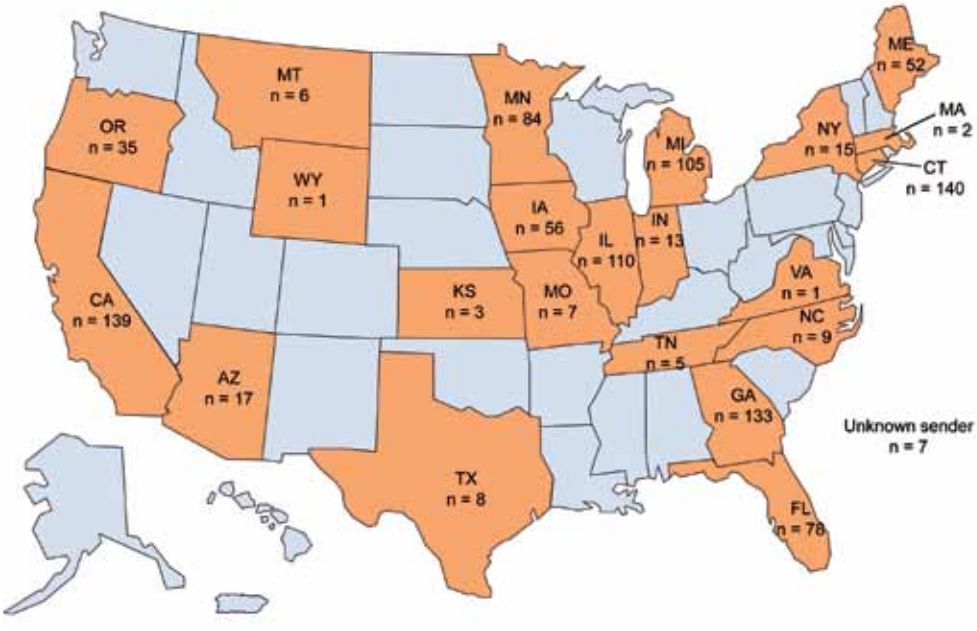

Figure 1. Distribution of *Aspergillus fumigatus* isolates, United States, 2011–2013. A total of 1,026 clinical isolates were received from 22 states during October 2011–October 2013.

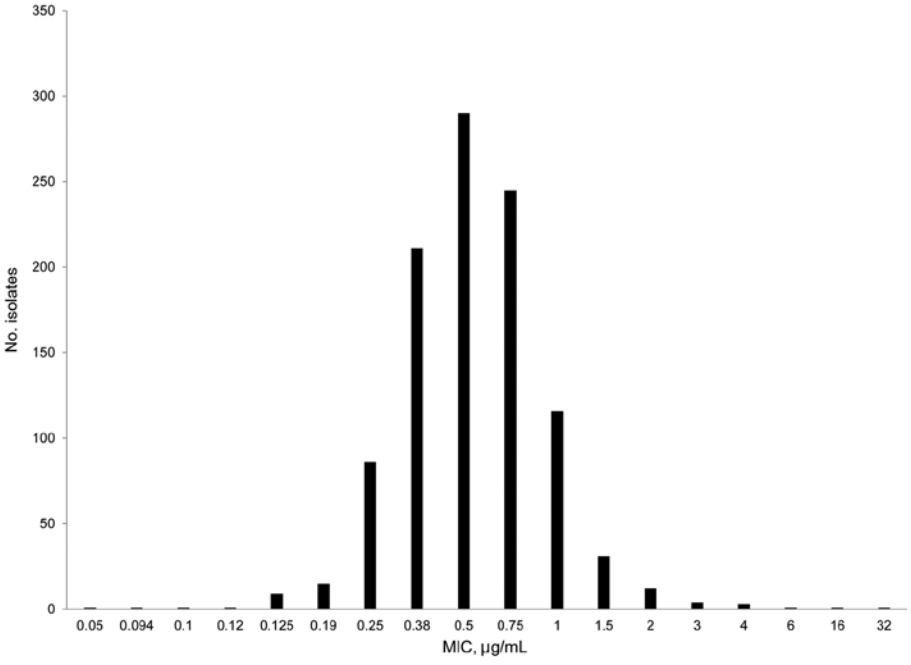

Figure 2. Itraconazole susceptibility profile for *Aspergillus fumigatus* isolates, United States, 2011–2013. The MIC (μg/mL) required by each isolate was determined by using the Etest method. Approximately 5% of the isolates require an MIC higher than the established epidemiologic cutoff value of 1 μg/mL.

susceptibility to these drugs has been reported (*3,7,15,18*). Many triazole-resistant isolates from aspergillosis patients who showed treatment failure with triazole therapy have mutations within the CYP51A protein (*31–35*), but this is not the only mechanism of resistance. The ECV was established to define the limits of the wild-type distribution of MICs. It does not define a breakpoint but it may help identify isolates harboring mutations. In *A. fumigatus*, 2%–20% of isolates, depending on the country, require MICs above the established ECVs for azoles (*2–4,31,32,36*). In this study, ≈5% of the isolates required an MIC greater than the established ECV for itraconazole. Isolates with no *cyp51A* mutations but which require high azole MICs are not uncommon. The mechanisms of resistance in these isolates are not precisely known but are assumed to be changes in drug transporter pumps (*37,38*).

Azole resistance is typically associated with *cyp51A* mutations, especially the TR_{34}/L98H and TR_{46}/Y121F/T289A dual mutations. A total of 26%–94% of azole-resistant isolates, especially in the Netherlands, have these mutations (*1,15,20,31*). However, we found no isolates harboring the TR_{34}/L98H mutation in this study. One isolate was found to have an amino acid substitution at M220 and an itraconazole MIC ≥32 μg/mL. Amino acid substitutions at the M220 residue in *A. fumigatus* have been shown to confer resistance to all 3 mold-active azoles and are known to be induced by prolonged use of azoles in individual patients (*39*). Another common mutation that was discovered in isolates that required increased MICs for itraconazole is I242V. The MIC required for isolates with this mutation ranges from 1 μg/mL to 4 μg/mL. There have been no reports linking this mutation to azole resistance and a relationship between this mutation and azole resistance has not been demonstrated experimentally. The mechanism causing increased MICs for other isolates is not known, a common problem for researchers working on drug resistance in *Aspergillus* spp. (*3,32*).

Genetic mutations in *A. fumigatus cyp51A* are believed to be associated with exposure to azole compounds (*19,34,37,39*). The TR_{34}/L98H and TR_{46}/Y121F/T289A mutations specifically are believed to have originated from exposure to DMI fungicides in the environment (*10,13,14,25*). According to the US Department of Agriculture, agricultural producers in the United States use a lower tonnage of DMI fungicides than their counterparts in Europe and Asia (*26*). During 2010–2012, combined DMI fungicide use from states that provided isolates for this study was 381,018 kg. Of these states, California was the highest user (84,051 kg). According to the European Centre for Disease Prevention and Control, the United Kingdom used 271,124 kg of DMI fungicides during 2006–2009 (*8*). Therefore, the observed low rate of *cyp51A* mutation–dependent resistance, specifically the absence of TR_{34}/L98H and TR_{46}/Y121F/T289A mutations in this study than in studies from countries in Europe, Asia, and the Middle East, might be caused by differences in the extent of *A. fumigatus* exposure to DMI fungicides. The low rate of resistance in *A. fumigatus* shown is supported by results of other smaller studies in the United States (*2,40*).

There were several limitations to our study. First, isolates were collected passively. Some areas of the United States were overrepresented and others, such as the

Midwest, were underrepresented. If the TR_{34}/L98H mutation is found only in isolates from specific localities in the United States, it might have been missed during this surveillance. Second, all isolates were accepted. If surveillance was aimed specifically at isolates from patients who showed therapy failure, there may have been few isolates but those received might have demonstrated a greater prevalence of *cyp51A* mutations. Third, because isolates were received without personal identifiers and little meta data, there was no way of knowing whether multiple isolates came from 1 person.

In conclusion, this surveillance study indicates that the TR_{34}/L98H mutant *A. fumigatus* that is now found throughout Europe has not yet emerged in the United States. Approximately 5% of *A. fumigatus* isolates from the United States required increased MICs for itraconazole, but most isolates did not have a detectable genetic mutation. In light of increasing reports of TR_{34}/L98H-mediated and other *cyp51A*-mediated drug resistance in Europe and Asia, further surveillance is warranted.

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Feeding Period Required by *Amblyomma aureolatum* Ticks for Transmission of *Rickettsia rickettsii* to Vertebrate Hosts

Danilo G. Saraiva, Herbert S. Soares, João Fábio Soares, and Marcelo B. Labruna

Rocky Mountain spotted fever is endemic to the São Paulo metropolitan area, Brazil, where the etiologic agent, *Rickettsia rickettsii,* is transmitted to humans by adult *Amblyomma aureolatum* ticks. We determined the minimal feeding period required by *A. aureolatum* nymphs and adults to transmit *R. rickettsii* to guinea pigs. Unfed nymphs and unfed adult ticks had to be attached to the host for >10 hours to transmit *R. rickettsii.* In contrast, fed ticks needed a minimum of 10 minutes of attachment to transmit *R. rickettsii* to hosts. Most confirmed infections of Rocky Mountain spotted fever in humans in the São Paulo metropolitan area have been associated with contact with domestic dogs, the main host of *A. aureolatum* adult ticks. The typical expectation that transmission of tickborne bacteria to humans as well as to dogs requires ≥2 hours of tick attachment may discourage persons from immediately removing them and result in transmission of this lethal bacterium.

The tickborne bacterium *Rickettsia rickettsii* is the etiologic agent of the deadliest known rickettsiosis, Rocky Mountain spotted fever (RMSF). RMSF is referred to as Brazilian spotted fever in Brazil, where case-fatality rates are 20%–40% (*1*,*2*). The known distribution of *R. rickettsii* is restricted to the Americas, where different tick species have been implicated as vectors. The ticks *Dermacentor andersoni* and *D. variabilis* are the main vectors in the United States, and ticks of the *Amblyomma cajennense* complex are the most common vectors in Central and South America (*3*,*4*). The tick *Rhipicephalus sanguineus* has also been implicated as a vector for *R. rickettsii* in a few areas of Mexico and the state of Arizona in the United States (*5*,*6*). In the state of São Paulo, southeastern Brazil, there are 2 distinct epidemiologic scenarios of RMSF. Although *A. cajennense* is the identified vector in the countryside of the state of São Paulo (*1*,*4*), the tick *A. aureolatum* is the main vector in the metropolitan area of the city of São Paulo (*7*). A recent study on experimental infection of *A. aureolatum* with *R. rickettsii* demonstrated that the agent was preserved between life stages (transstadial maintenance) and by transovarial transmission in 100% of the *A. aureolatum* ticks for several consecutive generations; in addition, larvae, nymphs, and adults transmitted *R. rickettsii* to susceptible guinea pigs (*8*). Figure 1 illustrates an *A. aureolatum* adult tick.

The life cycle of ticks in the hard tick family, Ixodidae, is characterized by a short parasitic phase and a long nonparasitic or free-living phase. The former consists of few days or weeks for the feeding of each of the ticks at the larval, nymphal, and adult stages; the free-living phase varies from several months to years, encompassing the off-host developmental stages (egg laying and incubation, molting), and the host-seeking period of unfed ticks (*9*). Unfed ticks are known for their capacity to survive extremely long fasting periods of months to years until they find a suitable host on which to start a new parasitic phase (*9*). During the fasting period, metabolic activity of salivary glands, midgut, reproductive organs, the excretory system, and circulation system of the tick are at much lower levels than they are during feeding periods (*9*).

Spencer and Parker (*10*) postulated that virulence of *R. rickettsii* in tick vectors is linked directly to the physiologic state of the tick. In fasting ticks, virulent *R. rickettsii* lose their pathogenicity and virulence for guinea pigs; however, incubation of infected fasting ticks at elevated temperature (37°C) for 24 to 48 h or allowing them to feed for >48 h induces *R. rickettsii* to revert to a virulent state (reactivation). This reactivation process, or restoration of virulence,

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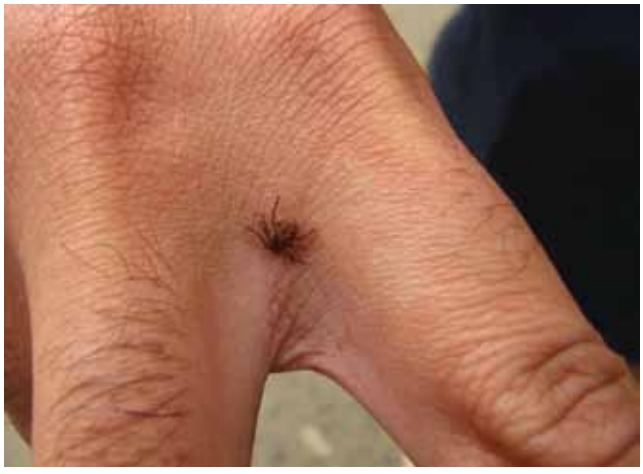

Figure 1. An adult male *Amblyomma aureolatum* tick attached to the hand of a person who became infested while in direct contact with a naturally infested dog in the metropolitan area of São Paulo, Brazil.

is accompanied by a series of changes in the surface structure of *R. rickettsii,* demonstrated by an ultrastructure study of the bacterium in *D. andersoni* ticks (*11*). In addition, a recent study demonstrated that the expression of some *R. rickettsii* genes is modulated by the physiologic state of the host, such as a fasting or feeding *A. aureolatum* tick (*12*); however, specific genes responsible for rickettsial reactivation remain unknown.

Earlier studies by Ricketts (*13*) and Moore (*14*) reported that adult *D. andersoni* ticks usually required a 10-hour feeding period to transmit *R. rickettsii* to vertebrate hosts, although a minimal period of 1 hour and 45 minutes was demonstrated for ticks that had previously fed on another host. Spencer and Parker (*10*) reported that this period would be >48 hours for unfed *D. andersoni* ticks. In Brazil, Magalhães (*15*) reported that *R. rickettsii*–infected *A. cajennense* adult ticks required 36 hours of feeding to transmit the agent to guinea pigs. The current literature, including medical textbooks, guidelines, and reviews on RMSF (*16*,*17*), has repeatedly advised that an infected tick requires a minimum feeding period varying from 2 to 10 hours to transmit *R. rickettsii* to humans. On the basis of this information, gathered from the above-mentioned earlier studies during the first half of the 20th century, it is widely recommended that adult persons entering wooded or grassy areas should inspect themselves and their children frequently for ticks and remove the parasites before they could efficiently transmit *R. rickettsii* (*17*).

Herein, we determined the minimal feeding period required by nymphs and adult male *A. aureolatum* ticks to transmit *R. rickettsii* to guinea pigs, since no such data have been reported for *A. aureolatum*. Male ticks were tested instead of adult female ticks because male *Amblyomma* ticks are highly motile on hosts, constantly seeking attached females (*9*). In addition, male *Amblyomma* ticks typically outnumber female ticks on hosts because male ticks can stay on hosts for a much longer period (*18*,*19*). Therefore, adult male *A. aureolatum* ticks, hereafter referred to as adult ticks, would be more likely to transmit *R. rickettsii* to humans.

Materials and Methods

We collected 4 engorged female *A. aureolatum* ticks from dogs in São Bernardo do Campo, São Paulo metropolitan area, and brought them to the laboratory of the University of São Paulo, where we placed them in an incubator at 24°C and 95%–100% relative humidity for egg laying. We indirectly found the female offspring to be free of *Rickettsia* infection by testing the collected female ticks after oviposition by PCR, targeting a 401-bp fragment, the rickettsial *gltA* gene, as previously described (*20*). For acquisition feeding, the first generation larval progeny were allowed to feed on 5 *R. rickettsii*–infected guinea pigs previously inoculated with *R. rickettsii* strain Taiaçu, as described (*8*,*20*). This rickettsial strain had been isolated from an *A. aureolatum* tick from an RMSF–endemic area in the São Paulo metropolitan area (*21*). Recovered engorged larvae molted to nymphs; using the PCR method referenced above, we found that 10 nymphs that comprised a random sample were infected by *R. rickettsii*. Previous studies have shown that this acquisition protocol usually results in the infection of 100% of *A. aureolatum* ticks, which are capable of sustaining the rickettsial infection by transstadial maintenance and transovarial transmission (*8*,*20*).

For determination of the minimal feeding period required by an *A. aureolatum* unfed nymph to transmit *R. rickettsii* to a vertebrate host, we used 32 guinea pigs (nos. 1–32). Each guinea pig was infested by 10 *A. aureolatum* unfed nymphs, which were placed within a cotton sleeve glued to the shaved back of the animal, as described (*20*). Each of the 32 guinea pigs had a specific period in which the nymphs were allowed to feed; however, for each feeding period, we used 2 or 4 guinea pigs to replicate a given feeding period. For example, on guinea pigs 3 and 4 (Table 1), nymphs were allowed to feed for 4 hours. In this case, when the first nymph was seen attached to the skin of each animal, we started counting the feeding period. Four hours after the attachment of the first nymph, all 10 nymphs were manually removed from the guinea pig and stored frozen at –80°C until further analysis. The same procedure was used for the remaining guinea pigs, with variation of 2- to 48-hour feeding periods (Table 1). On guinea pigs 31 and 32, unfed nymphs were allowed to feed until they detached naturally as engorged nymphs, which varied from 4 to 7 days. Additional guinea pigs were infested by infected nymphs that were left to molt into adults to obtain unfed adults to be used in the following infestations.

Table 1. Fever, seroconversion to *Rickettsia rickettsii* antigens, and ear and/or scrotal lesions in guinea pigs exposed to *R. rickettsii*–infected *Amblyomma aureolatum* unfed nymphs through different feeding periods, Brazil

Guinea pig no.	Tick feeding period, h*	Fever†	Anti–*R. rickettsii* antibody titers‡	Ear and/or scrotal lesions§
1	2	No	<1:64	No
2	2	No	<1:64	No
3	4	No	<1:64	No
4	4	No	<1:64	No
5	6	No	<1:64	No
6	6	No	<1:64	No
7	8	No	<1:64	No
8	8	No	<1:64	No
9	8	No	<1:64	No
10	8	No	<1:64	No
11	10	No	<1:64	No
12	10	No	<1:64	No
13	12	Yes	¶	Yes
14	12	No	<1:64	No
15	12	No	<1:64	No
16	12	No	<1:64	No
17	14	Yes	2,048	Yes
18	14	No	256	No
19	16	Yes	512	No
20	16	Yes	512	No
21	18	Yes	8,192	Yes
22	18	Yes	256	No
23	24	Yes	4,096	Yes
24	24	Yes	8,192	Yes
25	24	Yes	4,096	Yes
26	24	Yes	512	No
27	36	Yes	16,384	Yes
28	36	Yes	8,192	Yes
29	48	Yes	4,096	Yes
30	48	Yes	8,192	Yes
31	>96	Yes	16,384	Yes
32	>96	Yes	16,384	Yes

*Number of hours that infected nymphs were allowed to feed on each guinea pig before ticks were manually removed from host.
†Rectal temperature >39.5°C during 21 d after tick infestation.
‡Anti–*R. rickettsii* IgG endpoint titers determined 21 d after tick infestation.
§Ear or scrotal lesions (edema, necrosis) during the febrile period within 21 d after tick infestation.
¶Guinea pig died during the febrile period, before the 21st d after tick infestation; its lung was PCR-positive for rickettsiae.

To determine the minimal feeding period of *A. aureolatum* unfed adult ticks required to enable transmission of *R. rickettsii* to a vertebrate host, we used 24 guinea pigs (nos. 33–56). Each guinea pig was infested by 1 *A. aureolatum* unfed adult tick, as described for nymphs. Each of the 24 guinea pigs was assigned a specific feeding period in which the adult tick was allowed to feed. For example, on guinea pigs 39 and 40 (Table 2), adult ticks (1 per guinea pig) were allowed to feed for 8 hours. In this case, when the single adult tick was seen attached to the skin of each animal, we started counting the feeding period. Eight hours after attachment of the adult tick, it was manually removed from the guinea pig, and stored frozen at −80°C until further analysis. The same procedure was adopted for the remaining guinea pigs, except for the period in which the adult ticks were allowed to feed, which varied from 2 to 48 hours (Table 2). Unfed adult ticks were allowed to feed on 2 guinea pigs (nos. 55 and 56) for 7 days (168 hours), to simulate a feeding period that would last at least 7 days under natural conditions.

To determine the minimal feeding period required by a previously fed *A. aureolatum* adult tick to transmit *R. rickettsii* to a vertebrate host, we first allowed adult male ticks to feed with adult female ticks for 48 hours on the shaved back of tick-naïve domestic rabbits (*Oryctolagus cuniculus*), as described (*8*). Then, the fed ticks were removed from the rabbits and immediately used to infest 34 guinea pigs (nos. 57–90), as described above, except that the period in which the adult ticks were allowed to feed varied from 1 minute to 168 hours (Table 3).

Every guinea pig or rabbit used in this study was tick naive; these animals were provided by a private laboratory that raised the animals under proper sanitary conditions. The rectal temperatures of guinea pigs and rabbits were measured daily from the day of infestation through 21 days afterward. These animals were considered febrile if rectal temperature reached values >39.5°C (guinea pigs) or >40°C (rabbits). All animals were tested for seroconversion to *R. rickettsii* antigens. For this purpose, we collected blood samples at 0 and 21 days postinfestation and tested for anti–*R. rickettsii* reactive antibodies by immunofluorescence assay, as described (*8*,*22*). Animals were considered seronegative if their serum was not reactive at the 1:64 dilution. Some infested guinea pigs that died before day 21 postinfestation were not tested by immunofluorescence assay because a second blood sample was not obtained; however, we submitted a fragment of their lung tissue to DNA extraction using the DNeasy Tissue Kit (QIAGEN, Chatsworth, CA, USA) and tested the samples by the same PCR protocol referenced above. Clinical alterations, such as ear or scrotal necrosis, were noted when observed. We tested all nymphal and adult ticks that were manually removed from the infested guinea pigs individually by the same PCR protocol referenced above.

Results

All PCRs performed on the DNA of nymphal and adult ticks that fed on guinea pigs for different periods resulted in amplicons compatible with *R. rickettsii,* indicating that all 90 guinea pigs in this study were exposed to *R. rickettsii*–infected ticks. Among guinea pigs exposed to *R. rickettsii*–infected unfed nymphs, animals remained afebrile and seronegative when nymphs fed for ≤10 hours (Table 1). When nymphs fed for 12 hours on 4 guinea pigs, 3 of these animals (nos. 14–16) remained seronegative and afebrile, but the fourth animal (no. 13) became febrile and died on the second week, when ear and scrotal necrosis were evident. Its lung tissue sample was PCR–positive for rickettsiae. All guinea pigs on which nymphs fed for 14 to ≥96

Table 2. Fever, seroconversion to *Rickettsia rickettsii* antigens, and ear and/or scrotal lesions in guinea pigs that were exposed to *R. rickettsii*–infected *Amblyomma aureolatum* unfed adult male ticks, Brazil

Guinea pig no.	Tick feeding period, h*	Fever†	Anti–*R. rickettsii* antibody titers‡	Ear and/or scrotal lesions§
33	2	No	<1:64	No
34	2	No	<1:64	No
35	4	No	<1:64	No
36	4	No	<1:64	No
37	6	No	<1:64	No
38	6	No	<1:64	No
39	8	No	<1:64	No
40	8	No	<1:64	No
41	10	No	<1:64	No
42	10	No	<1:64	No
43	12	Yes	4,096	Yes
44	12	Yes	256	No
45	16	Yes	2,048	Yes
46	16	Yes	1,024	Yes
47	20	Yes	512	Yes
48	20	Yes	2,048	Yes
49	24	Yes	2,048	Yes
50	24	Yes	¶	Yes
51	36	Yes	2,048	Yes
52	36	Yes	4,096	Yes
53	48	Yes	¶	Yes
54	48	Yes	8,192	Yes
55	168	Yes	16,384	Yes
56	168	Yes	16,384	Yes

*Number of hours that an infected male adult tick was allowed to feed on each guinea pig before the tick was manually removed from the host.
†Rectal temperature >39.5°C during 21 days after tick infestation.
‡Anti–*R. rickettsii* IgG endpoint titers determined 21 days after tick infestation.
§Occurrence of ear or scrotal lesions (edema, necrosis) during the febrile period within 21 days after tick infestation.
¶Guinea pig died during the febrile period, before the 21st day after tick infestation; its lung tissue was PCR-positive for rickettsiae.

hours seroconverted to *R. rickettsii,* and fever developed in all but 1 (no. 18). Of 16 these animals, 5 did not show ear or scrotal lesions.

Among the 24 guinea pigs exposed to *R. rickettsii*–infected unfed adult ticks, 10 animals remained afebrile and seronegative when ticks fed for ≤10 hours (Table 2). Fever developed in the 14 guinea pigs on which the male ticks fed for 12–168 hours. Seroconversion to *R. rickettsii* was demonstrated in 12 of the 14 febrile guinea pigs. Two guinea pigs, nos. 50 and 53, died before 21 days; their lung tissue specimens were PCR–positive for rickettsiae. Thirteen of these febrile animals showed ear and scrotal lesions.

Of the 2 rabbits on which adult *A. aureolatum* ticks fed for 48 hours, 1 became febrile at day 5 and the other at day 7 postinfestation; ear necrosis developed in both rabbits, and blood samples seroconverted to *R. rickettsii* with endpoint titers of 8,192 or 16,384. When exposed to the *R. rickettsii*–infected adult ticks that had fed for 48 hours on rabbits, guinea pigs remained afebrile and seronegative when the ticks fed for ≤5 minutes (Table 3). Of 2 guinea pigs on which adult ticks fed for 10 minutes (nos. 63 and 64), no. 63 remained seronegative and afebrile, but no. 64 became febrile and seroconverted. Fever developed in all 26 guinea pigs on which fed adult ticks fed for 20 minutes to 168 hours; of these, 21 had ear or scrotal lesions, or both. Thirteen animals seroconverted to *R. rickettsii*, and 14 died during the febrile period; their lungs were positive for rickettsiae by PCR.

The infection with *R. rickettsii* in guinea pigs was confirmed by seroconversion (nonfatal cases) or by PCR on lung tissue (fatal cases). Fever onset was registered between 5 and 9 days (mean 6.8) postinfestation with nymphs, between 4 and 8 days (mean 5.6) postinfestation with infected unfed adult ticks, and between 4 and 11 days (mean 6.7) postinfestation with prefed adult ticks. Among the 17 guinea pigs that became infected by *R. rickettsii* after being exposed to unfed nymphs, only 1 died of spotted fever (6% fatality rate). Among the 14 guinea pigs that became infected after being exposed to unfed adult ticks, 2 (14% fatality rate) died of spotted fever. When guinea pigs were exposed to infected ticks that had previously fed on rabbits (prefed adult ticks), the fatality rate rose to 52% (14/27).

Discussion

This work showed that unfed nymphs and unfed adult male ticks of *A. aureolatum* needed to be attached for >10 hours on the host, to successfully transmit a virulent strain of *R. rickettsii.* In contrast, fed adults needed only up to 10 minutes of attachment for transmission of *R. rickettsii* to the host. The >10-hour feeding period observed for unfed ticks is similar to the 10-hour period previously reported for *D. andersoni* ticks in 2 earlier studies (*13*,*14*); albeit much lower than the periods previously reported for *D. andersoni* (>48 hours) in another study (*10*) and for *A. cajennense* ticks (36 hours) in Brazil (*15*). Regarding fed ticks, the 10-minute period herein observed for *A. aureolatum* ticks is much shorter than the 1 hour and 45 minutes previously reported for prefed *D. andersoni* ticks (*14*). It is possible that different tick species require different feeding periods for effective inoculation of *R. rickettsii* into the host; however, it is clear that prefed ticks require much shorter periods than unfed ticks. This difference should be related to the reactivation phenomenon; i.e., *R. rickettsii* was in a nonvirulent state in unfed nymphal and adult *A. aureolatum* ticks and in its virulent state (reactivated) in the prefed adult ticks used to infest guinea pigs.

Adult *A. aureolatum* ticks feed chiefly on Carnivora species (mostly domestic dogs), but immature ticks (larvae, nymphs) generally feed on passerine birds and a few rodent species (*7*,*23*). Humans have reported being attacked only by adult ticks, and usually by a single tick (*24*), because the population density of *A. aureolatum* ticks is usually low (*18*). In southeastern Brazil, the distribution of *A. aureolatum* populations is restricted to

Table 3. Fever, seroconversion to *Rickettsia rickettsii* antigens, and ear and/or scrotal lesions in guinea pigs that were infested by previously fed *R. rickettsii*–infected *Amblyomma aureolatum* adult male ticks through different feeding periods, Brazil

Guinea pig no.	Tick feeding period*	Fever†	Anti–*R. rickettsii* antibody titers‡	Ear and/or scrotal lesions§
57	1 min	No	<1:64	No
58	1 min	No	<1:64	No
59	3 min	No	<1:64	No
60	3 min	No	<1:64	No
61	5 min	No	<1:64	No
62	5 min	No	<1:64	No
63	10 min	No	<1:64	No
64	10 min	Yes	1,024	No
65	20 min	Yes	1,024	No
66	20 min	Yes	512	No
67	40 min	Yes	1,024	No
68	40 min	Yes	4,096	Yes
69	1 h	Yes	4,096	Yes
70	1 h	Yes	8,192	Yes
71	2 h	Yes	¶	Yes
72	2 h	Yes	512	No
73	4 h	Yes	¶	Yes
74	4 h	Yes	16,384	Yes
75	6 h	Yes	¶	Yes
76	6 h	Yes	¶	Yes
77	8 h	Yes	¶	Yes
78	8 h	Yes	¶	Yes
79	12 h	Yes	¶	Yes
80	12 h	Yes	¶	Yes
81	18 h	Yes	¶	Yes
82	18 h	Yes	8,192	Yes
83	24 h	Yes	8,192	Yes
84	24 h	Yes	16,384	No
85	36 h	Yes	¶	Yes
86	36 h	Yes	¶	Yes
87	48 h	Yes	¶	Yes
88	48 h	Yes	¶	Yes
89	168 h	Yes	16,384	Yes
90	168 h	Yes	¶	Yes

*Number minutes or hours that an infected adult male tick was allowed to feed on each guinea pig before the tick was manually removed from the host. All ticks had previously fed on rabbits for 48 h.
†Rectal temperature >39.5°C during 21 days after tick infestation.
‡Anti–*R. rickettsii* IgG endpoint titers determined at 21 days after tick infestation.
§Ear or scrotal lesions (edema, necrosis) during the febrile period within 21 days after tick infestation.
¶Guinea pig died during the febrile period, before day 21 after tick infestation; its lung was PCR-positive for rickettsiae.

Atlantic rainforest fragments where optimal conditions of high humidity and cool temperatures prevail throughout the year (*7*,*18*). Therefore, infestations occur typically on domestic dogs that are reared unrestrained, with access to Atlantic rainforest fragments (*18*,*25*). However, to our knowledge, *A. aureolatum*–human infestation acquired in the forest has not been studied and documented. In fact, in an Atlantic rainforest reserve in the state of São Paulo, 4 *Amblyomma* tick species (including *A. aureolatum*) were collected in wild animal trails during a 4-year period (*26*), when *A. aureolatum* was the only 1 of the 4 tick species that was not reported to have attached to researchers during their field activities in the forest (*27*). Thus, we hypothesize that many of the RMSF-confirmed cases in the São Paulo metropolitan area were transmitted by *A. aureolatum* ticks that had fed on domestic dogs. In this case, the domestic dog would have become infested in the forest and brought an infected tick indoors, where it came into direct contact with humans (Figure 2). This statement is corroborated by a study that reported that 69% of the RMSF cases in the São Paulo metropolitan area occurred in children and women, who usually did not enter the forest (habitat of *A. aureolatum*) as frequently as did adult men (*28*). In addition, 93% of the cases in this area have been associated with direct contact with dogs (*29*).

In this study, the fatality rate for guinea pigs exposed to prefed adult ticks (52%) was much higher than the rate for guinea pigs exposed to unfed ticks (14%). A recent study reported that the fatality rate for patients with RMSF in a region of the São Paulo metropolitan area (transmission by *A. aureolatum* ticks) was 62.5% during 2003–2010, which was substantially higher than the 33.3% fatality rate observed in a region of the countryside of the state of São Paulo (transmission by *A. cajennense* ticks) during a similar period (*29*). Similarly to the situation with the guinea pigs in this study, this marked difference among RMSF case-patients could be related to the reactivation state of *R. rickettsii* in the tick vector, since we postulated above that infestation by fed ticks would predominate in the metropolitan area of São Paulo. In the countryside, acquisition of *R. rickettsii* infection could be predominantly related to infestations by unfed *A. cajennense* ticks acquired directly in the field, since such infestations are commonly reported in this area (*4*,*30*,*31*).

According to results of this study, a fed *A. aureolatum* tick could transmit *R. rickettsii* to a human in as few as 10 minutes of parasitism. Because this route of transmission seems to be common in the metropolitan area of São Paulo, health authorities must be aware that current textbooks and guidelines that indicate that an infected tick takes 2 to 10 hours to transmit *R. rickettsii* to humans (*16*,*17*) do not apply to the São Paulo metropolitan area.

In the eastern United States, *R. rickettsii* is transmitted to humans typically by the *D. variabilis* tick in the adult stage, commonly known as the American dog tick, which feeds chiefly on domestic dogs (*17*). Similarly to the circumstances in the São Paulo metropolitan area, most of the RMSF cases in the eastern United States have occurred in children and women (*32*,*33*), and infections in canines have been associated repeatedly with an increased risk for disease in owners (*34*). Because numerous reports of infected humans were associated with tick-infested dogs or tick removal within 4 weeks of disease onset, researchers have proposed that many of these cases were a result of direct contact with rickettsiae from tick body fluids during tick removal (*34*,*35*). Although this postulated mechanism cannot

Figure 2. A typical area where infection with *Rickettsia rickettsii* occurs, manifested as Rocky Mountain spotted fever, in the metropolitan area of São Paulo, Brazil. Humans have constructed their homes in the Atlantic rainforest fragment (habitat of the *Amblyomma aureolatum* tick, a vector of *R. rickettsii*), where many dogs are unrestrained. Dogs frequently enter the forest, become infested by adult *A.aureolatum* ticks, and bring them into homes, allowing the direct transfer of feeding ticks from dogs to humans.

be discarded (including in the São Paulo metropolitan area), the current literature has considered that an attached tick needs several to many hours of attachment for a successful inoculation of rickettsiae into human skin. Once it is forcibly removed from a host, a partially fed tick loses its discriminatory senses and strives to feed wherever possible on any available vertebrate animal (*36*). Thus, it is reasonable to consider that tick removal habits in RMSF-endemic areas could have implications for the transmission of *R. rickettsii,* not only caused by potential direct contact with tick fluids, but also, as shown in this study, because detached ticks could readily attach to humans and inoculate them with rickettsiae within few minutes.

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This study was approved by the Ethics Committee on Animal Research for the Faculty of Veterinary Medicine of the University of São Paulo.

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Methicillin-Sensitive *Staphylococcus aureus* CC398 in Intensive Care Unit, France

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During testing for *Staphylococcus aureus* in an intensive care unit in France in 2011, we found that methicillin-sensitive *S. aureus* clonal complex 398 was the most frequent clone (29/125, 23.2%). It was isolated from patients (5/89, 5.6%), health care workers (2/63, 3.2%), and environmental sites (15/864,1.7%). Results indicate emergence of this clone in a hospital setting.

Livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type (ST) 398, which belongs to clonal complex (CC) 398, is an emergent zoonotic agent responsible for massive colonization of livestock and food products and infections in humans worldwide (*1*). Recently, emergence of animal-independent methicillin-sensitive *S. aureus* (MSSA) ST398 has been reported in China (*2*), France (*3,4*), the Netherlands (*5,6*), Spain (*7*), and North America (*8–11*). MSSA ST398 has been reported in colonized (*5,7,8,10,11*) and infected (*2,5,6,8,10,11*) patients. These isolates have been characterized as having staphylococcal protein A (spa) type t571, being sensitive to all antimicrobial drugs except macrolides, and having variable presence of Panton-Valentine leukocidin (*2,3,8*). In France, an increasing incidence of MSSA ST398 bacteremia has been observed since 2007 (*3,4*).

During a systematic, molecular, epidemiologic survey of *S. aureus* in an intensive care unit (ICU) in France, *S. aureus* CC398 was isolated from patients, health care workers (HCWs), and environmental sites. We conducted a study to describe the spread and characteristics of *S. aureus* CC398 in this setting.

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The Study

A prospective molecular epidemiologic study of *S. aureus* was performed in a 12-bed ICU at the University Hospital in Montpellier, France, during 5 months in 2011. *S. aureus* nasal carriage was investigated at admission and weekly in 89 patients and monthly in 63 volunteer health care workers (HCWs). Simultaneously, all *S. aureus* isolates from clinical samples were obtained from the hospital laboratory of bacteriology and clinical data were recorded.

Pneumonia was diagnosed on the basis of clinical, biologic, and radiologic criteria, and a colony count $\geq 10^4$ CFU/mL in bronchoalveolar fluid culture or $\geq 10^7$ CFU/mL in sputum cultures. Bronchial colonization was defined as a colony count $<10^7$ CFU/mL in sputum cultures in asymptomatic patients.

Random sampling of surfaces was performed monthly in all rooms of the ICU (864 environmental sites). Isolates were characterized by using multilocus sequence typing, double-locus sequence typing (DLST), and accessory gene regulation (agr) typing. Resistance to antimicrobial drugs was detected by using the disk-diffusion method. Virulence genes and *ermA*, *ermC*, *ermT*, and *msrA* genes were screened for by using PCRs.

During the survey period, the number of samples obtained ranged from 1 to 32 per patient and from 1 to 3 per HCW. Of these samples, 125 *S. aureus* isolates (110 MSSA and 15 MRSA) were obtained from 33 patients, 26 HCWs, and 36 environmental sites; these isolates belonged to 28 STs and 12 CCs. Among these 125 isolates, 12 isolates from 5 patients, 2 isolates from 2 HCWs, and 15 isolates from 15 environmental sites belonged to CC398 (Figure 1; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/9/13-0225-Techapp1.pdf). The 29 strains were MSSA and belonged to ST398 (n = 25) or to a new ST submitted to the MLST Database (http://www.mlst.net/) as ST2658 (n = 4). ST398 and CC398 were the most prevalent genotype and clonal complex identified: 25/125 (20%) and 29/125 (23.2%) isolates, respectively (Figure 2).

The prevalence of MSSA CC398 carriage was 3.2% (2/63) in HCWs and 5.6% (5/89) in patients. The prevalence of MSSA CC398 infection was 2.25% (2/89 patients) (Figure 1). These patients were hospitalized during the same period; nosocomial pneumonia developed after nasal colonization, and was associated with bacteremia in 1 case. Demographic and clinical characteristics were similar in patients colonized or infected with MSSA CC398 or with other genotypes (Table 1). No history of contact with livestock was found for patients and HCWs. The prevalence of MSSA CC398 environmental contamination was 1.7% (15/864 samples). Genotype CC398 was found more

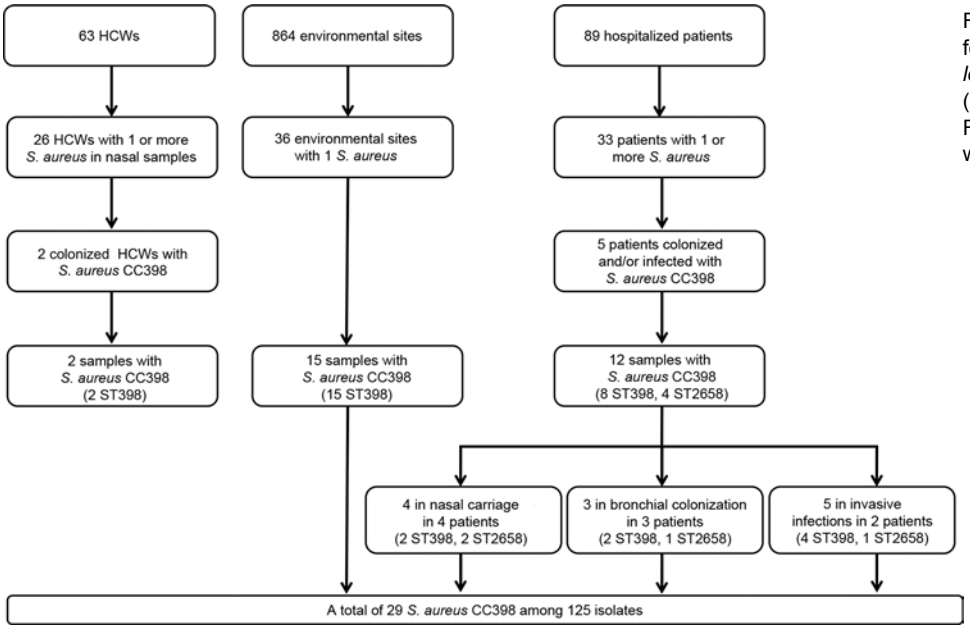

Figure 1. Flowchart of selection for methicillin-sensitive *Staphylococcus aureus* clonal complex (CC) 398 from intensive care unit, France, 2011. HCWs, health care workers; ST, sequence type.

frequently in the ICU environment (15/36, 41.7%) than in patients (5/33, 15.2%; χ^2 4.7, p = 0.03) and HCWs (2/26, 7.7%; χ^2 7.1, p = 0.007) (online Technical Appendix).

Molecular typing of CC398 strains and microbiologic results are shown in Table 2. Four strains belonged to the new ST2658, which differed from ST398 by a synonymous mutation (A→G) at position 198 of the *pta* gene. These 4 strains were isolated from nasal carriage samples (n = 2), bronchial colonization samples (n = 1), and pneumonia testing samples (n = 1) from 2 patients hospitalized at the same time. All MSSA CC398 strains were agr type 1, spa type t571 (determined by using DNAGear software; http://w3.ualg.pt/~hshah/DNAGear/), and DLST type 144–186 (DLST spa 186 corresponding to spa type t571). Genes encoding Panton-Valentine leukocidin, toxic shock syndrome toxin 1, and staphylococcal enterotoxin A were not detected. Sensitivity testing of MSSA CC398 isolates showed that all isolates were resistant to erythromycin and had an inducible macrolide–lincosamide–streptogramin B phenotype. In addition, resistance to penicillin and amoxicillin caused by b-lactamase production was observed in 41.4% (12/29) of the strains. Resistance to kanamycin, tobramycin, and gentamicin was observed in 24.1% (7/29) of the strains; all 7 strains were isolated from environmental samples. Analysis of genes encoding antimicrobial drug resistance identified the *erm*T gene in all the CC398 strains and a variable distribution of *erm*A and *erm*C genes.

Conclusions

Identification of MSSA CC398 in HCWs, patients without exposure to livestock, and the environment in an ICU indicates emergence of this clone in a hospital in France. The prevalence of nasal carriage in HCWs and patients was high (≤5.6%) in the context of the ICU, where these persons have frequent contact with each other. The small number of patients colonized or infected with *S. aureus* CC398 limits statistical comparison of the 2 groups and identification of risk factors for infection.

Despite the monocentric nature and the short period of the study, which limit extrapolation of our results to other settings, our study underlines the capacity of MSSA CC398 to circulate among and between patients,

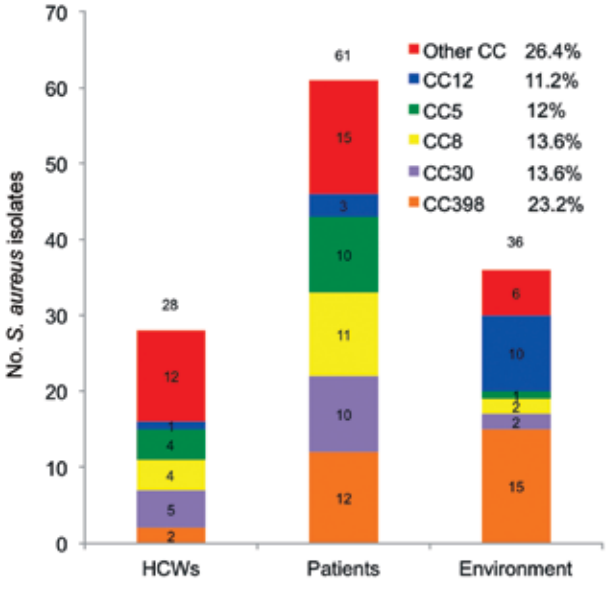

Figure 2. Principal clonal complexes (CCs) among 125 isolates of *Staphylococcus aureus* from intensive care unit, France, 2011. HCWs, health care workers.

Table 1. Demographic and clinical characteristics of 33 patients colonized or infected with *Staphylococcus aureus*, intensive care unit, France, 2011*

Characteristics	*S. aureus* CC398 (n = 5 patients)	Another genotype of *S. aureus* (n = 28 patients)	p value
Demographic data			
M:F ratio	5	1.5	
No. (%) men	5 (100)	17 (60.7)	0.14
Mean age, y	53.4	53.9	0.96
Concurrent conditions, no. (%)			
Diabetes	0	6 (21.4)	0.55
COPD/CRF	1 (20)	5 (17.8)	1
Cancer/hematologic disease	1 (20)	1 (3.6)	0.28
Chemotherapy/IS	0	3 (10.7)	1
HIV infection	0	0	1
Risk factors for *S. aureus* colonization, no. (%)			
Hospitalization >48 h to <1 y	2 (40)	15 (53.6)	0.66
History of *S. aureus* carriage or infection	0	7 (25)	0.56
Antimicrobial drug therapy for <3 mo	2 (40)	13 (46.4)	1
Residence in long-term care facility	0	0	1
Surgery/invasive procedure within 1 y	2 (40)	7 (25)	0.61
Chronic skin wounds	0	2 (7.1)	1
Colostomy	0	0	1
Indwelling urinary catheter	0	1 (3.6)	1
Tracheotomy	0	1 (3.6)	1
Medical data related to ICU, no. (%)			
Median length of ICU stay, d	38.4	13	0.09
Mechanical ventilation	5 (100)	16 (57)	0.13
Median length of invasive ventilation , d	33.8	18.3	0.14
Severity score on admission (SAPS II)	41.6	37.6	0.64
Use of vasoactive drugs	3 (60)	13 (46.4)	0.66
Extrarenal replacement	0	2 (7.1)	1
Deaths in ICU	2 (40)	5 (17.8)	0.28

*COPD, chronic obstructive pulmonary disease; CRF, chronic respiratory failure; IS, immunosuppressive therapy; ICU, intensive care unit; SAPS II, simplified acute physiology score II.

HCWs, and the ICU environment. Slingerland et al. reported prolonged survival of bovine MSSA ST398 strain in the human nose after artificial inoculation, which suggested that competition with human strains might facilitate its spread (*12*). Identification of ST2658 in 2 patients hospitalized at the same time reinforces the hypothesis of an increased capacity of transmission of this clonal complex between patients.

Person-to-person spread of MSSA ST398 has been reported within community households (*8,10)* and more recently in a hospital (*11*) and an urban jail (*9*), in which a high proportion of detainees sharing a holding tank were colonized with MSSA ST398 (*9*). These findings contrast with limited transmissibility of livestock-associated MRSA ST398, which is partially explained by molecular signatures of bacterial host adaptation identified only in the MSSA ST398 genome, such as different composition of adhesion genes that result in enhanced adhesion to human skin (*10*).

All strains were spa type t571, which is the major spa type associated with MSSA ST398 (*2,3,5*–*7*). There are other similarities between our strains and strains from China, Spain, Belgium, and the United States. (*2,6,7,11*), such as agr type 1, the presence of the *erm*T gene, tetracycline susceptibility, and macrolide–lincosamide–streptogramin B phenotype.

In ICUs, colonized or infected patients constitute the main reservoir of *S. aureus* (*13*). The association of MSSA CC398 with the ICU environment suggests that this environment could play a role as a bacterial reservoir as described (*14*). One hypothesis for such an association is the capacity to form a biofilm, which could be correlated with the *S. aureus* genetic background (*15*). Our findings emphasize potential hospital-adapted characteristics of *S. aureus* CC398, which is supported by others studies (*6*,*11*), and indicate that surveillance programs are needed to determine the role of this clonal complex, particularly in the hospital setting.

Acknowledgments

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Table 2. Microbiological characteristics of 29 *Staphylococcus aureus* clonal complex 398 isolates from intensive care unit, France, 2011

Source*	Place of isolation†	Date of isolation	MLST‡	Resistance phenotype§	MLS resistance genes¶			
					*erm*A	*erm*C	*msr*A	*erm*T
P1	S	Jan 31	ST2658	BL, iMLS	–	–	–	+
P1	N	Jan 31	ST2658	BL, iMLS	–	–	–	+
P2	N	Feb 14	ST2658	BL, iMLS	–	–	–	+
P2	S#	Feb 21	ST2658	BL, iMLS	–	–	–	+
P3	N	Feb 28	ST398	BL, iMLS	–	–	–	+
P4	BAL#	Apr 6	ST398	iMLS	–	+	–	+
P4	B#	Apr 8	ST398	iMLS	–	–	–	+
P4	BAL#	Apr 8	ST398	iMLS	–	–	–	+
P4	N	Apr 11	ST398	iMLS	–	–	–	+
P4	S#	Apr 11	ST398	iMLS	–	–	–	+
P4	S	May 9	ST398	iMLS	–	–	–	+
P5	S	June 14	ST398	BL, iMLS	–	–	–	+
HCW1	N	Feb 13	ST398	BL, iMLS	–	–	–	+
HCW2	N	Feb 14	ST398	iMLS	–	–	–	+
E	HCW kitchen, microwave	Mar 2	ST398	BL, iMLS	–	+	–	+
E	Care room no. 1, telephone	Mar 2	ST398	iMLS	–	+	–	+
E	Doctor's telephone	Apr 5	ST398	iMLS	–	+	–	+
E	HCW kitchen, lunch table	Apr 5	ST398	BL, iMLS	–	+	–	+
E	Staff room, notebook	Apr 5	ST398	BL, iMLS	–	–	–	+
E	Medical room, telephone	Apr 5	ST398	iMLSB, KTG	–	+	–	+
E	Material room, telephone	Apr 5	ST398	iMLSB, KTG	–	+	–	+
E	Refrigerator in office	Apr 6	ST398	iMLSB, KTG	–	+	–	+
E	Bedroom no. 7, table	Apr 6	ST398	iMLSB, KTG	–	+	–	+
E	Bedroom no. 8, care card	Apr 6	ST398	iMLSB, KTG	–	+	–	+
E	Bedroom no. 10, infusion manifold	Apr 6	ST398	iMLSB, KTG	–	–	–	+
E	Care room no. 3, telephone	Apr 6	ST398	iMLSB, KTG	–	–	–	+
E	Care room no. 2, furniture	Apr 6	ST398	BL, iMLS	+	–	–	+
E	Bedroom no. 1, infusion manifold	May 9	ST398	iMLS	+	–	–	+
E	Bedroom no. 12, negatoscope	May 9	ST398	BL, iMLS	–	–	–	+

*P, patient; HCW, health care worker, E, environment.
†S, sputum; N, nose; BAL, bronchoalveolar lavage fluid; B, bloodstream.
‡MLST, multilocus sequence type; ST, sequence type.
§BL, β-lactamase (resistance to penicillin and amoxicillin); iMLS, inducible macrolide–lincosamide–streptomycin B; KTG, kanamycin, tobramycin, gentamicin.
¶–, negative; +, positive.
#Strains isolated from infected patients.

Montpellier 1, and the Laboratory of Bacteriology (Arnaud de Villeneuve Hospital, Montpellier).

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Household-Level Spatiotemporal Patterns of Incidence of Cholera, Haiti, 2011

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A cholera outbreak began in Haiti during October, 2010. Spatiotemporal patterns of household-level cholera in Ouest Department showed that the initial clusters tended to follow major roadways; subsequent clusters occurred further inland. Our data highlight transmission pathway complexities and the need for case and household-level analysis to understand disease spread and optimize interventions.

The 2010–2011 Haiti cholera epidemic was one of the largest worldwide in recent history. Before the initial outbreak, cholera had not been reported in Haiti for at least 100 years (*1*). Multiple factors likely contributed to the magnitude and spread of the early outbreak, including lack of prior exposure to cholera among the population, genetic characteristics of the *Vibrio cholerae* strain, and the consequences of the January 2010 earthquake, which included mass destruction of the infrastructure of Haiti and displacement of 1.5 million persons. The water and sanitation infrastructure in Haiti were inadequate before the 2010 earthquake; much of the population had no access to treated drinking water (48%) or sanitation facilities (75%) (*2*). Many of the limited services were destroyed by the 2010 quake (*3*).

As of February 28, 2013, the Ministry of Public Health and Population (MSPP) of Haiti and the National Directorate for Water Supply and Sanitation, working with the Pan American Health Organization, announced an ambitious plan to eradicate cholera from Haiti and Hispaniola: the plan calls for aggressive efforts to improve sanitation and to vaccinate the entire Haitian population (*4*). Designing this plan for optimal operation requires an understanding of cholera transmission within the population, and the development of models that permit assessment of the impact of proposed interventions on disease incidence. However, epidemiologic studies on the Haiti cholera outbreak have focused on the diffusion of the disease by using aggregated data, such as those from arrondissements (*5*) and communes (*6*). To evaluate cholera case clustering and provide a basis for modeling and intervention design, we conducted a spatiotemporal analysis of household-level data in the Ouest Department in Haiti during 2010–2011.

The Study

We used data from the Collaborative Cholera Mapping Project (CCMP), a Web-based dataset (no longer online) of household-level cholera cases captured through the United Nations Children's Fund water, sanitation, and hygiene program in the Leogane/Petit Goave area and Sustainable Aid Supporting Haiti (https://www.facebook.com/SAS-Haiti/info), a private nongovernmental organization (NGO) working in the area. The CCMP contains case data from 4 communities that include 3 urban areas, Petit Goave, Grand Goave, and Leogane (Figure 1, panel A). The fourth community in the CCMP is La Source, a small community in the west on Highway 7 (not mapped). Case data are summarized in the Table; full details on the urban structure of each community are provided in the Detailed Description of the Four Communities in the Collaborative Cholera Mapping Project (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/9/13-1882-Techapp1.pdf).

The CCMP compiled global positioning system (GPS) data on household locations of patients who reported to cholera treatment centers during January–August 2011. Households were visited by community health workers employed by NGOs in the region. Persons in each household were educated about cholera, and surfaces in the house were disinfected. The GPS coordinates were entered into the dataset by NGO personnel.

Data were provided by CCMP with exemption from the University of Florida Institutional Review Board. Participants were not identified. We constructed a geographic information system, or GIS, database of household cases by date for each of the 3 communities on Route 2 and evaluated them separately. We plotted CCMP cases against daily case incidence reported to MSPP for the Ouest Department to evaluate the temporality of CCMP data.

We evaluated space-time clustering of cases for each community by season (winter or summer), using the spatial scan statistical tool in SaTScan (http://www.satscan.

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org). We used the retrospective space-time permutation model (*7*), which does not require data for population at risk, a key factor given the lack of reliable post-earthquake population data for Haiti (online Technical Appendix, Geospatial Analyses). Households were used as case locations. Clusters were identified as a maximum cluster size of 50% of case data and a maximum temporal window of 50% of the study period (online Technical Appendix, Geospatial Analyses).

There was close agreement in the trend of daily CCMP and MSPP data (Figure 1, panel B), illustrating 2 seasonal peaks for the epidemic, with fewer total cases in the dryer winter months and very close agreement in the timing of seasonal peaks between CCMP and MSPP cases. Datasets were not complete for all time periods for all sites (Table), reflecting some element of bias in identification of households related to operational factors such as availability of personnel to follow-up cases and enter case data into the dataset. Nonetheless, there was a clear pattern of disease movement from CCMP data, with winter/spring cases seen in La Source, Petit Goave, and Grand Goave, followed by ongoing summer cases in Grand Goave, and movement of the epidemic eastward into Leogane, with some early spring cases in Leogane.

In Grand Goave, long lasting (≈20 days) winter case clusters appeared in early January at the confluence of a natural waterway and Route 2, the major highway into the southern peninsula (Figure 2, panel A). This was followed by springtime cases further inland/upland in rural areas and along waterways (Figure 2, panel B). Summer clusters that followed were in rural, inland/upland areas, with no clusters identified in the urban center. In Leogane (summer), clusters were again identified along Route 2, beginning south of the city and in the urban center (Figure 2, panel C). Later clusters appeared the mountains, in communities

Table. Case count and date ranges of cholera case reports by community, Haiti, 2011

Community	Total no. cases	Date range of cases (no.)
Petit Goave	612	Jan 1–May 6 (612)
Grand Goave	549	Jan 1–Apr 7 (348); May 1–Aug 15 (201)
Leogane*	344	Feb 7–May 7 (61); Jun 7–Jul 26 (283)
La Source	25	Jan 10–Feb 22 (23); Apr 23 (2)

*Cases in Leogane were few for the reporting period of Feb 7–May 7; related data were not included in further space-time analysis.

along the Momance River, with subsequent clusters following the river toward the Caribbean Sea. Four of 6 clusters in Petit Goave were situated between 2 major highways with 2 clusters near the urban center (clusters 5, 6) (Figure 2, panel D). The first 2 clusters appeared east (cluster 1) and west (cluster 2) of the urban center in semirural regions along natural waterways.

Conclusions

Here we provide an initial spatiotemporal assessment of household-level cholera following the introduction of *V. cholerae* to Haiti. Our 2011 winter/spring cases occurred in the initial larger epidemic wave, followed by additional peaks in cases in summer, with the onset of the rainy season. Our data support the hypothesis that initial case transmission followed roadways, particularly Route 2. In Petit and Grand Goave, transmission along roadways was followed by disease movement into rural/inland areas. After initial urban case clusters occurred in Leogane, cases appeared in the mountains, with clusters then appearing along the Momance River, consistent with the hypothesis that the river provided a transmission route for *V. cholerae*. Supporting this observation, toxigenic *V. cholerae* O1 was recently recovered from the Momance River (*8*).

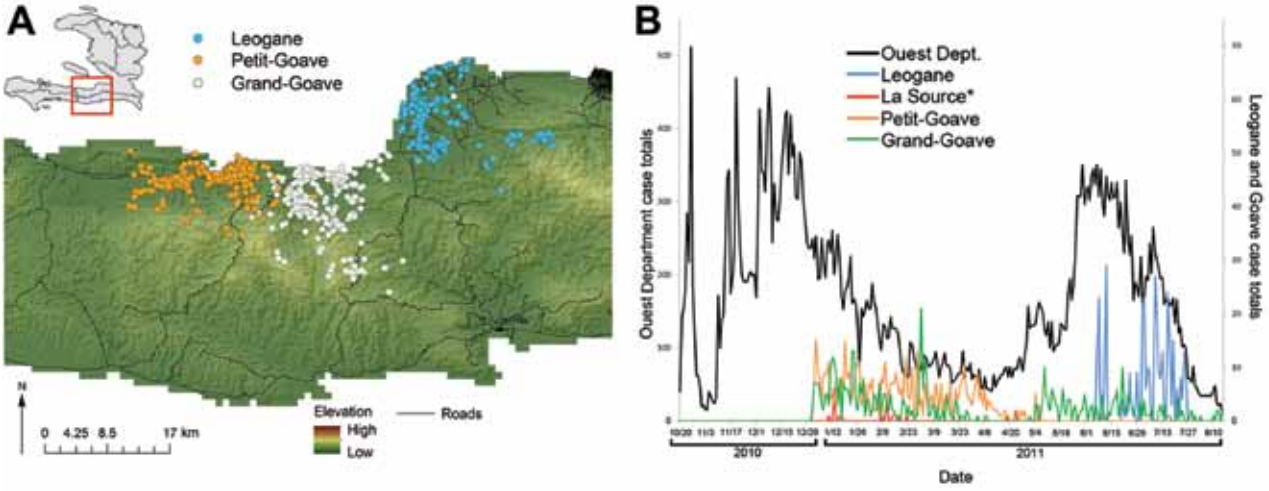

Figure 1. Findings of the Cooperative Cholera Mapping Project in Haiti, 2011. A) Geographic distribution of household cholera cases per day. B) Temporal pattern, color-coded by community, compared with reported cases in the Ouest Department (black). Color coding of map symbols in A correspond to line colors in B. La Source cases (n = 25) are plotted but not mapped.

There is increasing recognition that cholera has 2 routes of transmission, one involving movement through waterways (e.g., surface waters, rivers) and the other related to more direct transmission from person to person (*9*,*10*). In keeping with recent mathematical models (*11*), our data support the hypothesis that both routes are important to transmission in Haiti. The inland/river movement in both Petit Goave and Leogane occurred during the summer rainy season, consistent with a link between transmission involving surface waters and seasonal rainfall. These data were collected early in the course of the epidemic and limited to a small proportion of the total reported cases. Multiple years of observation are necessary to confirm these patterns; however, models in this and other regions already suggest the development of a seasonal pattern of illness linked with rainfall (*12*).

Generally, human mobility, such as urban/rural or rural/urban migrations can influence disease patterns (*13*).

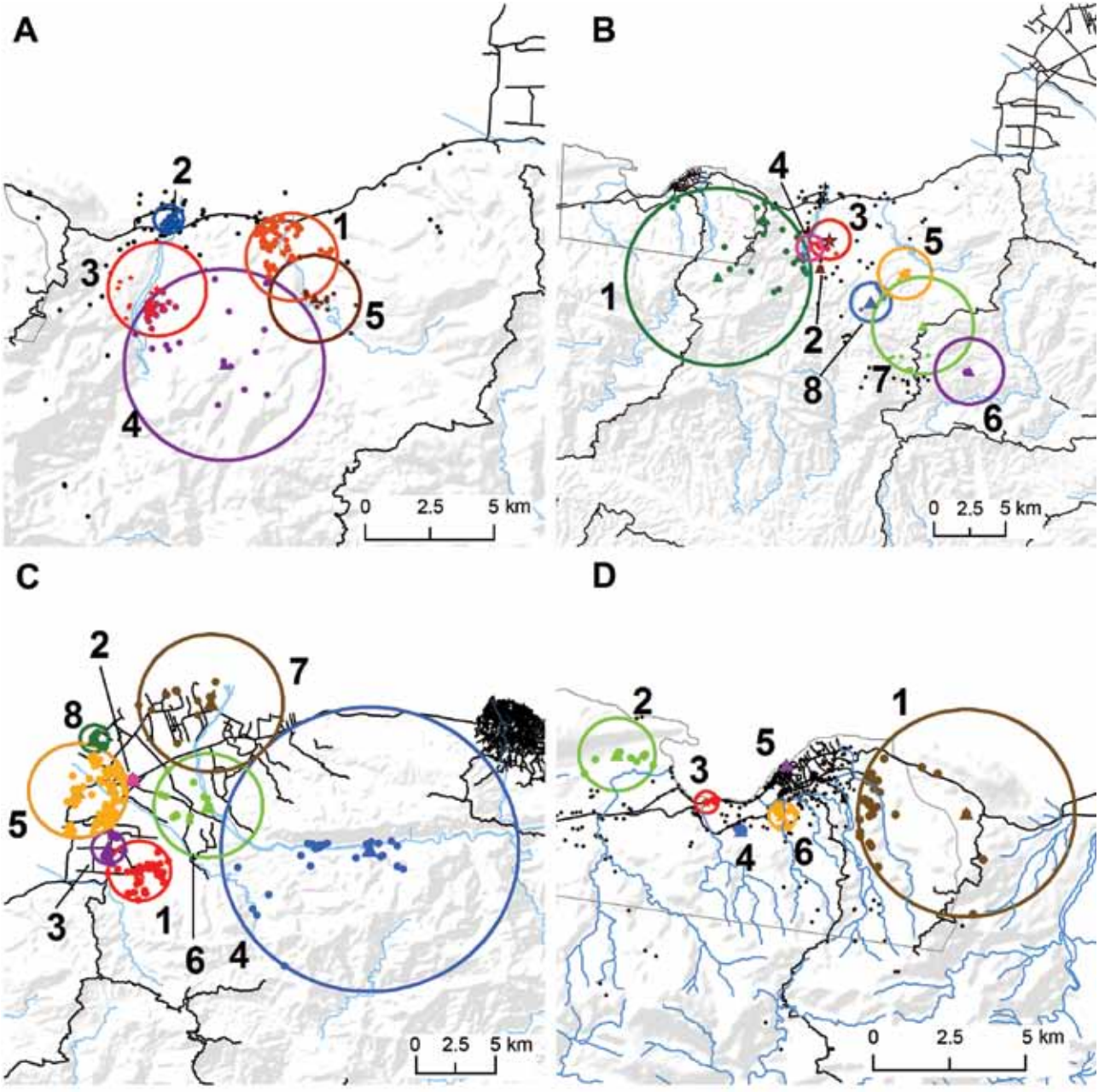

Figure 2. Space-time clusters of cholera in 4 communities in Haiti, 2011. A) Grand Goave, winter; B) Grand Goave, summer; C) Leogane, summer; and D) Petit Goave, summer. Stars represent primary cluster centers and triangles, secondary cluster centers. Dots represent approximate locations of households within clusters. Clusters are numbered sequentially by order of date of occurrence.

Our results suggest that such mobility is a factor of epidemic cholera transmission in Haiti. Recent models suggested human movevent out of damaged areas was substantial, but ultimately, persons attempt to return to the areas that formed the basis of their predisaster social networks (*14*). This study identifies key geographic areas for improved data collection. It also highlights the need for careful targeting of interventions that are shaped by ongoing data collection and analysis at local levels. Transmission routes can differ across space and time, and only by understanding these local differences can cost-effective disease control methods be identified and implemented.

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Dr Blackburn is an assistant professor of geography and principal investigator at the Emerging Pathogens Institute, University of Florida. His research interests are focused on the spatiotemporal patterns and ecology of pathogens found in environmental reservoirs; particularly, those that cause zoonoses.

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Incidence of *Cronobacter* spp. Infections, United States, 2003–2009

Mary E. Patrick, Barbara E. Mahon, Sharon A. Greene, Joshua Rounds, Alicia Cronquist, Katie Wymore, Effie Boothe, Sarah Lathrop, Amanda Palmer, and Anna Bowen.

During 2003–2009, we identified 544 cases of *Cronobacter* spp. infection from 6 US states. The highest percentage of invasive infections occurred among children <5 years of age; urine isolates predominated among adults. Rates of invasive infections among infants approximate earlier estimates. Overall incidence of 0.66 cases/100,000 population was higher than anticipated.

Cronobacter spp. are gram-negative bacteria mainly perceived to cause serious infections in infants (*1*). Cases are most common among newborns or young infants; estimated mortality rates are as high as 80% (*2*). Infections also occur in older children and adults (*3*). In adults, *Cronobacter* spp. cause septicemia, pneumonia, osteomyelitis, wound infections, and splenic abscesses (*4*).

Little is known about reservoirs or routes of transmission other than ingestion of contaminated powdered infant formula (*5*–*7*). However, organisms have been isolated from other foods and environmental sources, and infections have occurred among persons who did not consume or handle formula (*8*,*9*).

In the United States, the incidence of *Cronobacter* spp. infection is unknown, but evidence suggests it is low. In 1998, only 1 case was found among 10,660 hospitalized infants of low birth weight (*10*). In 2002, the Foodborne Diseases Active Surveillance Network (FoodNet) estimated an incidence of 1 case per 100,000 infants (*11*). However, these estimates might be unreliable because populations studied were small and the disease is rare. There is no national surveillance for *Cronobacter* spp. infections; such infections are reportable only for infants in Minnesota. To increase understanding of the public health effects and demographic distribution of *Cronobacter* spp. infections, we investigated incidence of laboratory-confirmed infection and characteristics of infected persons.

The Study

FoodNet is a collaborative program among the Centers for Disease Control and Prevention, 10 state health departments, the US Department of Agriculture, and the Food and Drug Administration. FoodNet conducts active, population-based surveillance for selected laboratory-confirmed enteric infections. In 2010, clinical laboratories within FoodNet were asked to query records for all laboratory-confirmed isolations of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) reported from January 1, 2003, through December 31, 2009, or for as many months in this period as was feasible. These isolations are subsequently referred to as *Cronobacter* cases. Participating laboratories were in California, Colorado, Maryland, Minnesota, New Mexico, and Tennessee. We recorded information on patient age, isolation site, and date (month and year) of specimen collection. We defined invasive infection as isolation of the organism from blood or cerebrospinal fluid. We conducted descriptive analyses and calculated incidence rates within age groups and overall by using Excel (Microsoft Corp., Redmond, WA, USA) and SAS version 9.3 (SAS Institute, Cary, NC, USA).

For incidence calculations, we estimated the number of persons served by each laboratory by calculating the proportion of isolates of other enteric pathogens (*Campylobacter*, *Listeria*, *Salmonella*, *Vibrio*, and *Yersinia* spp. and Shiga toxin–producing *Escherichia coli*) that each laboratory reported to FoodNet, by age group, for each year that the laboratory was surveyed. We applied this proportion to US census data for that age group, year, and FoodNet site and summed values from all laboratories to obtain an age-group denominator for 2003–2009. We then calculated age-specific incidence rates by dividing the number of *Cronobacter* cases for each age group by the overall age-group denominator and multiplying by 100,000. Similarly, we calculated the overall incidence rate by dividing the total number of *Cronobacter* cases by the sum of the age-group denominators and multiplying by 100,000.

We identified 544 *Cronobacter* cases from participating laboratories at 6 FoodNet sites. The overall median patient age was 59 years (range 1 day–100 years); by state, median ages ranged from 52 years (Tennessee) to 71 years (Colorado). Of the 544 patients, 198 (37%) were

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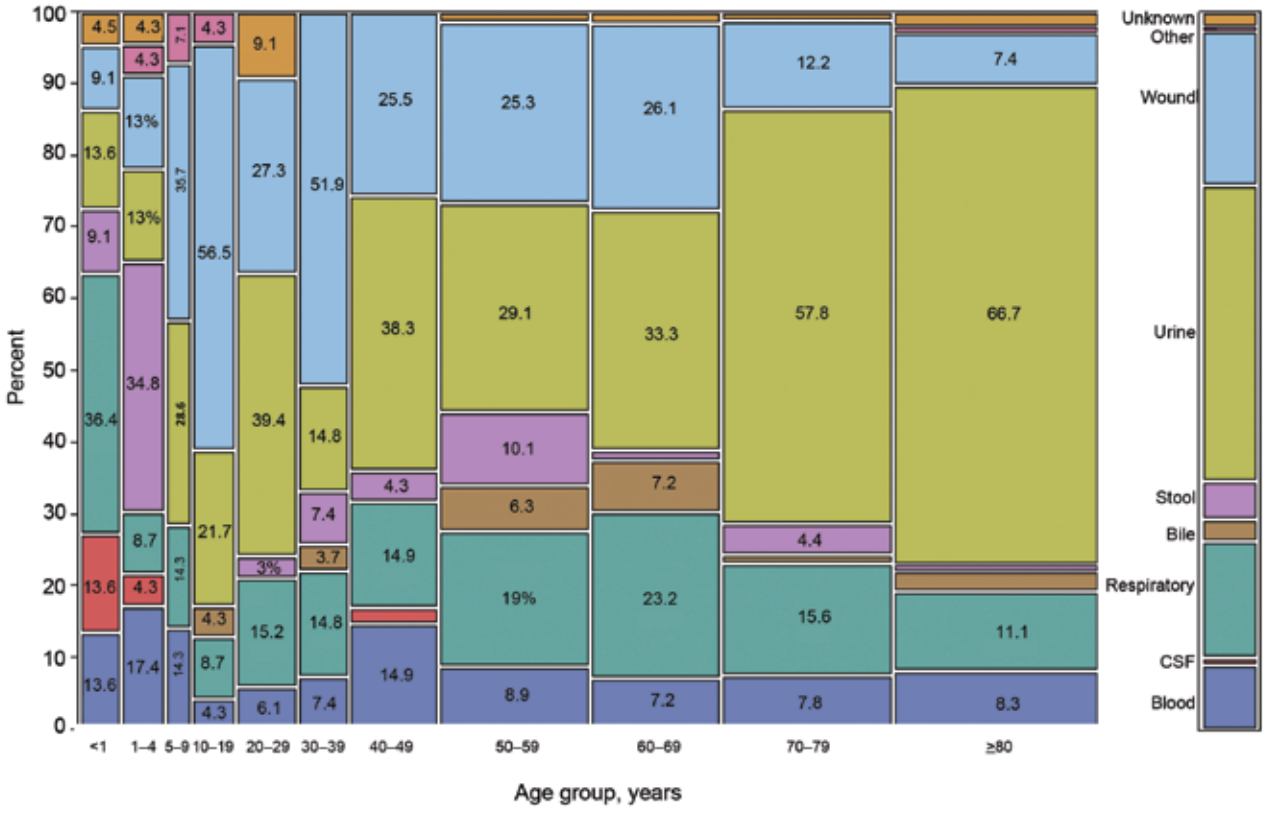

Figure 1. Isolations of *Cronobacter* spp., by specimen source and patient age group, Foodborne Diseases Active Surveillance Network (FoodNet), 2003–2009. Data are based on a sample from laboratories in 6 states (California, Colorado, Maryland, Minnesota, New Mexico, and Tennessee) in the FoodNet catchment area and are reported for 535 of 544 patients (age information missing for 9 patients). Width of the column is proportional to the number of isolations. CSF, cerebrospinal fluid.

>70 years of age and 22 (4%) were infants <1 year of age. *Cronobacter* spp. was most frequently isolated from urine (221 [41%] isolates); wound, abscess, or surgical site (115 [21%]); respiratory tract (sputum, pharyngeal swab, or tracheal aspirates) (87 [16%]); blood (51 [9%]); feces (31 [6%]); bile (16 [3%]); cerebrospinal fluid (5 [1%]), and other or unknown sources (18 [3%]). The highest percentage of invasive infections occurred among infants (6 [27%] of 22) and children 1–4 years of age (5 [22%] of 23), and isolates from urine predominated among those in most adult age categories (Figure 1). Other than a slight increase in the number of isolations in April, no seasonl patterns were detected.

The overall incidence rate was 0.66 cases per 100,000 population; rates varied by age, state, and invasiveness. The highest rates occurred among persons ≥80 years of age (3.93 cases/100,000 population), followed by persons 70–79 years of age (2.11) and infants (1.81) (Figure 2). Rates of invasive infection were 0.07 cases per 100,000 population overall and were highest among infants (0.49) and persons ≥80 years of age (0.33) (Figure 2, panel A). Overall incidence rates among infants were highest in Minnesota, and rates among persons ≥80 years of age were highest in Colorado. Rates of urinary tract isolation were 0.27 isolates per 100,000 population overall and highest among persons ≥80 (2.62) and 70–79 (1.22) years of age.

This analysis provides population-based incidence rates of *Cronobacter* spp. infection for all age groups in the United States. Overall rates were similar to those reported for vibriosis and yersiniosis (*12*). The rates for infants in our study were similar to those previously estimated for infants in the United States (*11*). The rates of bacteremia that we found among infants and older adults in the United States were higher than those reported in the Philippines and the Netherlands and similar to those reported in the United Kingdom (*13*,*14*).The higher rates for Minnesota might be explained by the reporting requirements in that state; however, high rates among adults in other states suggest that the geographic variation may be real.

Although rates were highest among persons at age extremes, we documented isolations for persons in all age groups and from a variety of clinical specimen sources. Although infants accounted for only a small percentage of

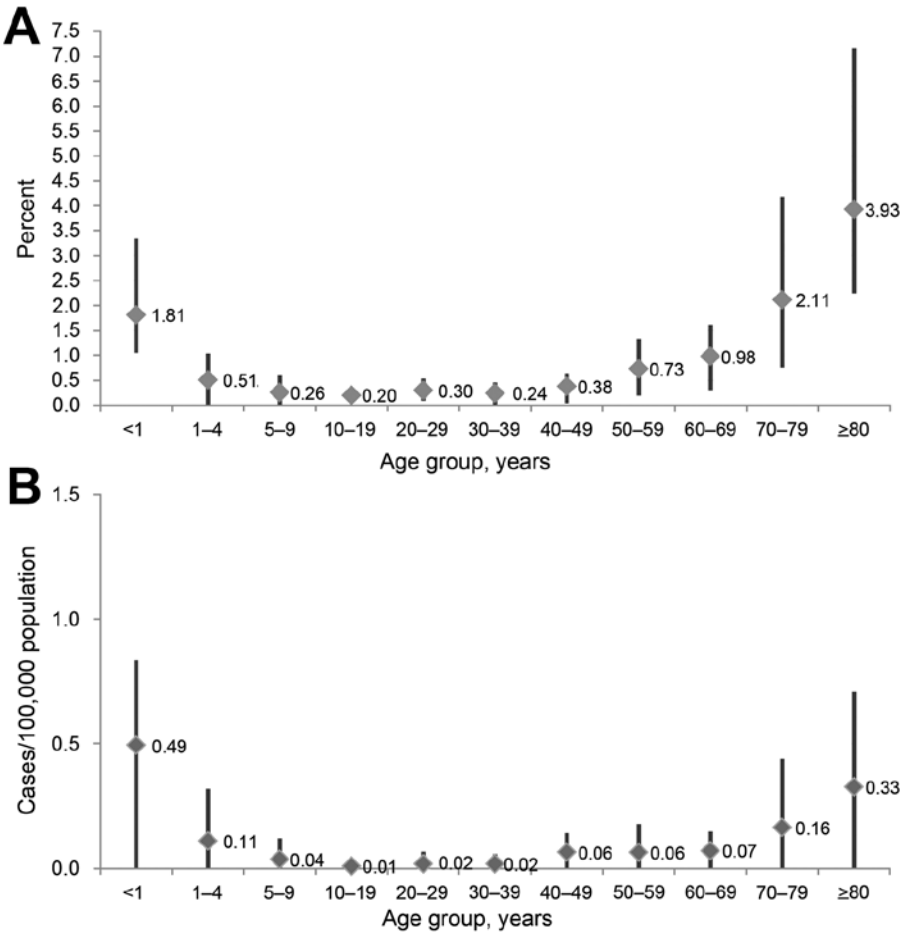

Figure 2. A) *Cronobacter* spp. incidence rates, by age group (overall and range by site) in the Foodborne Diseases Active Surveillance Network (FoodNet), 2003–2009. B) *Cronobacter spp.* incidence rates for invasive isolates by age group (overall and range by site), FoodNet, 2003–2009. Data are based on a sample from laboratories in 6 states (California, Colorado, Maryland, Minnesota, New Mexico, and Tennessee) in the FoodNet catchment area and are reported for 535 of 544 patients (age information missing for 9 patients).

isolates, they also accounted for the highest rate of invasive infections. More than a third of isolates from infants came from the respiratory tract and might represent colonization rather than infection. Rates for adults ≥80 years of age were higher than rates for any other age group and twice as high as rates for infants; however, we lack information to distinguish infection from colonization.

Half of the isolates in our survey came from urine, and these were mainly from older adults. Although others have documented urinary tract infections caused by *Cronobacter* spp. *(3,15),* our data suggest that urine might be a more common site of infection than previously thought. Studies are needed to understand the contribution of *Cronobacter* spp. to the prevalence and costs associated with urinary tract infections, which are common in older adults.

Our findings are subject to limitations. Only a third of laboratories that participate in FoodNet provided data for this study, and reporting varied by state and year. Our sample might not be representative of the states surveyed or the United States as a whole. Because we did not review charts or interview patients, we did not have information on underlying conditions, clinical signs and symptoms, patient sex, or possible exposures. Isolates were not available for speciation or subtyping.

Conclusions

Although rates of invasive *Cronobacter* spp. infections among infants in our study approximated earlier estimates, overall rates of isolation of *Cronobacter* spp. from ill persons in the United States were higher than we anticipated, and the very young and very old were disproportionately affected. A substantial proportion of isolates came from the urinary tract, raising questions about risk factors for transmission and clinical manifestations. Routine, systematic surveillance and special studies will be essential for understanding these findings, identifying reservoirs of infection and vehicles of transmission, and developing effective prevention and control measures.

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We thank Jillian Friedman and Suzanne Segler for gathering survey data from participating laboratories.

Ms Patrick works as the project coordinator for FoodNet. Her research interests include *Campylobacter* spp. epidemiology, infectious disease surveillance, and geospatial analysis.

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Investigation and Control of Anthrax Outbreak at the Human–Animal Interface, Bhutan, 2010

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In 2010, we investigated anthrax outbreak in Bhutan. A total of 43 domestic animals died, and cutaneous anthrax developed in 9 persons, and 1 died. All affected persons had contact with the carcasses of infected animals. Comprehensive preparedness and response guidelines are needed to increase public awareness of anthrax in Bhutan.

Anthrax, an acute infectious disease caused by infection with *Bacillus anthracis*, can affect almost all warm-blooded animals, including humans (*1*). Animals become infected through contact with soilborne *B. anthracis* spores; humans become infected only incidentally through contact with diseased animals or with the carcasses or by-products of diseased animals (*1*). Anthrax is widespread. Sporadic outbreaks and epizootics occur among livestock and wild animals in the United States, Canada, and southern and eastern Europe, and outbreaks at the animal–human interface are reported from countries in Africa, the Middle East, and Asia (*1*–*7*). In southern Asia, anthrax is highly endemic in India and Bangladesh, and frequent outbreaks and cases are reported among animals and humans (*6*–*8*).

In Bhutan, sporadic anthrax outbreaks occur annually among animals, posing health risks to persons who come into contact with the infected animals (*9*). We present the findings of an epidemiologic investigation of a major anthrax outbreak that occurred at the human–animal interface in a remote area of central Bhutan.

The Study

During July–September 2010, an outbreak of anthrax occurred among animals in Kaktong, a remote village in Zhemgang District in central Bhutan. The outbreak later spread to 8 neighboring villages, where humans also became infected. The outbreak began after a period of heavy rainfall, which may have brought spores to the soil surface, where they could be ingested by ruminants grazing in the area.

A multisectoral team from animal and public health offices in Bhutan visited the outbreak area to investigate and to establish a control program. A case of anthrax was suspected if an animal had signs or symptoms of infection (e.g., sudden death, bloated carcass, bleeding of unclotted blood from natural orifices); a case of anthrax was confirmed if rod-shaped bacilli were found by blood smear examination. Additional samples from animals with positive blood smears were referred to the US Centers for Disease Control and Prevention (Atlanta, GA, USA) for culture and isolate characterization. *B. anthracis* was isolated from 3 samples (2 ear tips and 1 nasal swab) from 3 cattle (from 3 separate villages).

All isolates were characterized by multilocus variable-number tandem repeat analysis, and 1 isolate was analyzed by whole-genome sequencing and single-nucleotide polymorphism analysis (*10*,*11*). Other strains from nearby Bangladesh and India were recently characterized and belong to the more widely dispersed A lineage. However, isolates from the Bhutan outbreak were found to be part of the multilocus variable-number tandem repeat analysis B1 lineage (genotype 83) and canonical single-nucleotide polymorphism subgroup B.Br.001/002 (Figure 1) (*10*–*13*). The B lineage is less widespread and primarily associated with South Africa, but it has been reported in parts of the United States, Europe, and Asia, including the Caucasus region in a recent report (*10*–*14*). The team in Bhutan investigated the mode of *B. anthracis* transmission and spread among livestock and humans in outbreak areas and implemented control measures.

In Kaktong, the index village, a cow had suddenly died after a brief illness; the animal exhibited bleeding of unclotted blood from nostrils, and its carcass was bloated (Figure 2, panels A–C) (*1*). The owner of the affected herd had opened the carcass and dressed the meat, which he shared or sold within the village for human consumption. Transportation of infected meat to neighboring villages resulted in the spread of disease and death among animal herds in 8 other villages; like the index animal, the animals that died were dressed out for human consumption. In some instances, horses that were used to carry contaminated meat became infected and died of anthrax. During July–September, a total of 43 animals in 9 villages died: 25 cattle, 8 horses, 4 pigs, and 6 cats. The infected cats were possibly exposed to *B. anthracis* through the ingestion of meat from infected carcasses. The infected pigs were fed with *B. anthracis*–contaminated kitchen waste.

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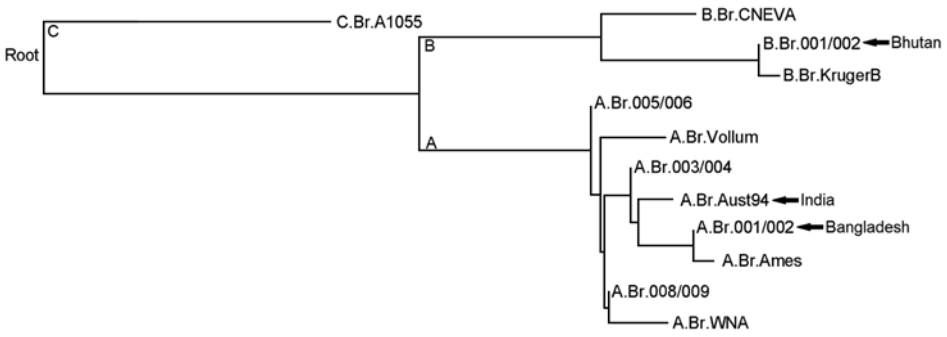

Figure 1. Phylogeny of major *Bacillus anthracis* groups as determined by using canonical single nucleotide polymorphisms as described by Van Ert et al. (*12*). Arrows indicate the lineages/groups of genotyped *B. anthracis* isolates from India (*12*), Bangladesh (*11*,*13*), and Bhutan.

The investigation showed that within 1 week of exposure, skin lesions developed on 9 humans (8 male; 1 female) who handled and dressed the animal carcasses. The lesions were black eschars, typical of anthrax, and occurred on the patients' necks, fingers, arms, feet, legs, or cheeks (Figure 2, panels D–G). Of the 9 persons with cutaneous anthrax, 1 (the female villager) died. Symptoms consistent with gastrointestinal anthrax, including abdominal cramps, vomiting, and respiratory distress, developed in this person after she ingested contaminated meat. A detailed investigation of the case was not conducted because of the remote location of the village.

To stop the spread of disease, animal and public health authorities initiated various prevention and control measures: a campaign to create awareness among villagers and students; ring vaccination of cattle against anthrax (≈445 animals in 11 villages); treatment of sick animals with antimicrobial drugs ; disposal of carcasses in deep burial pits; recall, collection, and disposal into burial pits of all potentially infected meat and hides from cattle that died of suspected or confirmed anthrax; and monitoring and treatment of persons in whom cutaneous anthrax developed. These control measures eventually contained the outbreak.

In the remote villages of Bhutan, meat from dead animals is usually consumed by the villagers because they live in poverty and lack slaughterhouse facilities and education regarding diseases that might harm them. As in other remote villages, the farmers in Kaktong and neighboring villages affected by this outbreak were unaware of anthrax in animals and of the public health implications of this disease. Our investigation showed that all affected persons had handled and/or consumed meat from animals with suspected or confirmed anthrax. While we were conducting the outbreak investigation and control programs, we held an education meeting to make villagers and students aware of the risks associated with anthrax. The villagers cooperated in response activities, including the disposal of carcasses

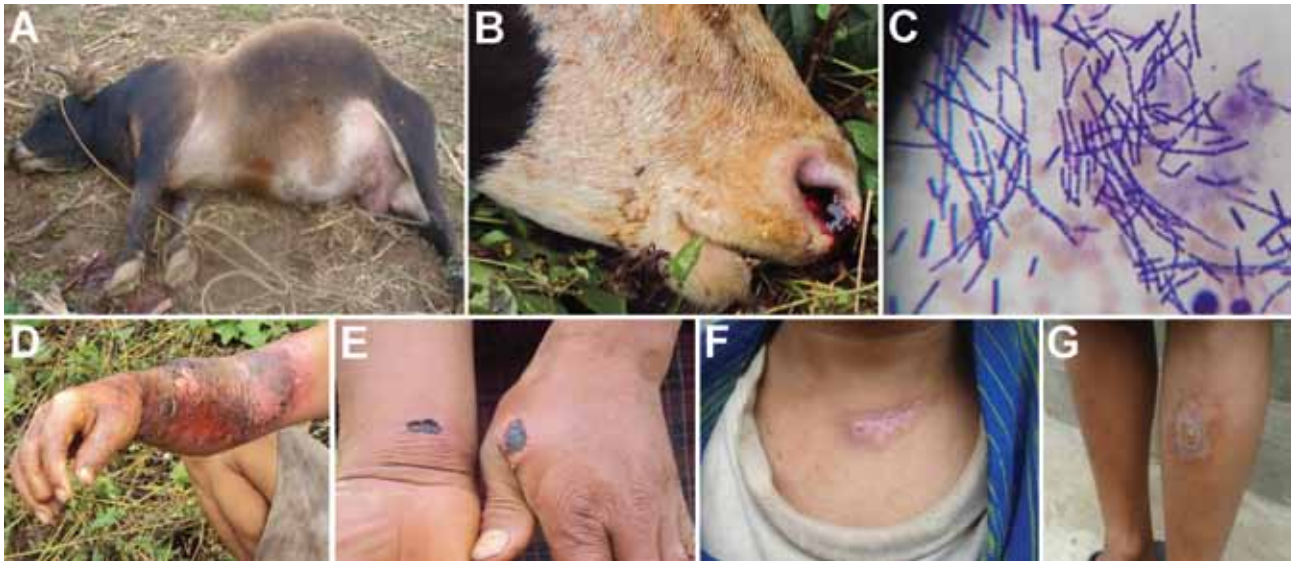

Figure 2. Signs of anthrax in infected animals (A–C) and humans (D–G), Bhutan, 2010. A) The carcass of an affected bull, showing bloating. B) Bleeding of unclotted blood from a cow's nostril. C) Rod-shaped *Bacillus anthracis* bacilli from 1 of the infected animals. Cutaneous anthrax causing severe inflammation of the arm (D) and typical black eschars on the hand and wrist (E), neck (scar) (E), and leg (G) of persons who had contact with *B. anthracis*–infected animals and carcasses.

and recall and disposal of meat from the carcasses that had been kept for human consumption, and they participated in control activities, including the treatment of affected animals and ring vaccination of animals that had been in contact with infected animals.

In Bhutan, sporadic anthrax cases in animals are detected and reported every year; such cases pose risks to humans (*9*). The sudden emergence in 2010 of an anthrax outbreak in remote villages in Bhutan could be linked to heavy rainfall, which may raise *B. anthracis* spores to the soil surface, where they can be ingested by animals. Cutaneous anthrax cases similar to those reported here have been reported at the human–animal interface in other countries. For example, >6,000 anthrax cases in humans were reported in Zimbabwe in 1979 and 1980; the cases were associated with the slaughter of *B. anthracis*–infected cattle (*4*). In addition, 25 cutaneous anthrax cases occurred in humans in Paraguay in 1987 after the slaughter of a single *B. anthracis*–infected cow (*5*), and many cases of cutaneous anthrax have occurred in humans following the slaughter of sick or dead animals in India (*6*), Bangladesh (*8*), and China (*15*).

For humans, the major sources of exposure to *B. anthracis* are direct or indirect contact with infected animals or contaminated animal products. Persons at risk for exposure should be made aware of those risks and of the public health implications of zoonotic diseases such as anthrax (*1*). From this outbreak investigation and our experiences in Bhutan, we recommend the following measures for this country: development of comprehensive guidelines for anthrax surveillance among humans and animals; establishment of surveillance for anthrax hot-spot areas; and development of education programs to teach persons at high risk (e.g., butchers) about anthrax transmission, the care of skin abrasions, and disease-prevention measures, including personal hygiene practices and refusal to eat meat from dead or sick animals.

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Family Cluster of Middle East Respiratory Syndrome Coronavirus Infections, Tunisia, 2013

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In 2013 in Tunisia, 3 persons in 1 family were infected with Middle East respiratory syndrome coronavirus (MERS-CoV). The index case-patient's respiratory tract samples were negative for MERS-CoV by reverse transcription PCR, but diagnosis was retrospectively confirmed by PCR of serum. Sequences clustered with those from Saudi Arabia and United Arab Emirates.

As of May 23, 2014, a total of 635 laboratory-confirmed human cases of Middle East respiratory syndrome coronavirus (MERS-CoV) infections had been reported to the World Health Organization; the epidemic has subsequently accelerated (*1*). Of these patients, 193 (30%) died. This new virus causes disease similar to that caused by severe acute respiratory syndrome coronavirus, but MERS-CoV is genetically distinct (*2*). We investigated a cluster of 3 MERS-CoV cases in 1 family in Tunisia.

The Cases

Patient 1, the index case-patient, was a 66-year-old Tunisian man with a 4-year history of untreated diabetes mellitus. During March 20–April 27, 2013, he visited his daughter (patient 2) in Qatar for 5 weeks (Figure 1), 1 week of which they spent on a Muslim pilgrimage to Mecca, Kingdom of Saudi Arabia. On April 18, results of a physical examination (including chest radiograph) for a visa extension in Qatar were unremarkable. On the day of arrival back in Tunisia (April 28), the patient experienced chills, followed by arthralgia, dry cough, and fever. The daughter reported that her father had had no direct contact with camels during his stay in Qatar or Saudi Arabia. One of his children (patient 3, a nurse) gave him acetaminophen and aspirin for 3 days and then intravenously administered dexamethasone (4 mg) twice a day for 2 days. On May 6, patient 1 experienced worsened dyspnea and he sought care at the Centre Hospitalier-Universitaire Fattouma Bourguiba Hospital (Monastir, Tunisia) emergency department, where he received a fifth injection of dexamethasone. Chest radiograph showed left lower lobe infiltrate (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/20/9/14-0378-Techapp1.pdf). The patient was first admitted to the pulmonary ward, where he received amoxicillin-clavulanate (1 g) 3 times daily; however, on May 8, respiratory failure and peripheral signs of shock necessitated admission to the intensive care unit (ICU), where he was positioned prone and given noradrenalin infusion and mechanical ventilation with additional nitric oxide.

Mini (<10 mL fluid injected) bronchoalveolar lavage recovered a liquid of low cellularity; cultures for bacteria and fungi were negative. Serologic tests for common respiratory viruses were negative. The patient was first given amoxicillin-clavulanate, ciprofloxacin, and rifampin. On his second day in ICU, oseltamivir was added. The lavage fluid was then tested in the Tunisia National Reference Laboratory (TNRL) for MERS-CoV by using real-time reverse transcription PCR (rRT-PCR) upE (region upstream of the E gene), open reading frame (ORF) 1a, and ORF1b assays. These assays were developed in house according to the Corman et al. protocol (*3*); results were negative. On May 10, patient 1 died of multiple organ failure. Because nasopharyngeal swab samples from his 2 adult children were positive for MERS-CoV, the case of patient 1 was reported to the World Health Organization as probable MERS-CoV infection (*4*).

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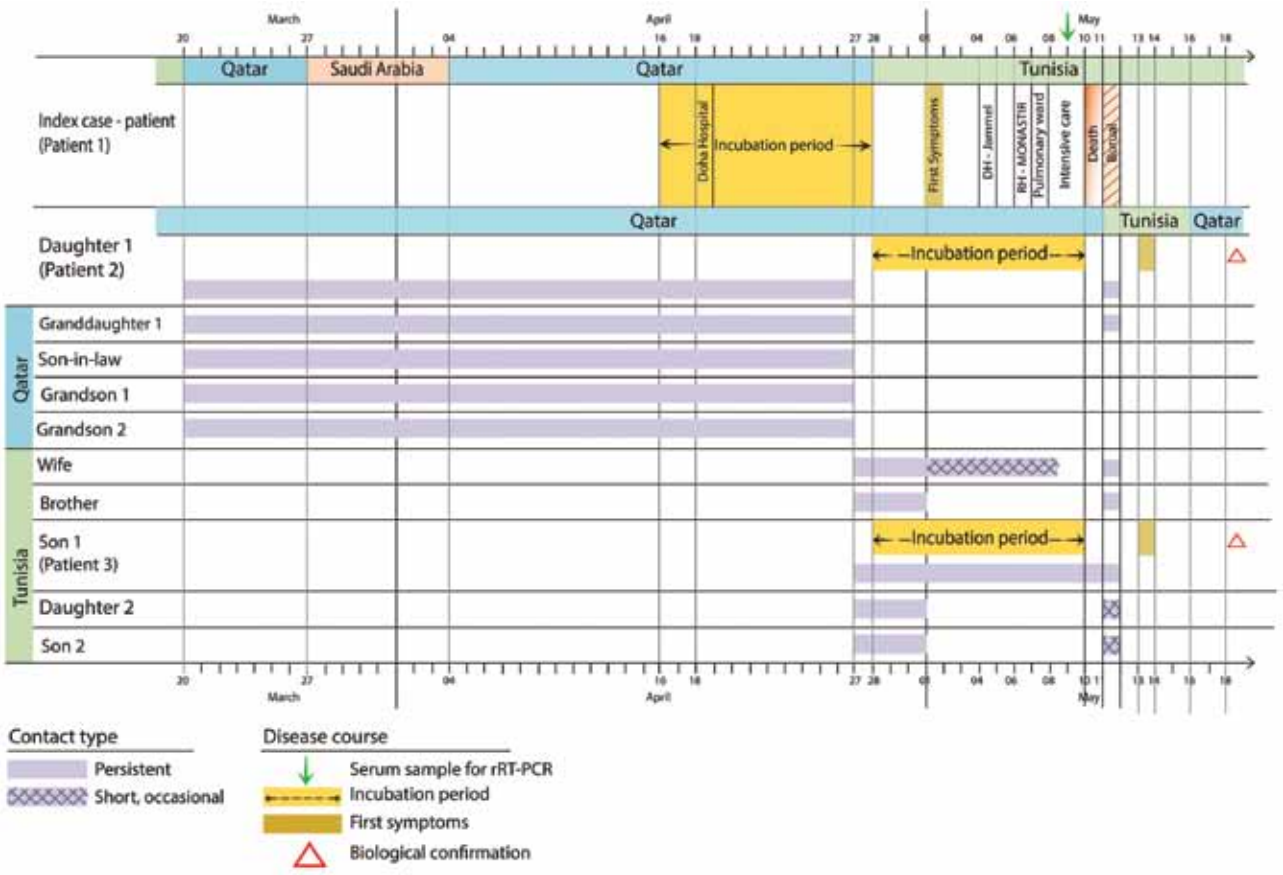

Figure 1. Clinical course of disease for patients with confirmed Middle East respiratory syndrome coronavirus Infection, Tunisia, 2013. RH, regional hospital; DH, district hospital; rRT-PCR, real-time reverse transcription PCR.

On August 5, 2013, the Centers for Disease Control and Prevention (CDC) tested a serum sample collected from the index-patient on May 9. Independent rRT-PCRs were positive for MERS-CoV (*5*); targets were upE (cycle threshold [C_t] 30.27) and nucleocapsid protein (N)2 (C_t 30.46). Sequences of the full N and spike (S) protein coding regions were submitted to GenBank (accession nos. KF811035 and KF811036, respectively). Nucleotide/predicted amino acid sequence identities with published MERS-CoV sequences for the N and S gene coding regions ranged from 99.2%–100% to 99.0%–100% and from 99.4%–99.9% to 99.4%–99.8%, respectively. Phylogenetic relationships between this virus (designated *Tunisia-Qatar_2013*) and other published MERS-CoV sequences showed clustering with geographically diverse sequences from Saudi Arabia and the United Arab Emirates (Figure 2).

Patient 2 was the 30-year-old daughter who had accompanied the index case-patient to Mecca. She remained in Qatar until she attended her father's funeral in Tunisia on May 11, 2013, when she reported sore throat, cough, and fever. On May 13, a chest radiograph showed bronchial thickening. A nasopharyngeal swab sample collected on May 16 was positive for MERS-CoV by rRT-PCR performed at the TNRL: upE C_t 27.5, ORF1a C_t 27.46, and ORF1b C_t 37.55. Testing at CDC detected a C_t of 28.46 for upE and negative results for N2 and N3 (*5*). A few days after she received oseltamivir, the patient's symptoms resolved.

Patient 3 was the 34-year-old son of the index case-patient, a nurse in the ICU where his father had been admitted. He had not traveled outside the country during the incubation period, and his first contact with the index case-patient was after his father's return to Tunisia and illness onset. He cared for his father at home during the initial phase of illness and thereafter in the pulmonology department and ICU. Patient 3 reported a sore throat on the day after his father's funeral. A nasopharyngeal swab sample obtained on May 16 was positive for MERS-CoV by rRT-PCR performed at TNRL: upE C_t 21.56, ORF1a C_t 27.6, and ORF1b C_t 31.39. At CDC, the nasopharyngeal swab sample was positive for MERS-CoV by 3 independent rRT-PCRs (*5*): C_t 21.67 for upE, 34.51 for N2, and 32.32 for N3. Patient 3 recovered without treatment.

Contact tracing involved the 4 remaining family members. Nasopharyngeal and/or throat swab samples were

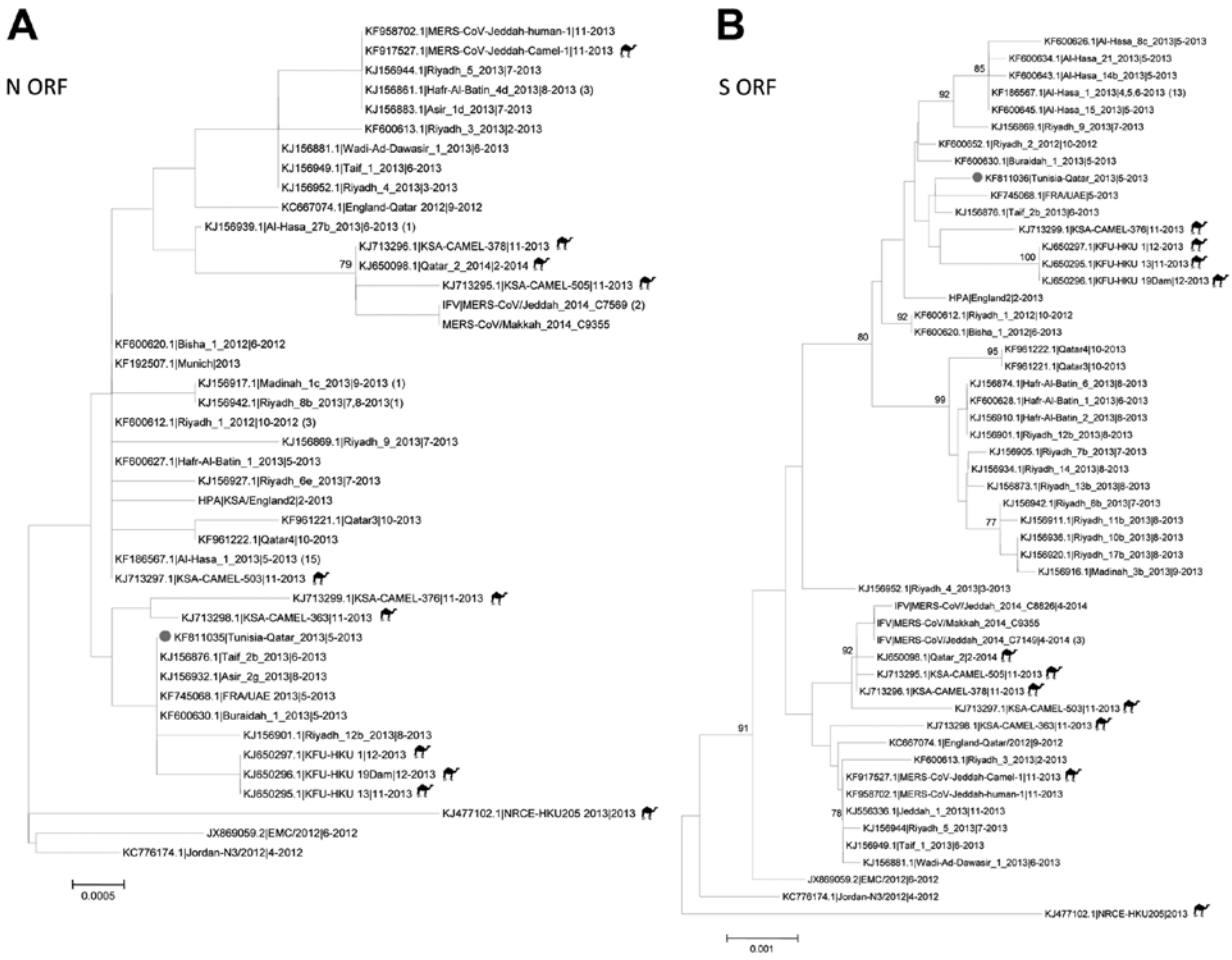

Figure 2. Midpoint-rooted phylogenetic trees of the full-length nucleocapsid (N) (panel A) and spike (S) (panel B) open-reading frames (ORFs) of isolates obtained from index case-patient with Middle East respiratory syndrome coronavirus (MERS-CoV) infection, Tunisia, 2013. Serum and available nucleotide sequences from GenBank and Public Health England (http://www.hpa.org.uk/webw/HPAweb&HPAwebStandard/HPAweb_C/1317136246479) and the Institut Für Virologie (http://www.virology-bonn.de/index.php?id = 46). The estimated neighbor-joining trees were constructed from nucleotide alignments by using MEGA version 6.06 (http://www.megasoftware.net). Sequence names are written as GenBank accession number|virus strain name|month-year of collection. Numbers in parentheses denote the number of additional sequences from viruses isolated from humans that are identical to the listed sequence. Solid circles indicate sequences from MERS-CoV from Tunisia, 2013. Camel icons indicate MERS-CoV sequences derived from isolates from camels. Bootstrap support values (1,000 replicates) ≥75% were plotted at the indicated internal branch nodes. Scale bars indicate number of nucleotide substitutions per site.

collected a mean of 5 weeks after contact from the other 2 (not ill) children of patient 1, his spouse, and the spouse of patient 3. Health care workers who had been in contact with the index case-patient in the pulmonology ward (n = 2) or ICU (n = 6) and who had reported sore throat, hyperthermia, and/or diarrhea (1 worker) were also investigated. All respiratory samples from contacts were negative for MERS-CoV by rRT-PCR.

Conclusions

The fact that the diagnosis for the index case-patient was made by PCR of a serum sample collected 10 days after symptom onset and tested several weeks later highlights the value of testing serum samples for MERS-CoV RNA. This finding also provides valuable information about viremia in MERS CoV–infected patients, contributing to our understanding of the natural history of MERS-CoV infection and kinetics of virus shedding (*7*).

Given the incubation period of the disease (up to 15 days), the father most likely acquired his infection in Qatar (*8*,*9*). Patient 3, who had not traveled outside Tunisia, could have been exposed during the 11 days he cared for his father at home and in the hospital. The history of patient 2 is less clear; she might have acquired the virus from the same source as her father in Qatar, or she might have been secondarily infected by contact with him before he

left Qatar, given that her illness began almost 12 days after her father's.

Patient 1 was severely ill at the time of ICU admission; in <3 days, his condition rapidly evolved to multiple organ system failure and death. Although we cannot account for the diabetes or corticosteroid contributions to his disease severity, we can speculate that they might have worsened his outcome. Other MERS CoV patients who have died had concurrent conditions (*2*), and corticosteroids are thought to worsen the outcomes for patients with influenza A(H1N1) virus infection (*10*)**.**

The contact tracing results shed light on the potential for person-to-person transmissibility of MERS-CoV. Only 2 family members who had been in close and prolonged contact with the index case-patient became infected. Infection was not acquired by the case-patient's wife, his 2 children who did not live with him, or the ICU workers who had short-term close contact with him. However, these results should be interpreted cautiously because only nasopharyngeal swab samples obtained 5 weeks after contact with the index case-patient were tested. In addition, serologic testing, which was not performed in the present investigation, could have shed more light on person-to-person MERS-CoV transmissibility.

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Pork Consumption and Seroprevalence of Hepatitis E Virus, Thailand, 2007–2008

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The nationwide seroprevalence of hepatitis E IgG was determined among young men in Thailand. Overall seroprevalence was 14% (95% CI 13%–15%); range by province was 3%–26%. Seroprevalence was lowest in the south, an area predominantly occupied by persons of the Islam religion, whose dietary laws proscribe pork.

Hepatitis E virus (HEV), the etiologic agent of hepatitis E, is a nonenveloped, positive–sense single-stranded RNA virus, ≈7.2 kb in length, of the family *Hepeviridae*. HEV, identified in 1990, was a major cause of what was previously called non-A, non-B hepatitis (*1*,*2*). Hepatitis E occurs worldwide in ≈20 million persons annually, causing ≈70,000 deaths (*3*). Clinical signs of hepatitis E are similar to those of other hepatitis virus infections; it is a usually self-limiting illness in healthy persons, who have mild symptoms or asymptomatic disease. Occasionally, fulminant hepatitis develops (*1*–*3*). The overall mortality rate of hepatitis E is 0.5%–4%, but it increases to 20% among pregnant women (*3*).

In Thailand, hepatitis E outbreaks have not been reported; however, sporadic cases have been reported from many areas (*4*). The reported annual incidence of hepatitis E in Thailand ranged 0.05–0.09 per 100,000 persons during 2008–2012; no deaths were reported (*4*). The low incidence of hepatitis E testing in Thailand may underestimate virus exposure among its population (*5*). Hepatitis E IgG provides evidence of individual HEV exposure: titers peaked ≈7–8 weeks after infection (*1*). Fourteen years after an outbreak of hepatitis E in Kashmir, 21 of 45 patients (47%) had positive results for IgG anti-HEV by using ELISA (*6*). The hepatitis E IgG seroprevalence range was 9%–23% in studies of small or specific populations in Thailand (*7*–*10*). To identify areas of HEV circulation in the country, we conducted a nationwide hepatitis E seroprevalence study of young men in Thailand.

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The Study

We collected serum specimens after gaining informed consent and permission for future studies as part of HIV-1 surveillance among young men in Thailand who were entering the Royal Thai Army (RTA) during 2007–2008. The RTA uses a lottery system to select ≈60,000 young Thai men at the district level of their family residence for enlistment annually. The men enlisted comprise approximately 10% of young men at the district level in Thailand. Sample sizes were calculated to detect a seroprevalence of ≈50% in each province to within 10% of the true value with 95% confidence. A total of 7,760 stratified randomized samples were chosen on the basis of the reported province of residence of the men before RTA entry.

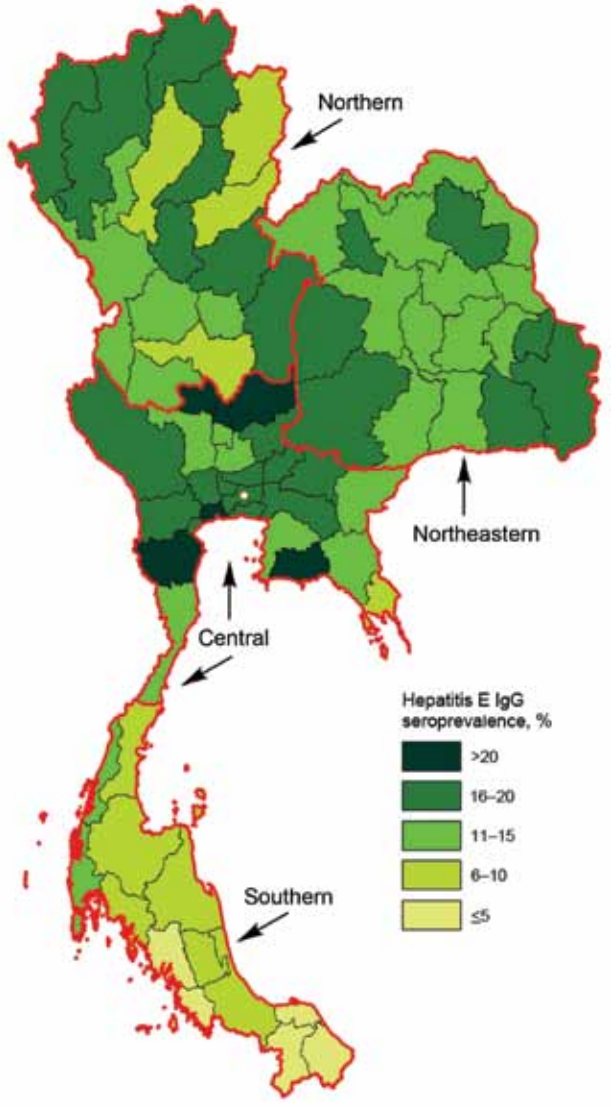

Figure. Map of Thailand, showing hepatitis E IgG seroprevalence in young Thai men, 2007–2008, grouped by reported province of residence during the 2 years before entry in to the Royal Thai Army. Circle indicates Bankok, the capital.

Table 1. Univariate analysis results of demographic variables associated with hepatitis E IgG seroprevalence in young men, Thailand, 2007–2008

Demographic characteristics	Study subjects, no. (%)*	Hepatitis E IgG seroprevalence, % (95% CI)
Total	7,760 (100)	14 (13–15)
Age group, y†		
18–20	1,164 (15)	18 (15–20)
21	5,359 (70)	13 (12–14)
22–30	1,150 (15)	14 (12–16)
Marital status		
Single	6,067 (80)	14 (13–15)
Married	1,509 (20)	14 (12–16)
Education level		
Primary school or less	2,121 (27)	14 (12–15)
Middle school	2,641 (34)	14 (13–15)
Senior high school and vocational	1,920 (25)	14 (12–15)
Diploma and high vocational	756 (10)	13 (10–15)
Bachelor's degree	305 (4)	15 (11–19)
Residential area		
Urban	2,503 (39)	14 (13–16)
Rural	3,896 (61)	13 (12–14)

*Number in each demographic characteristic does not add to the total number of study subjects because of missing data; the number of study subjects with data missing for age group, marital status, education level, and residential area are 87, 184, 17, and 1,361, respectively. Study subjects include all social/religious groups.

We measured hepatitis E IgG antibody using a commercial IgG ELISA kit following the manufacturer's instructions (DIA.PRO, Milan, Italy). We tested associations between demographic characteristics and hepatitis E prevalence using the χ^2 2-tailed test; p value <0.05 was considered statistically significant. We performed the analyses using SPSS version 12 (SPSS Inc; Chicago, IL, USA).

The study population is described in Table 1. Most of the men were 21 years of age, unmarried, had graduated from middle school, and lived in rural areas. The sample size per province was 69–130 persons. The overall crude seroprevalence of IgG against hepatitis E virus was 14% (95% CI 13%–15%). We generated a spatial distribution map of hepatitis E seroprevalence across Thailand by province of residence using ArcView 8.3 (SPSS Inc) (Figure). In the univariate analysis, seropositivity for hepatitis E IgG was associated with age group and residence region, but not with education level, marital status, or residential area (Tables 1,2).

The hepatitis E IgG seroprevelence was lowest in the southern region. This region has the highest percentage of persons whose religion is Islam, known as Muslims, in Thailand. Because consumption of pork is proscribed by the religion of Islam, the region had the lowest pork consumption and pork production (Table 2) (*11*).

This study determined the nationwide seroprevalence of hepatitis E IgG in Thailand in young men from all 76 provinces. The overall hepatitis E seroprevalence at 14% (95% CI 13%–15%) is similar to results of previous hepatitis E seroprevalence studies in Thailand (9%–23%) (*7–10*). However, these comparisons are limited by differences in study design and the performance of the ELISA kits used (*12*). Our study and previous studies used an ELISA to measure antibodies against HEV capsid protein antigens. The range of hepatitis E seroprevalence by province was 3%–26%, indicating HEV exposure of young men across the country. The province of residence was defined as the main province of residence during the 2 years before military service entry. Young men from Thailand typically do not migrate: according to a 2008 national migration report, the percentage of migration between regions by Thai men 20–24 years of age was 8.5% (*13*). Our study population was similar; 10.6% and 9.7% reported a difference between birth and residence at the province and region level, respectively.

HEV is transmitted by the enteric route and is thought to have human and zoonotic reservoirs (*1–3*). In Thailand, the route of HEV transmission has not been clearly identified. Studies in Thailand suggested the association of hepatitis E transmission with pork contact and pork consumption (*9*,*10*,*14*). Previous studies noted higher hepatitis E

Table 2. Hepatitis E IgG seroprevalence and average percentage of population who are Muslim and follow Islamic dietary laws that proscribe pork consumption, by region, Thailand, 2007–2008

Region of residence*	Hepatitis E IgG seroprevalence, % (95% CI)	Median % Muslim residents (Q1, Q3)†
North	14 (12–15)	0.10 (0.00, 0.20)
Northeast	14 (13–16)	0.10 (0.10, 0.10)
Central	17 (16–19)	0.70 (0.20, 2.80)
South	7 (6–9)	20.15 (10.90, 67.80)
Average	14 (13–15)	0.20 (0.10, 2.67)

*χ^2 test statistically significant (2-sided p<0.05).
†Q1, first quartile; Q3, third quartile.

seroprevalence in persons who frequently consume pig organ meat and in swine farmers than in poultry farmers and government officers (*9*,*10*). Moreover, HEV isolated from recovered Thai patients was shown to be closely related to HEV isolated from swine in Thailand (*14*).

Conclusions

In this study, hepatitis E seroprevalence was not associated with education level, marital status, or type of residence; but was associated with residential region (Table 1) and subsequently, consumption of pork. Hepatitis E seroprevalence level was lowest in the south, especially in the most southern areas where the highest percentage (≥67%) of the non-pork–eating Muslim population resides, such as Yala, Narativat, Satul, and Pattani, which showed a hepatitis E seroprevalences of 4%, 4%, 3%, and 3%, respectively (*11*). These findings support the association of HEV transmission with pork consumption in Thailand (*10*). Given the previously reported differential exposure risk between large-scale farms and medium-sized farms (*15*), it is not unexpected that this study did not find any association between hepatitis E seroprevalence and the number of swine per province (data not shown).

HEV has 1 serotype with 4 major genotypes (G1–G4) (*1*). G1 has been associated with cases in developing countries in Asia and Africa (*1*). G2 was reported in outbreaks in Mexico and West Africa (*2*). G3 was found in sporadic cases from industrialized countries associated with consumption of HEV-contaminated food and is the most prevalent genotype in swine infections worldwide (*2*). The G4 genotype was identified in infected persons and swine in Asia (*1*).

In Thailand, reported swine HEVs belong to the G3 genotype and were genetically closely related to HEV isolates from patients (*14*). HEV G3 may be the main genotype circulating in Thailand. HEV G3 typically induces asymptomatic disease (*1*). This may be a reason for detecting a higher seroprevalence of HEV IgG compared with that of reports of hepatitis E in Thailand. To clarify these findings and identify circulating genotypes, we recommend a nationwide hepatitis E seroprevalence study of the general population to identify areas of HEV circulation.

This study revealed evidence of widespread HEV circulation in Thailand. Hepatitis E is not currently a major public health problem in Thailand, but outbreak reports in many countries in Asia, including the neighboring countries of Vietnam and Myanmar, suggest that outbreak prevention and disease awareness of hepatitis E in Thailand should be enhanced (*2*). Prevention of widespread HEV infection in Thailand may be accomplished by providing information to the general population about proper sanitation and adequate cooking of pork and to the food industry regarding biosafety practices while handling and slaughtering potential reservoir animals.

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We thank the Royal Thai Army Medical Department for specimen collection and Nucharee Thongsen for specimen processing and creating hepatitis E IgG seroprevalence choropleth map of Thailand.

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Asymptomatic, Mild, and Severe Influenza A(H7N9) Virus Infection in Humans, Guangzhou, China

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Targeted surveillance for influenza A(H7N9) identified 24 cases of infection with this virus in Guangzhou, China, during April 1, 2013–March 7, 2014. The spectrum of illness ranged from severe pneumonia to asymptomatic infection. Epidemiologic findings for 2 family clusters of infection highlight the importance of rigorous close contact monitoring.

During February–May 2013, the initial outbreak of human infection with avian influenza A(H7N9) virus in China resulted in 133 cases (*1*). Influenza A(H7N9) virus reemerged in southern China in October 2013 and had caused 85 laboratory-confirmed cases of infection in Guangdong Province as of March 7, 2014. In response to the outbreak, targeted surveillance programs were established in April 2013 in Guangzhou, the provincial capital of Guangdong Province. Here, we report results of this surveillance program through early 2014.

The Study

Since 2004, all clinical facilities in Guangzhou (population 13 million in 2013) have been required by the China National Health and Family Planning Commission to report any patient who meets the criteria of having pneumonia of unknown etiology (PUE): fever (≥38°C), radiologic characteristics consistent with pneumonia, low-normal leukocyte count or low lymphocyte count in early-stage disease, and no alternative etiology (*2*). Upper or lower respiratory samples from these patients are collected for identification of possible causative pathogens. In response to the influenza A(H7N9) outbreak, PUE surveillance was enhanced in April 2013 by implementing laboratory testing specific for influenza A(H7N9) virus (*3*). Specimens are initially screened for influenza A and B viruses by real-time reverse transcription PCR (rRT-PCR); samples positive for influenza A are then subtyped as H1N1, H3N2, H5N1, or H7N9.

Surveillance for influenza-like illness (ILI) was initially conducted in 4 sentinel hospitals in Guangzhou and expanded to 19 hospitals in November 2013. Each hospital collects 10–20 convenience throat swab specimens weekly from ILI patients visiting the hospitals within 3 days of illness onset. The same laboratory screening protocols were adopted as for PUE surveillance.

Surveillance for influenza A(H7N9) virus was established in 24 live poultry markets (LPMs) in April 2013 and expanded to 42 LPMs in November 2013, covering all 12 districts in Guangzhou. From each LPM, 10–30 environmental samples are collected biweekly and tested by rRT-PCR. When human influenza A(H7N9) infection is confirmed, additional environmental sampling from epidemiologically linked LPMs is immediately launched to trace the possible source of infection. All poultry workers linked to influenza A(H7N9) virus–contaminated LPMs (i.e., LPMs with ≥1 virus-positive environmental samples identified) are placed under medical observation for 7 days. Throat swab specimens are collected within 24 hours for detection of influenza A(H7N9) infection and second swab specimens are collected if symptoms appear.

Close contacts of influenza A(H7N9) case-patients are defined as any family member who shares residence, social contacts who visit, and health care workers who provide medical services without effective personal protection (*4*) during the period from 1 day before illness onset to isolation (*5*). All close contacts are monitored for 7 days for any symptoms. Pared serum samples, tested by hemagglutinin inhibition assay (6), and throat swab specimens are collected to detect possible secondary cases.

From April 1, 2013, through March 7, 2014, a total of 47,937 patients with pneumonia were reported in Guangzhou (Table 1). Of these, 1,923 (4.0%) met PUE criteria, and respiratory specimens were collected and tested. An influenza A(H7N9) case in Guangzhou was confirmed on January 10, 2014 (Figure 1); since then, an additional 15 patients with PUE were confirmed as influenza A(H7N9) case-patients. All were adults; 11 (69%) were ≥60 years of age. Recent poultry exposure history was available for 14 (88%) patients (Table 2).

During the same period (April 1, 2013–March 7, 2014), a total of 4,149 throat swab specimens were collected from 349,712 ILI patients (Table 1); 3 (0.1%) specimens were positive for influenza A(H7N9) virus. All 3 patients were young urban residents who had mild upper respiratory

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Table 1. Investigation of weekly reported number of patients with pneumonia, PUE, and ILI and confirmed cases of influenza A(H7N9) virus infection, Guangzhou, China, April 1, 2013–March 7, 2014*

	PUE surveillance			ILI surveillance		
Year, wk	No. patients with pneumonia	No. (%) patients with PUE	No. confirmed influenza A(H7N9) infections	No. patients with ILI	No. (%) samples tested	No. confirmed influenza A(H7N9) infections
2013						
14	951	22 (2.3)	0	8,089	47 (0.6)	0
15	996	28 (2.8)	0	8,555	65 (0.8)	0
16	1,021	36 (3.5)	0	8,698	55 (0.6)	0
17	1,087	44 (4.1)	0	8,759	63 (0.7)	0
18	1,118	48 (4.3)	0	9,852	43 (0.4)	0
19	1,146	62 (5.4)	0	8,682	68 (0.8)	0
20	1,238	65 (5.4)	0	9,621	55 (0.6)	0
21	1,197	55 (4.6)	0	10,248	64 (0.6)	0
22	1,121	48 (4.3)	0	11,264	83 (0.7)	0
23	1,166	41 (3.5)	0	9,546	82 (0.9)	0
24	1,041	37 (3.6)	0	9,962	81 (0.8)	0
25	1,075	42 (3.9)	0	8,910	96 (1.1)	0
26	1,032	35 (3.4)	0	7,735	68 (0.9)	0
27	976	31 (3.2)	0	7,431	80 (1.1)	0
28	922	28 (3.0)	0	7,567	83 (1.1)	0
29	945	26 (2.8)	0	7,306	80 (1.1)	0
30	908	28 (3.1)	0	6,998	82 (1.2)	0
31	887	20 (2.3)	0	7,824	76 (1.0)	0
32	911	17 (1.9)	0	7,484	74 (1.0)	0
33	848	19 (2.2)	0	7,176	82 (1.1)	0
34	925	11 (1.2)	0	8,018	85 (1.1)	0
35	883	16 (1.8)	0	8,186	84 (1.0)	0
36	856	11 (1.3)	0	8,768	85 (1.0)	0
37	833	13 (1.6)	0	9,549	86 (0.9)	0
38	821	12 (1.5)	0	8,788	82 (0.9)	0
39	773	14 (1.8)	0	7,217	73 (1.0)	0
40	844	17 (2.0)	0	6,448	68 (1.1)	0
41	755	12 (1.6)	0	5,513	63 (1.1)	0
42	721	10 (1.4)	0	5,284	79 (1.5)	0
43	733	16 (2.2)	0	5,746	73 (1.3)	0
44	766	18 (2.4)	0	6,599	70 (1.1)	0
45	803	26 (3.2)	0	5,655	75 (1.3)	0
46	846	28 (3.3)	0	4,234	83 (2.0)	0
47	815	42 (5.2)	0	5,054	82 (1.6)	0
48	859	40 (4.7)	0	4,683	95 (2.0)	0
49	935	46 (4.9)	0	5,493	101 (1.8)	0
50	947	51 (5.4)	0	5,488	112 (2.0)	0
51	1,004	43 (4.3)	0	4,151	117 (2.8)	0
52	1,066	52 (4.9)	0	4,840	119 (2.5)	0
2014						
01	1,124	62 (5.5)	0	6,497	113 (1.7)	0
02	1,091	70 (6.4)	1	7,313	121 (1.7)	0
03	1,198	79 (6.6)	3	6,401	126 (2.0)	0
04	1,257	91 (7.2)	0	6,089	118 (1.9)	0
05	1,286	86 (6.7)	1	6,048	105 (1.7)	1
06	845	32 (3.8)	1	4,656	68 (1.5)	1
07	1,036	86 (8.3)	5	4,172	98 (2.4)	1
08	1,122	70 (6.2)	4	4,340	113 (2.6)	0
09	1,123	77 (6.9)	1	6,850	111 (1.6)	0
10	1,084	63 (5.8)	1	5,925	117 (2.0)	0
Total	47,934	1923 (4.0)	16	349,712	4,149 (1.2)	3

*Real-time reverse transcription PCR testing for influenza A(H7N9) virus was implemented in April 1, 2013 (2013 week 14). Samples were collected from all patients with PUE and were laboratory tested. PUE, pneumonia of unknown etiology; ILI, influenza-like illness.

symptoms (Table 2). As a safety measure, these patients were isolated and treated with oseltamivir. All 3 patients recovered quickly (within 5–7 days) and were discharged after test results for throat swab samples were negative for 2 successive days.

During April–October 2013, 3 of 3,355 environmental samples collected from 24 LPMs were positive for influenza A(H7N9) virus, all on May 16. In contrast, of the 5,220 samples collected from 48 LPMs during November 2013 through March 7, 2014, a total of 141 (2.70%) samples were positive (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/9/14-0424-Techapp1.pdf). A total of 375 poultry workers from 24 influenza A(H7N9) virus–contaminated LPMs were recruited and monitored, and

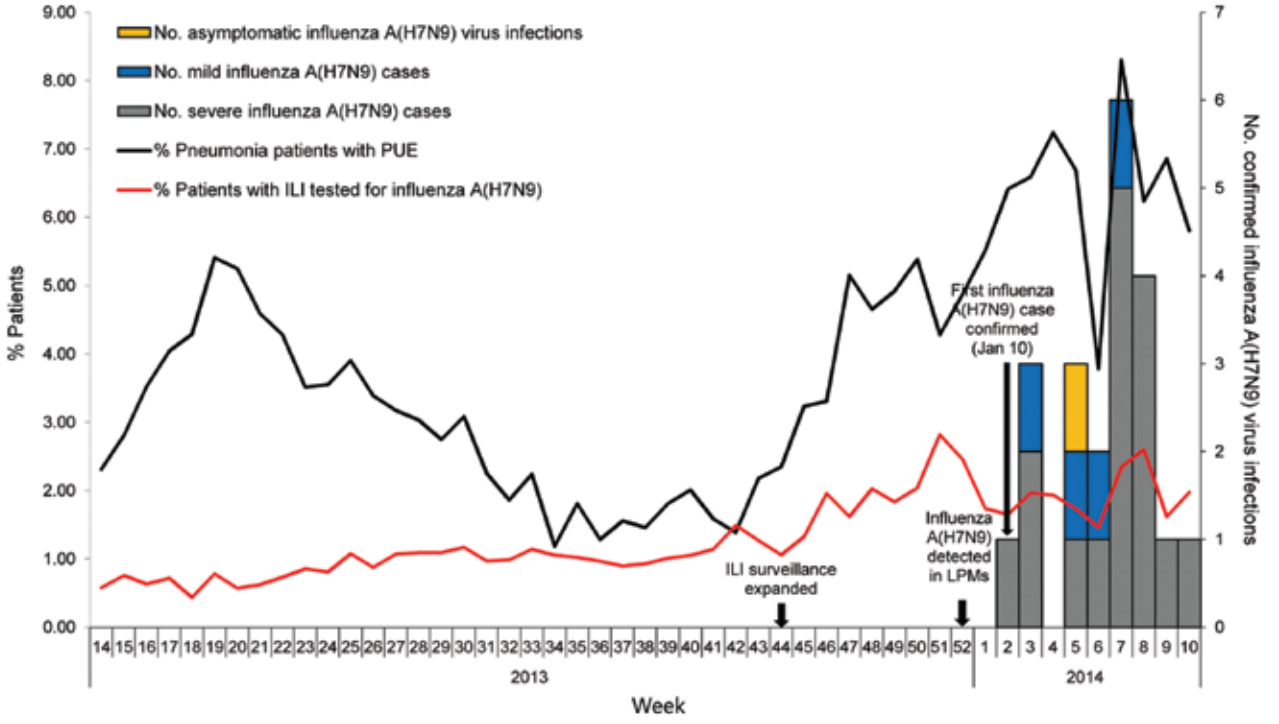

Figure 1. Weekly number of confirmed influenza A(H7N9) cases detected by real-time reverse transcription PCR, percentage of pneumonia patients with pneumonia of unknown etiology (PUE), and percentage of patients with influenza-like illness (ILI) tested for influenza A(H7N9), Guangzhou, China, April 1, 2013–March 7, 2014. For PUE and ILI surveillance in Guangzhou, laboratory testing for influenza A(H7N9) virus using real-time reverse transcription PCR was implemented in week 14 of 2013 (April 1, 2013). ILI surveillance was expanded to 19 sentinel hospitals in week 44 (November 2013), according to the requirements of the public health authority of Guangdong Province after 4 confirmed influenza A(H7N9) cases were reported in Guangdong.

381 throat swab specimens were collected; repeat specimens were collected from 6 workers who showed symptoms. Asymptomatic influenza A(H7N9) virus infection was detected in 1 worker who managed a live poultry stall and had daily direct contact with live poultry. Two environmental samples collected from his stall on January 27, 2014, and a throat swab sample collected from the worker on January 28 were positive for influenza A(H7N9) virus. The worker was isolated, but test results for 3 consecutive throat swab specimens collected on January 30 and 31 and February 6 were negative, and in the absence of any symptoms or abnormal chest radiograph findings, he was discharged.

A total of 361 pairs of serum samples and 411 throat swab specimens were collected from 384 close contacts of influenza A(H7N9) case-patients; 2 family clusters were detected. In family cluster 1 (Figure 2), influenza A(H7N9) infection was laboratory confirmed in the index case-patient on January 10 and in 1 close contact (his daughter) on January 14 by positive test results on 2 throat swab specimens. The daughter showed mild respiratory symptoms and recovered quickly. She had no known history of poultry exposure before illness onset but had close, prolonged, and unprotected contact with her sick father. In family cluster 2, the index case-patient slaughtered a live chicken on February 1, became ill on February 3, and had influenza A(H7N9) infection laboratory confirmed on February 10. Three asymptomatic close family contacts of this patient had influenza A(H7N9) infection confirmed by a 4-fold rise in HI titer, although test results on throat swab specimens were negative. All 3 of these contacts had been involved in buying, slaughtering, or handling chickens and had close and unprotected contact with the index case-patient before he was isolated (Figure 2).

Conclusions

Human infection with influenza A(H7N9) virus has been characterized by severe illness, in particular, rapidly progressive pneumonia and acute respiratory distress syndrome (*7*). However, the 21 case-patients with laboratory-confirmed influenza A(H7N9) that we identified in Guangzhou showed a wider spectrum of illness, ranging from severe pneumonia to mild ILI to asymptomatic infection. Clinical signs and symptoms differed notably across age groups; all mild cases occurred in those <20 years of age, whereas most severe cases occurred in older

Table 2. Demographic, epidemiologic, and clinical characteristics of patients with severe and mild influenza A(H7N9) cases, Guangzhou, China, April 1, 2013–March 7, 2014*

Characteristic	Severe cases, n = 16	Mild cases, n = 4†
Sex ratio, M:F	11:5	1:3
Age, y, median (range)	66 (29–83)	5 (4–17)
Age group, y		
0–14	0	3 (75)
15–59	5 (31)	1 (25)
≥60	11 (69)	0
Type of residence		
Urban	12 (75)	4 (100)
Rural	4 (25)	0
Occupation		
Retired	11 (69)	0
Housewife	2 (13)	0
Farmer	2 (13)	0
Tofu vendor in retail wet market	1 (6)	0
Kindergarten student	0	3 (75)
Primary or high school student	0	1 (25)
History of poultry exposure‡		
Direct contact	6§ (38)	0
Indirect contact	9¶ (56)	1# (25)
Unknown	3 (19)	3 (75)
Underlying medical conditions**	12 (75)	0
Preliminary diagnosis at the first hospital visit		
Influenza	0	3 (75)
Upper respiratory tract infection	6 (38)	1 (25)
Pneumonia	10†† (63)	0
Required hospitalization	16 (100)	1 (25)
Illness progressed to pneumonia	16 (100)	0
Received oseltamivir treatment	15 (94)	4 (100)
Admitted to intensive care unit	14 (88)	0
No. hospitals visited, median (IQR)	3 (1–4)	1 (1–2)
Time from illness onset to first medical care, median (IQR)	5 d (1–12 d)	6h (3–24h)
For patients in ≥60 y age group	7 d (2–12 d)	
Time from illness onset to antiviral therapy, median (IQR)	7 d (3–12 d)	1 d (6 h–4 d)
Time from illness onset to laboratory confirmation, median (IQR)	8 d (7–13 d)	3 d (2–5 d)
Length of illness, median (IQR)	24 d (11–32 d)	7 d (6–8 d)
Outcome		
Recovered and discharged	4 (25)	4 (100)
Still in hospital‡‡	1 (6)	0
Died	11 (69)	0

*Values are no. (%) patients except as indicated. LPM, live poultry market; IQR, interquartile range.
†Includes the confirmed mild case detected from follow-up of close contacts.
‡Direct contact: bought poultry, slaughtered poultry, handled poultry meat, raised backyard poultry. Indirect contact: visited LPMs.
§Four case-patients raised backyard chickens, 3 slaughtered live chickens, and 1 handled chicken meat.
¶Five case-patients visited LPMs daily; 4 visited LPMs several times.
#Case-patient's father managed a live poultry stall in a contaminated LPM; case-patient visited the stall several times.
**Diabetes, hypertension, uremia, chronic obstructive pulmonary disease, and coronary heart disease.
††Nine case-patients were ≥60 y of age.
‡‡As of April 7, 2013.

patients, similar to findings from previous studies (*8,9*). The age variances may be attributed to more frequent poultry exposure, more co-existing chronic diseases, or delayed medical admission and antiviral treatment among older patients.

Evidence shows the potential for influenza A(H7N9) virus transmission from person to person (10,11). In particular, epidemiologic findings of the father-daughter cluster indicate that person-to-person transmission may occur among family members after prolonged and intimate contact, consistent with findings in several other family clusters (8,12,13). However, no widespread mild influenza A(H7N9) infection was detected through ILI surveillance, which indicates that the likelihood of community-level transmission is low.

Subclinical influenza A(H7N9) virus infections of poultry workers have been identified by serologic testing (*14*). However, the possibility of cross-reactivity with other antigenically similar viruses cannot be ruled out. Using rRT-PCR, our surveillance identified a poultry worker with asymptomatic influenza A(H7N9) virus infection, providing further evidence for an occupational risk for asymptomatic infection.

Our study is limited by potential underreporting and by the increased use of PUE and ILI surveillance during the study period compared with previous periods. However,

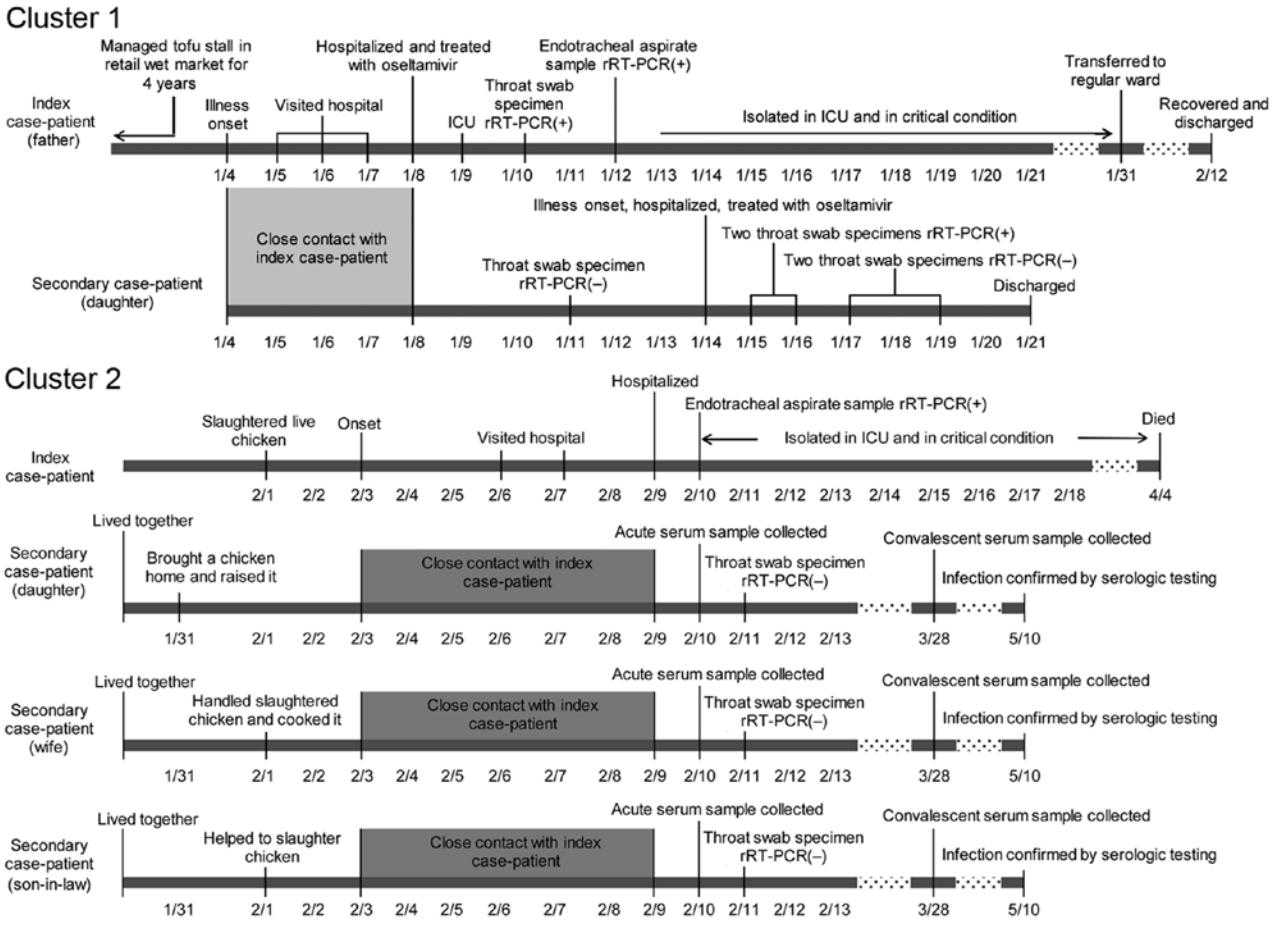

Figure 2. Timeline of illness for 2 family clusters of persons with confirmed influenza A(H7N9) virus infection, Guangzhou, China, 2014. ICU, intensive care unit; rRT-PCR, real-time reverse transcription PCR.

our results show that targeted surveillance during a period of elevated disease activity improved identification of mild or asymptomatic infections.

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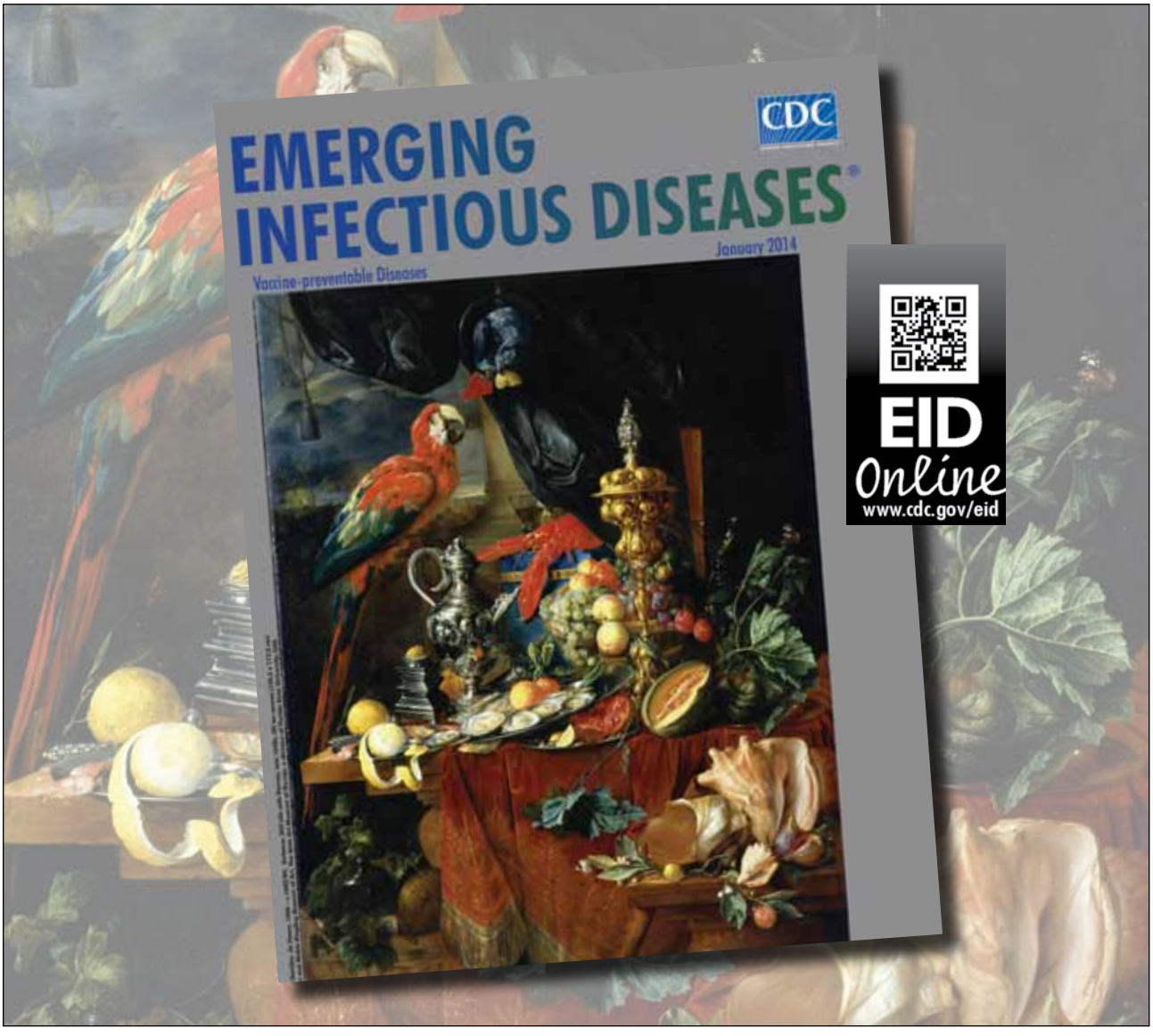

Mutations of Novel Influenza A(H10N8) Virus in Chicken Eggs and MDCK Cells

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The recent emergence of human infection with influenza A(H10N8) virus is an urgent public health concern. Genomic analysis showed that the virus was conserved in chicken eggs but presented substantial adaptive mutations in MDCK cells. Our results provide additional evidence for the avian origin of this influenza virus.

Influenza A virus remains a major threat to public health worldwide. The 2000s witnessed the epidemic of human infections with the avian influenza A(H5N1) and A(H7N9) viruses in China (*1*,*2*) and a global pandemic of human influenza caused by a novel swine-origin influenza A(H1N1) virus (*3*). More recently, the first human case of a novel influenza A(H10N8) virus infection was reported in China, and 2 additional human cases have been confirmed in the same province (*4*,*5*). The emergence of the novel influenza A(H10N8) virus has become an urgent public health concern (*6*).

A preliminary genomic analysis showed that the emerging influenza virus was genetically distinct from the avian influenza A(H10N8) viruses previously identified in China, and scientists have postulated that the virus resulted from multiple reassortments of subtype H9N2 strains that circulated widely in poultry in China (*4*). Nevertheless, no identical influenza A(H10N8) virus was detected in the live-poultry market visited by the first patient before the onset of her illness, and the origin of the novel A(H10N8) virus remains unclear. We compared the genomic mutations of the virus cultured in embryonated chicken eggs and in MDCK cells in an attempt to find additional evidence to support the possible avian origin of the virus.

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The Study

We obtained a tracheal aspirate specimen from the lower respiratory tract of the patient with the first reported A(H10N8) virus infection on day 7 after illness onset (*4*). The tracheal aspirate specimen was inoculated on monolayers of MDCK cells and into the allantoic cavities of 10-day-old embryonated chicken eggs. The isolated novel A(H10N8) virus strain IPB13 was serially passaged in MDCK cells every 72 hours for a total of 3 passages. After 1 blind passage, cytopathic effects were clearly visible in ≈80% of the cells. The supernatants of the second and third passages in MDCK cells and the allantoic fluids of the inoculated chicken eggs were individually harvested and processed for full genome sequencing. We extracted total RNA from each sample using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and amplified it by sequence-independent PCR with a OneStep RT-PCR Kit (QIAGEN). We determined the viral genome sequence of each isolate using an ABI 3730xl automatic DNA analyzer (Life Technologies, Grand Island, NY, USA). Potential heterogeneous sites in each genome were initially identified by manual inspection of the sequence trace data and confirmed by clone sequencing as previously described (*7*).

In addition, the tracheal aspirate sample was processed for direct deep sequencing as previously described (*8*) but with a slight modification. We used an Illumina/HiSeq2500 sequencer (Illumina, San Diego, CA, USA) to generate 100-bp single-end reads according to the manufacturer's instructions. The deep sequencing reads were screened for quality control and removal of human contamination as previously described (*9*). The valid reads were aligned to the previously determined genome sequence of the novel A(H10N8) virus strain JX346 (GISAID [Global Initiative on Sharing All Influenza Data] accession no. EPI497477–84) by using the BWA program with default parameters (*10*). We obtained a total of 2,629,199 reads that were successfully aligned to the JX346 genome. Then, we used the SAMtools package to detect possible polymorphic or heterogeneous sites from the alignment (*11*). We deposited the complete genome sequences of all isolates in GenBank under accession nos. KJ406531–KJ406562.

The change of host environment from the in vivo human respiratory tract to the in vitro cell lines might introduce mutations in the virus genome during culture. Indeed, in our previous study, we observed dozens of point mutations in the genome of novel A(H7N9) virus obtained from embryonated chicken eggs compared with that derived

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Table. Mutations and heterogeneities of influenza A(H10N8) virus detected in a tracheal aspirate sample from a human, the first passage in embryonated chicken eggs, and the second and third passages in MDCK cells*

Segment	Nucleotide position†	Direct sequencing	Embryonated chicken eggs P1	MDCK cells P2	MDCK cells P3	Amino acid change‡
PB2	1,879	A→G	A→G	A	A	E627K§
PA	1,689	G	G	A→G	A→G	–
PA	1,995	G	G	A→G	A→G	–
PA	1,997	T	T	C→T	C→T	L666P
HA	688	A	A	G→A	G→A	R220G
NP	583	C	C	A→C	A→C	–
NP	1,405	G	G	A→G	A→G	E469K
NA	739	C	C	C→T	C→T	A246V§
NA	877	G	G	A→G	A→G	R292K
NA	1,187	C	C	C→T	C→T	–
NS1	641	G→A	G→A	A→G	A→G	E208K

*HA, hemagglutinin; NA, neuraminidase; NP, nucleocapsid protein; NS, nonstructural protein; PA, polymerase acidic; PB, polymerase basic; –, synonymous substitution without amino acid change.
†Numbered from the first nucleotide of the determined genomic sequence.
‡Site positions are numbered from the start codon (M); H3 and N2 numbering for HA and NA, respectively.
§Potential amino acid change caused by the minor nucleotide in the heterogeneous site.

directly from clinical sample (*8*). However, in this study, we obtained exactly the same genome sequences of the novel A(H10N8) virus from the clinical sample and that cultured in chicken eggs (Table). Moreover, the 2 heterogeneous sites in the clinical sample were retained during culture in embryonated chicken eggs. In contrast, 10 heterogeneous sites, 8 of which resulted in point mutations, were observed in the virus genomes derived from cultures in MDCK cells (Table). Additionally, no genomic difference was visible between viruses in the second and third passages in MDCK cells.

The heterogeneous site in polymerase basic (PB) 2 resulted in a mixture of glutamic acid and lysine at residue 627 of the PB2 protein in the clinical sample (Table); this mixture might have partially contributed to the severity of the patient's illness (*4*). Indeed, the heterogeneity in this site vanished during culture in MDCK cells, leading to complete lysine substitution at residue 627 of the PB2 protein as previously observed (*12*). This finding is consistent with the established hypothesis that the E627K substitution in PB2 was associated with the increased transmissibility and pathogenicity of avian influenza viruses in mammals (*13*,*14*). Notably, a heterogeneous site in the neuraminidase (NA) segment led to a mixture of arginine and lysine at residue 292 (N2 numbering) of the NA protein in the viruses cultured in MDCK cells (Table). The R292K substitution in the NA protein of avian influenza A(H7N9) virus was able to reduce the antiviral efficacy of NA inhibitors, especially oseltamivir (*15*). Therefore, although the novel A(H10N8) virus was sensitive to oseltamivir in vitro (*4*), its potential to develop antiviral drug resistance in mammalian cells requires further attention.

Conclusions

We investigated the genomic mutations and heterogeneities of the novel influenza A(H10N8) virus during culture in embryonated chicken eggs and MDCK cells compared with the genome sequence obtained directly from the clinical specimen. The viral genome was highly conserved during culture in embryonated chicken eggs, and no mutations were identified. This result suggests that the novel A(H10N8) virus might have been highly adapted to an avian-like host before it was transmitted to the human host (i.e., the first patient). In contrast, substantial genetic mutations were observed in the viral genome during culture in MDCK cells; this finding implies an ongoing adaptive microevolution of the virus in a mammalian environment. Taken together, our results favor the proposal that the novel influenza A(H10N8) virus has an avian origin; however, more research is required to establish the definite origin of the emerging influenza virus. Furthermore, the substitutions E627K (in the PB2 protein) and R292K (in the NA protein) observed in the cultures of the MDCK cells indicate that the virus might be undergoing rapid adaptation to mammals and developing antiviral drug resistance. Although only 3 human cases of infection with the novel A(H10N8) virus have been reported, the potential for this virus to threaten public health should not be underestimated.

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Genetic Variation among African Swine Fever Genotype II Viruses, Eastern and Central Europe

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African swine fever virus (ASFV) was first reported in eastern Europe/Eurasia in 2007. Continued spread of ASFV has placed central European countries at risk, and in 2014, ASFV was detected in Lithuania and Poland. Sequencing showed the isolates are identical to a 2013 ASFV from Belarus but differ from ASFV isolated in Georgia in 2007.

African swine fever (ASF) is a devastating disease of domestic and wild suids, and there is no vaccine to protect against the disease. ASF is caused by a DNA arbovirus, African swine fever virus (ASFV), belonging to the family *Asfaviridae* (*1*); the virus genome is 170–192 kb long. ASF is endemic in sub-Saharan countries and in Sardinia (Italy) and has become more prevalent in Russia and the Caucasus region (*2*) since its spread from eastern Africa to Georgia (in the Caucasus region) in 2007 (*3*). The ongoing spread of ASFV to adjacent eastern European countries, such as Ukraine (*4*,*5*) and Belarus (*6*), and the uncontrolled spread of the disease in Russia have placed the bordering areas of the European Union at high risk for the introduction of ASFV. In early 2014, the first cases of ASF in the European Union were reported; the cases occurred in 4 wild boars in areas of Lithuania and Poland that border the eastern European country of Belarus (*7*,*8*) (Figure 1, http://wwwnc.cdc.gov/EID/article/20/9/14-0554-F1.htm). To further our knowledge of the epidemiology and spread of ASFV, we determined the virus sequences of the ASFVs isolated in Poland and Lithuania by using international standardized procedures (*9*) and by the analysis of an additional ASFV genome marker region characterized by the presence of tandem repeat sequences (TRSs). We report the genetic characterization of these ASFVs.

The Study

On January 24, 2014, the European Commission and the World Organisation for Animal Health received reports from Lithuanian authorities of 2 cases of ASF in wild boars. One of the infected animals was found in Salcininkai and the other in Varena, 5 km and 40 km, respectively, from the Belarus border (*7*). Then, on February 14 and 17, 2014, reports of 2 cases of ASF in wild boars were received from northeastern Poland (Sokolka County, Podlaskie Province). One of the infected animals in Poland was found in the municipality of Szudzialowo; the other was found in Kruszyniany, a forest area (*8*). The 2 wild boars in Poland were found dead ≈900 m and ≈200 m, respectively, from Poland's border with Belarus.

ASFV-positive clinical samples (spleen, kidney, lung, bone marrow) from the 4 infected wild boars were sent to the European Union reference laboratory for ASF, Centro de Investigación en Sanidad Animal (CISA-INIA), Madrid, Spain, for confirmatory testing and genetic characterization. After the presence of ASFV was confirmed in samples, initial genetic characterization was performed by using standardized genotyping procedures on virus DNA extracted directly from homogenized tissues and from bone marrow samples. These analyses included the C-terminal end of the *p72* gene, the full sequence of the *p54* gene, and the central variable region within the *B602L* gene (*9*). We also included in the study 21 genotype II ASFVs that were isolated from wild and domestic pigs in Russia and the Caucasus region during April 2007–June 2013 (Table).

We compared the nucleotide sequences obtained from the p72- and p54-based PCRs with those of previously described representative isolates (*10*). We used Clustal Omega (http://www.clustal.org/) to perform multiple sequence alignments. Minimum evolution trees, rooted at the midpoint, were constructed by using MEGA V6.0 (http://www.megasoftware.net/) with the *p*-distance nucleotide substitution

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Table. African swine fever virus isolates from eastern Europe selected for a study of the genetic variation among genotype II viruses in eastern and central Europe, 2007–2014*

Isolate	Source country, area	Host	Onset of outbreak	GenBank accession no.		
				p72 gene	P54 gene	CVR
Abk07	Georgia, Abkhazia Republic, Gulripish	DP	2007 Jul 04	JX857509	JX857495	JX857523
Arm07	Armenia, Dilijan	DP	2007 Aug 07	JX857508	JX857494	JX857522
Che07	Russia, Chechnya Republic, Shatoysky	EWB	2007 Dec 04	JX857510	JX857496	JX857524
Az08D	Azerbaijan, Qebele District	DP	2008 Jan 22	JX857515	JX857501	JX857529
Az08B	Azerbaijan, Qebele District	DP	2008 Jan 22	JX857516	JX857502	JX857530
Ing08	Russia, Ingushetia Republic, Sunzhensky	EWB	2008 Jul 21	JX857511	JX857497	JX857525
Oren08	Russia, Orenburg Oblast, Chernorechye	DP	2008 Jul 10	JX857512	JX857498	JX857526
NO08/Av	Russia, Republic of North Osetia, Vladikawkaz	DP	2008 Jul 18	JX857513	JX857499	JX857527
NO08/Ap	Russia, Republic of North Osetia, Prigorodni	DP	2008 Jul 21	JX857514	JX857500	JX857528
Dagestan09	Russia, Dagestan Republic, Tarumovsky, District	EWB	2009 Sep 11	JX857517	JX857503	JX857531
StPet09	Russia, Leningradskaya Oblast, Kirovsky	DP	2009 Oct 01	JX857520	JX857506	JX857534
Kalmykia09	Russia, Republic of Kalmykia, Yashaltinsky district	DP	2009 Oct 10	JX857519	JX857505	JX857533
Rostov09	Russia, Rostov Oblast, Krasnosulinsky District	DP	2009 Oct 20	JX857518	JX857504	JX857532
Tver0511/Torjo	Russia, Tver Oblast, Torjo	DP	2011 May 31	KJ627208	KJ627186	KJ627197
Tver0312/Novo	Russia, Novozavidovskii, Tver region	DP	2012 Mar 14	KJ627212	KJ627190	KJ627201
Tver0312/Torjo	Russia, Torjo, Tver region	EWB	2012 Mar 28	KJ627211	KJ627189	KJ627200
Tver0712/Les	Russia, Lesnoi, Tver region	DP	2012 Jul 16	KJ627210	KJ627188	KJ627199
Ukr12/Zapo	Ukraine, Zaporozhye region	DP	2012 Jul 30	JX857521	JX857507	JX857535
Tver0812/Bolo	Russia, Bologovskii, Tver region	EWB	2012 Aug 15	KJ627209	KJ627187	KJ627198
Tver1112/Zavi	Russia, Zavidovo, Tver region	EWB	2012 Nov 20	KJ627214	KJ627191	KJ627202
Bel13/Grodno	Belarus, Grodno region, Lelyukinskiy District of Ivye	DP	2013 Jun 19	KJ627215	KJ627192	KJ627203
LT14/1490	Lithuania, Šalčininkai District Municipality	EWB	2014 Jan 21	KJ627216	KJ627193	KJ627204
LT14/1482	Lithuania, Alytus County, Varėna District Municipality	EWB	2014 Jan 21	KJ627217	KJ627194	KJ627205
Pol14/Sz	Poland, Szudzialowo, Sokolka County, Podlaskie Province	EWB	2014 Feb 14	KJ627218	KJ627195	KJ627206
Pol14/Krus	Poland, Kruszyniany, Sokolka County, Podlaskie Province	EWB	2014 Feb 17	KJ627219	KJ627196	KJ627207

*CRV, central variable region; DP, domestic pig; EWB, European wild boars.

model. The 2014 ASFVs from Lithuania (LT14/1482, LT14/1490) and Poland (Pol14/Sz and Pol14/Krus) clustered, as expected, within p72 genotype II (Figure 2) and showed 100% nucleotide identity with all compared ASFV isolates from eastern Europe across the 478-bp C-terminal *p72* gene and the 558-bp full length *p54* gene. We obtained the same result by sequencing the central variable region within the *B602L* gene, revealing 10 copies of amino acid tetramer repeats that were 100% identical and unique to those of the ASFV circulating in the Caucasus regions since 2007 (*11*).

Although the central variable region has proven useful for resolving epidemiologic complexities at the genotype (*12*), country (*13*), and region levels, additional genome markers are required to determine the origin and to map the spread of closely related ASFV isolates circulating in eastern Europe. Thus, we designed a set of primers, named ECO1A (5′-CCATTTATCCCCCGCTTTGG-3′ binding site 172,270–172,290) and ECO1B (5′-TCGTCATCCT-GAGACAGCAG-3′ binding site 172,616–172,626), to amplify a 356-bp fragment located between the *I73R* and *I329L* genes and characterized by the presence of TRS (*14*). Primer binding sites were based on the genome of the ASFV from Georgia (GenBank accession no. FR682468.1). Using the same reaction conditions as used for full *p54* gene amplification (*10*) and an annealing temperature of 60°C, we generated 367-bp amplicons from isolates from Ukraine, Belarus, Lithuania, and Poland. The estimated size of the remaining isolates from eastern Europe that were included in the study was 356 bp (data not shown). Nucleotide sequence analysis of the PCR products revealed that the size difference was caused by the insertion of an additional TRS (GGAATATATA) at nt 136 (Figure 3). All sequences generated in this study were submitted to GenBank under accession nos. KJ620028–51.

Conclusions

Current available molecular data derived by using standardized genotyping procedures (*9*) have indicated the presence of only 1 ASFV variant. That variant belongs to p72 genotype II, which has been circulating in eastern European countries since the introduction of ASFV into Georgia in 2007 (*11*). In agreement with those findings, results from our analysis of the 3 independent regions included in the classical genotyping showed that sequences for ASFV isolates from Lithuania and Poland were 100%

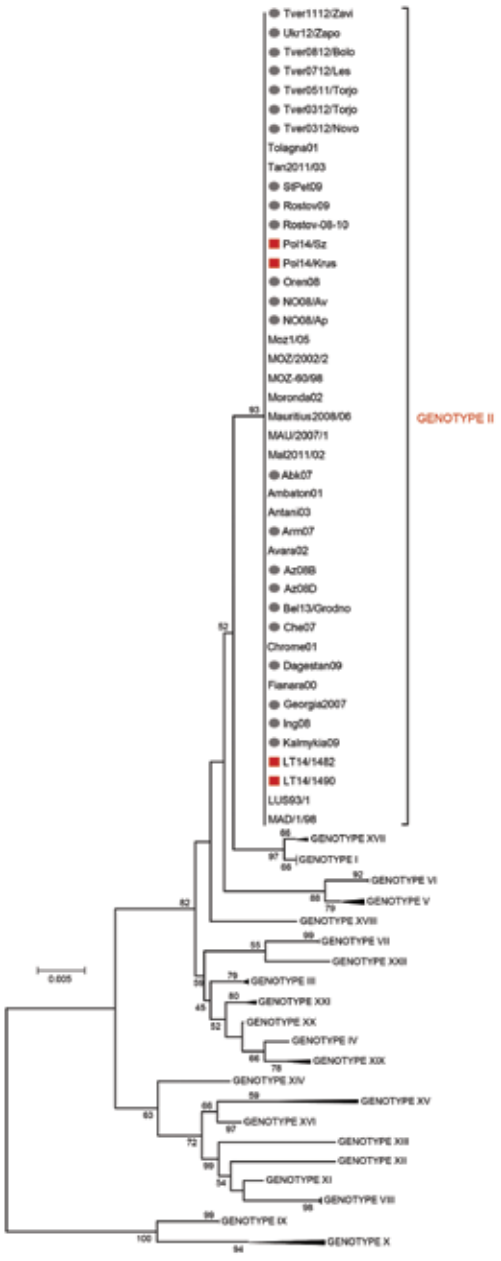

Figure 2. Minimum evolution (ME) phylogenetic tree of African swine fever virus (ASFV) isolates from Lithuania and Poland based on the C-terminal end of the *p72* coding gene relative to the 22 p72 genotypes (labeled I-XXII), including 88 nt sequences. The tree was inferred by using the ME method (http://www.megasoftware.net/mega4/WebHelp/part_iv___evolutionary_analysis/constructing_phylogenetic_trees/minimum_evolution_method/rh_minimum_evolution.htm) following initial application of a neighbor-joining algorithm. The phylogenetic tree was rooted by the midpoint method. The percentage of replicate trees >50% in which the associated taxa clustered together by bootstrap analysis (1,000 replicates) is shown adjacent to the nodes. The robustness of the ME tree was tested by using the close-neighbor-interchange algorithm at a search level of 1. Squares indicate ASFV isolates from Lithuania and Poland that were genotyped in this study; circles indicate ASFV isolates during 2007–2013 from the Caucasus region. Scale bar indicates nucleotide mutations per site.

homologous with those for ASFVs from eastern Europe. However, the long-term presence of ASFV in Russia and the Caucasus regions and the rapid spread of the virus to neighboring countries highlight the need for finding additional ASFV genome markers capable of discriminating among circulating virus isolates so that we may better determine their source and evolution.

The whole-genome sequence analysis of ASFV has identified some regions that contain tandem repeat arrays that have proven useful for discriminating between closely related ASFVs (*15*). Thus, the approach described in our study focused on analysis of the TRS in the intergenic region between the *I73R* and *I329L* genes at the right end of the genome (*14*). The results showed that the viruses from Poland and Lithuania had a TRS insertion identical to that present in ASFV isolates from Belarus and Ukraine. This TRS insertion was absent in the remaining viruses from eastern Europe, including those obtained in Tver Oblast, Russia, in 2012 and in Georgia in 2007. These molecular data, together with the epidemiologic findings, confirmed that the ASFVs detected in Poland and Lithuania most probably originated from Belarus. However, knowledge of the epidemiology of ASF and a full understanding of the evolution and spread of ASFV in this region require additional sequence analysis of ASFVs currently circulating in Russian regions bordering Belarus and Ukraine.

Our results show the genetic variability among ASFVs circulating in eastern Europe and describe a new method that can be useful for distinguishing between closely related ASFV isolates. Such genetic data are essential for determining the source and studying the evolution of ASFV isolates and to fully elucidate the spread of ASFV in the eastern and central European countries.

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Figure 3. Partial nucleotide sequence alignment of the intergenic region between *I73R* and *I329L* in African swine fever virus (ASFV) isolates from eastern and central Europe, including a virus isolated in 2007 in Georgia (Georgia2007; GenBank accession no. FR682468.1). The mutation that results in the insertion of a single nucleotide internal repeat sequence (GGAATATATA) in the ASFVs from Belarus, Ukraine, Lithuania, and Poland is indicated by gray shading.

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Another Dimension

The EID journal accepts thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Novel Circovirus from Mink, China

Hai Lian,[1] Ye Liu,[1] Nan Li, Yuying Wang, Shoufeng Zhang, and Rongliang Hu

A long-established epidemic of enteritis, caused by an unidentified pathogen distinct from parvovirus, has now been recognized in mink. In 2013, we identified a novel circovirus by degenerate PCR and fully sequenced its genome. This virus differs substantially from currently known members of the genus *Circovirus* and represents a new species.

Members of the family *Circoviridae* are nonenveloped, icosahedral viruses with diameters of 16–26 nm and a small, circular, single-stranded DNA genome (the smallest known autonomously replicating viral genome) (*1*). The family *Circoviridae* is currently composed of 2 genera, *Gyrovirus* and *Circovirus*, each of which has been associated with diseases in birds, pigs, and dogs (*1*,*2*). Among birds, circovirus infections have been associated with beak abnormalities, lethargy, and anorexia in parrots and pigeons and production losses and death in geese (*3*,*4*). Among pigs, porcine circovirus type 2 (PCV-2) causes respiratory and enteric diseases, dermatitis, and reproductive problems, resulting in major economic losses to the pork industry (*5*,*6*). A proposed new genus (*Cyclovirus*) within the family *Circoviridae* consists of cycloviruses. Genomes of cycloviruses have been identified in human and chimpanzee feces and human cerebrospinal fluid (*7*,*8*). Recently, circoviruses have been found in bats and have shown high genetic diversity (*9*,*10*). We describe a novel circovirus in mink (mustelids). This mink circovirus (MiCV) was found in the liver, digestive tract, and fecal specimens from mink with diarrhea as their main clinic sign.

Mink enteritis is caused by a parvovirus (*11*). However, around 1978, on some mink farms in Dalian, Liaoning Province, China, ≈7% of mink that had received the inactivated parvovirus vaccine had diarrhea and anorexia that resulted in death. The farmers called this refractory diarrhea. According to the observations of the farmers, the incubation period of this disease was <10 days. The preliminary clinical signs included lethargy, anorexia, pale muzzle, and unkempt fur. The feces were initially white and then became jelly red or yellow. Several days after the appearance of red or yellow feces, ≈7% of affected mink died. All mink from farms with disease seemed to have been affected; 70%–80% showed clinical signs, but most recovered.

Within a few years of farmers first noticing the disease, a major outbreak occurred during 1985–1990 in Dalian and on mink farms in the surrounding mountain regions. The mink in this area are pedigree breeds with premium quality fur; all other farms introducing stock from this area were also affected. Spread of the infection seemed to be horizontal because mink were usually affected after a mink on the same farm became ill. However, the epidemic now seems to be limited, potentially in part because of the practice of autogenous vaccination that uses formalin-inactivated supernatant from tissue suspension from affected minks.

The Study

During September–October 2013, we collected liver, digestive tract (gut), and fecal samples from 43 mink (26 with diarrhea and 17 healthy) ≈6–10 months of age from 3 mink farms in Dalian, China. Using PCR-based methods, we excluded the following as causative agent: mink enteritis virus, canine distemper virus, Aleutian mink disease virus, mink orthoreovirus, and mink coronavirus. To identify the causative agent, we separately homogenized fecal, liver, and gut samples from diseased unvaccinated mink in phosphate-buffered saline and then submitted the supernatants to negative staining and observation under an electron microscope. We found circovirus-like particles, but not in typical lattice arrangement, in liver and gut samples. We performed PCRs with degenerate primers (CV-F1, CV-R1, CV-F2, and CV-R2; Table 1) based on highly conserved amino acid motifs in the Rep proteins of circoviruses and cycloviruses. Products of ≈400 bp were purified and sequenced by using primer CV-R2.

All samples from mink with diarrhea were positive by PCR for the same circovirus-like Rep sequence; all samples from healthy mink were negative (Table 2), showing a strong relationship between the identified virus and disease. A BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) showed the sequence to be an authentic circovirus sequence, with closest similarity (73%) to the bat circavirus (BtCV) from the *Rhinolophus ferrumequinum* group XO bat (XOR) genome, recently identified in bats by metagenomic analysis of tissue samples (*10*).

To obtain the complete genome sequence of MiCV, we used primers CV-F3, CV-R3, CV-F4, and CV-R4 (Table 1) for inverse PCR, together with Premix PrimeSTAR HS DNA polymerase (Takara Bio, Inc., Dalian, China). The product was sequenced in duplicate. The complete

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Table 1. Oligonucleotide sequences of primers used in study of novel circovirus isolated from mink, Dalian, China

Primer	Oligonucleotide sequence, 5′→3′	Reference
CV-F1	GGIAYICCICAYYTICARGG	(*7*)
CV-R1	AWCCAICCRTARAARTCRTC	(*7*)
CV-F2	GGIAYICCI CAYYTICARGGITT	(*7*)
CV-R2	TGYTGYTCRTAICCRTCCCACCA	(*7*)
CV-F3	GCCCGCTTAAACGGCTCAAACCGCATTTTC	Designed for this study
CV-R3	TGGGAGGGGCCTGAGGGATTACGTCATACA	Designed for this study
CV-F4	GCAGTAAGTCTCCCCCTTTACTGCAATATC	Designed for this study
CV-R4	CTTGCTGAATAATGGCGGAACAATGACTGA	Designed for this study

circular genome of MiCV-DL13 contained 1,753 nt (GenBank accession no. KJ020099); highest similarity was 64.1% with the genome of BtCV-XOR. The putative Rep protein of MiCV-DL13 was 297 aa, and it shared 50.7%, 51.6%, 55.4%, and 79.7% aa identity with the Rep protein of pig (PCV2 AUT1, AY424401), bird (GoCV, AJ304456), dog (DogCV-1698, NC_020904) (*2*), and bat (BtCV-XOR, JX863737), respectively, circoviruses. The results of phylogenetic analyses based on the amino acid sequence of the Rep protein are shown in the Figure. The deduced capsid protein is 227 aa; highest similarity is 47.3% with porcine circovirus 2 (EU-RO-WB2006–38, JN382157) from wild boars in Romania (*12*).

The International Committee for the Taxonomy of Viruses has suggested criteria for circovirus species demarcation: genome nucleotide identities of <75 % and capsid protein amino acid identities of <70 % (*1*). The MiCV reported here therefore represents a new circovirus species.

Conclusions

Apart from the well-documented pathogenicity of porcine circovirus in pigs, of dog circovirus in dogs, and of other circoviruses in birds, the biological significance of widespread cyclovirus, circovirus, and circovirus-like virus infections in other domesticated and wild animals remains unknown. In this study, the novel circovirus, MiCV, was identified in liver and digestive tract samples from mink with diarrhea. No MiCV DNA was found in any healthy mink, thereby indicating a strong relationship between the isolated virus and disease. Whether other mustelids (e.g., weasels, badgers, and ferrets) or even humans in close contact with infected mink are susceptible to this virus merits further study.

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Table 2. Prevalence of mink circovirus DNA in mink, Dalian, China

Specimen tested	No. mink	Age of mink, mo. (no. mink)	Farm*	Positive for mink circovirus
Mink with diarrhea				
Liver	12	6–7 (8), 7–8 (3), 9–10 (1)	4 from farm 1, 5 from farm 2, 3 from farm 3	12
Gut	12	6–7 (8), 7–8 (3), 9–10 (1)	4 from farm 1, 5 from farm 2, 3 from farm 3	12
Feces	19	6–7 (12), 7–8 (5), 9–10 (2)	7 from farm 1, 8 from farm 2, 4 from farm 3	19
Healthy mink				
Liver	9	6–7 (6), 7–8 (3)	4 from farm 1, 3 from farm 2, 2 from farm 3	0
Gut	9	6–7 (6), 7–8 (3)	4 from farm 1, 3 from farm 2, 2 from farm 3	0
Feces	11	6–7 (7), 7–8 (3), 9–10 (1)	4 from farm 1, 5 from farm 2, 2 from farm 3	0

*Farms 1 and 2 located in Zhuanghe County, Dalian, China; farm 3 located in Pulandian County, Dalian

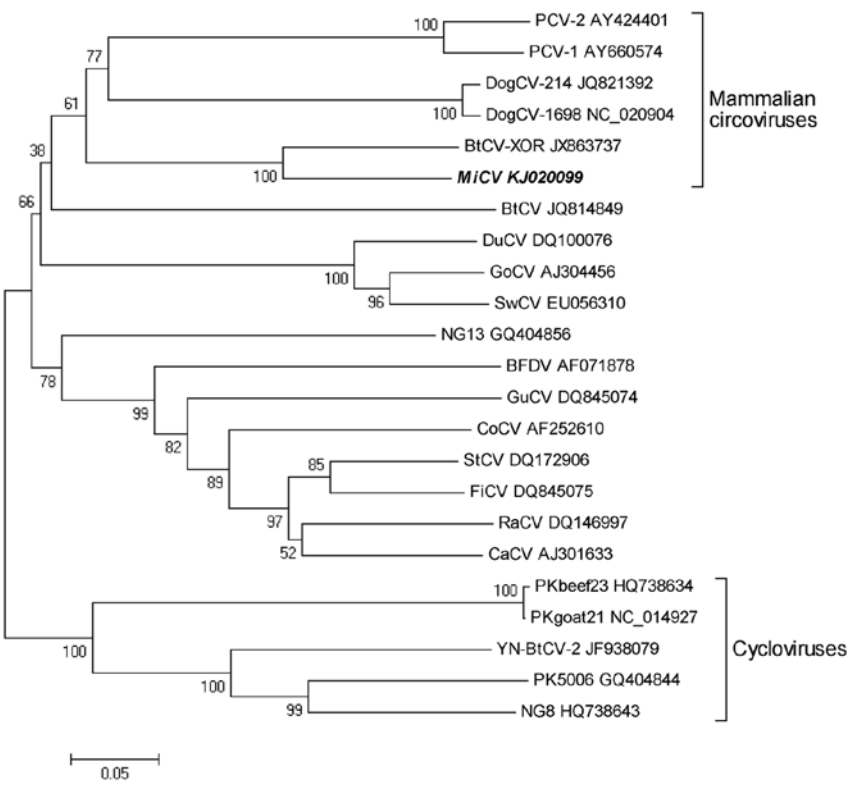

Figure. Phylogenetic tree constructed on the basis of the Rep protein sequence of the mink circovirus by using the neighbor-joining method in MEGA5 (http://www.megasoftware.net). Representative members of the genera *Circovirus* and *Cyclovirus* were included in the analysis, and GenBank accession numbers are indicated. Numbers at nodes indicate bootstrap values based on 1,000 replicates. Scale bar indicates nucleotide substitutions per site. The strain sequenced from the mink in Dalian, China, during 2013 (this study) is indicated in boldface italics. PCV-2, porcine circovirus type 2, PCV-1, porcine circovirus type 1, DogCV, dog circovirus; BtCV, bat circovirus; MiCV, mink circovirus; DuCV, duck circovirus; GoCV, goose circovirus; SwCV,swan circovirus; NG13, human stool-associated circular virus NG13; BFDV, beak and feather disease virus; GuCV,gull circovirus; CoCV, columbid circovirus; StCV, starling circovirus; FiCV, finch circovirus; RaCV, raven circovirus; CaCV, canary circovirus; PKbeef23, cyclovirus PKbeef23/PAK/2009; PKgoat21, cyclovirus PKgoat21/PAK/2009; PK 5006, cyclovirus PK5006; NG8, cyclovirus NGchicken8/NGA/2009.

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Factors Contributing to Decline in Foodborne Disease Outbreak Reports, United States

Maho Imanishi, Karunya Manikonda, Bhavini P. Murthy, and L. Hannah Gould

The number of foodborne disease outbreaks reported in the United States declined substantially in 2009, when the surveillance system transitioned from reporting only foodborne disease outbreaks to reporting all enteric disease outbreaks. A 2013 survey found that some outbreaks that would have been previously reported as foodborne are now reported as having other transmission modes.

Since 1973, the Centers for Disease Control and Prevention (CDC) has collected data on foodborne disease outbreaks submitted by all states, the District of Columbia, and US territories through the Foodborne Disease Outbreak Surveillance System. In 2009, existing foodborne and waterborne disease outbreak surveillance systems were transitioned to an enhanced reporting platform, the National Outbreak Reporting System (NORS), which also collects reports of enteric disease outbreaks transmitted through person-to-person contact, contact with animals, environmental contamination, and indeterminate means (*1*). A new electronic reporting form and data entry interface were also introduced. In 2009, the number of reported foodborne disease outbreaks declined 32% compared with the mean of the preceding 5 years (*2*); the number also remained below the pre-2009 average during 2010–2012 (*2*,*3*) (Figure). The decline was largely observed among outbreaks attributed to norovirus (Figure), which can be transmitted through many routes: in comparison, the number of outbreaks attributed to *Salmonella* spp., which is usually transmitted through food, remained relatively constant (*1*,*2*).

We considered 3 possible reasons for the decline in the number of reported foodborne disease outbreaks: 1) classification of some outbreaks that previously would have been reported as foodborne as caused by another mode of transmission; 2) technical issues associated with the introduction of the new system; and 3) staffing and resource limitations related to the influenza A(H1N1)pdm09 virus pandemic. Clarification of how these factors might have affected reporting would provide accurate conclusions about trends in foodborne disease outbreaks. In 2013, we conducted a survey to identify possible reasons for the decline in the number of foodborne disease outbreak reports that started in 2009.

The Study

In January 2013, we conducted a voluntary, anonymous, internet-based survey of public health officials who are responsible for entering foodborne disease outbreak data into NORS at US state and territory health departments. The survey contained 33 questions in multiple choice, rating scale, or text formats. The questions asked about reporting procedures that might influence data quality and completeness, challenges and practices when determining the mode of transmission for each outbreak, the usability of the online reporting interface, and resource limitations.

Of the 133 public health officials in 56 jurisdictions who received the link to the survey, 50 (38%) from 39 (70%) jurisdictions completed the survey in whole or in part. The denominator varied for different questions because of nonresponse and exclusion of responses when "don't know" was selected. Also, some respondents had not used the previous reporting system. Most respondents (36/43, 84%) assigned a high priority to entering foodborne disease outbreak data, rating outbreak reporting as 4 or 5 on a scale of 1 to 5, where 1 indicated low priority and 5 high priority. Similarly, 38/47 (81%) of respondents reported that 90%–100% of foodborne disease outbreaks investigated in their health departments were entered into NORS; 5/47 (11%) reported entering <50% of outbreaks.

The survey included 1 question to determine whether respondents had experienced difficulties identifying the primary mode of transmission for some outbreaks and 1 question to understand which modes of transmission they found difficult to distinguish from foodborne transmission. Many respondents (35/47, 74%) reported sometimes having difficulties in determining an outbreak's primary mode of transmission. More than half (26/47, 55%) of respondents reported that, since 2009, they had used the newly established category of indeterminate/other/unknown to report an outbreak for which the mode of transmission was not clear. Twenty (80%) of 25 respondents reported that they had experienced difficulty distinguishing between foodborne and person-to-person transmission (Table). In comparison, determining whether an outbreak was caused by transmission of the infectious agent through food or by animal contact was a problem for only 6 of 21 (29%) respondents. Respondents who reported difficulty distinguishing between

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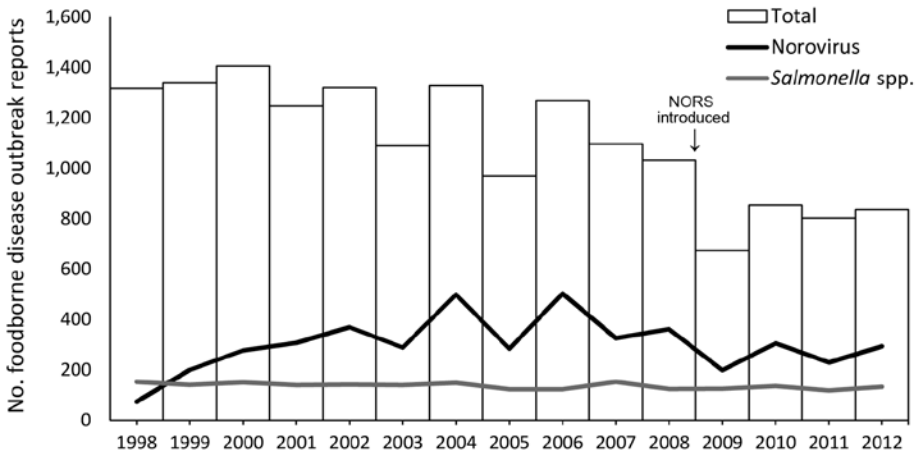

Figure. Total number of foodborne disease outbreaks and number caused by norovirus and *Salmonella* spp. as reported to the Foodborne Disease Outbreak Surveillance System, United States, 1998–2012. Data current as of April 22, 2014.

foodborne and another mode of transmission were asked if they would have reported the outbreak as foodborne to the previous reporting system (before 2009). Most respondents indicated that an outbreak was very likely or likely to have been reported to the previous system as a foodborne disease outbreak if there was a problem determining whether an outbreak was caused by foodborne or person-to-person transmission (15/20 respondents, 75%); by foodborne or environmental contamination (8/11 respondents, 73%); or if a specific mode of transmission could not be determined with confidence (13/19 respondents, 68%).

Regarding usability of the NORS reporting interface, most respondents (36/37, 97%) reported that usability of the new interface was the same as or better than that of the previous system. Most respondents (26/31, 84%), reported that technical issues with the NORS system did not prevent them from entering outbreak reports; only 2 respondents reported that >10% of outbreaks were not entered because of technical issues. When asked if their health departments experienced decreased resources available to work on foodborne disease outbreaks in 2009 while dealing with influenza A(H1N1)pdm09 virus, 19 (57%) of 35 respondents reported decreased resources for foodborne disease outbreak investigations; 14 (44%) of 32 respondents reported decreased resources for outbreak detection (e.g., laboratory capacity); and 14 (38%) of 37 respondents reported decreased resources for outbreak data entry and reporting.

Conclusions

Clarifying the factors that affect foodborne disease outbreak surveillance enables accurate interpretion of observed changes over time. The findings of this survey suggest that the large decline in the number of foodborne disease outbreaks reported in 2009 was likely a combined result of the following: 1) a surveillance artifact, in that some outbreaks previously reported as foodborne are now attributed to other modes of transmission; and 2) limited availability of resources to detect, investigate, and report foodborne disease outbreaks during the influenza A(H1N1) pdm09 pandemic. The total number of outbreaks reported increased after 2009 but remained below pre-2009 numbers, which suggests that the effect of the surveillance artifact is persistent and that outbreaks are now being more accurately categorized by mode of transmission. Although we hypothesized that technical issues with the new reporting interface might have affected reporting, this explanation appears less likely.

Limitations of the survey included the length of time between the transition to NORS and the survey, which meant that some survey participants who are current NORS users had not used the previous reporting system or worked on foodborne disease outbreaks in 2009. This limitation explains the low number of responses to survey questions that required knowledge of practices before 2009. Also, the overall response rate for the survey was low. Possible explanations include staffing and resource

Table. Number of survey respondents reporting difficulty in distinguishing between foodborne disease outbreaks and outbreaks caused by other modes of transmission in the National Outbreak Reporting System, United States, 2013

Mode of transmission	No. (%) respondents	
	Experienced difficulty in distinguishing outbreak type	If experienced difficulty, likely reported as foodborne to previous system
Foodborne vs. person-to-person, n = 25	20 (80)	15 (75)
Foodborne vs. indeterminate/other/unknown, n = 25	19 (76)	13 (68)
Foodborne vs. environmental contamination, n = 22	11 (50)	8 (73)
Foodborne vs. water, n = 23	8 (35)	2 (25)
Foodborne vs. animal contact, n = 21	6 (29)	1 (17)

limitations, but the survey was voluntary and anonymous, and no follow-up efforts were made to determine reasons for the low response rate. In addition, because some jurisdictions have >1 reporting administrator, personnel in some jurisdictions may have compiled a single response. On the other hand, the experiences of some health departments that did not compile responses might have been overrepresented. Further, the survey was not designed to measure the proportion of reported outbreaks affected by the introduction of NORS. Other potential reasons for the decline in the number of reported foodborne disease outbreaks, such as resource limitations and loss of public health positions resulting from budget cuts during the recession (*4*), were not explored.

In summary, the results of this survey provide unique insights into the decline in the number of foodborne outbreak reports submitted in 2009 and thereafter. NORS provides more comprehensive surveillance of outbreaks and a better understanding of the epidemiology of pathogens with multiple transmission pathways (*1*). These findings may be useful to improve guidance and training for outbreak reporting, particularly in reporting of the mode of transmission when multiple pathways exist for a pathogen.

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Risk Factors for Severe Influenza A Virus Pneumonia in Adult Cohort, Mexico, 2013–14

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During the 2013–14 influenza season, we assessed characteristics of 102 adults with suspected influenza virus pneumonia in a hospital in Mexico; most were unvaccinated. More comorbidities and severity of illness were found than for patients admitted during the 2009–10 influenza pandemic. Vaccination policies should focus on risk factors.

During 2009, Mexico reported 3 outbreaks of influenza A(H1N1)pdm09 infection (*1*); during the 2011–12 winter, a fourth pandemic wave of illness attributed to the virus was reported (*2*). Many patients had severe pneumonia related to influenza A(H1N1)pdm09 virus; the death rate was higher in Mexico than in most countries (*3*–*5*).

Beginning December 5, 2013, admissions of adults with acute respiratory infections increased at Hospital Central "Dr Ignacio Morones Prieto" (Hospital Central), in San Luis Potosí, Mexico; 102 persons were admitted with suspected influenza pneumonia. A high proportion of patients required mechanical ventilation (MV) and intensive care. The number of acute respiratory infection–associated admissions of young children and older adults did not increase. This pattern, occurring nearly 5 years after the initial influenza A(H1N1)pdm09 outbreak, resembles that of the initial outbreak in San Luis Potosí in April 2009 (*6*). This raises questions regarding possible changes in the viral strain or the presence of large numbers of susceptible persons not exposed to, or vaccinated against, this virus. We analyzed characteristics of 102 patients with confirmed or suspected influenza pneumonia admitted during a 2-month period and compared them with those of 100 patients with confirmed A(H1N1)pdm09 infection hospitalized during the 2009–10 pandemic.

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The Study

During December 5, 2013–February 7, 2014, 102 patients, 17–79 years of age, were admitted to Hospital Central with acute onset of cough or rhinorrhea plus dyspnea, fever, and radiographic evidence of pneumonia and were considered to have possible influenza-related pneumonia. We compared data for the 2013–14 cohort with data from patients 15–71 years of age with confirmed influenza A(H1N1)pdm09 infection admitted to Hospital Central during the 2009–10 pandemic (*7*). Patients were evaluated according to a clinical questionnaire (*6*,*7*); blood samples (laboratory tests and cultures), sputum samples or tracheal aspirates (Gram stain and culture), and chest radiographs were also evaluated. Nasopharyngeal or tracheal samples were analyzed to detect influenza virus by using real-time reverse transcription PCR (Laboratorio Estatal de Salud Pública, Servicios de Salud del Estado de San Luis Potosí) or sequence-specific primer PCR (Universidad Autónoma de San Luis Potosí) with previously reported primers (*8*). Empiric treatment (ceftriaxone and clarithromycin or ceftriaxone and levofloxacin) was administered, and a course of oseltamivir was started within 6 hours after admission at doses of 75–150 mg 2× daily.

Influenza A(H1N1)pdm09 virus was detected in 47 patients and influenza A (not subtyped) in 8 patients; samples were not submitted for virologic testing for 8 patients. In 28 (71.8%) of 39 patients with negative results by real-time and sequence-specific primer reverse transcription PCR, samples were collected >5 days after onset of symptoms or patients had received oseltamivir during ≥1 day at the time of sample collection; therefore, test results for those patients were not conclusive, and the patients were included in the cohort. Signs and symptoms of the remaining 11 patients with negative results and of the 8 untested patients were suggestive of influenza infection; therefore, they were included in the cohort.

Mean age of the 102 patients was 44.6 years; 52 (51%) were female, and 82 (80.4%) had risk factors for complications. The most common concurrent health conditions were obesity (63[62%]) and diabetes mellitus (23 [23%]). Eight (8%) patients had been vaccinated for 2013–14 seasonal influenza. Radiologic manifestations were bilateral ground glass opacities and consolidations. Most patients had severe illness; mean partial pressure arterial oxygen and fraction of inspired oxygen ratio (on admission was 175.6 (reference value 400; SD 74.4); 52 (50.9%) patients required MV.

Demographic and clinical characteristics of patients are shown in Table 1. The 52 patients receiving MV were

Table 1. Characteristics of patients with severe pneumonia who required mechanical ventilation compared with patients who did not require mechanical ventilation, San Luis Potosi, Mexico, 2013–2014*

Characteristic	Without mechanical ventilation, n = 50	With mechanical ventilation, n = 52	p value
Age, y, mean (SD)	42.8 (13.6)	46.2 (13.3)	
Sex, no. (%)			
M	25 (50)	25 (48.1)	0.21
F	25 (50)	27 (51.9)	0.85
Presence of ≥1 underlying condition, no. (%)	38 (76)	44 (84.6)	0.27
Obesity†	23 (46.9)	39 (76.5)	0.002
Diabetes mellitus	12 (24)	11 (21.2)	0.73
Immunosuppression‡	4 (8)	5 (9.6)	1.0
Asthma-COPD-bronchiectasis	8 (16)	1 (1.9)	0.01
Other§	9 (18)	3 (5.8)	0.06
Underlying disorders, excluding obesity, no. (%)	27 (54)	21 (40.4)	0.17
Influenza vaccination during most recent season, no. (%)	8 (16)	0	0.002
Signs and symptoms, no. (%)			
Fever	49 (98)	52 (100)	0.49
Headache	45 (90)	49 (94.2)	0.48
Cough	50 (100)	52 (100)	NA
Myalgias	46 (92)	46 (88.5)	0.74
Dyspnea	50 (100)	52 (100)	NA
Blood-streaked sputum	14 (28)	15 (28.8)	0.92
Diarrhea	9 (18)	6 (11.5)	0.36
Clinical findings, mean (SD)			
Body mass index†	29.6 (4.8)	31.9 (3.9)	0.01
Respiratory rate, bpm	26.4 (3.3)	30.3 (5)	<0.001
Cardiac rate, bpm	102.6 (12.4)	109.2 (16.9)	0.03
Mean blood pressure, mm Hg	80.6 (11.3)	74.2 (14.6)	0.01
Temperature, °C	38.5 (0.5)	38.5 (0.5)	0.42
PaO_2/FiO_2¶	236 (49.2)	119.8 (43.8)	<0.001
Laboratory findings, mean (SD)			
Total leukocytes, $\times 10^3$ cells/μL†	8.1 (4.3)	7.4 (3.9)	0.39
Lymphocytes, $\times 10^3$ cells/μL†	1.2 (0.75)	0.84 (0.64)	0.01
Platelet count, $\times 10^3$/μL†	223.5 (93.1)	212.4 (125.4)	0.62
C-reactive protein, mg/dL†	12.9 (8.8)	19.7 (11.6)	0.001
Lactate dehydrogenase, U/L#	406.8 (416.6)	694.6 (547.4)	0.006
Creatine phosphokinase, U/L**	349.5 (760.1)	575.9 (544.4)	0.1
Symptom onset			
Duration of symptoms before dyspnea, d, mean (SD)	2.8 (2.8)	3.1 (3.3)	0.67
Dyspnea duration before admission, d, mean (SD)	3.9 (2.8)	3.7 (2.7)	0.75
Duration of symptoms before hospital admission, d, mean (SD)	6.7 (3.8)	6.6 (3.5)	0.99

*Statistical analyses used included Fisher exact test, χ^2 test, and Student t test. COPD, chronic obstructive pulmonary disease; NA, not applicable; PaO_2/FiO_2, partial pressure arterial oxygen and fraction of inspired oxygen ratio.
†Body mass index >30. Data available for 100 patients.
‡Patients with autoimmune disorders (4 cases; 1 patient had undergone renal transplantation), HIV infection (3 cases), lymphoma (1 case), and chronic renal failure (1 case).
§Other disorders: cardiopathy (5 cases), Down syndrome (3 cases; 1 with cardiopathy), hypothyroidism (3 cases), pregnancy (1 case), and obstructive sleep apnea syndrome (1 case)
¶Data available for 98 patients.
#Data available for 93 patients.
**Data available for 91 patients.

more likely to be obese (body mass index [BMI] >30) than those who did not require it (76.5% versus 46.9%, respectively; p = 0.002). Lactate dehydrogenase concentrations (406.8 mg/dL versus 694.6 mg/dL; p = 0.006) and C-reactive protein level (12.9 mg/dL versus 19.7 mg/dL; p = 0.001) were higher in patients who required MV.

To assess severity of illness and possible associated factors, we compared characteristics of the 55 patients with confirmed influenza A infection and 100 patients with A(H1N1)pdm09 infection admitted during the 2009–10 pandemic (Table 2). Patients in the 2013–14 cohort were somewhat older and heavier than those in the 2009–10 cohort: mean age of patients admitted during 2009–10 was 38.9 y and that of patients admitted during 2013–14 was 46.1 y (p = 0.002; Figure 1). Mean BMI of the 2009–10 cohort was 28.9, versus 31.9 for the 2013–14 cohort (p<0.001). Mean partial pressure arterial oxygen and fraction of inspired oxygen ratio was not significantly different for the 2 groups; however, proportion of those needing MV was significantly higher in the 2013–14 cohort (36/55 [65.5%] than in the 2009–10 cohort (47/100 [47%]; p = 0.03).

By the time of this report, 29 of the 102 patients admitted during 2013–14 had died, 69 had been discharged, and

Table 2. Demographic features of adult patients admitted with confirmed influenza virus pneumonia during December 5, 2013–February 7, 2014, compared with patients with confirmed influenza A(H1N1)pdm09 infection during the 2009–10 pandemic period, San Luis Potosí, Mexico*

Characteristics	2009–10, n = 100	2013–2014, n = 55	p value
Age, y, mean (SD), median (range)	38.9 (13.1), 37 (15–71)	46.1 (14), 45 (19–79)	0.002
Sex, no. (%)			
M	52 (52)	28 (50.9)	0.89
F	48 (48)	27 (49.1)	
Presence of ≥1 underlying condition, no. (%)†	68 (68)	47 (85.5)	0.017
Obesity (body mass index>30)‡	39 (39.8)	40 (74.1)	<0.001
Diabetes mellitus	20 (20)	15 (27.3)	0.3
Immunosuppression	8 (8)§	3 (5.5)¶	0.75
Asthma-COPD-bronchiectasis	9 (9)	2 (3.6)	0.33
Other#	19 (19)	4 (7.3)	0.049
Any underlying disorder excluding obesity, no. (%)	45 (45)	23 (41.8)	0.7
Influenza vaccination during most recent season, no. (%)**	4 (4)	3 (5.5)	0.7
Clinical findings			
Body mass index, mean (SD) †	28.9 (5.9)	31.9 (3.9)	<0.001
PaO_2/FiO_2, mean (SD) ††	152.1 (102.5)	156.2 (69.3)	0.32
Total leukocytes, K/μL, mean (SD)†	8.4 (5.3)	6.4 (3.2)	0.02
Lymphocytes, K/μL, mean (SD) ‡‡	0.94 (0.5)	0.85 (0.72)	0.37
C-reactive protein, mg/dL, mean (SD)§§	14.1 (9.8)	17.9 (10.9)	0.03
Lactate dehydrogenase, U/L, mean (SD)¶¶	1,099.4 (769.6)	581.8 (387.9)	<0.001
Creatine phosphokinase, U/L, mean (SD)##	530 (820.6)	480.6 (513.9)	0.69
Duration of symptoms before dyspnea, days, mean (SD)***	2.1 (3.5)	3 (3.4)	0.13
Dyspnea duration before admission, days, mean (SD)†††	2.7 (2.7)	3.8 (2.6)	0.03
Duration of symptoms before hospital admission, d, mean (SD)**	4.7 (6.6)	6.6 (3.8)	0.003
Other features			
Requirement for mechanical ventilation, no. (%)	47 (47)	36 (65.5)	0.03
Death within 5 d after admission, no. (%)	6 (6)	6 (10.9)	0.35

*Statistical analyses used included Fisher exact test, χ^2 test, Student *t* test, and Mann Whitney u test. COPD, chronic obstructive pulmonary disease; PaO_2/FiO_2, partial pressure arterial oxygen and fraction of inspired oxygen ratio.
†Some patients had >1 underlying condition: heart failure (6 cases), pregnancy/puerperium (6 cases), hypothyroidism (4 cases), seizure disorder (2 cases), ischemic cardiopathy (1 case), microcephaly (1 case), Down syndrome (1 case), obstructive sleep apnea (1 case).
‡Body mass index >30. Data was available for 152 patients.
§Includes chronic renal failure (4 cases), chronic steroid use (3 cases), and leukemia (1 case).
¶HIV infection (1 case), autoimmune disorder (2 cases; 1 patient had undergone renal transplant),
#Down syndrome (2 cases), hypothyroidism (1 case), cardiopathy (1 case).
**Data available for 154 patients.
††Data available for 147 patients.
‡‡Data available for 151 patients.
§§Data available for 144 patients.
¶¶Data available for 135 patients.
##Data available for 131 patients.
***Data available for 139 patients.
†††Data available for 138 patients.

4 remained hospitalized. Of 29 deaths, 11 (37.9%) occurred within 5 days of admission. The rate of deaths within 5 days of admission was 10.9% among patients with confirmed influenza infection admitted during 2013–14, and 6% among those admitted during 2009–10 (p = 0.35). In contrast to findings with the 2009–10 cohort, duration of symptoms among the 2013–14 cohort at the time of admission did not appear to affect the outcome (Figure 2).

In Mexico, the first 3 influenza A(H1N1)pdm09 waves were characterized by a high proportion of hospitalizations among persons 5–59 years of age (*1*,*9*); in the fourth wave, the proportion of hospitalizations increased in young children and older adults (*2*). The 2013–14 influenza season data resemble those of the initial A(H1N1)pdm09outbreak in the high number of severe cases among adults <80 years and, although this observation has been described in other pandemics (*10*), possible explanations for this epidemiologic pattern should be analyzed. The following characteristics of the 2013–14 influenza season cohort could explain this demographic situation: 1) low vaccination coverage of high-risk populations; 2) differences in the size of the high-risk susceptible population; 3) high prevalence of diabetes mellitus and obesity; 4) antigenic drift; and 5) unusually low temperatures during the 2013–14 winter season. The severity of pneumonia cases (MV requirement, low lymphocyte counts, and high C-reactive protein and lactate dehydrogenase concentrations) among adults <80 years of age during the 2013–14 influenza season could be explained by a high prevalence of concurrent health conditions (especially obesity), delay in health care access, or a change in virulence of the current influenza strain.

Conclusions

Because of the high number of unvaccinated patients in this study cohort, the need to modify the intended

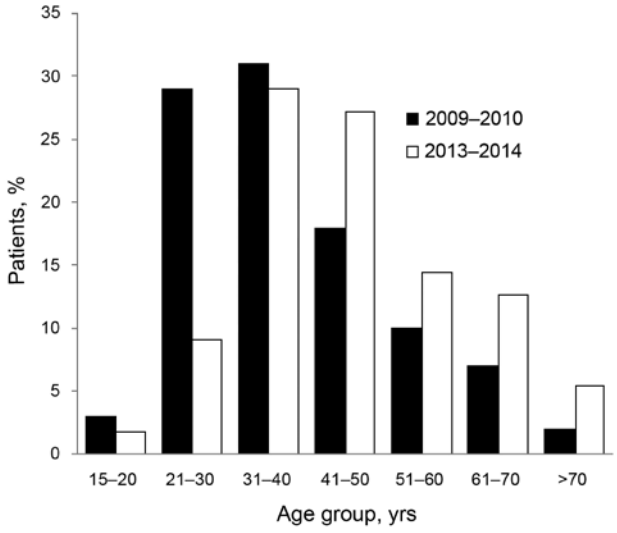

Figure 1. Patients with confirmed influenza pneumonia admitted to "Dr Ignacio Morones Prieto" (Hospital Central), in San Luis Potosí, Mexico during 2009–10 and 2013–14, according to age group.

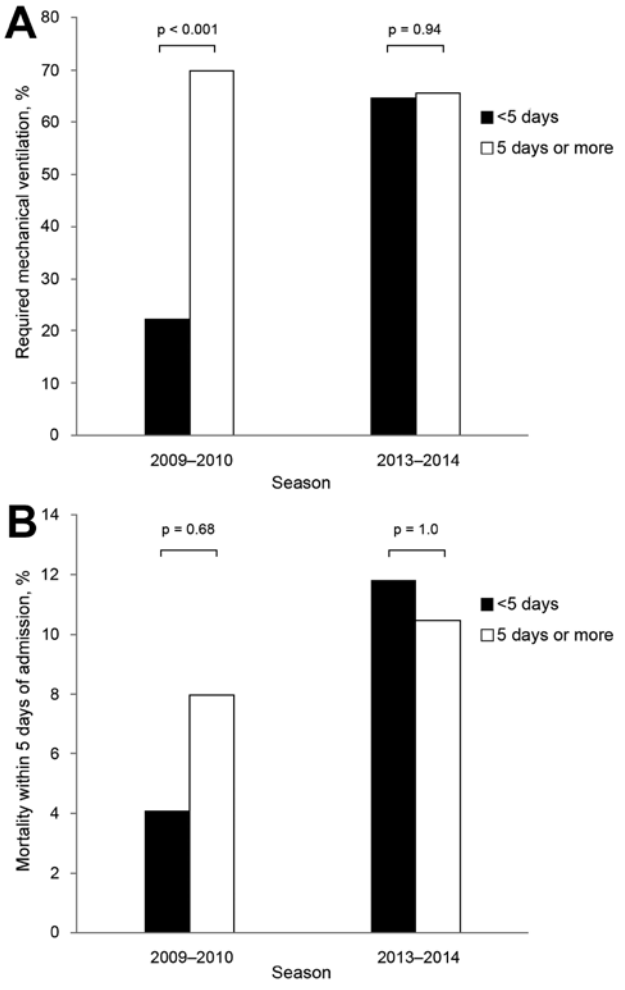

Figure 2. A) Proportion of patients with confirmed influenza pneumonia that required mechanical ventilation according to the duration of symptomatology on admission (0–4 days versus >5 days) during the 2009–10 pandemic and 2013–14 season. B) Mortality rate within 5 days of admission according to the duration of symptomatology on admission during the 2009–10 pandemic and 2013–14.

population for influenza vaccination policies should be assessed. The vaccination rate was substantially lower in patients who required MV compared with those who did not; the overall vaccination rate among hospitalized patients contrasts with the high vaccination rates reported recently in Mexico for high-risk groups (71.7%–101.9%) (*11*,*12*). The association between severe influenza infection and obesity has been increasingly documented since the 2009 pandemic (*13*). Therefore, this group should be included among persons at high risk for influenza complications and the BMI range of subjects that require vaccination should include not only those with morbid obesity, but also those weighing >30 kg/m^2.

To improve outcomes, additional measures could be implemented such as campaigns and educational programs for the public and physicians that advocate early diagnosis, treatment, and identification of influenza-associated complications. In summary, to establish appropriate preventive measures, the epidemiologic characteristics of influenza outbreaks should be used to identify risk factors for severe infections.

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Common Exposure to STL Polyomavirus During Childhood

Efrem S. Lim, Natalie M. Meinerz, Blake Primi, David Wang, and Robert L. Garcea

STL polyomavirus (STLPyV) was recently identified in human specimens. To determine seropositivity for STLPyV, we developed an ELISA and screened patient samples from 2 US cities (Denver, Colorado [500]; St. Louis, Missouri [419]). Overall seropositivity was 68%–70%. The age-stratified data suggest that STLPyV infection is widespread and commonly acquired during childhood.

Polyomaviruses are nonenveloped double-stranded circular DNA viruses that infect a wide range of hosts, including humans. The capsid of the virus comprises primarily 72 pentamers of the major coat protein, VP1. Human polyomaviruses have been associated with several diseases (*1*). BK polyomavirus (BKPyV) has been associated with nephropathy in renal transplant recipients and JC polyomavirus (JCPyV) with progressive multifocal leukoencephalopathy in immunocompromised persons (*2*,*3*). Trichodysplasia spinulosa–associated polyomavirus (TSPyV) infection is linked to a rare skin disease in immunocompromised patients called trichodysplasia spinulosa (*4*). Furthermore, infection with Merkel cell polyomavirus (MCPyV) in rare instances leads to Merkel cell carcinomas, an aggressive form of skin cancer (*5*). Other polyomaviruses, including WU polyomavirus (WUPyV), KI polyomavirus (KIPyV), human polyomavirus 6, human polyomavirus 7, human polyomavirus 9, MW polyomavirus (MWPyV), STL polyomavirus (STLPyV), and human polyomavirus 12, have been identified in specimens from humans, but their role in disease remains to be defined (*1*).

Seroepidemiology has played an important role in establishing the link between human polyomaviruses and disease and in understanding infection dynamics. The seroprevalences of BKPyV and JCPyV range from 70% to 90% and 40% to 60%, respectively, with an age profile indicating high frequency of early-age infections and lifelong persistence (*6*–*8*). Seropositivity for MCPyV in healthy persons ranges from 25% to 64%; all patients with Merkel cell carcinoma are seropositive (*6*,*9*).

STLPyV was recently identified from fecal specimens from a child in Malawi (*10*). Viral DNA also was detected in fecal specimens from the United States and The Gambia, and STLPyV has been found in a surface-sanitized skin wart surgically removed from the buttocks of a patient with a primary immunodeficiency called WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome (*11*). These observations suggest that STLPyV might infect humans. We defined the seropositivity rate of STLPyV in humans using serum from 2 independent US sites (Denver, Colorado, and St. Louis, Missouri).

The Study

To determine the seropositivity for STLPyV, we developed a capture ELISA using recombinant glutathione S-transferase–tagged STLPyV VP1 capsomeres (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/9/14-0561-Techapp1.pdf). Electron microscopy of the STLPyV capsomeres showed 10-nm pentamers characteristic of polyomaviruses (Figure 1, panel A). We assessed the specificity of the STLPyV ELISA by preincubating 24 serum samples in the presence and absence of soluble STLPyV VP1 pentamers before addition to the immobilized STLPyV glutathione S-transferase–tagged VP1. The ELISA signal intensity was markedly reduced when serum was pre-incubated with STLPyV VP1 pentamers (Figure 1, panel B; compare white bars to gray bars). This result indicates the ELISA seroreactivity could be self-competed with soluble STLPyV pentamers. STLPyV shares 55% aa identity in the VP1 region with its next most closely related polyomavirus, MWPyV (*10*). Therefore, we examined whether cross-reactivity existed between STLPyV and MWPyV VP1 capsomeres. Competition assays with soluble MWPyV VP1 pentamers showed limited interference with the ELISA seroreactivity (Figure 1, panel C; compare white bars to gray bars). This result indicates that there was no significant cross-reactivity between STLPyV and MWPyV VP1 capsomeres. Taken together, these data demonstrate that the ELISA was specific to STLPyV VP1.

We screened 500 serum specimens collected from children and adults in Denver for antibodies against STLPyV. The overall seropositivity for STLPyV was 68.0% (Figure 2, panel A). Children 1–3 years of age had the lowest seropositivity rate (23.8%). In contrast, seroprevalence of children 4–20 years of age ranged from 61.1% to 70.8%. Similar seropositivity rates persisted in adults (>21 years of age), ranging from 68.8% to 74.2%.

We next examined a panel of 419 serum specimens from St. Louis that had a higher resolution of age-stratification in young infants. The overall seropositivity for STLPyV was 70.0% (Figure 2, panel B), similar to that

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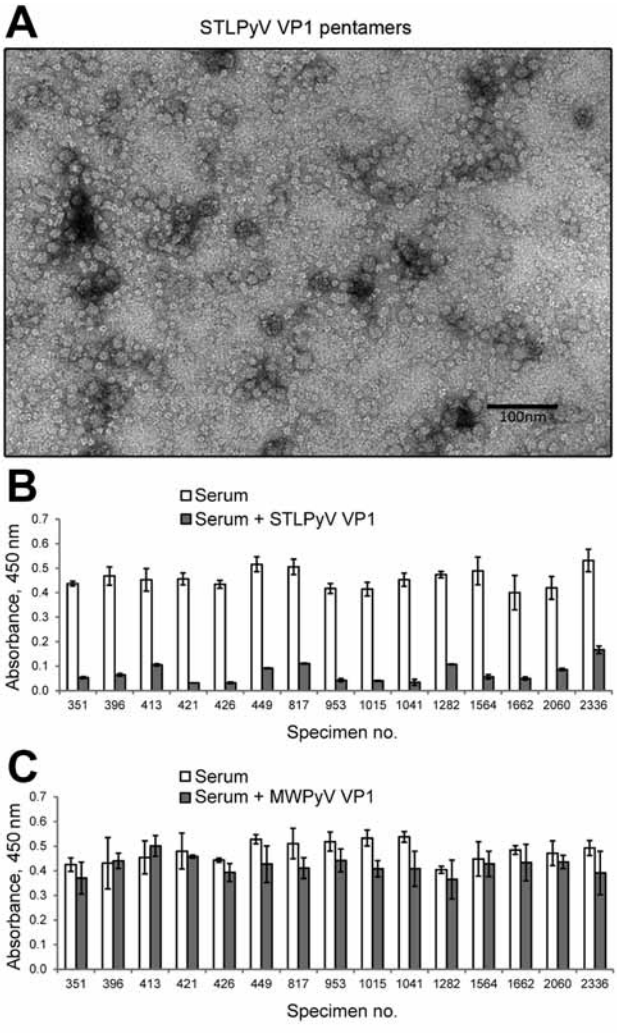

Figure 1. STL polyomavirus (STLPyV) ELISA. A) Electron microscopy image shows purified STLPyV VP1 capsomeres. Scale bar = 100 nm. B) Serum samples were pre-incubated in the absence (white bars) or presence of soluble STLPyV VP1 pentamers (gray bars), followed by the STLPyV-capture ELISA. Serum was tested in triplicate, and average absorbance values are shown. Error bars indicate SD. Representative data are shown. C) Seroreactivity to STLPyV in the absence (white bars) or presence of competition with MW polyomavirus (MWPyV) VP1 pentamers (gray bars) are shown. Serum was tested in triplicate, and average absorbance values are shown. Error bars indicate SD. Representative data are shown.

in the specimens from Denver (68.0%). Seropositivity for STLPyV in infants dropped from 53.3% in the <0.5-year age group to 37.9% in the 0.5–1-year age group, with the lowest seropositivity of 22.6% in the 1–2-year group. In contrast, seropositivity rates for children >2 years of age were higher, ranging from 60.0% to 85.3%. Finally, seropositivity in adults (>21 years of age) ranged from 91.2% to 95.2%. Thus, these data indicate that exposure to STLPyV occurs during early childhood and that immune responses to STLPyV are maintained in adults.

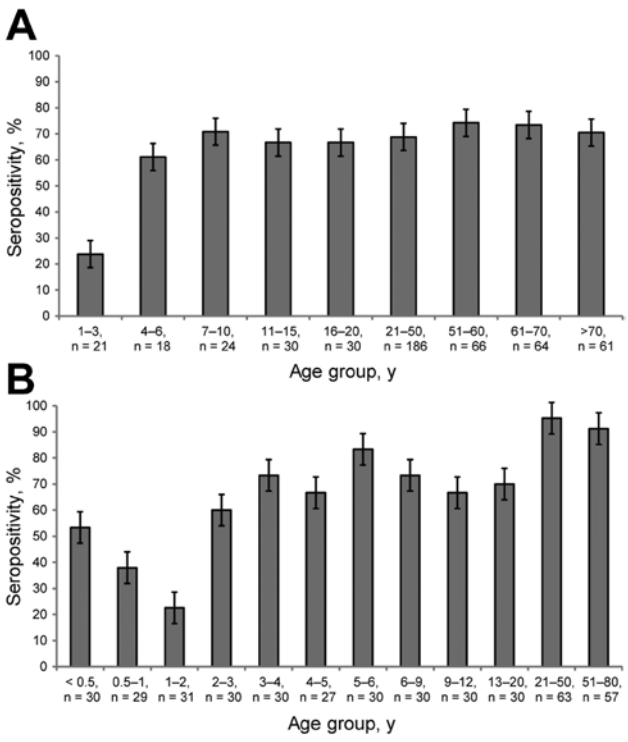

Figure 2. Age-specific seropositivity for STL polyomavirus (STLPyV) from serum specimens collected in Denver, Colorado, USA (A), and St. Louis, Missouri, USA (B). A total of 500 serum specimens from Denver and 417 from St. Louis were tested for seroreactivity to STLPyV VP1 proteins. Overall seropositivity in Denver was 68% and in St. Louis, 70%. Error bars indicate SD.

Conclusions

In our analysis of the seroepidemiology of STLPyV in 2 areas of the United States, we found that prevalence of the virus was similar (68.0%–70.0%). This prevalence is slightly higher than the 41.8% for MWPyV, the polyomavirus most closely related to STLPyV (*12*). However, the seropositivity of STLPyV is comparable to other human polyomaviruses (>60%), such as BKPyV, KIPyV, WUPyV, MCPyV, human polyomavirus 6, and TSPyV (*6*,*8*). We found no cross-reactivity with MWPyV VP1, the most closely-related polyomavirus that shares 55% aa identity in the VP1 region. Thus, the seroepidemiology strongly supports the notion that STLPyV is a bona fide infectious agent of humans.

Age stratification of the seropositive specimens suggested an initial waning of immune response followed by rapid seroconversion during childhood. In the St. Louis specimens, seropositivity was higher for the <0.5-year and 0.5–1-year age groups (53.3% and 37.9%, respectively) than for the 1–2-year group (22.6%). This observation was followed by an increase in STLPyV seropositivity in the 2–3-year group and older age groups (60.0%). Specimens from Denver were too few to reliably stratify the data to

the same resolution. Nonetheless, we observed a similar trend in specimens from Denver where the seropositivity for the 1–3-year age group (23.8%) was lower than for the 4–6-year group (61.1%). These data indicate that immune responses to STLPyV decreased in the first 2 years of age, which suggests waning maternal antibodies. However, seropositivity was rapidly acquired thereafter, indicating high exposure of STLPyV in children. Because the specimens were selected from hospital-associated blood draws of children of unknown health status, seropositivity rates for healthy children might have varied somewhat from the results we obtained. Nonetheless, the trends we observed with STLPyV are similar to profiles that have been reported for JCPyV, BKPyV, TSPyV, MCPyV, WUPyV, and KIPyV (*6*,*13*–*15*).

STLPyV has not been clearly associated with any disease. However, the high overall seropositivity rate suggests widespread infection in the population at large. None of the pathogenic human polyomaviruses (JCPyV, BKPyV, MCPyV, TSPyV) have been clearly associated with acute disease at the time of initial infection. Rather, immunosuppression is a critical co-factor that is coupled to the ability of polyomaviruses to persist throughout life or to integrate into the genome, as in the case of MCPyV, ultimately leading to disease. Our study demonstrates that a large segment of the general population has been infected by STLPyV and might harbor persistent STLPyV infection, assuming the persistence paradigms of JCPyV and BKPyV hold true for STLPyV. A recent report identified STLPyV DNA in a skin wart specimen from an adult with primary immunodeficiency (*11*). Thus, it is critical to determine whether human diseases exist that are caused by STLPyV, especially in immunocompromised persons.

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Enhanced MERS Coronavirus Surveillance of Travelers from the Middle East to England

Helen Lucy Thomas, Hongxin Zhao, Helen K. Green, Nicola L. Boddington, Carlos F.A. Carvalho, Husam K. Osman, Carol Sadler, Maria Zambon, Alison Bermingham, and Richard G. Pebody

During the first year of enhanced MERS coronavirus surveillance in England, 77 persons traveling from the Middle East had acute respiratory illness and were tested for the virus. Infection was confirmed in 2 travelers with acute respiratory distress syndrome and 2 of their contacts. Patients with less severe manifestations tested negative.

The first reported case-patient infected with Middle East respiratory syndrome coronavirus (MERS-CoV) died in June 2012 in the Kingdom of Saudi Arabia and was reported on September 20 (*1*). The second case reported globally was in a Qatari national patient who had been transferred from Qatar to a hospital in England; preliminary data sharing on September 23 indicated that isolates from the second case-patient had 99.5% identity with the virus identified in the first case. (*2*). On September 24, 2012, Public Health England (PHE) (formerly the Health Protection Agency [HPA]) established an enhanced surveillance system to rapidly detect and investigate possible cases of MERS-CoV infection among travelers to England from the Middle East. The first 12 months of surveillance in England identified 1 additional case of MERS-CoV in a traveler returning from the Middle East and 2 cases among family contacts of this second case.

Definitions for possible and confirmed cases were established. Possible cases were defined by clinical and epidemiologic criteria. Clinical criteria specified acute respiratory syndrome (including fever ≥38°C or history of fever and cough) requiring hospitalization and clinical or radiologic evidence prompting suspicion of lower airway involvement not explained by another etiology. Epidemiologic criteria specified travel to or residence in an area where infection with MERS-CoV could have been acquired during the 10 days before onset of illness. At the time these criteria were initiated, the Kingdom of Saudi Arabia and Qatar were the 2 areas indicated. A confirmed case was defined by respiratory samples testing positive for MERS-CoV by at least 2 specific PCR assays targeting different regions of the MERS-CoV genome.

Because MERS-CoV is an emerging pathogen, case definitions were, and continue to be, revised in response to new information (*2*), in agreement with World Health Organization case definitions (*3*–*5*). Substantial revisions included extension of the geographic areas where infection could have occurred to include all countries neighboring those where infection could have been acquired (November 29, 2012), the recommendation to test patients with the appropriate clinical and epidemiologic criteria if they had an alternative etiology which did not fully explain their clinical manifestation (February 12, 2013), and extension of the incubation period to 14 days (June 21, 2013).

The Study

Enhanced surveillance involved the collection of a minimum dataset for each possible case, including demographic data, clinical symptoms, travel and contact history, and results of testing for respiratory pathogens (*6*). Nose and throat swab specimens and, when possible, lower respiratory tract specimens, were tested at 1 of 4 regional laboratories. Although the testing guidelines recommended MERS-CoV testing after exclusion of alternative etiologies, other tests were conducted in parallel with MERS-CoV testing for most suspected cases.

During the first few days of surveillance, a pan-coronavirus assay conducted at the PHE National Reference Laboratory was used as a screening test; then the viral genome was fully sequenced. After the generation of MERS-CoV specific assays, a first-line screening assay targeting the viral genomic area upstream of the E gene (*7*) was conducted, followed by confirmatory testing at the HPA/PHE National Reference Laboratory. Results of MERS-CoV testing are reported regularly in the HPA/PHE Weekly Influenza Report (*8*).

A descriptive analysis included the number of persons tested and proportion positive for MERS-CoV by key demographic, epidemiologic, and clinical characteristics. The positive predictive value of different combinations of signs and symptoms was calculated as the proportion of persons who had signs and symptoms of MERS and tested positive for MERS-CoV and showed exact (Clopper-Pearson) binomial 95% confidence intervals.

During September 24, 2012–October 15, 2013, 77 travelers from the Middle East that met the possible case

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Table 1. Results of MERS coronavirus testing of 77 travelers from the Middle East to England by key clinical and epidemiologic characteristics where information available, September 2012–October 2013

Characteristics	No. tested	No. (%) MERS coronavirus–positive
Age group, y		
0–4	5	0
5–17	1	0
18–44	10	0
45–64	34	2 (6)
≥65	25	0
Unknown	2	0
Sex		
M	49	2 (4)
F	26	0
Unknown	2	0
Clinical history		
Fever	51	2 (4)
Cough	51	2 (4)
Pulmonary parenchymal involvement	34	2 (6)
Acute respiratory distress syndrome	7	2 (29)
Mechanical ventilation	15	2 (13)
Extracorporeal membrane oxygenation	2	2 (100)
Travel history in exposure period* before symptom onset†		
Israel	1	0
Jordan	1	0
Kingdom of Saudi Arabia	40	1 (3)
Qatar	7	1 (14)
United Arab Emirates	27	0
Yemen	1	0

*Exposure period was 10 days until June 2013, when it was increased to 14 days.
†Some patients had also traveled to countries outside the Middle East.

definition were tested for MERS-CoV. Seventy-five travelers tested negative on the screening assay, and 2 tested positive. Positive results on the screening assay were confirmed by positive results at the HPA/PHE National Reference Laboratory.

In addition to testing the 77 persons who met all of the possible case criteria, MERS-CoV testing was conducted on 13 patients who had severe acute respiratory disease but did not meet the travel requirements: 2 had a travel history outside the Middle East, 4 had no travel history in the relevant exposure period, and travel histories of the remaining 7 were unknown. MERS-CoV was not detected in any of these persons.

The clinical and epidemiologic characteristics of the 77 persons tested and their MERS-CoV test results are shown in Table 1. Those tested ranged in age from 3 months to 90 years; 34 (44%) had signs of pulmonary parenchymal involvement. The 2 confirmed cases were in male patients, 45 and 60 years of age; both had severe acute respiratory symptoms requiring treatment by extracorporeal membrane oxygenation; both subsequently died. MERS-CoV PCR testing was conducted on 53 contacts of the 2 confirmed case-patients in England; 2 of these contacts tested MERS-CoV positive (*9*,*10*).

The positive predictive value for MERS-CoV infection of different combinations of signs and symptoms is shown in Table 2. No case-patients who did not have pulmonary parenchymal involvement tested positive for MERS-CoV, and the positive predictive value of the clinical manifestations increased as the severity of disease increased. Of the 77 patients tested, 22 had positive results for alternative respiratory pathogens, including 10 with influenza (7 influenza A and 3

Table 2. Positive predictive value of signs and symptoms among 77 travelers from the Middle East tested for MERS-CoV, Enhanced MERS-CoV Surveillance System, England, September 2012–October 2013*

Signs and symptoms	No. MERS CoV–positive/no. tested	Positive predictive value, % (95% CI)
Fever, cough; no pulmonary parenchymal involvement	0/3	0 (0–71†)
Fever, cough, and pulmonary parenchymal involvement	2/18	11 (1–35)
Fever, cough, and pulmonary parenchymal involvement requiring mechanical ventilation	2/4	50 (7–93)
Fever, cough, pulmonary parenchymal involvement, and acute respiratory distress syndrome	2/4	50 (7–93)
Fever, cough, and pulmonary parenchymal involvement requiring extracorporeal membrane oxygenation	2/2	100 (16–100)

*MERS-CoV, Middle East respiratory syndrome coronavirus.
†1-sided, 97.5% CI.

influenza B); 1 of the influenza A–infected case-patients was later confirmed to also be infected with MERS-CoV. Two case-patients tested positive for *Legionella pneumophila*, 4 for rhinovirus, 3 for adenovirus, 1 for respiratory syncytial virus, and 1 for human metapneumovirus.

Conclusions

Unlike surveillance for established organisms, surveillance for a novel pathogen requires analysis of information collected from all patients tested, even from those that test negative, to build knowledge of the predictive value of different epidemiologic and clinical manifestations. This report on the characteristics of patients traveling to England from the Middle East and tested for MERS-CoV enables a first crude estimation of the positive predictive value of different signs and symptoms during the first year following the emergence of this pathogen.

Because this study is based on a cohort of 77 suspected case-patients, of whom only 2 laboratory-confirmed cases were identified during the surveillance period, estimates on the basis of identified symptoms are very imprecise (Table 2). However, in the context of an emerging pathogen, reporting such data progressively helps optimize case detection and surveillance systems.

During the 12-month surveillance period, no patients who had respiratory symptoms but no pulmonary parenchymal involvement were positive for MERS-CoV by PCR, and the positive predictive value of signs and symptoms increased with the severity of clinical manifestation. This suggests that the case definitions that were in use during this period (which recommended MERS-CoV testing only for patients who met the epidemiologic criteria and had a severe respiratory illness) were appropriate.

A range of respiratory pathogens were found in those patients that were MERS-CoV negative, highlighting the importance of looking for alternative diagnoses. However, the diagnosis of 1 of the MERS-CoV case-patients was delayed because of an initial diagnosis of influenza. The testing algorithm was subsequently changed to ensure that patients meeting the possible case definition were tested for MERS-CoV if they had an alternative etiology which did not fully explain their clinical manifestation.

The predictive value of the possible case definition depends on the incidence of infection and would be expected to vary across different population groups and change over time, especially in the context of an emerging pathogen. We encourage other countries to similarly report the characteristics of all patients tested for MERS-CoV to improve understanding of the predictive value of different clinical and epidemiologic manifestations in various populations at different times. This will help inform the evolving international public health response to this novel pathogen.

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Live Poultry Market Closure and Control of Avian Influenza A(H7N9), Shanghai, China

To the Editor: China reported its first human infections with avian influenza A(H7N9) virus in late March 2013 (*1*). In the following weeks, 131 human infections were confirmed; 33 occurred in Shanghai (http://www.moh.gov.cn). Because infection with this novel virus had a high fatality rate and posed a pandemic risk, Shanghai disease control authorities launched rapid investigations to identify the source of the infections. Migratory birds, mammals, poultry, and humans could be potential reservoirs of H7 subtype avian influenza viruses (*2*,*3*), so all of these possibilities were simultaneously evaluated immediately after the discovery of the novel virus.

To investigate human-to-human transmission, we evaluated 45 close contacts of the first 6 reported case-patients. The only suspected human-to-human transmission was in 1 family cluster with 2 confirmed cases. Intensive follow-up monitoring (20 contacts of this cluster and 25 of the other 4 case-patients) did not identify any further infections, which suggests that sustained human-to-human transmission did not occur.

Migratory birds are natural reservoirs for avian influenza viruses (*2*,*4*), and Shanghai is in the eastern Asia–Australian migratory shorebird flyway. Thus, transmission of these viruses from wild birds to humans is possible. The Shanghai forestry authority has conducted surveillance for influenza virus among migratory birds since 2004. During January 2010–April 2013, a total of 884 throat/cloacal swab, serum, and fecal samples from 496 birds were tested, and no infections with influenza viruses of subtypes H7 or N9 were found. After human infections with influenza A(H7N9) virus occurred, an additional 229 samples were collected from migratory birds at Chongming Island, Shanghai Zoo, and Shanghai Wildlife Park. All were negative for the virus.

Avian influenza surveillance in domesticated animals (e.g., minks, raccoons, tigers, and pigs) in high-risk regions of Shanghai has been conducted since 1995. During 2010–2012, a total of 13,691 samples from these animals were tested, and all were negative for H7 subtype influenza viruses. After human infections with influenza A(H7N9) virus were identified, another 1,129 samples were collected from domesticated mammals for enhanced surveillance; all were negative for H7 subtype influenza viruses.

Live poultry was also considered a potential source of influenza A(H7N9) infection because poultry in wet markets have been previously found to be infected with H7 subtype avian influenza viruses (*5*–*7*). Shanghai residents could be exposed to influenza A(H7N9) virus from live poultry in 2 ways: by raising poultry at home or by visiting live poultry markets (LPMs). To assess the risk from home-raised poultry, we collected 405 blood, cloacal/throat swab, and environmental samples from poultry raised in family courtyards or in communities surrounding the residences of the first 6 identified case-patients. The samples were tested for antibodies to H7 subtype viruses and RNA for subtype H7N9 virus, and all results were negative.

Previous studies have shown that LPMs are potential locations for virus transmission through human-poultry contact (*8*,*9*). Our field investigation of the 6 initially confirmed influenza A(H7N9) case-patients revealed that 2 had visited LPMs, 2 had direct contact with live poultry from LPMs, and 1 had experienced both exposures. To assess the risk for infection at LPMs, we collected 280 water, soil, cloacal swab, and throat swab samples from the LPMs surrounding the locations of the initial 6 cases. PCR testing found that 20 samples were positive for influenza A(H7N9) virus. These results indicate that the human case-patients were most likely infected from poultry in LPMs.

On the basis of the epidemiologic and laboratory evidence, the Shanghai municipal government temporarily closed all 464 LPMs on April 6, 2013. The markets were cleaned and disinfected, and relevant authorities in the neighboring provinces were notified of the action and requested not to transport live poultry to Shanghai. The municipal government reimbursed the poultry traders and raisers for these measures.

After the closure of LPMs, 4 additional human influenza A(H7N9) cases were detected in Shanghai within the first 7-day incubation period, and no new cases were detected during the rest of 2013, although other influenza virus subtypes continued to be detected. Meanwhile, in other provinces where closure of LPMs was not implemented in a timely manner, 31 cases were reported during the first incubation period and 13 during the second incubation period (Figure). In addition, Shi et al. reported that isolates from the LPMs showed 99.1%–99.9% nucleotide sequence homology with isolates from 2 of the first 6 human case-patients (*10*), which further indicates that the virus spread from infected poultry in the LPMs.

The closure of LPMs after epidemiology and laboratory investigations proved timely and effective in the control of human infection with this novel virus. In early 2014, Shanghai lifted the ban on LPMs. Eight new influenza A(H7N9) cases were identified in early January 2014, but LPMs were again closed on January 31, 2014, and no further cases have been reported. The long-term effectiveness of the LPM closures remains to be determined.

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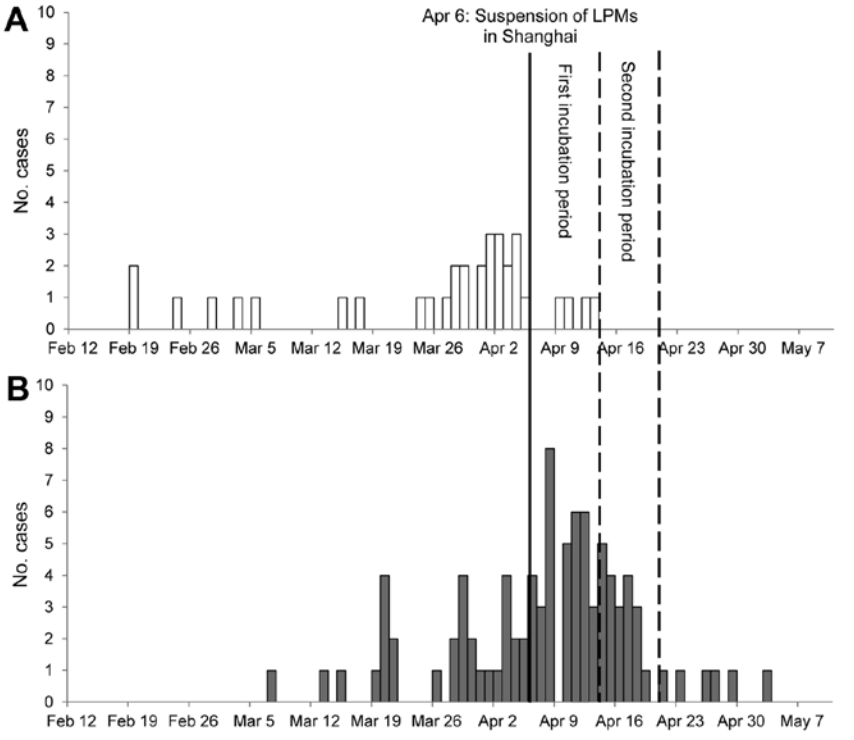

Figure. Illness onset dates for 33 confirmed cases of influenza A(H7N9) infection in Shanghai (A) and 78 cases in other provinces (B) in China, February 12–May 10, 2013. Solid vertical line indicates date live poultry markets (LPMs) in Shanghai were suspended (April 6, 2013). Dashed vertical lines delineate first and second incubation periods.

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Shiga Toxin 2A–Encoding Bacteriophages in Enteroaggregative *Escherichia coli* O104:H4 Strains

To the Editor: In 2011, enteroaggregative *Escherichia coli* (EAEC) O104:H4 strains that produce Shiga toxins (EAEC-STEC) caused an outbreak of hemorrhagic disease affecting nearly 4,000 patients in Europe (*1*). During 2001–2013, several countries reported infections caused by EAEC O104:H4 and EAEC-STEC O104:H4 strains (*1–9*). Genomic analysis of EAEC and EAEC-STEC O104:H4 strains revealed high similarity, and it has been suggested that EAEC-STEC O104:H4 strains evolved from EAEC O104:H4 strains by uptake of Shiga toxin 2 (Stx2)–producing bacteriophages (*3*,*4*).

We investigated Stx-2 subunit A (Stx-2A) bacteriophages in a group of epidemiologically unrelated EAEC-STEC O104:H4 strains isolated from animals and food in Germany (collection of the National Reference Laboratory for *Escherichia coli*). One phage genome (P13374) was sequenced (*2*). The Stx-2A bacteriophages were highly similar in morphologic features, restriction endonuclease profiles, chromosomal integration sites, and superinfection immunity (*2*,*3*) and showed <65% similarity to Stx phages from non-O104 strains. Major genetic differences between the bacteriophages we investigated and other Stx phages were found in the genes for DNA replication, DNA metabolism, and in the immunity region (*2*,*3*).

We identified 2 genes, *orf15* and cI_{P13374}, that were specific to Stx-2A bacteriophages found in EAEC-STEC O104:H4 strains (*10*). These genes were found in only 14 (5.8%) of 241 Stx-2A–positive non-O104 STEC strains. Viable Stx-2A bacteriophages isolated from 4 bovine non-O104 STEC strains were similar to Stx-2A bacteriophages from EAEC-STEC O104:H4 strains for all features described above (*10*). Similar to P13374, one of the bovine phages (P13803) lysogenized an Stx-negative EAEC O104:H4 strain and converted it into an EAEC-STEC–producing Stx-2A bacteriophage (*10*).

Our results provide experimental evidence that EAEC-STEC O104:H4 have evolved by uptake of a distinct type of Stx-2A bacteriophage. Bovine STEC harboring Stx-2A bacteriophages able to transduce Stx-2A genes to EAEC O104:H4 are found worldwide, and phage-mediated transfer of Stx-2A can occur in the environment (*10*). Thus, the emergence of EAEC-STEC O104:H4 does not appear to be the result of introduction of the strains from areas to which they are endemic. Instead, the process may have occurred spontaneously by phage transduction, which could explain why EAEC-STEC O104:H4 infections were found at different locations and at different times. Regardless of time or place, however, these strains show characteristic differences in their prophage and plasmid profiles, which may serve as indicators of epidemiologic origin (*1–4*). Investigation of EAEC-STEC O104:H4 strains from sporadic cases of human infection could reveal these markers and help differentiate between strains that were introduced from other areas and strains that were newly generated by phage transduction.

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Rio Mamoré Virus and Hantavirus Pulmonary Syndrome, Brazil

To the Editor: Hantavirus pulmonary syndrome (HPS) is an acute, severe, frequently fatal disease associated with cardiopulmonary failure; it is caused by hantaviruses naturally hosted by wild rodents. Rio Mamore virus (RIOMV) was first described in 1996 in Bolivia; it was associated with the small-eared pygmy rice rat, *Oligoryzomys microtis* (*1*). Subsequently, 1 strain of RIOMV was isolated from *O. microtis* rats in Peru, designated HTN-007 (*2*); and 2 strains were recovered in the Brazilian Amazon from *O. microtis* rats (RIOMV-3) and uncharacterized species of rodents of the genus *Oligoryzomys* (RIOMV-4) (*3*). Recently, HPS cases associated with RIOMV have been reported: 2 cases in Peru (*4*) and 1 case in French Guiana (caused by a variant named Maripa virus) (*5*). We report isolation of a strain of RIOMV from a patient with fatal HPS in Brazil.

In June 2011, a 28-year-old man was admitted to the Tropical Medicine Foundation Dr. Heitor Vieira Dourado, Amazonas State, with a 4-day febrile illness that included nonproductive cough, myalgia, and headache. Laboratory testing revealed hematocrit within reference range (43.9%), thrombocytopenia (27,000 cells/mm^3), elevated levels of liver enzymes (alanine transaminase 347 IU/L, aspartate transaminase 139 IU/L), creatinine (1.2 mg/dL), and urea (40 mg/dL). Laboratory testing ruled out malaria, leptospirosis, and dengue. About 24 hours after hospitalization, the patient experienced hypotension, progressive dyspnea, and acute respiratory distress. Thoracic radiographs revealed bilateral diffuse alveolar pulmonary infiltrates. Despite empirical treatment with antimicrobial drugs, mechanical ventilation, and inotropic therapy, the patient's clinical condition deteriorated and he died on day 6 after illness onset.

The patient, who had no history of travel, resided on a submerged region in the western floodplain of the Solimões-Amazon River, Amazonas, a state with low population density (6.2 persons/square mile), in a rural area of Careiro da Várzea Municipality (3°11′53″S, 59°52′18″W), where access is possible only by boat. He had a history of contact with rodents not only at home but also in the boat he used. A serum sample collected on day 6 after illness onset was evaluated for hantavirus by serologic and PCR testing. ELISA result was positive for IgM and IgG against recombinant nucleocapsid protein (N) of the Juquitiba virus (*6*). Viral genome was detected by reverse transcription PCR, and the complete genomic small segment sequence, designated LH60_11/Hu (GenBank accession no. KF584259), was determined (*7*). This sequence was compared with a reference panel of sequences that covered the diversity of most hantaviruses in South America and was subjected to phylogenetic analysis by MrBayes software version 3.1.2 (*8*). Nucleotide and amino acid sequence similarities between all taxa for the partial N gene were calculated by using MegAlign version 5.05 (DNASTAR, Inc.; Madison, WI, USA). The best-fit evolutionary model general time reversible + Γ + proportion invariant was determined by using MEGA version 5.2.2 (http://www.megasoftware.net), and the dataset compiled only 905 nt of the N gene to include sequences of Anajatuba and Rio Mearim viruses from Brazil for comparison.

Bayesian analysis indicated that strain LH60_11/Hu is closely associated with rodent-derived RIOMV-3/Olm strain (Itacoatiara, Amazonas State) and in a sister relationship with RIOMV-4/Olsp strain (Alto Paraíso, Rondônia State) from Brazil (Figure). Analysis of the partial sequence revealed 86.6%–95.4% of genetic identity with the strains recovered from rodents and 83.4% with the Maripa virus strain from humans. The sequence from the human patient in Peru was not available for comparison.

In July, the patient's house and environment were investigated; accumulation of garbage and other waste in homes that were still flooded was observed. We obtained and tested serum samples from 15 healthy residents (10 female, 5 male) with a recent history of acute fever; IgG against hantavirus was detected in samples from 3 women (17, 25, and 57 years of age).

This case report describes RIOMV as a highly pathogenic agent of HPS in Brazil. The location of the patient with this fulminant case of HPS, Careiro da Várzea, borders the Municipality of Itacoatiara, where RIOM-3–infected *O. microtis* rats and the first HPS case in Amazonas State, with no etiologic identification so far, have been reported (*3*,*9*). Careiro da Várzea is part of an area in which grain production is expanding, an activity that attracts rodents to human dwellings, especially those in lowland regions that are constantly flooded.

The close association between the sequences from the human and the *O. microtis* rat (>98% aa identity) suggests that the patient might have been infected as a consequence of close physical contact with an RIOMV-

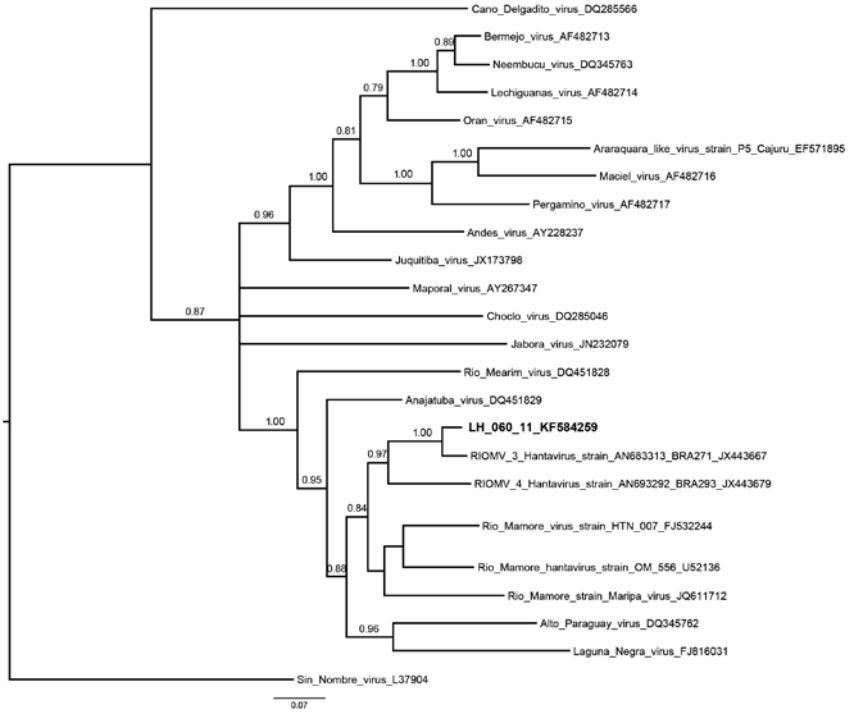

Figure. Phylogenetic relationships among hantaviruses were estimated by using the Bayesian Markov chain Monte Carlo method implemented in MrBayes version 3.1.2 (*8*). The relationships were based on the initial 905-nt fragment of the small segment. The numerical value ≥0.7 at the node indicates the posterior probability replicates that supported the interior branch. The branch labels include GenBank accession number and virus species or strain. Boldface indicates the reference sequence; scale bar indicates nucleotide substitutions per site. RIOMV, Rio Mamore virus.

infected *O. microtis* rat. The geographic distribution of these rats and, thus, the potential area at risk for transmission of RIOMV is vast, including 5 Brazilian states in the Amazon Basin and contiguous lowlands of Peru, Bolivia, and Paraguay (*10*).

This study confirms the notion that ROIMV is a highly pathogenic hantavirus. Recent recognition of RIOMV as a causative agent of HPS might be attributed to either increased awareness by local physicians or improved diagnosis of hantavirus infections. This finding emphasizes the need for extensive molecular investigation of undiagnosed infections because of the shared clinical features with other diseases endemic to this region (e.g., malaria and dengue).

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Reemergence of *Brucella melitensis* in Wildlife, France

To the Editor: Brucellosis is a worldwide zoonosis caused by *Brucella* spp. France has been free of bovine, ovine, and caprine brucellosis (caused by *B. abortus* or *B. melitensis*) since 2003 (*1*). In early 2012, an outbreak of bovine and human brucellosis caused by *B. melitensis* biovar 3 (*Bmel*3) occurred in a French Alp massif (mountainous region), where the last reported outbreak occurred in 1999 (online Technical Appendix Figure, http://wwwnc.cdc.gov.EID/article/20/9/13-1517-Techapp1.pdf) (*2*). This outbreak suggested the persistence or reemergence of *Brucella* spp. in livestock.

An extensive investigation was conducted that involved 40 animal herds with direct links to the outbreak. Six months later, blood samples from each adult animal in any herd (12,116 animals in 205 herds) that grazed during the summer of 2012 in the massif underwent serologic analysis. However, no other case was identified in this population (online Technical Appendix Table 1). Therefore, a potential wildlife source was investigated.

Wild ruminants in the study area were the following species: hunted red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), chamois (*Rupicapra rupicapra*), and protected Alpine ibex (*Capra ibex*). Although *B. abortus* and *B. suis* have been reported in numerous wildlife species (*3*), *B. melitensis* has rarely been isolated from wildlife, and only sporadic cases of infection have been reported in Europe, in chamois and Alpine ibex in the Alps (*4,5*) and in Iberian ibex (*Capra pyrenaica hispanica*) in the Pyrenees (*6*). These cases were considered to be caused by spillover from domestic ruminants, which suggests that these wild species are unable to sustain the infection (*3*).

We conducted our investigation during the fall–winter of 2012–2013 in the entire massif where the outbreak occurred. Blood, lung, spleen, and testes or uterus samples were obtained from all hunted animals. French Authorities authorized the killing of 12 seropositive or diseased Alpine ibex with clinical signs of brucellosis (i.e., arthritis or orchitis) among 30 captured animals.

All serum samples were tested according to requirements of the World Organisation for Animal Health for diagnosis of brucellosis in small ruminants by using by the Rose Bengal test (RBT) and the complement fixation test (CFT) (*7*), and by indirect ELISA (IDEXX, Montpellier, France) and competitive ELISA (cELISA; Ingenasa, Madrid, Spain). When blood samples were unsuitable for RBT or CFT or were missing, a lung extract was tested by only the 2 ELISAs. Culture was only performed on samples from seropositive animals (online Technical Appendix Table 1) (*8*). If bacteriologic results were negative, a *Brucella* genus–specific real-time PCR was also used (*9*).

A total of 129 hunted ruminants (55 chamois, 30 red deer, 44 roe deer) were tested. No clinical signs were observed, except for arthritis in the knee of 1 chamois. All ruminants were seronegative except for the chamois, which showed positive results in the RBT, CFT, and cELISA, and 1 red deer, which showed a weakly positive result in the cELISA, but negative results by culture and real-time PCR. *Bmel*3 was isolated from the chamois (online Appendix Table 1).

Among 289 Alpine ibex observed in the massif, 24 were killed (22 randomly sampled animals that showed 2 diagnostic lesions at necropsy [arthritis in the knee and mammary abscesses] and 2 diseased animals [arthritis in the knee and orchitis]), and samples from these animals were subjected to serologic analysis. Ten Alpine ibex (including the 2 diseased animals) showed positive results in the RBT, CFT, and both ELISAs, and 2 showed positive results only for both ELISAs. Thus, the prevalence of *B. melitensis* in randomly captured animals was 45% (10/22; 95% CI 24.6%–66.3%) (online Technical Appendix Table 1).

*Bmel*3 was isolated from 5 of 11 seropositive Alpine ibex (1 Alpine ibex was killed in an avalanche). Three seropositive but culture-negative ibex showed positive results by PCR (online Technical Appendix Table 2). Multilocus variable number tandem repeat analysis showed similarity among all strains isolated in this study and strains isolated from local domestic outbreaks >13 years ago (*10*).

Although persistence of *B. melitensis* in wild ruminants has not been reported, and these animals are considered an epidemiologic dead-end reservoir (*3*), the unexpected prevalence observed (≈50%) suggests that Alpine ibex could be the source of bovine brucellosis reemergence in the study area in France. Strict surveillance policies have prevented infection of domestic livestock with *B. melitensis* in the study area since 1999. However, cohabitation of domestic and wild ruminants on pastures during the summer is rare but possible. Clinical signs and lesions observed in chamois and Alpine ibex are consistent with those reported for chamois and Alpine ibex with brucellosis (*4,5*). However, positive cultures were obtained from conventional target organs (knee, testes, and lymph nodes) but also from urogenital fluids, which indicates the potential for excretion of the organism.

The fact that births occur during periods and in places where female Alpine ibex are not in close contact with other wild/domestic species (because of higher altitude or rocky peaks) could explain the low transmission rate of *B. melitensis* to these animals. It also suggests that the venereal route might contribute to transmission within Alpine ibex during the mating season in winter. This report demonstrates the need for maintaining an active/reactive surveillance system for livestock and wildlife in brucellosis-free regions.

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Clostridium tetani Osteitis without Tetanus

To the Editor: Posttraumatic osteoarticular infections caused by *Clostridium* spp. are rare, and their outcomes are often unfavorable because of the persistence of the bacteria in bone (*1*,*2*). In a recent series of 12 patients (*2*), only 1 case of posttraumatic osteoarticular infection was caused by *C. tetani* (fracture of the distal humerus with polymicrobial infection). However, no information was available about the production of tetanospasmin by the infecting strain.

To the best of our knowledge, the only case of *C. tetani* infection with a toxigenic strain but without tetanus or osteitis was a wound infection that quickly improved after administration of antitetanus vaccine, prophylactic immunoglobulins, flucloxacillin, and metronidazole (*3*). The absence of clinical signs of tetanus despite chronic *C. tetani* infection probably resulted from vaccine-induced immunity and the fact that the patient received a booster vaccination and prophylactic immunoglobulins as soon as *C. tetani* had been identified. Retrospective immunochromatic testing of the patient's serum seemed to confirm this hypothesis. We report a case of osteitis caused by *C. tetani* in which clinical signs of tetanus did not develop despite production of tetanospasmin by the infecting strain.

In August 2011, a 26-year-old man was admitted to Nord Hospital in Marseille, France, because of an open fracture of his left tibia and fibula, contaminated with soil. The patient had been vaccinated against tetanus in 1997 and worked in scraps recycling, He rapidly underwent osteosynthesis (locking plates). Despite receiving oral amoxicillin–clavulanate (1 g 2 times/day) for 7 days, he was readmitted 12 days later for fever and suppuration of the leg wound and underwent a second surgical debridement. A bone biopsy sample revealed *Enterococcus faecalis*, *Enterobacter cloacae*, and *C. tetani*. Identification of *C. tetani* was confirmed by 16S rRNA amplification and sequencing (99.8% identity to *C. tetani,* GenBank accession no. AE015927). The organism was susceptible to amoxicillin, rifampin, vancomycin, and metronidazole. Because antitetanus vaccine had not been administered at the time of his previous hospital admission, a dose of vaccine and prophylactic immunoglobulins were administered at this time. Treatment with intravenous imipenem (1 g 3 times/day) plus oral ciprofloxacin (500 mg 3 times/day) was initiated for 1 month, followed by oral amoxicillin–clavulanate (1 g 2 times/day) plus ciprofloxacin (500 mg 3 times/day) for 1 month and then oral amoxicillin (2 g 3 times/day) for 2 months.

In February 2012, because bone consolidation had not occurred, the patient underwent surgical revision to remove the locking plate, clean the wound, and insert an external fixator. Cultures of specimens collected during surgery were negative. Serologic qualitative immunochromatic test result was positive for *C. tetani*. The patient received intravenous vancomycin and imipenem (1 g 2 times/day each) for 1 month, followed by oral amoxicillin (3 g 2 times/day), rifampin (300 mg 3 times/day), and ciprofloxacin (500 mg 3 times/day) for 3 months.

In July 2012 (11 months after the accident), because of fistula persistence, the patient underwent ablation of a tibial sequestrum (Figure) and implantation of a temporary cement spacer containing gentamicin and vancomycin. The only bacterium isolated from a tibial biopsy sample was *C. tetani*.

The causative strain was referred to the Centre National de Référence des Bactéries Anaérobies et du Botulisme, Pasteur Institute, Paris, where presence of the *tetX* gene encoding the tetanus neurotoxin was confirmed. Oral treatment with clindamycin (2.4 g/day) for 4 months was prescribed. However, because of the unfavorable outcome despite multiple interventions and antimicrobial drug regimens, the left leg was amputated 17 months after the accident.

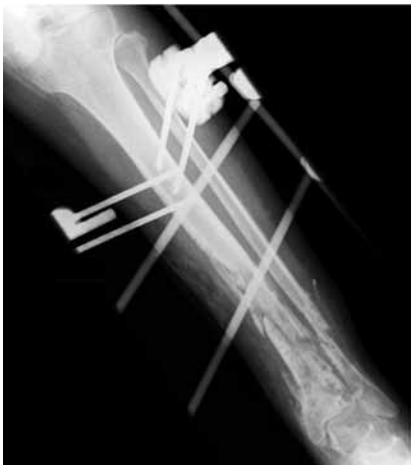

Figure. Radiograph of left leg of patient with *Clostridium tetani* infection, showing delayed bone consolidation 11 months after fracture.

The case reported here is remarkable because clinical tetanus did not develop despite the production of tetanospasmin by the infecting strain and because late relapse occurred despite adapted treatment. The persistence of infection might be explained by a questionable initial antimicrobial drug regimen but also by spore formation and/or poor diffusion of antimicrobial drugs, as suggested by the presence of necrotic tissues such as the bone sequestrum. However, surgical revision, notably the ablation of this defect, should have facilitated the recovery and decreased bacterial concentration. In the literature, 3 cases of relapsing *C. tetani* infections have been reported, but those patients had not received antitetanus vaccine and they did show signs of tetanus (*4*,*5*); 1 of these patients with mandible necrosis experienced relapse 8 months after discontinuation of metronidazole.

The pathogenesis of *C. tetani* has mainly been attributed to its toxin. Our report suggests that *C. tetani* can also cause focal infections, notably severe chronic osteitis after open fractures, especially because the anatoxin-based antitetanus vaccine does not prevent colonization and infection.

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Rate of Congenital Toxoplasmosis in Large Integrated Health Care Setting, California, USA, 1998–2012

To the Editor: Although congenital toxoplasmosis occurs throughout the United States, little information is available about the rates of diagnosed illness in most of the nation, including California. Infection usually occurs by ingestion of undercooked meat and unwashed fruits and vegetables or exposure to soil or water contaminated with cat feces. Congenital transmission can occur when a woman is infected with *Toxoplasma gondii* during, or just before, pregnancy. Approximately 91% of women of childbearing age in the United States are susceptible to *T. gondii* infection (*1*). The United States has a low prevalence of *T. gondii* infection compared with many areas of the world (*2*). Severe congenital toxoplasmosis can result in hydrocephalus, retinochoroiditis that affects vision, microcephalus, seizures, hepatosplenomegaly, icterus, psychomotor retardation, and other sequelae (*3*). Infants with congenital toxoplasmosis are most often asymptomatic at birth; however, when severe symptoms occur, they are usually recognized and the condition diagnosed by the time the child is 2 years of age (*3*).

Our goal was to determine the rate of clinically identified cases of congenital toxoplasmosis in children from birth to 2 years of age within the Northern California Kaiser Permanente Medical Care Program (KPNC) during a 15-year period. KPNC is a group health plan that provides care for >3.2 million residents of northern California. The KPNC membership represents ≈30% of the insured population in the region and is demographically similar to the residents of the counties served except that the very poor and very wealthy are underrepresented (*4*).

We studied live births and infants during 1998–2012, the most recent 15-year period for which records were available and considered complete. We identified potential cases from KPNC electronic medical record databases and confirmed them by reviewing electronic and paper records. The system documents outside services, identified by the corresponding diagnostic codes or laboratory test codes. Eligible case-patients were infants, defined as <24 months of age, at the time of meeting any potential case criterion. We identified all births in which ICD-9-CM diagnostic codes for the mother or the infant included the following: 130-130.9 (toxoplasmosis), 771.2a (a special KPNC subset code specifying toxoplasmosis), and those with the more general 771.2 (congenital infections specific to the perinatal period) code; for the latter, an external special test for toxoplasmosis was assessed). We also identified all infants for whom any toxoplasmosis laboratory test had been ordered that had ≥1 of 23 specific KPNC laboratory codes for related serologic and PCR tests. We considered clinically confirmed case-patients to be infants with positive *T. gondii* IgM and/or IgA tests at <6 months of age, persistent IgG at >12 months of age, PCR–positive results for *T. gondii*, or diagnosis and care of toxoplasmosis-related conditions. To calculate 95% confidence intervals for rates, we used the exact binomial method.

During the 15-year study period, there were 521,655 live births at KPNC facilities and 2,010 infants received ≥1 test for toxoplasmosis. Ten infants met the potential case criteria of diagnostic codes; no additional patients met any of the case criteria by age 2 years. After electronic and paper charts were reviewed, 2 cases of congenital toxoplasmosis were confirmed. One case was diagnosed in 2003, the other in 2011. Both case-patients were girls: 1 was of Hispanic ethnicity and the other was of

mixed Filipino-White heritage; both had IgG persistently detected beyond 12 months of age and were monitored clinically for retinochoroiditis. Their charts contained no information regarding maternal exposure or risk factors. During the 15-year period, the rate of diagnosed congenital toxoplasmosis was 3.8 (95% CI 1.5–9.2) per million live births. There were no infant deaths for which congenital toxoplasmosis was recorded as a cause. We were unable to study fetal deaths because the corresponding cause-of-death codes were not readily available.

Historically, the lowest prevalence of *T. gondii* infection has been recorded in the western United States (*5*). The rate of clinically apparent congenital toxoplasmosis in this study was lower than that found during the late 1980s through early 1990s in the New England Newborn Screening Program initially after birth (2 per 521,555 live births [3.8 per million] versus 5 per 635,000 live births [7.9 per million], respectively) (*6*). However, the prevalence of *T. gondii* infection has decreased in the United States since the 1990s (*1*).

Our study is subject to several limitations. Our approach would only detect clinically apparent cases, and the results should be considered a minimal estimate of congenital infection. Some cases may not have been recorded in the electronic system, but this omission is not likely for severe illness, repeated hospital or clinic visits, or outside consultation. The small number of cases makes the rate of diagnosed congenital toxoplasmosis somewhat imprecise; a few missed cases would increase the rate considerably. In addition, we were not able to evaluate fetal deaths; however, stillbirth is reportedly a rare complication of congenital toxoplasmosis (*7*). Although we found a low rate of diagnosed congenital toxoplasmosis in northern California, population-based studies to evaluate rates of the disease in other geographic areas would be beneficial.

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Invasive Infection Caused by Carbapenem-Resistant *Acinetobacter soli*, Japan

To the Editor: Infections caused by *Acinetobacter* spp., especially *A. baumannii*, have been increasingly documented in recent years. Carbapenems tend to be empirically prescribed as first-choice drugs for severe invasive infections caused by *Acinetobacter* spp. other than *A. baumannii* because these microbes are usually susceptible to carbapenems. However, infections with carbapenem-resistant *Acinetobacter* spp. have been increasingly reported during the past 15 years. In *A. baumannii*, carbapenems are usually inactivated by intrinsic oxacillinase (OXA)-51–like, acquired OXA-23–like, or OXA-58–like carbapenemases. Moreover, production of acquired metallo-β-lactamases (MBLs) of the Verona integron (VIM), imipenemase (IMP), or New Delhi (NDM) types has been detected among carbapenem-resistant *Acinetobacter* species, including *A. baumannii*, *A. junii*, *A. bereziniae*, *A. nosocomialis*, and *A. pittii* (*1*). We report a case of infection with carbapenem-resistant *A. soli* producing another

MBL type, Tripoli MBL 2 (TMB-2), in a man in Japan.

A man in his 60s who had mesenteric injury, pelvic fracture, and intestinal perforation from a traffic accident was admitted to Okazaki City Hospital in Aichi, Japan, on May 3, 2013. After surgery, cefmetazole was prescribed on May 6 (1 g 2×/d for 7 d). On May 12, symptoms of infection developed in the patient, and 2 sets of blood samples were drawn from different vessels for bacterial culture. The following day, cefmetazole was discontinued, and ciprofloxacin (0.3 g 2×/d) and piperacillin/tazobactam (4.5 g 2×/d) were started. *Acinetobacter* isolates resistant to piperacillin/tazobactam and carbapenems were then recovered from the blood samples, so piperacillin/tazobactam was discontinued on May 14. After that, ceftriaxone (2 g 2×/d) and gentamicin (0.04 g 2×/d) were successively prescribed, in addition to ciprofloxacin; the symptoms of infection improved, and all antimicrobial drugs were discontinued by May 26. Additional blood cultures performed on May 17, 21, and 28 yielded negative results for *Acinetobacter* spp. However, the patient's condition worsened on June 5. Meropenem (0.5 g 4×/d) was then given, but the patient died of multiorgan failure on June 7.

The bacterial isolates from the initial blood cultures were identified as *A. soli* by nucleotide sequencing of the *rpoB* and *gyrB* genes and assigned identification no. HK001. MICs of β-lactams, measured by the agar dilution method in accordance with the guideline M07-A9 of the Clinical and Laboratory Standards Institute (http://clsi.org), were as follows: sulbactam/ampicillin, >128 mg/L; piperacillin, >128 mg/L; tazobactam/piperacillin, >128 mg/L; cefotaxime, >64 mg/L; ceftazidime, >64 mg/L; aztreonam, 64 mg/L; cefmetazole, >128 mg/L; imipenem, 8 mg/L; meropenem, 32 mg/L; and doripenem, 32 mg/L. However, MICs of gentamicin, amikacin, levofloxacin, ciprofloxacin, colistin, and tigecycline were below the breakpoints of susceptibility as listed in Clinical and Laboratory Standards Institute document M100-S23. Carbapenem resistance was not transferred from *A. soli* HK001 to *Escherichia coli* strain CSH-2 (*metB* F^- NA^r Rif^s) by conjugation. A double-disk synergy test was initially performed by using sodium mercaptoacetic acid (SMA) (*2*) and ceftazidime and meropenem disks (Eiken Chemical Co., Ltd, Tokyo, Japan), and results suggested MBL production. The modified Hodge test was then performed, and ertapenem and meropenem disks gave clear positive results (data not shown). PCR was performed to detect bla_{OXA-23}–like, $bla_{OXA-24/40}$–like, bla_{OXA-51}–like, bla_{OXA-58}–like, bla_{IMP-1}, bla_{IMP-2}, bla_{VIM-1}, bla_{VIM-2}, bla_{NDM-1}, bla_{SMB-1}, and bla_{TMB-1} genes. Nucleotide sequence analyses showed that the *A. soli* isolate harbored bla_{TMB-2} and bla_{OXA-58}. The modified SMA-disk method (*3*) was reevaluated to determine whether it could successfully detect TMB-2 production in *A. soli* HK001. Apparent positive results were obtained when disks containing imipenem, meropenem, or ertapenem were used, particularly when the edge-to-edge distance between 2 disks containing SMA and a carbapenem, respectively, was kept at 5 mm (Figure, top row). However, when the distance between the ertapenem and SMA disks was ≥10 mm, MBL production was more difficult to detect (Figure, lower 2 rows). This finding may be the result of co-production of OXA-58 by the isolate.

More than 30 *Acinetobacter* species had been registered by January 2012 (*4*); *A. soli* was initially isolated from the soil of a mountain forest in South Korea in 2007 (*5*) and has been recovered from blood cultures of 5 neonates in Brazil (*6*). Carbapenem-resistant *A. soli* co-harboring bla_{IMP-1} and bla_{OXA-58}–like genes was identified in April 2011 in Japan and is frequently recovered from bacteremia patients (*7*). TMB-1 was reported in 2012 in an *Achromobacter xylosoxidans* isolate from a hospital in Tripoli, Libya (*8*); TMB-2 was later reported in Japan

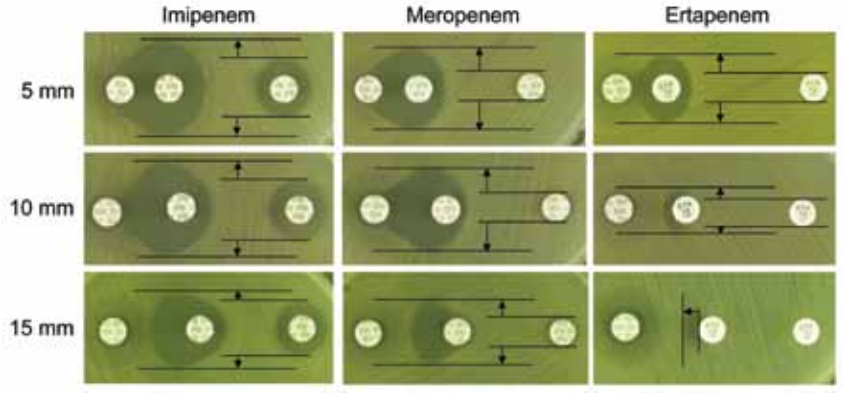

Figure. Results of double-disk synergy testing of the *Acinetobacter soli* isolate HK001 identified in a man in Japan. Testing was performed by using disks containing sodium mercaptoacetic acid (SMA) and the carbapenems imipenem, meropenem, and ertapenem. Apparent expansion of growth inhibition zone around a carbapenem disk placed near a SMA disk compared with that around a disk of carbapenem alone is seen on Mueller-Hinton agar if the isolate produces metallo-β-lactamases (*2*,*3*). When the edge-to-edge distance between 2 disks containing a carbapenem and SMA, respectively, was kept at 5 mm, expansion of the growth inhibition zone became clearer than for those kept at a distance of 10 mm and 15 mm, regardless of carbapenems used. Vertical expansion of growth inhibition zones by the effect of SMA is indicated by arrows; ertapenem gave the clearest result when the disk distance was kept at 5 mm (top right panel), even though *A. soli* HK001 co-produces oxacillinase 58–like carbapenemase, which is hardly inhibited by SMA.

(*9*). The TMB-2–producing *A. soli* strain that we isolated came from a blood culture, indicating that *A. soli* is a potential cause of bloodstream infections or bacteremia. *A. soli* has also been detected in lice and keds of domestic animals (*10*), indicating that *A. soli* may inhabit natural environments and that injuries and bites by arthropods might present a risk for invasive infections. Isolates of *Acinetobacter* species, particularly those recovered from blood culture, should be identified to species type to enable further evaluation of the clinical significance of carbapenem-resistant *A. soli* strains.

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Spread of Vaccinia Virus to Cattle Herds, Argentina, 2011

To the Editor: Since 1999, several zoonotic outbreaks of vaccinia virus (VACV) infection have been reported in cattle and humans in rural areas of Brazil. The infections have caused exanthematous lesions on cows and persons who milk them, and thus are detrimental to the milk industry and public health services (*1*,*2*). In Brazil during the last decade, VACV outbreaks have been detected from the north to the extreme south of the country (*1*–*4*). Because Brazil shares extensive boundaries with other South American countries, humans and cattle on dairy and beef-producing farms in those countries may be at risk of exposure to VACV. To determine if VACV has spread from Brazil to Argentina, we investigated the presence of VACV in serum samples from cattle in Argentina.

During 2011, we obtained serum samples from 100 animals (50 dairy and 50 beef cattle) on farms in Córdoba, Corrientes, Entre Ríos, and Santa Fe Provinces in Argentina (online Technical Appendix, panel A, http://wwwnc.cdc.gov/EID/article/20/9/14-0154-Techapp1.pdf). No VACV cases had been reported in humans or cattle in these provinces. However, Corrientes Province borders the Brazilian state of Rio Grande do Sul, where VACVs (Pelotas 1 and Pelotas 2 viruses) were isolated during an outbreak affecting horses in 2008 (*2*).

To determine the presence of neutralizing antibodies in the serum samples, we used an orthopoxvirus 70% plaque-reduction neutralization test as described (*4*). On the basis of previous studies that detected viral DNA in serum samples (*4*–*6*), we used real-time PCR to amplify the highly conserved orthopoxvirus vaccinia growth factor (*vgf*) gene DNA (P.A. Alves, unpub. methods).

To amplify the hemagglutinin (HA) gene DNA from the serum samples, we used real-time PCR with primers as described by de Souza Trindade et al. 2008 (*7*). The HA PCR products were directly sequenced in both orientations by using specific primers and capillary electrophoresis (Genetic Analyzer 3130; Applied Biosystems, Grand Island, NY, USA). We used ClustalW (http://www.clustal.org) and MEGA4 software (http://megasoftware.net/) to align nucleotide sequences and construct a phylogenetic tree (neighbor-joining method, 1,000 bootstraps) from the obtained HA fragment.

Of the 50 dairy cattle samples, 4 (8.0%) had neutralizing antibodies against orthopoxvirus; of these, 3 (75.0%) had titers of 100 neutralizing units (NU)/mL, and 1 (25.0%) had a titer of 400 NU/mL. Of the 50 beef cattle, 8 (16.0%) had antibodies to orthopoxvirus, 1 (12.5%) of which had a titer of 800 NU/mL. Most of the positive samples were from cattle in Corrientes and Entre Ríos Provinces (Table).

Of the 100 serum samples, 5 (3 from beef and 2 from dairy cattle) were positive for *vgf* by real-time PCR. HA DNA was amplified from 2 of the 3 *vgf* PCR–positive beef cattle samples; plaque-reduction neutralization test results were also positive for the 2 samples (Table).

Alignment of the HA fragment nucleotide sequence of the isolates from Argentina showed that the sequence was highly similar to that of the homologous gene of VACV isolates from Brazil. Furthermore, the sequences showed a signature deletion that is also present in the sequences of VACV isolates from Brazil. Compared with sequences for other VACV isolates, those from Argentina had 2 polymorphisms (online Technical Appendix, panel C). The HA sequences from the isolates from Argentina demonstrated 100% identity among themselves and exhibited higher identity with group 1 (98.2% identity) versus group 2 (93.6% identity) isolates from Brazil (online Technical Appendix, panel D). In the phylogenetic tree based on the HA nucleotide sequences (online Technical Appendix, panel B), the VACVs from Argentina clustered with several group 1 VACVs detected during outbreaks in Brazil.

Although no outbreaks of exanthematous VACV infection have been described in cattle or humans in Argentina, we detected neutralizing antibodies against orthopoxvirus and detected VACV DNA in serum samples from cattle in the country. Most of the seropositive samples were from cattle in Entre Ríos Province, which shares a border with Uruguay, and Corrientes Province, which shares a border with Rio Grande do Sul State in Brazil, where Pelotas VACVs have been isolated (*2*).

We believe that the seropositive cattle in this study may have been exposed to VACV, the only orthopoxvirus known to be circulating in South America (*1–4*,*8–10*). Despite veterinary surveillance efforts of border control organizations, VACV control may be hampered by the circulation of infected rural workers and the misdiagnosis of VACV infection; misdiagnoses occur because VACV lesions resemble those of other exanthematous diseases. Moreover, peridomestic rodents have been hypothesized to act as VACV hosts, and could facilitate the spread of VACV in border areas (*10*). In addition, we could not rule out the circulation of autochthonous VACV in Argentina, but this is a less likely explanation. Our findings suggest that cattle herds in areas of Argentina near the border with Brazil may be exposed to VACV from Brazil and, thus, may be at risk for VACV infection. Further research is needed to determine the risk factors for VACV infection and to assess the circulation of VACV in South America

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Table. Diagnosis of *Orthopoxvirus* infection in beef and dairy cattle during a study of the spread of vaccinia virus to cattle herds, Argentina, 2011*

Province	Cattle type	No. farms sampled	No. serum samples tested	No. positive samples, by level of NU/mL against orthopoxvirus†					No. (%) positive by real-time PCR	
				100	200	400	800	Total (%)	*vgf*	HA
Córdoba	Dairy	1	25	0	0	1	0	1 (4.0)	2 (8.0)	0
Santa Fe	Dairy	1	25	3	0	0	0	3 (12.0)	0	0
Corrientes	Beef	>2‡	8	0	1	1	0	2 (25.0)	1 (12,5)§	1 (12,5)§
Entre Ríos	Beef	5	42	2	2	1	1	6 (14.3)	2 (4,8)	1 (2,4)§
Total	Dairy and beef	>9	100	5	3	3	1	12 (12.0)	5 (5.0)	2 (2.0)

*HA, hemagglutinin gene DNA; NU, serum dilution at which 70% plaque reduction per mL occurs; *vgf*, orthopoxvirus vaccinia growth factor gene DNA.
†Determined by plaque-reduction neutralization test.
‡Samples were obtained from several farms in Corrientes Province.
§Animal was also positive by plaque-reduction neutralization test.

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Cerebellitis Associated with Influenza A(H1N1)pdm09, United States, 2013

To the Editor: Central nervous system (CNS) manifestations of influenza are uncommon, especially in adults (*1*,*2*), and influenza-associated cerebellitis is exceedingly rare: 8 cases have been reported (*3*–*7*; online Technical Appendix). We describe a case of cerebellitis caused by influenza A(H1N1)pdm09 in an adult woman.

The 37-year-old female patient who sought medical care in Florida, United States, on January 5, 2013, described a 4-day history of intermittent fever of 38.5°C, generalized fatigue, diffuse headache, mild nonproductive cough, 3 episodes of vomiting, and decreased oral intake. On January 4, she experienced acute onset of ataxia and dysarthric speech with slurred pronunciation. She reported no contact with sick persons, recent travel, or exposure to pets or birds. She had a medical history of asthma since childhood, controlled by using montelukast tablets and inhaled steroids. The patient denied having ever received an influenza vaccination.

The patient appeared ill; her oral temperature was elevated at 38.3°C, but other vital signs were within normal limits (blood pressure 109/70 mm Hg; pulse rate 88 beats/minute; respiratory rate 15 breaths per minute; and oxygen saturation 98% at room air). Mucosal membranes appeared normal. No neck stiffness or palpable lymph nodes were noted. Results of heart examination were normal. Lungs were clear to auscultation, and the abdomen was soft, indicating no hepatosplenomegaly or palpable masses. No rash was seen. The neurologic examination revealed normal mental status but moderate ataxic dysarthria. Her cranial nerves were intact, and motor strength was 5/5 throughout. Results of a sensory

examination were normal, and patient's reflexes were largely intact; Babinski sign was absent. However, her coordination was bilaterally impaired in finger-to-nose testing, and her gait was notably broad-based and ataxic.

Laboratory test results showed a leukocyte count of 13.72×10^3 cells/mm^3; percentages of neutrophils and lymphocytes were within reference limits at 59% and 25%, respectively. Levels of electrolytes, liver enzymes, and creatine phosphokinase were within reference ranges. C-reactive protein level was below the limit of detection. A nonenhanced brain computed tomographic scan revealed no pertinent findings. Brain magnetic resonance imaging (MRI) revealed enlarged bilateral cerebellar hemispheres with evidence of hypointensity of the affected thoracic vertebral segment on T1 image and hyperintensity on the T2 image (Figure). A lumbar puncture drained clear and colorless cerebrospinal fluid (CSF) with an opening pressure of 15 cm of H_2O. CSF analysis was pertinent, showing presence of erythrocytes (7.5/mm^3) and elevated number of leukocytes (330/mm^3 [13% neutrophils and 62% lymphocytes]). Glucose and protein levels in CSF were 61 mg/dL and 41 mg/dL, respectively. Blood and urine cultures were negative for pathogens. A chest radiograph did not show infiltrates. Bacterial culture, acid-fast smear, and culture of CSF were all negative. Blood and CSF tests for HIV syphilis, respectively, were nonreactive. However, reverse transcription PCR (RT-PCR) for influenza A(H1N1)pdm09 virus RNA was positive in the nasopharyngeal swab sample and CSF specimens, at a titer of 4.5×10^5 and 671 RNA copies/mL, respectively. RT-PCR of CSF was negative for viruses, including herpes simplex, Ebstein-Barr, cytomegalovirus, West Nile, and herpes zoster.

The patient was given oseltamivir, 75 mg orally twice daily for 5 days. She experienced a progressive improvement of ataxia and dysarthria during her hospital stay and was discharged after 1 week. At a follow-up visit 2 months later, the patient had remained healthy and neurologically stable.

Cerebellitis, or acute cerebellar ataxia, is an inflammatory process of the cerebellar white matter that occasionally is manifested after systemic viral or bacterial infections (*8*). The following pathogens are known to cause acute cerebellitis: viruses varicella-zoster, herpes simplex, Epstein-Barr, rotavirus, echovirus, coxsackie, mumps, measles, and rubella; and bacteria *Borrelia burgdorferi*, *Coxiella burnetii*, *Salmonella typhi*, and *Bordetella pertussis* (*8*). Although the condition is presumed to be more common in children, adult cases of cerebellitis have been well described (*8*).

Before this case, influenza cerebellitis had been diagnosed in 8 cases as of 2011 (*3*–*7*) (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/20/9/14-0160-Techapp1.pdf). Two cases were reported in adult women and the remaining were in children. Four had a probable diagnosis of influenza cerebellitis, although positive viral culture or RT-PCR was lacking (*4*). Seven case-patients had influenza-like illness preceding the neurologic symptoms (*3*–*6*). One case-patient showed evidence of pneumonia, and described the interval from respiratory illness onset to developing of cerebellar signs (*6*) Clinical sequelae, displayed in most case-patients affected by influenza cerebellitis (*3*,*4*,*6*,*7*), varied from complete recovery to development of serious complications such as hydrocephalus (*5*).

The pathogenic mechanism of influenza virus infection on the CNS can be either a direct invasion of the virus that causes acute illness or, more typically, a delayed autoimmune demyelinating postviral encephalopathy (*9*,*10*). Amplification of viral DNA in CSF is rare in most influenza-related CNS infections (*10*). In this case, the positive RT-PCR results for influenza A and the pertinent brain MRI findings, as well as the concurrent influenza prodromal symptoms, suggest that acute influenza cerebelllitis, rather than a postinfluenza encephalopathy, caused the associated neurologic findings.

The limitation of this report includes the lack of sequence data comparing the patient's viral RNA from the CSF and the nasopharynx and the absence of sequential sampling during the course of her illness. In conclusion, influenza virus, though rare, should remain a consideration in patients who have acute cerebellitis during influenza season.

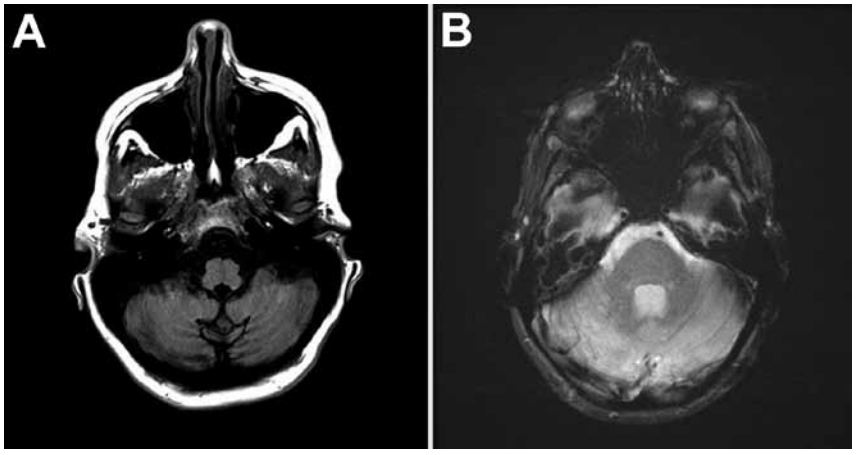

Figure. A) Magnetic resonance images of the brain of a woman with cerebellitis associated with influenza A(H1N1)pdm09, United States, 2013. T1-weighted axial MRI brain sequence showing hypo-intensity of bilateral cerebellar hemispheres. B) T2-weighted axial MRI brain sequence showing hyperintensity of bilateral cerebellar hemispheres.

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Potential Human Adaptation Mutation of Influenza A(H5N1) Virus, Canada

To the Editor: In December 2013, influenza associated with pandemic influenza A H5N1 was reported in Canada in a patient who had traveled to China; the patient died in January 2014. This case leaves unanswered questions.

In the absence of direct poultry contact by the patient, the possible route of transmission and infection, often influenced by receptor-binding properties of the virus, requires special attention. The full genome and phylogenetic analysis by Pabbaraju et al. (*1*) provides a summary of what can typically be deduced from the sequence. The authors also mention 2 novel mutations, R189K and G221R, in the hemagglutinin (HA) protein (R193K and G225R in H3 numbering, used hereafter). When mapped to the H5 HA protein structure by using FluSurver in GISAID (http://www.gisaid.org, http://flusurver.bii.a-star.edu.sg), both mutations are found in the immediate receptor-binding pocket, and G225R has been known to change specificity of an H3N2 virus toward human erythrocytes (*2*). The same position is also known for receptor recognition changes in the 2009 pandemic H1N1 virus (mutations D222G, D225G, or D239G in different numberings). Besides A/Alberta/01/2014 (clade 2.3.2.1c), the mutation G225R has been found in 3 other H5N1 sequences: A/duck/Hunan/15/2004 (clade 2.3.3), A/chicken/Xinjiang/53/2005, and A/chicken/Xinjiang/27/2006 (both clade 7, all lineage assignments made with LABEL, http://label.phiresearchlab.org/). Although few G225R mutations were found, they were all found in avian hosts, indicating that the mutation can occur sporadically and avian-like receptor-binding properties may not be fully abolished by G225R.

In the absence of glycan-binding data or crystal structures, which take longer to deduce, computational structural modeling is an efficient and safe alternative for fast preliminary assessment of these mutations in their natural structural context of H5N1 binding pockets. We have shown (*3*) that a method using the classical AMBER03 molecular mechanics force field (*4*) with an implicit solvation model in combination with short molecular dynamics simulations in YASARA (*5*) can reproduce relative preference for human-like α2,6-linked versus avian-like α2,3-linked sialic acid receptors. The interaction energies of all atoms in a system are described and combined with distance-dependent functions for different interaction types, including bonds, various angles, van der Waals, electrostatics, and solvent, which leads to consideration of the concerted effects of all residues in the binding pocket. By using this energy function, short molecular dynamics simulations enable all atoms to move for specified intervals within the constraints of their interactions. These simulations are used to minimize and finally predict the energies of the wild-type and mutant HA proteins for their ligand-bound and unbound states considering their respective ligands (see Methods section of [*3*] for details).

In this study, we further tested the computational structural model on mutations with known effect on receptor-binding properties (*2*,*3*,*6*–*9*) in H5N1 context based on recently resolved crystal structures and the respective ligand complexes (*9*). We limited this selection to mutations in the immediate vicinity of the crystallized receptor analog because the method should be most accurate for this scenario. The results showed that the binding preference of known mutations could be predicted at least qualitatively. Next, we tested the additional mutations found in A/Alberta/01/2014(H5N1). Our results (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/209/12-1200-Techapp1.pdf) suggest that G225R could incur a relative predicted increase in binding to the human-like receptors. Although the quantitative accuracy of computational methods in this regard is limited, the predicted numerical value suggests a possible similar extent of the effect to that of the well-known Q226L utation. It should also be noted that the predicted increase in binding to human-like receptors does not necessarily imply a concomitant loss of avian receptor binding. The role of R193K is less clear with a slight predicted tendency of favoring avian-like receptors. These preliminary findings highlight the necessity of verifying not only the receptor-binding properties of this virus through experimentation, but given the predicted increased preference for human receptors, also verifying potential roles in altered mammalian transmissibility.

These receptor-binding pocket mutations of the virus were not seen in the most closely related Asian H5N1 sequences of clade 2.3.2.1c (*1*), and no human contacts were known to be affected. From the epidemiologic perspective of this isolated human case, it is possible that this variant arose in the patient after initial infection and contributed to prolonged and severe infection and to the more unusual spread to brain tissue. If more avian strains with G225R mutations are found, the example of Q226L in H7N9 indicates that relative receptor-binding changes alone do not necessarily imply immediate mammalian transmissibility (*10*). It should also be noted that G225R was not among the mutations identified by recent controversial mammalian adaptation studies, (*7*,*8*) indicating that there may be more H5N1 host specificity markers than have been identified. Consequently, the functional roles of G225R in avian influenza should be further analyzed by conducting secure experiments and, pending verification, checking closely for its potential as an avian influenza host specificity marker.

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Genetic Changes of Reemerged Influenza A(H7N9) Viruses, China

To the Editor: From March 30, 2013, through April 8, 2014, a total of 401 human infections with novel avian influenza A (H7N9) virus were reported in China (*1*). In the initial wave from February through May 2013, cases were laboratory confirmed for 133 patients (45 died), mainly in eastern China. From June through early October 2013, only 2 laboratory-confirmed cases were reported in China. One of these, identified on August 10, 2013, was the first case of influenza A(H7N9) virus infection in Guangdong Province (strain A/Guangdong/HZ-01/2013). However, a second wave of influenza A(H7N9) virus infection began on October 14, 2013 (*2*). As of April 8, 2014, a total of 266 laboratory-confirmed cases had been reported, mainly in Zhejiang Province in eastern China (92 cases, 37 deaths) and Guangdong Province in southern China (99 cases, 30 deaths).

Previous sequencing studies suggested that 6 of the 8 influenza A(H7N9) virus RNA segments were acquired from influenza A(H9N2) virus. This acquisition process involved at least 2 steps of sequential reassortment; the most recent event most likely occurred in the Yangtze River Delta area of eastern China (*3*–*5*). To date, nearly all analyses have been performed by using sequences obtained from viruses isolated during the first wave of infection; changes associated with viruses isolated during the second wave are largely unknown (*6*). We therefore conducted phylogenetic analyses of whole-genome sequence data for 15 influenza A(H7N9) viruses isolated from human patients in Guangdong from November 4, 2013, through January 15, 2014.

We estimated maximum-likelihood trees for all 8 RNA segments by using MEGA version 5.2 and the general time-reversible model (*7*). RNA segments encoding the hemagglutinin, neuraminidase, and matrix genes of A/Guangdong/H7N9 viruses isolated after November 2013 were genetically similar to those of A/Guangdong/HZ-01/2013 and H7N9 strains from the first wave of influenza (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/9/14-0250-Techapp1.pdf). An additional 4 RNA segments (nonstructural protein [NS], nucleocapsid protein [NP], polymerase basic proteins [PB] 1 and 2) of A/Guangdong/H7N9 influenza viruses isolated after November 2013 were clustered with A/Guangdong/HZ-01/2013 virus and were divergent from all currently sequenced subtype H7N9 viruses from the first wave in eastern China. The only exception was the NP segment of A/Guangdong/SZ-026/2014, which was found segregated into a separate cluster with subtype H9N2 viruses from Shandong Province. Moreover, analyses showed that RNA segments encoding NS, NP, PB1, and PB2 of A/Guangdong/H7N9 isolated after November 2013 were most similar to the same segments from influenza A(H9N2) viruses that had recently circulated in Guangdong (online Technical Appendix Figure, panels D–G). That is, NS, NP, PB1, and PB2 showed greater similarity to local subtype H9N2 viruses from Guangdong than to subtype H7N9 viruses from the first wave of influenza.

Notably, 2 separate clusters were observed for the phylogenetic tree of the RNA segment encoding the polymerase acidic gene (online Technical Appendix Figure, panel H). A/Guangdong/HZ-01/2013-like viruses clustered with subtype H7N9 viruses from the first wave of influenza. However, A/Guangdong/DG-02/2013-like viruses were clustered with subtype H9N2 influenza viruses circulating in Guangdong, suggesting that recent reassortment with circulating subtype H9N2 viruses occurred after the first case of infection with influenza A(H7N9) virus reported in Guangdong (online Technical Appendix Figure, panel H).

This study provides evidence that influenza A(H7N9) viruses isolated during the second wave of influenza in Guangdong differ genetically (in 5 of the 8 RNA segments) from that of influenza A(H7N9) viruses isolated during the first wave. High similarity of these 5 segments with those of locally circulating subtype H9N2 viruses suggests that rapid and continued reassortment with circulating subtype H9N2 viruses occurred during the second wave of the influenza A(H7N9) virus epidemic. Because reassortment and genetic changes can contribute to host fitness and infection capacity of reemerged influenza A(H7N9) viruses, studies of pathogenicity and transmission, to reveal the exact role of each genetic alteration, are needed.

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Carbapenem-Resistant *Enterobacter cloacae* Isolates Producing KPC-3, North Dakota, USA

To the Editor: Carbapenem-resistant *Enterobacteriaceae* (CRE) continue to emerge as a serious public health threat throughout the world (*1*). CRE infections in the United States are often mediated by acquisition of *Klebsiella pneumoniae* carbapenemase (KPC) expressed by *Klebsiella* spp., although KPC is also found in other genera (*2*). The spread of KPC-producing, gram-negative bacteria in hospitals has been linked to severity of illness, co-existing medical conditions, exposure to antimicrobial drugs, and need for chronic care (*3*).

After reporting of CRE ifections to the North Dakota Department of Health became mandatory in 2011, a total of 20 CRE cases were noted in 12 of 53 counties (2.9 cases/100,000 population [*4*]). Most cases involved infection with *Enterobacter cloacae* and occurred in Cass County, where the state's largest city, Fargo, is located. We describe an outbreak of clonal carbapenem-resistant *E. cloacae* in a health care system in Fargo.

Sanford Health is a 583-bed, acute-care facility, representing ≈70% of acute-care beds in Fargo. The hospital handles ≥27,000 admissions/year and serves as a referral center for a large area of the state, and the only long-term acute-care (LTAC) facility in the eastern half of the state operates on its campus. During December 2011–December 2012, all isolates of *Enterobacteriaceae* with reduced susceptibility to ertapenem (MIC ≥1 μg/mL) identified at the hospital's clinical microbiology laboratory were screened for carbapenemase production by using the modified Hodge test (mHT), according to Clinical and Laboratory Standards Institute recommendations (*5*). Identification and susceptibility testing were done with the MicroScan system (Siemens Healthcare Diagnostics, Tarrytown, NY, USA); MICs of carbapenems were confirmed with Etest (bioMérieux, Durham, NC, USA). Three carbapenem-resistant *E. cloacae* isolates from documented cases of CRE infection at the hospital during 2010 were analyzed for comparison.

To characterize carbapenem-resistant and mHT-positive isolates, we used PCR to amplify and sequence the carbapenemase genes bla_{IMP}, bla_{NDM}, bla_{VIM}, and bla_{KPC} by using established methods (*6*). The upstream sequence of bla_{KPC}-positive strains was analyzed to determine the isoform of the transposon Tn*4401* that harbored bla_{KPC} (*7*). We investigated genetic similarity among isolates by repetitive sequence-based PCR; isolates with >95% similarity were considered clonal (*6*). We also sequenced the highly conserved *hsp60* gene (*8*) and attempted conjugative transfer of the bla_{KPC} gene by growing KPC-producing *E. cloacae* along with sodium azide–resistant *Escherichia coli* J-53. As part of the study, we examined records of patients from whom carbapenem-resistant *E. cloacae* was isolated. The study was approved by the Institutional Review Board at Sanford Health.

During December 2011–December 2012, a total of 19 single-patient *E. cloacae* isolates and 1 *E. aerogenes* isolate had positive mHT results. bla_{KPC} was detected in 17 of the 19 *E. cloacae* isolates and in the 3 carbapenem-resistant *E. cloacae* isolates from 2010. For all 20 of those isolates, sequencing revealed bla_{KPC-3} in association with isoform d of the transposon Tn*4401*, and all isolates were clonally related (Figure). All 20 isolates also had an identical *hsp60* sequence belonging to cluster VI in the Hoffman and Roggenkamp scheme (*8*). Conjugation of a bla_{KPC}-containing plasmid into *E. coli* J-53 was successful for 1 strain.

All 20 of the patients from whom KPC-producing CRE isolates were obtained (17 from this study, 3 from 2010) had been hospitalized at Sanford Health during the 3 months before CRE isolation; 13 (65%) were admitted to intensive care. In addition, 13 (65%) patients had been admitted to the LTAC during the year before CRE isolation. Co-colonization with multidrug-resistant bacteria was documented in 16 (80%) patients, including extended-spectrum β-lactamase–producing and carbapenem-resistant organisms in 4 and 2 patients, respectively. Seven (35%) patients died; 3 (15%) deaths were attributed to CRE infection. One of the patients was a neonate 30 days of age.

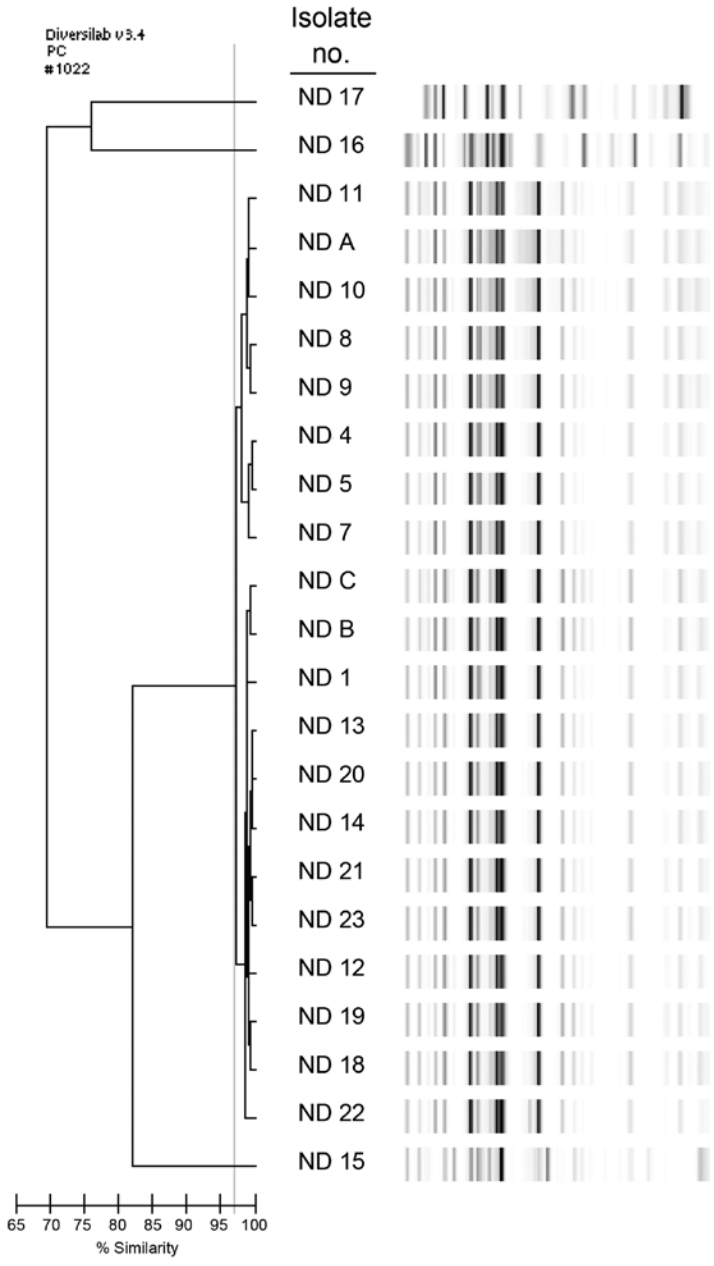

Figure. Genetic typing of carbapenem-resistant *Enterobacter cloacae* identified from patients at Sanford Health in Fargo, North Dakota, USA. Repetitive sequence–based PCR was used. The dendrogram at left displays the percentage similarity among band patterns shown at right. Isolate numbers ND 1, ND 4–5, ND 7–14, and ND 18–23 indicate *Klebsiella pneumoniae* carbapenemase (KPC) 3–producing *E. cloacae* isolates isolated during December 2011–December 2012; ND A–C indicate KPC-3–producing *E. cloacae* isolated during 2010. All KPC-3–producing *E. cloacae* isolates share >97% similarity, indicating a clonal strain. ND 15 and 16 are *E. cloacae*, and ND 17 is *E. aerogenes*, genetically distinct and without carbapenemases.

The finding of KPC-3–producing *E. cloacae* in North Dakota contrasts with the predominant epidemiology of CRE across the United States. Most CRE cases nationwide are caused by KPC-producing *K. pneumoniae* (*2*). KPC-type β-lactamases were previously identified in diverse strains of *Enterobacter* spp. from an urban health care system in Detroit, accounting for ≈15% of CRE (*9*). In contrast, our genetic analysis reveals a uniform genetic background among KPC-producing *E. cloacae*, which suggests horizontal dissemination of an outbreak strain.

Because active surveillance programs do not exist at our facility, this study probably underestimates the extent of CRE spread. We found that patients with KPC-producing *E. cloacae* in this sample were exposed to an LTAC and concomitantly were colonized or infected with other multidrug-resistant organisms (*9*). Although the spatio-temporal origin of the outbreak (acute care vs. LTAC) remains undefined, these findings likely reflect longer exposure to the continuum of care and higher rates of co-existing conditions within the LTAC population. This outbreak of KPC-producing *E. cloacae* infections in a health care system in North Dakota highlights the infection control challenges of long-term care facilities and the potential role they play in CRE dissemination.

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Urethritis Caused by Novel *Neisseria meningitidis* Serogroup W in Man Who Has Sex with Men, Japan

To the Editor: We report a case of urethritis caused by a novel multilocus sequence type (ST), 10651, of the ST11/electrophoretic type (ET)–37 complex *Neisseria meningitidis* serotype W. The patient was a man who has sex with men. We also report on the patient's male partner, who was colonized with the same bacteria.

In March 2013, a 33-year-old Japanese man sought medical care at Shirakaba Clinic (Tokyo) after experiencing a urethral discharge for 4 days. The man was HIV positive (CD4 count 649 cells/mL) but was not receiving antiretroviral therapy. Physical examination showed a mucous urethral discharge. Gram staining of a sample revealed many gram-negative diplococci phagocytosed by polymorphonuclear leukocytes. Eleven days before seeking care, the patient had oral and anal intercourse with his male partner. A diagnosis of suspected urethritis caused by *Neisseria gonorrhoeae* was made, and a sample of the urethral discharge was sent for culture and testing (Strand Displacement Amplification) for *N. gonorrhoeae* and *Chlamydia trachomatis*. The patient was intravenously administered a single dose of ceftriaxone (1 g) (intramuscular administration of ceftriaxone is not approved in Japan). He was also given a single dose of azithromycin (1g orally) for possible *C. trachomatis* urethritis (*1*).

Six days after receiving treatment, the patient showed improvement. Results of the Strand Displacement Amplification test were negative for *N. gonorrhoeae* and *C. trachomatis*. Eight days after the patient received treatment, the culture for the urethral discharge sample was shown

to be positive for *N. meningitidis*. Urine culture was negative 20 days after treatment.

The 33-year-old male partner of the case-patient was originally from the United States and had been living in Japan for 4 years. Because of his history of sexual contact with the case-patient, he was advised to undergo a screening test for HIV and *N. meningitidis*. The man underwent a physical examination at our clinic 40 days after the case-patient received treatment; findings were unremarkable, and the result for HIV testing done 2 days earlier was negative. Throat and urine samples were obtained for culture, and the man was intravenously administered ceftriaxone (1 g). The urine sample culture was negative, but the throat sample culture was positive for *N. meningitidis*. The throat culture result was negative 10 days after the patient's treatment.

We performed cultures and tests to identify *N. meningitidis*, and we conducted multilocus sequence typing (MLST), serotyping, PorA typing, and pulsed-field gel electrophoresis (PFGE) as described elsewhere (*2*). Isolates from both men were identified as serotype W and PorA type P1.5, 2. MLST showed that the strains were ST10651 (genes analyzed: *aroE*:3, *adk*:4, *fumC*:3, *gdh*:8, *pdhC*:4, *pgm*:6, and *abcZ*:662). Although *abcZ*:662 was a novel allele, ST10651 belongs to the ST11/ET37 complex (*3*). We used PFGE with restriction enzyme *Nhe*I to compare the *N. meningitidis* strains from the case-patient and his partner; the isolates had the same PFGE pattern (Figure). Both isolates were confirmed to be a novel multilocus ST, 10651, of the ST11/ET37 complex; however, novel MLST types frequently occur. By using the E-test (Sysmex bioMérieux, Tokyo, Japan), we determined that the 2 isolates required the same minimum inhibitory concentrations (MICs) for the following antimicrobial drugs: penicillin (MIC 0.125 mg/L), ceftriaxone (MIC 0.004 mg/L), ciprofloxacin (MIC 0.004 mg/L), and azithromycin (MIC 0.25 mg/L) (*4*).

Urethritis caused by *N. meningitidis* infection in men who have sex with men (MSM) has been reported, as has an association between urethritis and oral sex (*5*,*6*). Most previously reported urogenital isolates of *N. meningitidis* have belonged to serogroups B (*5*,*6*), Y (*5*,*6*), and C (*5*). Among 115 cases of *N. meningitidis* infection in Japan during the last 9 years, 22 (19.1%) were caused by serogroup B and 18 (15.7%) were caused by serogroup Y; only 3 (2.6%) cases were caused by serotype W (*7*).

N. meningitidis ST11/ET37 complex is a hyperinvasive lineage. During the 1990s, the serogroup C ST11/ET37 complex was prominent in Europe and North America. However, in 2000, an outbreak of *N. meningitidis* serotype W infections occurred among Hajj pilgrims (*8*), and this serotype has now spread worldwide (*3*,*8*).

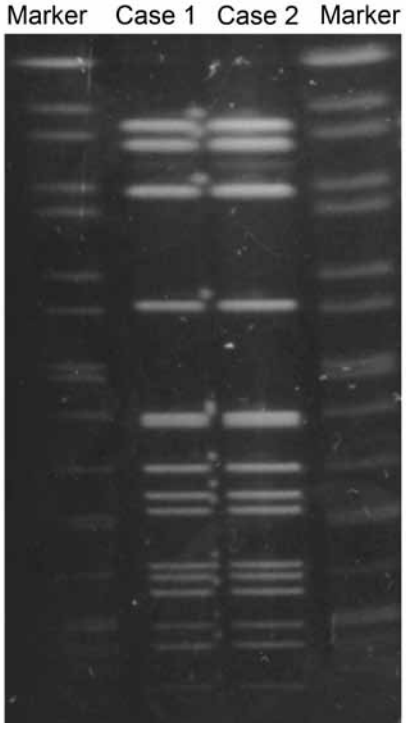

Figure. Pulsed-field gel electrophoresis (PFGE) patterns for *N. meningitidis* strains isolated from a man with urethritis (case 1) and his male sex partner (case 2), Japan. PFGE was performed with the restriction enzyme *Nhe*I. Results showed the same PFGE pattern for both isolates. *Salmonella enterica* serovar Braenderup strain H9812 was used as the PFGE size marker strain; it was digested with *Xba*I and resolved by PFGE.

Chemoprophylaxis is indicated for persons who have close contact with someone with invasive meningococcal infection (*9*), but there is uncertainty regarding the treatment of asymptomatic persons who have contact with someone with *N. meningitidis* urethritis. To avoid a reinfection cycle between the men in this study, we treated the asymptomatic, *N. meningitidis*–colonized male partner.

Since the early 2000s, and especially since 2012, outbreaks of invasive serogroup C, ST11/ET37 complex meningococcal disease causing high rates of death have been reported among MSM in the United States and Europe (*10*). These outbreaks have raised policy questions concerning vaccination recommendations for HIV-infected persons and for the MSM population (*10*). In Japan, meningococcal vaccination has not been officially approved, and neither of the men in this study had been vaccinated against *N. meningitidis*.

A diagnosis of urethritis is often based on Gram staining or nucleic acid amplification tests (*1*). However, Gram staining cannot differentiate *N. meningitidis* from *N. gonorrhoeae*, and amplification tests only detect *N. gonorrhoeae*. This practice makes it difficult to diagnose and access the number of cases of *N. meningitidis* urethritis.

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Highly Pathogenic Avian Influenza A(H5N8) Virus from Waterfowl, South Korea, 2014

To the Editor: To date, 18 hemagglutinin (HA) subtypes and 11 neuraminidase (NA) subtypes have been identified in influenza A viruses (*1*–*4*). Influenza A viruses containing HA subtypes 1–16 circulate in aquatic birds (*1*,*2*), whereas those harboring HA subtypes 17 and 18 are found in bats (*3*,*4*).

On January 18, 2014, the government of South Korea reported an outbreak of highly pathogenic avian influenza A(H5N8) virus in breeding ducks in the southern part of Jeollabuk-Do Province (*5*). More than 12 million poultry have since been culled, but the spread of the virus continues in duck and chicken farms. We report the genetic characterization of this virus.

On February 15, 2014, a total of 200 fecal samples were collected from waterfowl in the Pungse River in Chungnam Province, which is geographically close to Jeollabuk-Do Province. All samples were inoculated into hens' eggs, and influenza A viruses were confirmed by PCR by using influenza A–specific nucleoprotein (NP) primers. We obtained 1 isolate, A/waterfowl/Korea/S005/2014 (H5N8), and sequenced the full regions of all 8 genes as described (*6*). These sequences were deposited into GenBank under accession nos. KJ511809–KJ511816.

We conducted a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi, http://platform.gisaid.org/epi3/frontend#4ead5c) to identify the closest gene sequences to those of A/waterfowl/Korea/S005/2014 (H5N8) (Table). Sequences for polymerase basic (PB) 2 (99% homology), HA (97% homology), and NP (99% homology) genes were closely related to those of A/wild duck/Shandong/628/2011 (H5N1). Sequences for PB1 (99% homology), polymerase acidic subunit (PA) (98% homology), matrix (M) (99% homology), and nonstructural (NS) (99% homology) genes were closely related to those of A/duck/Jiangsu/1-15/2011 (H4N2). Sequences for the NA (98% homology) gene were closely related to that of A/duck/Jiangsu/k1203/2010 (H5N8). Phylogenic analysis showed that all 8 genes of A/waterfowl/Korea/S005/2014 (H5N8) belonged to the Eurasian lineage, and that the HA gene clustered with clade 2.3.4 (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/20/9/14-0390-Techapp1.pdf).

We further analyzed the amino acid sequences of the virus isolate (online Technical Appendix Table 1). Positions 138 and 160 of the HA protein (H3 numbering) contained an alanine (A) residue, which was previously found to be related to enhanced binding to the human influenza receptor (*7*). The connecting peptide of HA contained an insertion of 4 basic amino acids (arginine-arginine-arginine-lysine), which is the same as in the HA of A/duck/Korea/Buan2/2014 (H5N8), an isolate from a duck farm in South Korea (GenBank accession no. KJ413839.1–KJ413846.1). Aspartic acid was found in M1 at position 30 and alanine at position 215; this combination has been connected with increased virulence in mice (*8*). The NS1 sequence contained serine at position 42, which is related to the enhanced pathogenicity in mice, but a truncation of the amino acids at positions 218–230 that has been linked with reduced pathogenicity in mice (*9*) was not identified. Asparagine was identified at position 31 of M2, which is the same in M2 of A/duck/Korea/Buan2/2014 (H5N8) and confers resistance to amantadine and rimantadine (*10*).

Because all 8 genes of A/waterfowl/Korea/S005/2014 (H5N8) are closely related to those of the A/duck/

Table. Nucleotide homology of genes of influenza virus strain A/waterfowl/Korea/S005/2014 (H5N8) to the closest related influenza virus strains*

Gene	Closest related virus strain	Nucleotide identity, %
PB2	A/wild duck/Shandong/628/2011 (H5N1)	99
PB1	A/duck/Jiangsu/1-15/2011 (H4N2)	99
PA	A/duck/Jiangsu/1-15/2011 (H4N2)	98
HA	A/wild duck/Shandong/628/2011 (H5N1)	97
NP	A/wild duck/Shandong/1/2011 (H5N1)	99
NA	A/duck/Jiangsu/k1203/2010 (H5N8)	98
M	A/duck/Jiangsu/1-15/2011 (H4N2)	99
NS	A/duck/Jiangsu/1-15/2011 (H4N2)	99

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

Korea/Buan2/2014 (H5N8) isolate that was obtained from a duck farm, it is likely that A/waterfowl/Korea/S005/2014 (H5N8) originated from infected waterfowl that had visited poultry on an infected farm (online Technical Appendix Figure 1). Our laboratory has studied the feces of wild birds in Chungnam Province since 2009, surveying >20,000 fecal samples from wild birds in this area each year, but we had not previously isolated avian influenza A(H5N8) virus from any samples.

The genetic analysis of the A/waterfowl/Korea/S005/2014 (H5N8) isolate indicates that this novel strain may have been created by the reassortment of PB2, HA, and NP segments from H5N1-like avian influenza virus; PB1, PA, M, and NS segments from H4N2-like avian influenza virus; and NA segments from H5N8-like avian influenza virus (online Technical Appendix Figure 2). Most genes of the virus we isolated are related to those of avian influenza viruses isolated in China, but the HA gene of A/waterfowl/Korea/S005/2014 (H5N8) showed only 97% homology to the closest HA gene in GenBank, which indicates that this gene may have been created in poultry in South Korea. To our knowledge, no outbreak of this virus in poultry farms in China has been reported, and we found no previous reports in the literature that migratory birds could carry the virus. Taken together, our data suggest that A/waterfowl/Korea/S005/2014 (H5N8) may have been reassorted in a duck farm in South Korea.

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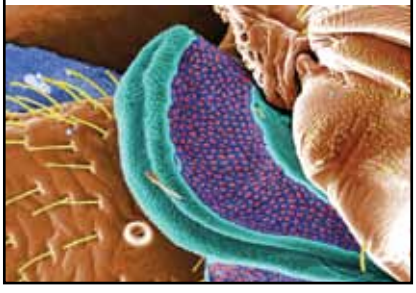

Worker Health and Safety Practices in Research Facilities Using Nonhuman Primates, North America

To the Editor: Since 1975, federal quarantine regulations (*1*) have restricted nonhuman primate importation to scientific, educational, or exhibition purposes to limit risks for disease introduction (*1*,*2*). Infectious diseases resulting from importation of nonhuman primates need to be prevented to ensure that colonies of these animals are available for research and to protect persons working with them from exposure to established and emerging zoonotic diseases (*2,3*).

Most imported nonhuman primates are bred for research and undergo standard screening and conditioning before shipment, which substantially reduce importation-associated health risks (*4*). However, many zoonotic agents can be difficult to exclude from even meticulously controlled breeding facilities (*3,5*). Nonhuman primates are commonly imported from regions with a high prevalence of potentially zoonotic diseases, such as tuberculosis and meliodosis, in humans and animals. Diagnosing tuberculosis in nonhuman primates can be difficult; inadvertent colony and human exposures can occur through undiagnosed cases (*6*). Similarly, *Burkholderia pseudomallei*, the causative agent of meliodosis, can be carried asymptomatically for extended periods before illness onset, posing a persistent exposure risk for persons working with imported nonhuman primates from regions to which meliodosis is endemic (*7*). Finally, nonhuman primates are host to potentially zoonotic viruses, such as simian foamy virus, which has unknown pathogenic potential in infected persons (*8*), and Macacine herpesvirus 1, which causes severe, often fatal, neurologic disease in humans exposed to macaques with asymptomatic infection (*9*).

Quarantine and testing of imported nonhuman primates, rigorous hygiene at research facilities, and strict personal protection equipment (PPE) standards are important to protect the health of nonhuman primate colonies and persons working with the animals (*4*). Importers must register with the Centers for Disease Control and Prevention (CDC) and implement disease control measures, including a 31-day quarantine for newly arrived animals (*1*). Specific PPE is mandated for quarantine facility staff, but individual facilities determine PPE standards after the animals are released from CDC-mandated quarantine (*4*).

To better understand occupational health and safety practices at facilities housing nonhuman primates, in December 2012, the Association of Primate Veterinarians, with technical support from CDC, surveyed primate veterinarians in North America about animal handling practices and PPE standards at their institutions. The Association of Primate Veterinarians received completed surveys and removed identifying information before providing data to CDC for analysis.

CDC and the University of Guelph (Guelph, ON, Canada) determined that the survey did not qualify as human subjects research. Information collected applied to the institution, not the individual respondent. Respondents were informed that participation was voluntary and anonymous, refusal carried no repercussions, and results would be presented in aggregate.

Of 149 facilities, 7 (5%) indicated they were not currently housing nonhuman primates, and 26 (17%) provided completed surveys. Most responding facilities were university or private/contract research facilities (16 [62%] and 5 [19%] facilities, respectively). Most (18 [69%]) facilities maintained ≤500 nonhuman primates, primarily rhesus or cynomolgus macaques. Nineteen (73%) facilities acquired imported nonhuman primates during 2010–2012. During this period, 47,876 nonhuman primates were imported, of which 90% were cynomolgus macaques. Fewer nonhuman primates were acquired from domestic sources (1,877 animals; see also [*10*]).

In a free-text field, we asked about quarantine and testing policies for newly acquired nonhuman primates. Most facilities reported applying standard health requirements to newly acquired animals, regardless of source, and requiring additional quarantine periods before moving new animals into the facility population or assigning them to a study.

The number of staff working near nonhuman primates differed among facilities. Ten (38%) facilities reported that ≥30 staff members handle or work in close proximity to nonhuman primates for cleaning or observation each day (Table). All facilities required PPE for routine handling of animals, including use of surgical masks or N95 respirators; goggles, safety glasses, or full-face shields; specialized clothing (e.g., laboratory coat, scrubs, or coveralls); gloves; and either shoe covers, reusable boots, or facility-designated shoes (Table).

Twenty-one (81%) facilities reported routinely handling conscious nonhuman primates by using specialized safety equipment (e.g., pole and collar or restraint chair). Four (15%) facilities reported manually capturing conscious animals ("hand-catching"); 2 (8%) facilities performed intrapalpebral tuberculin skin tests on conscious animals (Table).

All facilities reported routinely performing postmortem examinations. Five facilities specified that complete necropsies were performed only on animals found dead or euthanized because of illness or injury; for animals euthanized at study completion, targeted tissue specimens were collected to fulfill research objectives (Table).

These results suggest that responding facilities generally maintained high standards for health and safety and

Table. Health and safety practices reported by 26 research facilities that use nonhuman primates, North America, December 2012*

Characteristic	No. (%) facilities
Average no. staff working daily with or near nonhuman primates	
1–5	8 (31)
6–10	3 (12)
11–15	1 (4)
16–20	3 (12)
21–30	1 (4)
>30	10 (38)
Required personal protection equipment*	
Respiratory protection used	
Surgical mask	21 (81)
N95 respirator	8 (31)
Powered air-purifying respirator	4 (15)
Eye protection	
Goggles/safety glasses	19 (73)
Full face shield	24 (92)
Protective clothing	
Laboratory coat/scrubs	17 (65)
Reusable coveralls	7 (27)
Disposable coveralls	15 (58)
Head covering/cap/bonnet†	8 (31)
Extra gown layer/arm covers†	5 (19)
Gloves	
Latex or nitrile gloves	26 (100)
Double gloves†	4 (15)
Leather gloves†	2 (8)
Footwear	
Shoe covers	24 (92)
Reusable boots	10 (38)
Shoes designated for use in facility only†	6 (23)
Handling of animals	
Manually capture conscious animals ("hand-catch")‡	4 (15)
Handle conscious animals with special equipment (e.g., pole and collar, chair)	21 (81)
Conduct tuberculin skin tests on conscious animals	2 (8)
Routinely conduct necropsy on nonhuman primates that die or are euthanized§	26 (100)

*Because respondents could select >1 option, percentages will not total 100% within each personal protection equipment category.
†This answer choice was not one provided in the answer options but was provided in the associated free-text field for "other." The number provided reflects the number of respondents who volunteered this answer under "other."
‡One respondent skipped this question. Percentages calculated with 25 facilities as the denominator.
§Five respondents provided a written caveat that all animals that die spontaneously or are euthanized specifically because of health concerns routinely undergo necropsy but noted that animals euthanized at study completion often undergo study-specific tissue collection that might not include a complete necropsy.

are aware of disease risks. However, this survey has limitations for assessing the effectiveness of risk mitigation policies. Although a variety of facilities responded, response biases cannot be excluded. Additionally, these results summarize occupational health and safety standards on record but cannot address compliance or employee attitudes toward health and safety concerns in working with nonhuman primates. Facilities maintaining nonhuman primates need to strive for strict enforcement of occupational health and safety requirements; consider requiring regular continuing education about human health risks associated with working closely with animals; and consider the degree of risk pertaining to specific activities, particularly those generating infectious aerosols.

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Mortierella wolfii–Associated Invasive Disease

To the Editor: In January 2013, a 34-year-old man was admitted because of severe shock to the intensive care unit (ICU) at University Hospital of Liege (Liege, Belgium) on day 10 after nonfamilial hematopoietic stem cell transplantation (HSCT). X-linked *gp91phox* gene mutation chronic granulomatous disease (CGD) had been diagnosed when he was 4 years of age. The patient had a history of recurrent pulmonary aspergillosis (*Aspergillus fumigatus* infection) and anaphylaxis to lipid-based formulations of amphotericin B (AmB). During the year before HSCT, he had received voriconazole for possible recurrent aspergillosis until 2 cavitary necrotic pulmonary lesions prompted prolonged combined treatment with caspofungin. No fungus was yielded before transplant.

On day 9 after HSCT, a computed tomographic (CT) scan of the abdomen showed multiple hypodense lesions in the hepato-splenic parenchyma, and the patient was admitted to the ICU. Antifungal drug administration were escalated (voriconazole 6 mg/kg twice daily, caspofungin 70 mg/day), taking into account patient's history of anaphylaxis. The patient recovered from aplasia within 12 days (*1*), and CT-guided liver biopsy was performed on day 20 after HSCT.

Microbiological cultures of the liver biopsy sample were performed on 5% sheep blood agar and Sabouraud agar medium supplemented with chloramphenicol. The plates were incubated at 30°C and 37°C, respectively, for 10 days. Subcultures were performed on malt agar, Takashio, and agar-agar medium and incubated at 30°C, 37°C, and 42°C, respectively, to obtain sporulation. Blood cultures were collected on the same day the biopsy was performed on BacT/ALERT FAN aerobic and anaerobic medium bottles (bioMérieux, Durham, NC, USA). Antifungal susceptibility testing was performed by Etest. Histopathologic examination was conducted after the liver sections were stained with hematoxylin and eosin, periodic acid–Schiff, and Gomori methenamine silver.

We sequenced regions of internal transcribed spacers (ITS) 1 and 2 of the rRNA genes (*2*).The ribosomal target of the large subunit RNA gene (D1–D2 region) was used to confirm the first results. Sequences were aligned by using the GeneStudio Pro software (http://genestudio.com/) and identified in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and in the CBS database for filamentous fungi (http://www.cbs.knaw.nl/collections/BioloMICSSequences.aspx).

Histologic examination of the liver biopsy sample showed fungal filaments of variable diameter, without septa, and with bulbous dilations suggesting mucormycosis amid necrotic parenchyma (Figure). A white filamentous mold grew after the sample was incubated for 2 days on Sabouraud agar at 30°C and 5% sheep blood agar medium at 37°C. Microscopic examination of the culture confirmed a fungus belonging to the mucoromycotina. Nonseptate hyphae of irregular width were visible and marked by short lateral extensions distributed at right angles along the filaments. Despite subcultures on different media and incubation at 30°C, 37°C, and 42°C, no sporulation was observed. The fungus did not grow on Sabouraud agar supplemented with cycloheximide. One blood culture was positive after 3 days' incubation at 37°C, and the subculture grew the same fungus. In vitro susceptibility testing showed high MICs (≥32 mg/L) for voriconazole and caspofungin and lower MICs for AmB (1 mg/L) and posaconazole (0.012 mg/L). The 3 targeted sequences of ITS2 alone (GenBank accession no. KJ825897), ITS1–ITS2 (GenBank accession no. KJ82598), and D1–D2 region of the rRNA genes (GenBank accession no. KJ825899) identified *Mortierella wolfii* with 98% and 100% similarity with the reference sequences in the CBS database.

CGD is a primary immunodeficiency syndrome characterized by impaired phagocytic activity of intracellular pathogens and fungi. Invasive fungal infections account for one third of deaths attributed to CGD, and lung involvement is predominant (*3*). To our knowledge, the 2 reports of human cutaneous infection with *M. wolfii* are considered inconclusive (*4*). It is a mold belonging to the order Mortierellales according to the most recent taxonomy (*5*) and is considered a pathogen solely of animals. It causes abortion, encephalitis, and pneumonia in cattle in specific geographic locations (Australia, North

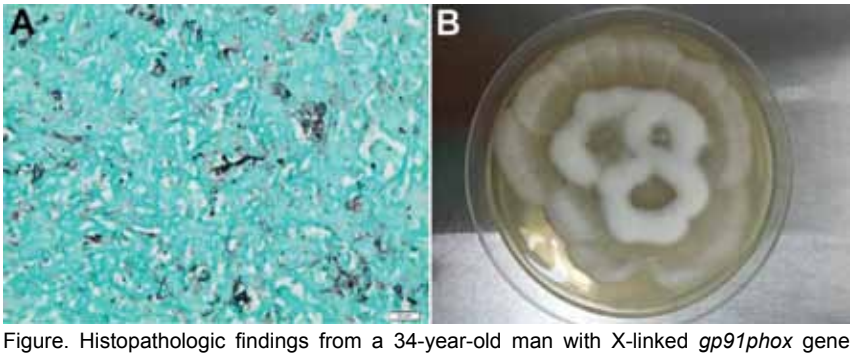

Figure. Histopathologic findings from a 34-year-old man with X-linked *gp91phox* gene mutation chronic granulomatous disease. A) Liver biopsy results showing numerous irregular and nonseptate hyphae with bulbous dilations. Gomori methenamine silver stain. Original magnification ×400. B) Culture of *Mortierella wolfii*, after 3-days of incubation at 30°C on Sabouraud agar medium.

America, and Japan) (*6*). The natural habitats of *M. wolfii* include moldy grass in silage, and infected animals might inhale spores from contaminated silage or acquire them through digestive tract ulcerations after ingestion of semen (*4*). Thus, possible transmission routes of *Mortierella* sp. in this patient include airborne exposure to mulch or ingestion of contaminated imported food during pressure-selection azole prophylaxis and inflammatory bowel disease. After being ingested or inhaled, this weakly virulent mold must have remained quiescent until a few months before HSCT. We suspect that it was responsible for the necrotic cavitary pneumonia for which no fungus was identified before transplant. *M. wolfii* eventually emerged during a profound iatrogenic neutropenic period (*1*). Because death partly correlates with dissemination, preemptive and adequate antifungal treatment is of utmost importance in mucormycosis. In this patient, who died 11 days after ICU admission, past anaphylaxis precluded prompt initiation of a lipid-based formulation of AmB, which remains the best choice for treating invasive mucormycosis (*7*). Posaconazole, a second-choice drug, has shown efficacy in CGD patients who had invasive mucormycosis resistant to first-line treatment (*8*). Allogeneic donor–matched HSCT has a curative potential in CGD patients with refractory fungal infections (*9*). Several other authors have pointed to the emergence of rare new fungi in CGD, as well as reclassification of misdiagnosed fungi, identified by sequence-based analysis (*10*).

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Antibody against Arenaviruses in Humans, Southwestern United States

To The Editor: Woodrats (*Neotoma* spp.) are natural hosts of Whitewater Arroyo virus (WWAV) and other Tacaribe serocomplex viruses (family *Arenaviridae*) in the western United States and northern Mexico (*1*). The results of a previous study (*2*) suggested that WWAV or Tacaribe serocomplex viruses antigenically closely related to WWAV are etiologic agents of severe febrile illnesses in humans in the United States. We note that Junín virus and other South American Tacaribe serocomplex viruses are etiologic agents of hemorrhagic fever in humans (*3*).

To further our knowledge of the epidemiology of the North American Tacaribe serocomplex viruses, we tested serum samples from hospitalized persons in a study of thrombocytopenic febrile illnesses that mimicked hantavirus pulmonary syndrome for IgG against arenaviruses. The 173 study participants were hospitalized during 1993–2001 in Arizona and New Mexico, United States. The study protocol was approved by the University of New Mexico Human Research Review Committee and the Navajo Nation Institutional Review Board. Ages of the study participants ranged from 9 to 86 years (mean 40 years). Virtually all serum samples were acute-phase

specimens, and a specific diagnosis was achieved for only 55 (31.8%) of the 173 study participants.

Serum samples were tested for IgG against WWAV, Amaparí virus (AMAV), an arenavirus that is antigenically closely related to the Tacaribe serocomplex viruses known to cause hemorrhagic fever (*4*), and lymphocytic choriomeningitis virus (LCMV), the prototypical arenavirus and member of the Lassa–lymphocytic choriomeningitis serocomplex, by using an ELISA. (*5*). Briefly, we tested serial 4-fold dilutions (1:80–1:5,120) of each sample and compared results with results for negative control antigens. The adjusted optical density (AOD) of a sample-antigen reaction was the OD associated with the test antigen minus the OD associated with the corresponding control antigen. A sample was considered positive if the AOD at 1:80 was ≥0.250, the AOD at 1:320 was ≥0.250, and the sum of the AOD for the series of 4-fold dilutions was ≥0.750. The criteria for positivity were based on results of ELISA for serum samples from febrile persons who did not participate in this study and were negative for IgG against WWAV, AMAV, and LCMV.

The IgG titer against a test antigen in a positive sample was the reciprocal of the highest dilution for which the AOD was ≥0.250. Titers <320 were considered to be 160 in comparisons of titers for WWAV, AMAV, and LCMV in individual samples. The apparent homologous virus in a positive sample was the virus associated with the highest titer if the absolute value of the differences between the highest titer and titers for the 2 other viruses were ≥4-fold.

IgG against WWAV was found in acute-phase samples from 8 (4.6%) of the 173 study participants. None of the 173 study participants were positive for IgG against AMAV or LCMV. The IgG titers against WWAV in the positive samples were 320 (n = 1), 1,280 (n = 3), and ≥5,120 (n = 4). WWAV was the apparent homologous virus in the 7 persons with antibody titers ≥1,280. The apparent homologous virus in the person with the titer of 320 could not be determined from ELISA data. The presence of IgG against WWAV in acute-phase serum samples (all collected within 10 days of illness onset) implied past infection with WWAV or an arenavirus antigenically closely related to WWAV.

The state of residence (2 from Arizona, 6 from New Mexico), sex ratio (4 male patients:4 female patients), and mean age (36 years, range 16–47 years) of antibody-positive persons reflected the characteristics of the entire study population. The clinical features in each of the antibody-positive persons included fever, headache, myalgia, and thrombocytopenia. The diagnoses given for these persons were acute parvovirus infection (n = 1) by IgM assay, adult respiratory distress syndrome (n = 1) by clinical progression, and not determined (n = 6).

The results of this study indicate that a small fraction of the adult population in the southwestern United States has been infected with North American Tacaribe serocomplex virus(es). We note that the dominant epitopes in ELISA for IgG against arenaviruses are associated with the viral nucleocapsid (N) protein, and that amino acid sequence of the N protein of WWAV and amino acid sequences of N proteins of other Tacaribe arenaviruses from Arizona or New Mexico showed differences as high as 15.1% in a previous study (*1*).

It might be the case that human IgG against some Tacaribe serocomplex viruses in the southwestern United States does not react strongly against WWAV in ELISA. If so, the true prevalence of antibody against North American Tacaribe serocomplex viruses in this study might be >4.6%. Accordingly, future work should include development of broadly reactive assays for detection of human IgM and human IgG against North American Tacaribe serocomplex viruses, including those associated with wild rodents in Mexico (*6,7*).

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Porcine Coronavirus HKU15 Detected in 9 US States, 2014

To the Editor: Porcine coronavirus (PorCoV) HKU15 is a single-stranded, positive-sense, enveloped RNA virus belonging to the genus *Deltacoronavirus* (family *Coronaviridae*). PorCoV HKU15 was first identified in 2012 in a surveillance study from China (*1*). Until February 2014, however, the role of this virus strain in clinical diseases of pigs had not been reported.

We recently reported the detection of PorCoV strain HKU15-OH1987 in feces samples from sows and intestine samples from piglets in Ohio, United States; the infected animals were from swine farms where outbreaks of diarrheal disease had occurred in late January and early February 2014 (*2*). Genetic analysis showed that HKU15-OH1987 is closely related to 2 deltacoronavirus strains that were detected in Hong Kong, China, in 2012: HKU15-155 and HKU15-44 (*2*). We also demonstrated the presence of histopathologic lesions in the small intestines of PorCoV HKU15–infected piglets with diarrhea (L. Wang, unpub. data). In April 2014, a novel swine enteric coronavirus disease caused by PorCoV HKU15 or porcine epidemic diarrhea virus was reported to the World Animal Health Organization by the US Department of Agriculture (http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=15133).

PorCoV HKU15 is now recognized as a key pathogenic cause of diarrheal diseases in pigs in the United States. However, the geographic distribution and genotype diversity of PorCoV HKU15 in this country are still not clear. To further our knowledge of the virus, we analyzed swine samples that had been submitted for diagnosis of diarrheal disease from farms in 10 US states. We report the detection and phylogenetic analyses of PorCoV HKU15 strains from these samples.

Between February 7, 2014, when PorCoV HKU15-OH1987 was first identified in Ohio (*2*), and April 9, 2014, the Animal Disease Diagnostic Laboratory of the Ohio Department of Agriculture received >2,000 swine samples from farms in 10 US states for diagnosis of diarrheal disease. The states from which samples had been submitted were Minnesota, South Dakota, Nebraska, Illinois, Indiana, Michigan, Kentucky, Pennsylvania, Maryland, and Ohio. Among those samples, 435 were selected to be tested for the presence of PorCoV HKU15. A real-time reverse transcription PCR assay targeting the membrane protein gene was used to identify PorCoV HKU15. Samples with a cycle threshold value of <35 were considered positive on the basis of validation data using the cloned membrane protein gene (data not shown). Of the 435 samples, 109 (25%) from 9 states (all states mentioned above, excluding Maryland) were positive for PorCoV HKU15 by real-time reverse transcription PCR (Figure). Of those 109 samples, 19 (17%) were also positive for porcine epidemic diarrhea virus. This result suggests that PorCoV HKU15 is prevalent among pig populations in the major pig-producing US states.

To determine the genetic diversity of PorCoV HKU15 strains from the 9 states, we conducted whole-genome sequencing for 1 strain from each state by using 16 pairs of previously described, overlapping primers (*2*). Strain names were designated by the state abbreviation and case number. The complete sequence for HKU15-OH1987 was reported previously (*2*). Sequence analysis showed that strains from South Dakota (SD3424), Nebraska (NE3579), Illinois (IL2768), Indiana (IN2847), Kentucky (KY4813), Michigan (MI6148), and Pennsylvania (PA3148) have the same genome size (25,422 nt) as OH1987, and whole-genome pairwise comparison showed that they share a high nucleotide similarity (≥99.8%). Furthermore, all of the isolates share high nucleotide similarity (98.9%–99.2%) with the 2 PorCoV HKU15 strains in GenBank, HKU15-155 and HKU15-44.

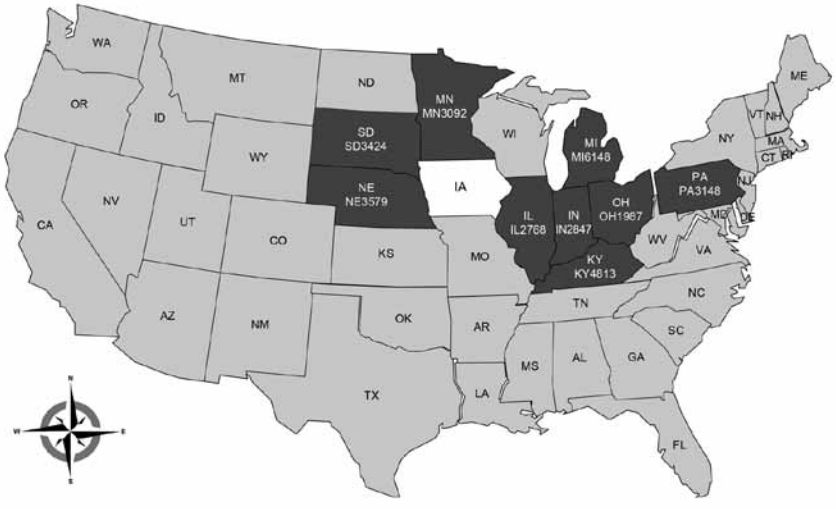

Figure. US states with swine samples positive for porcine coronavirus (PorCoV) HKU15, between February and April 2014. A total of 435 samples from 10 states were selected to be tested for the presence of PorCoV. Of those samples, 109 (25%) from 9 states (dark gray) were positive for PorCoV HKU15. Another recent article reported the presence of PorCoV HKU15 in Iowa (white) (*3*). Strain names are indicated below state abbreviations.

A phylogenetic tree constructed by using the entire sequence showed that all PorCoV HKU15 strains from the United States clustered together in 1 clade of the genus *Deltacoronavirus* with HKU15-155 and HKU15-44 (online Technical Appendix Figure, panel A, http://wwwnc.cdc.gov/EID/article/20/9/14-0756-Techapp1.pdf). This finding indicates that 1 genotype of PorCoV HKU15 is currently circulating in multiple US states. This result was further supported by phylogenetic trees constructed by using the full-length amino acids of spike and nucleocapsid proteins (online Technical Appendix Figure, panels B, C). Because of limitation of the samples received, only a partial genome sequence was determined for strain MN3092 from Minnesota. However, on the basis of the spike and nucleocapsid protein sequence analyses, it is highly likely that the entire genome of the Minnesota strain is genetically identical to that of the other 8 strains (online Technical Appendix Figure, panels B, C).

In addition to the 9 states reported in this study, Iowa has also had a recent detection of PorCoV HKU15 (*3*). Thus, PorCoV HKU15 has been detected in 10 of the 50 US states, and those 10 states mainly cluster in the midwestern United States (Figure). As with data collected for porcine epidemic diarrhea outbreaks by the US National Animal Health Laboratory Network (http://www.nahln.org/default/), data collected on the geographic location and numbers of PorCoV HKU15 cases is also required to be reported weekly.

Earlier reports support avian coronaviruses as the gene source for *Deltacoronavirus* spp. (*1,4*). To confirm this, surveillance for PorCoV should be carried out among birds. Moreover, effective control strategies, including vaccine development, should be in place for prevention and control of infections caused by PorCoV HKU15.

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Schistosomiasis Haematobium, Corsica, France

To the Editor: In Europe, urinary schistosomiasis (*1*) has previously been detected only in Portugal, where this focus disappeared during the 1950s (*2*). However, freshwater snails of the species *Bulinus contortus*, *B. truncatus*, and *Planorbarius metidjensis*, which are recognized intermediate hosts for *Schistosoma haematobium* trematodes, have been found in Portugal (*3*), Spain (*4*), and Corsica (*5,6*). This finding suggested that autochthonous schistosomiasis could re-emerge in southern Europe if these mollusks become infected. We report a probable focus for transmission of schistosomiasis haematobium in Corsica, France.

In March 2014, a 4-year-old girl (index case-patient) from France was referred to the Toulouse University Hospital (Toulouse, France), with gross hematuria. Ultrasonography and cystoscopic examination of the bladder detected a polyp. Examination of the polyp for parasites identified bodies that were consistent with schistosome eggs. Parasitologic examination of urine confirmed schistosomiasis by detecting viable *S. haematobium* eggs.

The parents of the girl (family A) did not report any stay or travel in an area to which urinary schistosomiasis was endemic; they reported summer holidays only in Mallorca in the Balearic Islands (Spain) and Corsica. However, her father reported that since 2012, he had experienced gross hematuria that had been evaluated by standard urologic investigations but not by cystoscopy; no etiology was determined. Parasitologic urinalysis in our hospital department showed numerous viable *S. haematobium* eggs in the father's urine.

The parents of the index case-patient also reported that an 8-year-old boy in a friend's family (family B), who shared summer vacations with

them had exhibited gross hematuria since February 2013. A third family (family C) was also investigated because they also spent holidays in Corsica with families A and B. Families B and C had also spent a summer in Mallorca, but they denied any contact with freshwater. Of 11 French native-born members of the 3 families, 6 had positive results for *S. haematobium* by urine examination. All case-patients had specific positive immunodiagnostic results by an ELISA that used *S. mansoni* extracts and by indirect hemagglutination. In addition, 2 family members who had a negative result by urine examination had a positive serologic result.

Spending summer vacations in the same village in Corsica (Sainte-Lucie de Porto-Vecchio), where members of the 3 families had bathed at least once per holiday period in the Cavu River, was the epidemiologically prominent feature that linked these persons. Families A and C were in Sainte-Lucie de Porto-Vecchio in August 2011, and families A, B, and C were in the same location in August 2013.

During these investigations, we were contacted by the Department of Tropical Medicine, Dusseldorf University Hospital (Dusseldorf, Germany), because a 10-year-old boy and his father had been given diagnoses of schistosomiasis haematobium on the basis of positive urinalysis results for *S. haematobium* eggs. Two other members of this family (5 persons) had a positive immunodiagnostic result. Locations of previous vacations for this family outside Germany included Spain (not the Balearic Islands) and Corsica, where they bathed frequently in the Cavu River. These epidemiologic findings provide strong circumstantial evidence supporting the presence of a previously unrecognized focus of urinary schistosomiasis in Corsica.

We performed molecular analysis of schistosomal miracidia DNA. The second internal transcribed spacer region of the ribosomal gene complex (*7*,*8*) was amplified and sequenced. Viable eggs obtained from the patients in France were those of *S. haematobium*. Additional molecular investigations are being conducted to assess genetic diversity of this isolate from Corsica and the geographic origin of the introduced parasite.

The malacologic situation in Sainte Lucie de Porto-Vecchio was investigated during May 12–19, 2014; three rivers (Figure) were included in the survey. Four sites were sampled in the Cavu River, and *B. truncatus* snails were found in 3 sites that corresponded to bathing areas (site 1: 41°43′53.57″N, 9°17′36.70″E; site 2: 41°43′22.13″N, 9°17′59.87″E; site 3: 41°42′8.40″N, 9°21′5.82″E). Snails were also found in the nearby Tarcu River (site 5) and Osu River (site 6). These findings confirmed previous data for the presence of *B. truncatus* snails in Corsica (*5*,*6*). Water temperature was recorded at 11:00 AM at these 3 sites (range 15°C–16°C). This temperature range is not optimal for the snail intermediate host stage of the parasite life cycle (*9*,*10*). Of 148 live snails that were obtained in the Cavu River, none were infected with schistosome cercariae.

Data from the field survey and epidemiologic information for the cases in France and Germany, indicated transmission of schistosomiasis haematobium in the Cavu River in southeastern Corsica in 2011 and 2013. Additional supportive evidence is the fact that the father of the index case-patient had gross hematuria in 2012 and 2013.

Two hypotheses are proposed to account for this situation. The first hypothesis is that the parasite (i.e., schistosome eggs) was transmitted

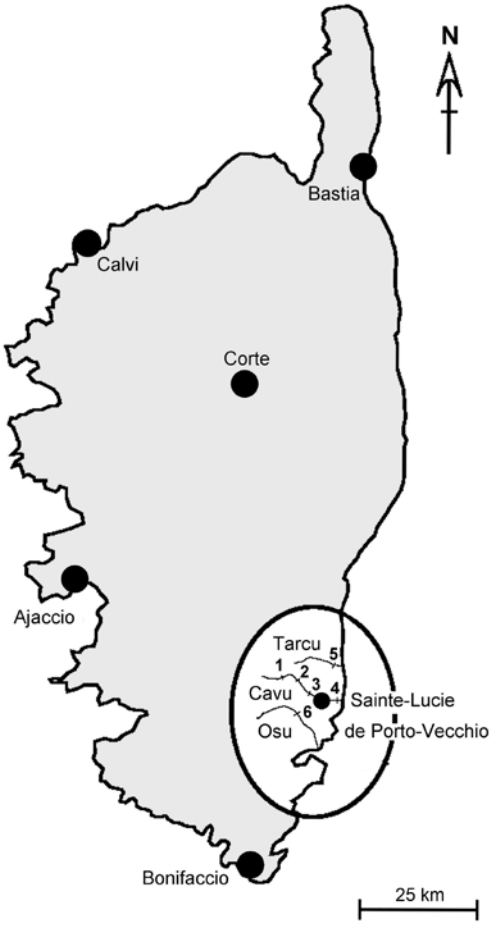

Figure. Corsica, France, showing malacologic survey sampling sites (oval) in 3 rivers (Tarcu, Cavu, and Osu). *Bulinus truncatus* snails were found at sites 1, 2, 3, 5, and 6.

by an infected person into the Cavu River in June or July 2011, when environmental conditions were favorable for snail infection. However, questions arise about survival of infected snails during the winter and their ability to reinfect the area during the following summers in 2012 and 2013. The second hypothesis is that schistosome eggs were spread by infected persons at the beginning of summer and caused a permanent transmission cycle in this focus. This situation would be difficult to control. Additional information should be obtained by a long-term malacologic survey to detect infected mollusks in this region.

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Pornchai Jaima (b. 1970) Integrated Farming (2013) (detail). Acrylic on canvas (39 1/4 × 31 1/2 inches/100 cm × 80 cm). Courtesy of the Artist

The Art of Intertwining Life and Work

Byron Breedlove and Komatra Chuengsatiansup

Pornchai Jaima grew up in rural northern Thailand, where village life, work, and worship were all intertwined. His parents were wood carvers at the local Buddhist temple, where at age 10 he began studying traditional northern Thai Buddhist scriptures. Upon entering the sixth school grade at age 12, Jaima became a Buddhist novice for three years of study and meditation.

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After completing high school, Jaima attended a local fine arts college in Chiangmai and began painting murals. Inspired after meeting the artist Chalermchai, he continued his studies at Silpakorn University. Before taking the admission examination, Jaima vowed to Khruba Srivichai, a Buddhist saint of northern Thailand, that if he passed, he would return home to build temples.

At the university, he began painting in the traditional northern Thai mural style, focusing on work, leisure, and customs of everyday village life in northern Thailand. Pornchai Jaima has exhibited his work in the United States,

France, the People's Republic of China, and Japan. He has won many awards, including the prestigious Silpathorn Award in Visual Arts. He regularly paints murals for temples and is building a meditation center that he will decorate with his own art for the community.

Integrated Farming, this month's cover image, reveals a lush tropical setting. Colorfully dressed villagers appear at a homestead, where there is space both for agricultural production and family activities. Pots, bowls, and cups cover the table. A woman carries a basket of mangoes, and flat baskets of peppers are drying on the lower roof. Bananas hang from the corner of the pavilion; papaya and mango trees are loaded with gold and green fruits. The shelves and tables are piled with foods.

As man tends a small fire, roasting glutinous rice in bamboo joints, 2 boys pretend to ride "horses" made from banana leaves and are greeted by the village dog. A girl plays with a walking toy made from coconut shells.

Two areca palms, laden with betel nuts, jut toward the canopy of tree tops where a riot of shapes and textures fans out like fireworks. Smoothly worn paths lead to a hut visible through the trees. Rice paddies glimpsed at the top of the painting reveal that the homestead is more extensive than first thought. Tools used for farming, harvesting, and cleaning stand idle as the villagers take time to prepare and enjoy a meal from the efforts of their own labors.

Hues of green, gold, and brown are the predominant colors. Jaima uses very little blue in this painting—there is not a glimpse of the sky. In keeping with the traditional painting technique of two-dimensional depiction, there are almost no shadows or reflections other than those under the ducks on the pale blue-gray water. A flock of birds swoops across the middle of the painting, disappearing into nests woven from dried leaves.

Jaima pays extraordinary attention to detail in this idyllic scene of everyday life. Joseph Campbell observed that in Buddhist art in Far Eastern countries the accent is often on "the rich garden of this beautiful world itself, where things comfortable in their places may be recognized as themselves divine in their very idiosyncrasies."

In this somewhat utopian depiction of traditional village life, the integration of agriculture and aquaculture offers an economically and environmentally viable means of sustenance. Fruit trees, livestock, vegetables, rice, fish, and water sustain the people. The various components of this system of mixed farming, in turn, support each another. The household poultry eat insects, fallen fruit, and weeds and provide fertilizer for the soil. Fish feed on vegetable matter and help fertilize the rice paddies. Bamboo and other trees provide fuel, materials, shelter, and tools.

Humans in rural settings have lived in close proximity with domestic and wild animals for millennia. These self-sufficient villages have sustained generations of people but may be susceptible to climate change and other threats.

The demand for wood and wood products, land for agricultural and living space, and minerals and other natural resources threatens the ecosystems across Southeast Asia, which also increases the potential exposure to emerging pathogens. Many emerging human, domestic animal, and wildlife diseases may infect multiple hosts.

While monocropping and modern agroindustrial practices raise concerns of emerging infectious diseases, remote and rural villages where mixed farming sustains people can become crucibles for emerging microbes and pathogens. One hopes that Jaima's wonderful paintings of rural Thai life are not someday merely retrospectives that show us a lost traditional way of life.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Resurgence of Cutaneous Leishmaniasis in Israel

Increased Pyrethroid Resistance in Malaria Vectors and Decreased Bed Net Efficacy, Burkina Faso

Person-to-Person and Nosocomial Transmission of Andes Hantavirus, Southern Chile

Shiga Toxin 1a–Producing *Shigella flexneri*

Age-Specific Modulation of Cytokines and Endothelial Markers Related to Ebola Hemorrhagic Fever Survival in Children

Malaria Control and Eradication in Venezuela, 1800s–1970s

Rickettsia parkeri and *Rickettsia montanensis*, Kentucky and Tennessee, USA

Differences in Influenza Seasonality by Latitude, Northern India

Human Granulocytic Anaplasmosis, South Korea, 2013

Knemidocoptic Mange in Wild Golden Eagles, California, USA

Human Infection with Highly Pathogenic A(H7N7) Avian Influenza Virus, Italy, 2013

Ongoing Epidemic of Cutaneous Leishmaniasis among Syrian Refugees, Lebanon

Novel Influenza A(H7N2) Virus in Chickens, Jilin Province, China, 2014

Treatment of Giardiasis after Nonresponse to Nitroimidazole, Israel

Evidence of Recombinant Strains of Porcine Epidemic Diarrhea Virus, United States, 2013

Pandemic Fear and Literature: Observations from Jack London's The Scarlet Plague

Imported Malaria in China, 2012

Probable Importation of Dengue Virus Type 4 to Angola from Brazil

Marburgviruses in Bats in Mine after Extermination Attempts, Uganda

Borrelia garinii and *Rickettsia monacensis* in *Ixodes ricinus* Ticks, Algeria

Mycobacterium marseillense and Pulmonary Disease, Italy

Complete list of articles in the October issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

2014

September 5–9, 2014
ICAAC 2014
Interscience Conference on Antimicrobial Agents and Chemotherapy
Washington, DC, USA
http://www.icaac.org

October 8–12, 2014
ID Week 2014
Philadelphia, PA, USA
http://www.idweek.org/

October 31–November 3, 2014
IMED 2014
Vienna, Austria
http://imed.isid.org

November 2–6, 2014
ASTMH
American Society of Tropical Medicine and Hygiene
63rd Annual Meeting
New Orleans, LA, USA
http://www.astmh.org/Home.htm

November 15–19, 2014
APHA 142nd Annual Meeting & Expo
New Orleans, LA, USA
http://www.apha.org/meetings
AnnualMeeting

November 30–December 4, 2014
ASLM 2014 International Conference
Cape Town International Convention Centre, South Africa
http://www.aslm2014.org/

2015

March 8–11, 2015
ICEID
International Conference on Emerging Infectious Diseases
Atlanta, GA, USA

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Article Title

Confirmed *Bacillus anthracis* Infection among Persons Who Inject Drugs, Scotland, 2009–2010

CME Questions

1. You are seeing a 33-year-old man in the emergency department. He complains of severe pain and redness on his left upper extremity after injecting heroin into an antecubital vein yesterday. A brief examination of the area reveals 8 cm of erythema, induration, and calor, with a central necrotic area. You consider whether this patient has injection anthrax; you also consider his prognosis. Which one of the following patient factors was most associated with a higher risk for mortality related to injection anthrax in the current study?

A. Younger age
B. Male sex
C. Excessive alcohol use
D. Cigarette smoking

2. Which one of the following variables at patient presentation was most associated with a higher risk for mortality in the current study of patients with injection anthrax?

A. Temperature less than 36˚C
B. Heart rate greater than 115 beats per minute
C. Respiratory rate less than 12 breaths per minute
D. Skin lesions on the upper extremity vs lower extremity

3. Which one of the following statements regarding treatment and mortality outcomes related to injection anthrax in the current study is most accurate?

A. Rates of initial diagnosis with *Bacillus anthracis* infection were similar among nonsurvivors and survivors
B. Compared with survivors, nonsurvivors were less likely to receive antibiotics at hospital admission
C. Compared with survivors, nonsurvivors were less likely to receive ciprofloxacin
D. Nonsurvivors and survivors were equally likely to require surgery

4. Which one of the following hospital events was more common among nonsurvivors vs survivors in the current study?

A. Application of anthrax immune globulin
B. Excessive bleeding during surgery
C. Longer duration until initial surgery
D. Initiation of renal replacement therapy

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

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to http://www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Pneumocystis jirovecii Pneumonia in Patients with or without AIDS, France

CME Questions

1. Your patient is a 42-year-old man with AIDS admitted to the hospital for respiratory distress and suspected *Pneumocystis jirovecii* pneumonia, also known as pneumocystis pneumonia (PCP). According to this prospective, multicenter observational study, which one of the following statements about presentations of PCP in patients with AIDS and with other immunosuppressive disorders is correct?

A. More than half of patients with PCP had AIDS
B. One third of patients with PCP had been taking PCP prophylaxis
C. Median time from the onset of respiratory tract symptoms to PCP diagnosis was 5 days (range, 1–15 days) in patients without AIDS and 21 days (range, 7–30 days) in patients with AIDS ($p<0.0001$)
D. Hypoxemia at presentation was more severe in patients with AIDS

2. According to this prospective, multicenter observational study, which one of the following statements about outcomes of PCP is correct?

A. Fewer patients without AIDS vs patients with AIDS required intensive care unit admission and ventilation
B. Hospital mortality rate was 17.4% overall, 4% in patients with AIDS, and 27% in patients without AIDS
C. Hospital mortality rates did not differ significantly between patients with AIDS and those without AIDS
D. Shock was more common in patients with AIDS and was significantly associated with pulmonary microbial coinfection

3. According to this prospective, multicenter observational study, which one of the following statements about risk factors for mortality in PCP, as determined by multivariable analysis, would most likely be correct?

A. Hospital mortality was not associated with age
B. Solid-organ transplantation was associated with twice the risk for hospital mortality
C. Hospital mortality was not associated with time to treatment
D. Patients receiving immediate oxygen had approximately 4-fold the risk for hospital mortality

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

Ticks and Lyme Disease

For more information about Lyme disease, visit http://www.cdc.gov/Lyme

How to prevent tick bites when hiking and camping

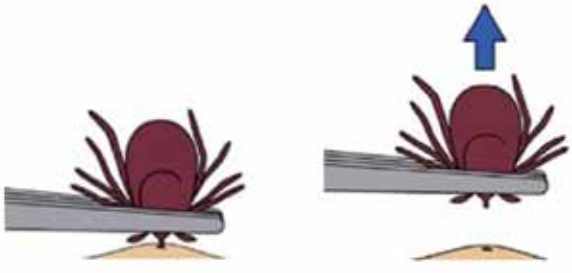
Ticks can spread disease, including Lyme disease. Protect yourself:

- Use insect repellent that contains 20 - 30% DEET.
- Wear clothing that has been treated with permethrin.
- Take a shower as soon as you can after coming indoors.
- Look for ticks on your body. Ticks can hide under the armpits, behind the knees, in the hair, and in the groin.
- Put your clothes in the dryer on high heat for 60 minutes to kill any remaining ticks.

How to remove a tick

1. If a tick is attached to you, use fine-tipped tweezers to grasp the tick at the surface of your skin.
2. Pull the tick straight up and out. Don't twist or jerk the tick—this can cause the mouth parts to break off and stay in the skin. If this happens, remove the mouth parts with tweezers if you can. If not, leave them alone and let your skin heal.
3. Clean the bite and your hands with rubbing alcohol, an iodine scrub, or soap and water.
4. You may get a small bump or redness that goes away in 1-2 days, like a mosquito bite. This is not a sign that you have Lyme disease.

Note: *Do not put hot matches, nail polish, or petroleum jelly on the tick to try to make it pull away from your skin.*



If you remove a tick quickly (within 24 hours) you can greatly reduce your chances of getting Lyme disease.

National Center for Emerging and Zoonotic Infectious Diseases
Division of Vector Borne Diseases | Bacterial Diseases Branch

CS232422D

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

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Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.