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August 2010

Influenza

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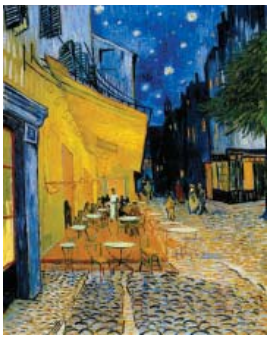

On the Cover

Vincent van Gogh (1853–1890)
Terrace of a Café at Night
(Place du Forum)
(c. 18 September 1888)
Oil on canvas (80.7 cm × 65.3 cm)

Courtesy of Kröller-Müller Museum,
Otterlo, the Netherlands

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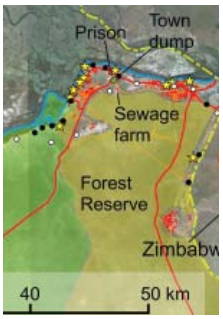

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Conference Summary

Hantavirus Infections in Humans and Animals, China

Yong-Zhen Zhang, Yang Zou, Zhen F. Fu, and Alexander Plyusnin

Hemorrhagic fever with renal syndrome (HFRS) is a serious public health problem in the People's Republic of China. Although 7 sero/genotypes of hantaviruses have been found in rodents, only Hantaan virus (carried by *Apodemus agrarius* mice) and Seoul virus (carried by *Rattus norvegicus* rats) reportedly cause disease in humans. During 1950–2007, a total of 1,557,622 cases of HFRS in humans and 46,427 deaths (3%) were reported in China. HFRS has been reported in 29 of 31 provinces in China. After implementation of comprehensive preventive measures, including vaccination, in the past decade in China, incidence of HFRS has dramatically decreased; only 11,248 HFRS cases were reported in 2007. Mortality rates also declined from the highest level of 14.2% in 1969 to ≈1% during 1995–2007. However, the numbers of HFRS cases and deaths in China remain the highest in the world.

During the past decade, hantaviruses have gained worldwide attention as emerging zoonotic pathogens (*1*–*3*). Hantaviruses, which belong to the family *Bunyaviridae*, genus *Hantavirus*, are enveloped, single-stranded, negative-sense RNA viruses. Transmission among rodents and from rodents to humans generally occurs through inhalation of aerosolized excreta (*4*). In their natural hosts (rodents of the families Muridae and Cricetidae), hantaviruses establish a persistent infection, which causes no apparent harm (*5*). In humans, however, hantaviruses cause 2 diseases: hemorrhagic fever with renal syndrome (HFRS) in Eurasia, and hantavirus (cardio)pulmonary syndrome in North and South America (*6*). Each year worldwide, 60,000–100,000 HFRS cases are reported, mostly from the People's Republic of China (*7*).

To date, 7 sero/genotypes of hantaviruses have been identified in China (*8*). Of these, only Hantaan virus (HTNV), carried by *Apodemus agrarius* mice, and Seoul virus (SEOV), carried by *Rattus norvegicus* rats, cause HFRS (*8*–*11*). Despite intensive measures implemented in the past 3 decades, HFRS remains a major public health problem in China (*10*).

Incidence and Mortality Rates

HFRS-like disease was described in Chinese writings ≈1,000 years ago. Then in the early 1930s, HFRS cases among Japanese soldiers in northeastern China were reported (*9*). Subsequently, HFRS cases have been reported each year in China, >30,000 cases during 1931–1949 (*9*). Since 1950, HFRS has been listed as a class B notifiable disease. Before 1982, HFRS cases were defined by a national standard of clinical criteria; and starting in 1982, cases were also confirmed by detection of antibodies against hantavirus in patients' serum samples. Serious epidemics occurred during the 1980s and 1990s (*9*,*10*). During the 58-year period of 1950–2007, a total of 1,557,622 HFRS cases were reported in China (Figure 1, panel A). Only a few cases were reported in the beginning of the 1950s, after which the number gradually increased. The first peak was reported in 1964 (3,520 cases; 0.5 cases/100,000 population), and then the number declined gradually to only 1,139 cases (0.1 cases/100,000 population) in 1969. HFRS cases again increased in the beginning of the 1970s. During 1970–1979, a total of 143,949 cases were reported, representing a >6-fold increase over the number reported in the 1960s (23,824). The actual number of HFRS cases might be even higher for these periods because the reporting system was suboptimal and knowledge of pathogen source, transmission

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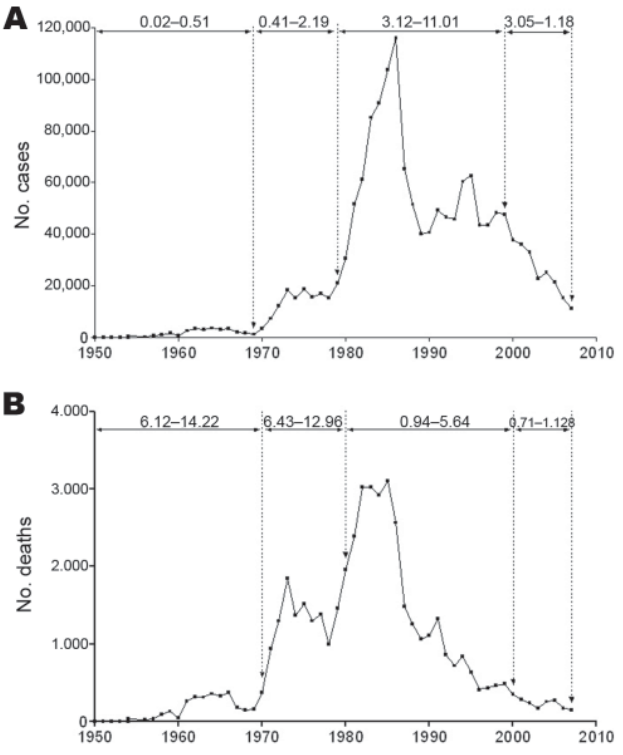

Figure 1. Annual numbers of hemorrhagic fever with renal syndrome (HFRS) cases (A) and HFRS-caused deaths (B) reported in China, 1950–2007. Incidence rates are cases/100,000 population. Mortality rates are shown at top.

routes, and diagnostics was poor. The third peak was reported in 1986, when 115,804 cases were reported (11.1 cases/100,000 population), the largest annual number of HFRS cases during the 58-year period. During 1980–1989, a total of 696,074 cases were reported. During the 1990s, the total number of cases was reduced to 488,135 (29.9% reduction from cases in 1980–1989); the annual number of cases fluctuated between 40,000 and 62,754. The fourth peak was reported during 1994–1995, at >60,000 cases/year. Since 2000, the annual number of HFRS cases has declined >3-fold, from 37,814 in 2000 to 11,248 in 2007.

The number of deaths from HFRS during these same 58 years, 1950–2007, totaled 46,427 (Figure 1, panel B); median death rate was 3%. Death rates varied substantially, from 14.2% in 1969 to 5.6% in 1981. The high death rates reflected not only the severity of HFRS caused by HTNV but also the poor knowledge of how to treat it. Death rates declined gradually, from 4.9% in 1982 to 2.7 % in 1991, then to 1% in 1995, and have remained at ≈1% during the past decade (1996–2007). The accumulated knowledge about HFRS and improved diagnostics and treatment have dramatically increased survival rates. In addition, the gradual change in the disease structure (proportions of mild and severe disease) might have contributed to the decreased mortality rates as well. In recent decades, as rats (*Rattus* spp.) followed human activities and migration from rural to urban areas during the fast socioeconomic development in China, the proportion of mild HFRS cases caused by SEOV steadily increased while the proportion of more severe cases associated with HTNV infection decreased (*9*,*10*,*12*–*14*). Increased awareness of diagnosis, treatment, and prevention also contributed to the decrease of the more severe cases.

Geographic Distribution

Before 1950, HFRS cases had been reported in only 2 provinces (*9*), Heilongjiang and Jilin, which are located in northeastern China and share borders with Russia and North Korea, respectively. By the end of the 1950s, sporadic HFRS cases were reported in 8 provinces, spreading southward from northeastern to eastern and central China. By the end of the 1960s, HFRS cases were noted in 18 provinces; by the end of the 1970s, in 19 provinces; and during the 1980s and 1990s, in 27 and 28 provinces in southern and southwestern China, respectively. Especially after the discovery of SEOV in 1981 (*15*), HFRS distribution became nationwide; only 3 provinces (Qinghai, Xizang, and Xinjiang) remained unaffected (Figure 2). During 2000–2007, HFRS cases declined dramatically (Figure 2, panel C). Since 2005, however, 2 HFRS cases have been reported in Qinghai Province, where HFRS had never been found before; further studies are needed to clarify whether these 2 cases were indigenous or imported.

Although HFRS cases have been found in 29 provinces, the disease remains more prevalent in Shandong, Heilongjiang, Jilin, Liaoning, Hebei, Jiangsu, Zhejiang, Anhui, Henan, Jiangxi, Hubei, Hunan, Shaanxi, Sichun, and Guizhou provinces (Figure 2). Cases in these 15 provinces account for ≈95% of all HFRS cases reported since 1950, and each province reported >1,000 cases during 1990–1999. Most HFRS cases occurred in Shandong Province, which had 23.7%, 7.9%, 36.1%, and 27% of cases in China in 1985, 1995, 1990 and 2000, respectively. Since implementation of comprehensive preventive measures in 1981, incidence of HFRS has substantially decreased in Anhui, Guizhou, Henan, Hubei, Hunan, Jiangsu, Jiangxi, Shangdong, and Sichuan provinces in the past 8 years but remains high in Heilongjiang, Jilin, Liaoning, Hebei, Shaannxi, Shangdong, Inner Mongolia, and Zhejaing provinces (Figure 2, panel C).

Although during the past decade, the annual incidence of HFRS has been gradually decreasing in China, the disease has emerged in areas where it had not been reported during the periods of high prevalence (1980–1990s), such as the Bayannaoer District of Inner Mongolia (*14*). In addition, incidence of HFRS caused by SEOV has been high in some cities, e.g., Beijing and Shenyang (*10*,*16*,*17*).

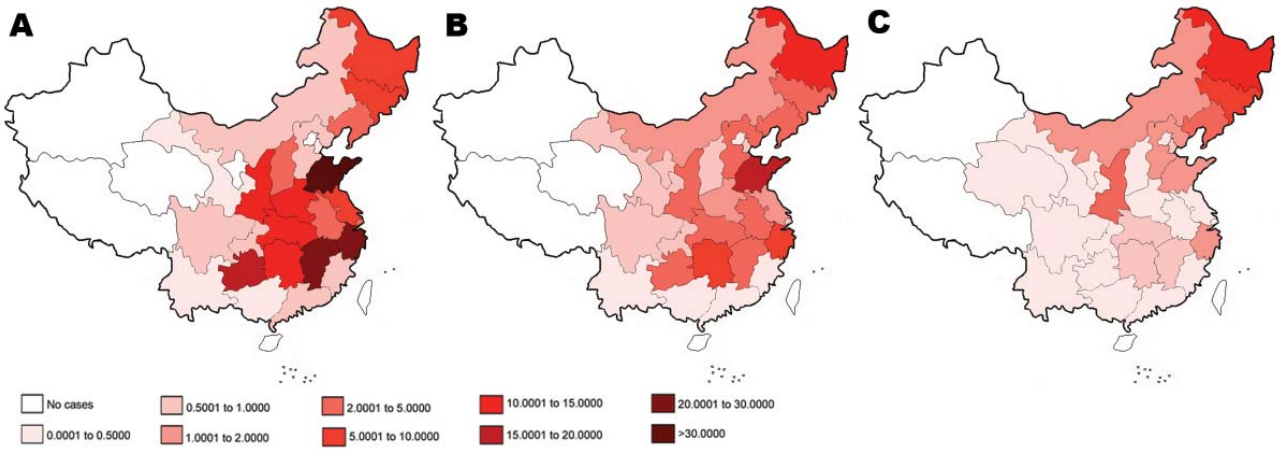

Figure 2. Geographic distribution and annual incidence of hemorrhagic fever with renal syndrome in China in 1986 (A), 1996 (B), and 2006 (C).

Epidemiology

Infections in Humans

For humans, individual HFRS cases as well as outbreaks are influenced by natural (e.g., ecologic) and occupational factors (*3*,*18*,*19*). Many hantavirus infections occur in persons of low socioeconomic status because of poor housing conditions (*6*). In China as well, occurrence and epidemics of HFRS are influenced by natural and social factors (*10*,*14*,*20*–*22*). HFRS cases occur mainly in the northeastern, eastern, central, and southwestern parts of China (humid and semihumid zones) and rarely in the northwestern part (arid zone) (Figure 2). Rural areas account for >70% of HFRS cases; i.e., mainly peasants are infected (*9*,*10*). Poor housing conditions and high rodent density in residential areas seem to be responsible for most HFRS epidemics.

The increase of HFRS from the end of the 1970s coincided with the fast socioeconomic development that started in 1978 in China. During the 1980s and 1990s, China underwent large changes such as agricultural development, irrigation engineering, urban construction, mining, and highway and railway construction. These activities might increase human contact with rodents. Because rats are more mobile than other hantavirus hosts (*4*), fast socioeconomic development also causes wide dispersion of rats and SEOV (*23*), which might subsequently lead to the high nationwide prevalence of SEOV infections. However, improved housing conditions, improved hygiene, and human migration from rural areas to cities might contribute the declining trend of HFRS cases since 2000.

Generally, HFRS cases occur throughout the year and increase in winter and spring (*9*,*10*,*12*). Early epidemiologic investigations found that the winter peak resulted from HTNV carried by striped field mice and that the larger spring epidemic was mainly caused by SEOV carried by Norway rats (*12*).

The age of HFRS patients is all inclusive (from infancy to >65 years), but mostly adolescents and young adults are affected (*9*,*10*,*12*). During 1997–2003, among 265,691 HFRS cases reported and confirmed by epidemiologic surveys, 4.2% were in children ≤14 years of age, 91.2% were in persons 15–64 years of age, ≈4.5% were in persons ≥65 years of age, and 0.1% were of persons of unknown age. Of these patients, 70.63% were male.

In addition to hantavirus transmission to humans from wild rodents, HFRS outbreaks associated with laboratory animals have been reported in China (*17*,*24*). In 1983, laboratory rats accounted for hantavirus transmission, resulting in 16 HFRS cases in 1983 in Shanxi Province (*25*). Since then, dozens of hantavirus infections associated with laboratory rodents have occurred (*17*) and increased during recent years (*26*). For example, in 2006 an outbreak of HFRS among students in Shenyang was caused by SEOV that had been circulating in local wild rats (*R. norvegicus*) and was transmitted to humans through laboratory rats (*17*). Because SEOV is prevalent in urban areas of China, surveillance of hantavirus infection in laboratory rodents and management of laboratory animal centers should be reinforced to prevent laboratory-associated cases of HFRS.

Infections in Animals

Species diversity of rodents and insectivores in China is remarkable (*27*). A total of 171 species of rodents, which belong to 10 subfamilies, have been found; the subfamilies Murinae and Microtinae contain 38 and 43 species, respectively. In addition, at least 32 species of insectivores are present. These rodent and insectivore species are distributed nationwide. In particularly, *A. agrarius* and *R. norvegicus* rodents, the reservoir hosts of HTNV and SEOV,

Table 1. Major rodent species captured in China, by location and years*

	Fields, %			Residential areas, %		
Species	1984–1990	1991–1995	1996–2000	1984–1990	1991–1995	1996–2000
Apodemus agrarius	57.362	44.508	37.012	3.425	4.411	3.944
Apodemus peninsulae	3.761	8.870	1.516	0.011	0.137	0.125
Apodemus chevrieri	0.318	2.475	4.053	NC	1.820	2.282
Rattus norvegicus	5.081	8.358	10.112	49.106	53.025	54.266
Rattus losea	7.302	6.401	22.457	0.141	0.289	0.087
Rattus flavipectus	1.089	2.674	2.284	13.527	10.969	9.395
Rattus nitidus	0.225	0.158	0.042	0.006	0.007	0.067
Niviventer confucianus	0.775	0.339	0.987	0.138	0.078	0.062
Niviventer fulvescens	1.062	0.164	0.184	0.001	NC	NC
Mus musculus	4.635	5.889	4.472	28.625	21.045	20.984
Cricetulus barabensis	3.883	3.946	1.781	0.427	0.171	0.067
Cricetulus longicaudatus	1.124	0.769	NC	0.033	NC	NC
Cricetulus migratorius	0.784	1.097	1.120	0.342	0.022	0.766
Cricetulus triton	7.532	8.065	6.282	1.458	3.911	2.657
Meriones meridianus	0.513	NC	NC	NC	NC	NC
Meriones erythrourus	0.445	0.070	0.884	0.053	NC	0.004
Myodes rutilus	0.230	0.091	0.558	0.030	0.022	0.017
Myodes rufocanus	0.185	0.319	0.194	0.035	0.037	0.092
Microtus fortis	0.469	1.340	1.368	0.005	NC	NC
Microtus maximowiczii	0.274	0.219	0.174	0.073	0.056	0.046
Microtus arvalis	0.176	0.155	0.052	NC	NC	0.208
Anourosorex squamipes	0.429	0.342	NC	0.005	2.024	3.919
Crocidrua lasiura	0.418	0.202	0.161	0.012	0.041	0.062
Crocidrua horsfieldi	0.006	0.544	0.026	NC	0.204	0.042
Suncus murinus	0.354	0.427	1.520	1.826	0.489	0.183
Sorex araneus	0.219	0.187	0.303	0.044	0.037	0.258

*Data from Chen and Luo (*23*). Hantavirus antigens had been identified in all species listed except *Anourosorex squamipes* mole shrews and *Microtus arvalis* voles. NC, not captured.

are the predominant species (Table 1) and have been found in 28 and 30 provinces, respectively, in China.

National HFRS surveillance data (1984–2000) and the nationwide geographic epidemiologic investigation of HFRS (1984–1987) have detected hantavirus antibodies or antigens in 67 species of vertebrates (*23*,*28*). Of those, 38 species of rodents and 8 species of insectivores had been found to contain hantavirus antigen. Hantavirus infection has been reported for several species of domestic animals (e.g., cats, pigs, rabbits, dogs) as well. Most recently, we found hantavirus antigen in lung tissue of midday jirds (*Meriones meridianus*), which belong to family Muridae, subfamily Gerbilinae, which have not been known to carry hantaviruses (*14*). Thus, yet-unidentified hantaviruses may be circulating in China.

During 1984–2000, a total of 167,540 small animals were trapped in the wild (mostly *A. agrarius* mice) and 184,096 rodents were trapped in residential areas (mostly

Table 2. Hantaviruses circulating in China, 1981–2008

Virus, rodent	Human disease*	Distribution within China
Hantaan		
Apodemus agrarius mouse	HFRS (severe)	Nationwide except Xinjiang and Jilin provinces
A. peninsulae mouse	HFRS (severe)	Nationwide except Xinjiang and Jilin provinces
Seoul		
Rattus norvegicus rat	HFRS (mild)	Nationwide except Qinhai, Xinjiang, Xizang provinces
R. rattus rat	HFRS (mild)	Eastern and southwestern parts
R. flavipectus rat	HFRS (mild)	Eastern, central, southern, and southwestern parts
R. losea rat	HFRS (mild)	Southern and southwestern parts
R. nitidus rat	HFRS (mild)	Eastern, central, southern, and southwestern parts
Da Bie Shan, *Niviventer confucianus* rat	Unknown	Anhui Province
Hokkaido, *Myodes rufocanus* vole	Unknown	Jilin Province
Khabarovsk, *Microtus maximowiczii* vole	Unknown	Inner Mongolia
Vladivostok, *Microtus fortis* vole	Unknown	Jilin and Liaoning provinces
Yuanjiang, *M. fortis* vole	Unknown	Hunan Province

*HFRS, hemorrhagic fever with renal syndrome.

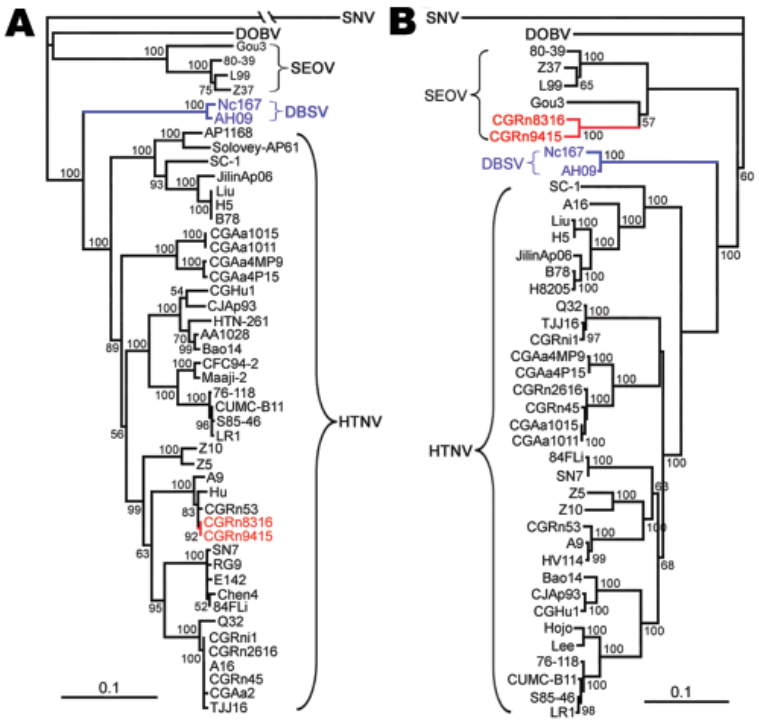

Figure 3. Phylogenetic trees of Hantaan virus (HTNV) variants according to the small segment (A) and medium segment (B) coding sequences. PHYLIP program package version 3.65 (http://helix.nih.gov/Applications/phylip.html) was used to construct the phylogenetic trees; the neighbor-joining method was used. Bootstrap values were calculated from 1,000 replicates; only values >50% are shown at the branch nodes. The trees constructed using the maximum-likelihood method (not shown) had similar topology. Scale bars indicate nucleotide substitutions per site. Colors (blue and red) highlight viruses of interest from China. SNV, Sin Nombre virus; DOBV, Dobrava-Belgrade virus; SEOV, Seoul virus; DBSV, Da Bie Shan virus.

R. norvegicus rats), as recorded by national surveillance centers (*23*) (Table 2). Of the small animals, 10,238 had hantavirus antigen. *A. agrarius* mice accounted for 44.9% (3,270/7,282) of hantavirus antigen–positive animals collected during 1984–1990, 59.55% (970/1,629) of those collected during 1991–1995, and 32% (424/1,327) of those collected during 1996–2000. *R. norvegicus* rats accounted for 37.2% (2,707/7,282), 23.9% (390/1,629), and 43 (570/1,327) of hantavirus antigen–positive animals during corresponding periods. Hantavirus-positive *A. agrarius* mice have been found in all parts of China except Xinjiang Province, and hantivirus-positive *R. norvegicus* rats have been found in most provinces except Qinghai, Xinjiang, and Xizang.

Hantavirus Isolates

Antigenic and genetic studies of hantaviruses isolated from HFRS patients and rodents in China found 3 hantaviruses in China: HTNV, SEOV, and Da Bie Shan virus carried by Chinese white-bellied rats (*Niviventer confucianus*) (Table 2, Figure 3) (*11*–*13*). Recently, we found Puumala virus-like Hokkaido virus in *Myodes rufocanus* voles (*29*), Khabarovsk virus in *Microtus maximowiczii* voles, Vladivostok virus in *Microtus fortis*, subspecies *pelliceus* voles (*30*), and a presumably novel Yuanjiang virus in *M. fortis*, subspecies *calamorum* voles (*8*). So far, only HTNV and SEOV are known to cause HFRS in China (*8*–*11*). Because *A. agrarius* and *R. norvegicus* rodents are the predominant carriers and are distributed nationwide, HTNV and SEOV are obviously the major threat for HFRS in China.

HTNV was first isolated from striped field mice in 1981 (*31*). Consistent with the geographic distribution of *A. agrarius* mice, HTNV has been found in all Chinese provinces except Xinjiang (*11*,*12*,*22*,*28*,*32*). In addition to *A. agrarius* mice, HTNV has been also found in *Apodemus peninsulae* mice in northeastern China (*33*). Genetic analysis of the small (S) and medium (M) genome segments suggested that at least 9 distinct lineages of HTNV are circulating in China (Figure 3) (*11*,*32*). Generally, HTNV variants display geographic clustering. Recently, we detected reassortment between HTNV and SEOV in *R. norvegicus* rats in Guizhou Province (Figure 3) (*34*), which indicates that genetic reassortment occurs naturally between 2 hantavirus types. Because reassortment is a way for segmented viruses to achieve high infectivity and adapt to new animal hosts, further studies are warranted to evaluate susceptibility of *A. agrarius* and *R. norvegicus* rodents to these unique reassortant viruses and to determine whether these reassortants can infect humans.

HFRS cases caused by SEOV were first reported in Henan and Shanxi provinces along the Yellow River in China (*15*). Subsequently, SEOV (strain R22) was isolated from *R. norvegicus* rats in Henan (*35*), and SEOV has been found in almost all provinces of China except Qinghai, Xinjiang, and Xizang (*11*,*14*,*23*,*28*). SEOV-associated

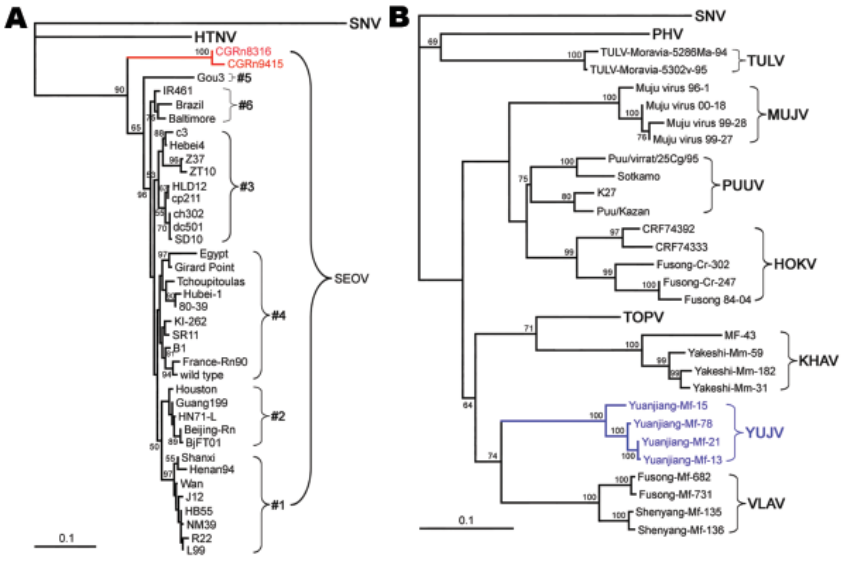

Figure 4. Phylogenetic tree of Seoul virus (SEOV) variants according to partial (nt 2001–2301) medium segment sequences (A). Phylogenetic tree of hantaviruses according to complete coding sequences of the medium segment (B). PHYLIP program package version 3.65 (http://helix.nih.gov/Applications/phylip.html) was used to construct the phylogenetic trees; the neighbor-joining method was used. Bootstrap values were calculated from 1,000 replicates; only values >50% are shown at the branch nodes. The trees constructed by using the maximum-likelihood method (not shown) had similar topology. Scale bars indicate nucleotide substitutions per site. Colors (blue and red) highlight viruses of interest from China. SNV, Sin Nombre virus; HTNV, Hantaan virus; PHV, Prospect Hill virus; SEOV, Seoul virus; TULV, Tula virus; MUJV, Muju virus; PUUV, Puumala virus; HOKV, Hokkaido virus; KHAV, Khabarovsk virus; YUJV, Yuanjiang virus; VLAV, Vladivostok virus.

HFRS seems to have recently spread to areas where it had not been reported during previous epidemics (*14*). Most known SEOV variants (from lineages 1–4 and 6), including those from China, Brazil, Japan, South Korea, North America, and the United Kingdom, are genetically homogeneous (Figure 4, panel A). Lineages 1–4 are widely distributed and do not follow a geographic clustering pattern. Thus, the variants from lineages 1–4 and 6 are closely related and may share a more recent common ancestor. Because *R. norvegicus* rats are distributed nationwide (*27*,*28*) and are more mobile than other hantavirus hosts (*4*), SEOV has become the largest threat for public heath in China and may bring even more potential threats to humans as rat species become more widespread along with globalization of the economy. Natural HFRS cases caused by SEOV have been found almost exclusively in China and other Asian countries. Lack of HFRS in other countries may result from better living conditions, low rat densities, and low SEOV-carrying rates by the rats.

In 2000, Da Bie Shan virus was isolated from Chinese white-bellied rats captured in the Da Bie San mountainous area of Anhui Province (*11*). Although white-bellied rats are widely distributed in China (*27*), Da Bie Shan virus has not yet been found outside this area. Serologic and genetic analyses have shown it to be distinct from HTNV and other known hantaviruses (*11*). According to current taxonomy, Da Bie Shan virus is a provisional, novel hantavirus species. Whether this virus can be transmitted to humans and cause HFRS remains unknown.

Previous investigations found hantavirus antigens in *M. rufocanus*, *M. fortis*, *M. maximowiczii*, and other voles (*9*,*12*,*23*,*28*). Recently, we recovered the S and M segment sequences from *M. rufocanus* voles trapped in Fusong, Jilin Province (Figure 4, panel B) (*29*). Phylogenetic analysis of these sequences revealed that they belong to Hokkaido virus, which was first identified in *M. rufocanus* voles in Hokkaido, Japan (*36*), and form a distinct lineage. Whether Hokkaido virus and other Puumala-like viruses (e.g., Muju virus) are pathogenic in humans is not known, but the possibility cannot be excluded because Puumala virus carried by bank voles (*M. glareolus*) in Europe causes a milder form of HFRS, nephropathia epidemica.

Our recent study found Khabarovsk and Vladivostok viruses in China (Figure 4, panel B) (*30*). The virus isolated from reed voles in Fusong County (Jilin Province) is closely related to Vladivostok virus, whereas the virus isolated from the Maximowiczi vole in Yakeshi (Inner Mongolia) is closely related to Khabarovsk virus. These results suggest that *M. fortis* voles are the natural host for Vladivostok virus and that *M. maximowiczii* voles are the natural host for Khabarovsk virus.

Further molecular investigation showed that hantaviruses detected in *M. fortis* (subsp. *dolichocephalus*) voles from Shenyang belong to Vladivostok virus and form a distinct lineage on the phylogenetic trees on the basis of the S and M segment sequences (Figure 4, panel B) (*8*). Complete S segment and partial large (L) segment sequences from the virus identified in *M. fortis* (subsp. *calamorum*) voles from Yuanjiang (Hunan Province) were distinct from those of Shenyang and Fusong variants; they had up to 18% nucleotide and 5% amino acid sequence divergences. Moreover, partial M segment sequences (nt 2676–3650) from the Yuanjiang variant were even more divergent from Shenyang and Fusong variants (>20% and 8%, respectively). Thus, our results suggest that the hantavirus from

M. fortis calamorum voles in Yuanjiang represents a novel hantavirus species, Yuanjiang virus. These data also demonstrate impressive genetic diversity and complexity of the *M. fortis* vole–associated hantaviruses in China.

Hantaviruses are thought to have coevolved with their respective hosts. Each serotype and/or genotype of hantavirus appears to be primarily associated with 1 (or a few closely related) specific rodent host species (*4*). As described above, >100 species of rodents and several dozens of insectivores are widely distributed in HFRS-endemic areas in China (*27*). Hantavirus-specific antibodies and/or antigens have been identified in at least 38 rodent species (*23*,*28*). Therefore, in addition to already known HTNV, SEOV, Da Bie Shan virus, Hokkaido virus, Khabarovsk virus, Vladivostok virus, and Yuanjiang virus, yet-unknown hantavirus species may be circulating in China. In-depth studies on hantavirus distribution through different geographic regions and hosts in China as well as genetic characterization of hantaviruses and elucidation of the relationship among them and between these viruses and other known hantaviruses should help prevent and control the diseases they cause.

Control and Prevention

To control and prevent HFRS in China, a comprehensive preventive strategy has been implemented and includes public health education and promotion, rodent control, surveillance, and vaccination (*10*). Surveillance of hantavirus infection in rodents could help with organization of an advanced warning service for possible increases in human infections. In 1984, a national surveillance system was established on mainland China (*12*). Each province to which HFRS is endemic has at least 1 surveillance laboratory; the number depends on the severity of HFRS. According to a request from the Chinese Center for Disease Control and Prevention (previously Chinese Academy of Preventive Medicine), studies have been conducted to determine 1) the number of HFRS cases, 2) the list of local small animal species (including their density in nature and in residential areas), and 3) hantavirus prevalence in rodent and human populations. The system has provided systemic epidemiologic knowledge of hantavirus infection in China.

Inactivated hantavirus vaccine was developed after HTNV and SEOV were successfully isolated and propagated in A-549 cells (*31*,*37*,*38*). Inactivated hantavirus vaccine was first approved in 1993 and, since 1995, has been used in areas where HFRS is highly endemic. Four hantavirus vaccines based on inactivated HTNV and SEOV have been widely used and demonstrated to be safe and efficacious (Table 3) (*39*). Every year, ≈2 million vaccine doses are used. Purified bivalent vaccine for HTNV and SEOV cultured in Vero cells has been used since 2003 (*40*). From 2008, hantavirus vaccine has been included in the national Expanded Program on Immunization. For persons in areas in which HFRS is highly endemic, the vaccination is free of charge.

The most effective way to control hantavirus diseases is to reduce human exposure to infected rodents and their excrements. Since the 1950s on mainland China, the rat population has been controlled by using poison bait or trapping around residential areas. During the 1980s and 1990s, deratization around residential areas effectively decreased both rodent density and incidence of HFRS, especially the disease caused by SEOV (*23*,*28*). In addition, the minimization of food availability for rodents around residential areas effectively reduced rodent populations.

Improving general awareness and knowledge of pathogen source, transmission routes (how to avoid contact with a pathogen), diagnostics, vaccination, and general hygiene is one of the most effective and economic ways to prevent infectious diseases. Since the 1970s, public education on HFRS and other infectious diseases has been conducted by all possible means in China, especially in rural areas.

Conclusions

At least 7 geno/serotypes of hantaviruses are circulating in rodents in China, and, as better tests are developed, more not-yet identified hantaviruses may be found in rodent or insectivore species. Therefore, a better understanding of hantavirus infection ecology and epidemiology would be beneficial for controlling the disease in humans.

Environmental and social economic changes may affect the geographic distribution, abundance, and dynamics of rodent carriers and, hence, the epidemiology of hantavirus infections. Over the past few decades, recognition and understanding of hantavirus infection in China have greatly improved. Although HFRS was highly epidemic during the 1980s and 1990s, the incidence has dramatically declined during the past 8 years as a result of comprehensive preventive measures and improved living conditions. HFRS-associated mortality rates also decreased dramatically. However, the total number of HFRS cases

Table 3. Inactivated hantavirus vaccines used in China, 1995–2010

Virus	Cell culture used	Vaccination procedure*	Protection, %
Hantaan	Mongolian gerbil kidney	3 + 1	>90
Seoul	Golden hamster kidney	2 + 1	>95
Hantaan	Suckling mouse brain	3 + 1	>90
Hantaan/Seoul	Mongolian gerbil kidney	2 + 1	>95
Hantaan/Seoul	Vero	2 + 1	>85†

*No. doses + no. boosters.
†Seroconversion rate of human hantavirus antibody determined by plaque-reduction neutralization test.

and the number of deaths are the highest in the world, and China still has a long way to go to control hantavirus infection in humans.

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Clostridium difficile Bacteremia, Taiwan[1]

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Learning Objectives

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

- Identify presenting symptoms of *Clostridium difficile* bacteremia (CDB).
- Specify the most common source of bacteremia in cases of CDB and incorporate that knowledge into the development of effective management plans..
- Describe the prognosis of CDB.

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To determine clinical characteristics and outcome of patients with *Clostridium difficile* bacteremia (CDB), we identified 12 patients with CDB in 2 medical centers in Taiwan; all had underlying systemic diseases. Five had gastrointestinal diseases or conditions, including pseudomembranous colitis (2 patients); 4 recalled diarrhea, but only 5 had recent exposure to antimicrobial drugs. Ten available isolates were susceptible to metronidazole and vancomycin. Five isolates had *C. difficile* toxin A or B. Of 5 patients who died, 3 died of CDB. Of 8 patients treated with metronidazole or vancomycin, only 1 died, and all 4 patients treated with other drugs died (12.5% vs. 100%; p = 0.01). *C. difficile* bacteremia, although uncommon, is thus associated with substantial illness and death rates.

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Clostridium difficile is well recognized as the etiologic agent of pseudomembranous colitis and has been implicated as the cause of 10%–25% of cases of antimicrobial drug–associated diarrhea (*1*). The pathogen has been responsible for numerous recent hospital-based epidemics and is also emerging in the community (*2*). The clinical features, disease spectrum and pathogenesis, and optimal treatments of *C. difficile*–associated diarrhea have been well studied. In contrast, reports of the isolation of *C. difficile* in body sites other than the intestines, or extraintestinal infections, have been anecdotal (*3*,*4*). Extracolonic manifestations of *C. difficile* infections reported were variable, including bacteremia, osteomyelitis, visceral abscess, empyema, reactive arthritis, pyelonephritis, prosthetic joint infection, and skin and soft tissue infection (*3*–*10*). Most

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cases of extracolonic *C. difficile* infections have been preceded by gastrointestinal events, either *C. difficile* colitis or surgical and anatomic disruption of the colon (*4*).

Recently, Libby and Bearman reviewed the literature on bacteremia caused by *C. difficile* (*6*). Most cases were identified from individual case reports. However, as the incidence of *C. difficile* infection increases, an increase in cases of *C. difficile* bacteremia (CDB) is likely (*10*). Knowledge of the clinical signs and symptoms of these extracolonic manifestations of bloodstream infections will be useful in patient care and could improve clinical outcomes (*4*). To outline the spectrum and clinical significance of CDB, we report 12 cases of CDB over a recent 10-year period at 2 medical centers in Taiwan and review the literature published in English.

Patients

Patients with blood cultures positive for *C. difficile* at 2 teaching hospitals (National Cheng Kung University Hospital, a 1,100-bed tertiary care hospital in southern Taiwan and National Taiwan University Hospital, a 2,800-bed tertiary care hospital in northern Taiwan) during January 1989 through February 2009, were retrospectively identified from laboratory records, and their medical records were reviewed. If a patient experienced >1 episode of CDB, only information about the first episode was included. Information about age, sex, underlying diseases, clinical course, antimicrobial drug therapy, and clinical outcome was recorded in a case-record form.

We conducted a literature review to find relevant articles published between January 1, 1962, and August 31, 2009, by querying the PubMed database using the keywords "*Clostridium difficile*," "bacteremia," "sepsis," and "bloodstream infection." The references of available articles were surveyed for additional cases.

Definitions

Underlying conditions were stratified on the basis of the McCabe and Jackson score (*11*) and the comorbidity score of Charlson et al. (*12*). Severity of illness was evaluated on the first day of bacteremia onset by means of the Acute Physiology and Chronic Health Evaluation II Score (*13*), Simplified Acute Physiology Score II (*14*), and the Pittsburgh bacteremia score (*15*). The physiologic response to bacteremia was categorized as severe sepsis if the patient met the criteria for severe sepsis specified by the American College of Chest Physicians/Society for Critical Care Medicine Consensus Conference Committee (*16*). Immunosuppressive therapy was defined as the receipt of corticosteroid treatment (10 mg/day or an equivalent dosage) for >2 weeks of antineoplastic chemotherapy or antirejection medication within 30 days before admission. Sepsis-related death was defined as the death of a patient with a clinical course that suggested persistently active infection if the patient had no other obvious explanation for the death.

Bacteremia was defined as the presence of an organism in a blood culture specimen. Clinically significant bacteremia was defined as ≥1 positive blood culture and clinical features compatible with fever and sepsis syndrome; patients with noteworthy bacteremia were included in this study. An episode of bacteremia was considered to be hospital acquired if a bacteremic episode was noted at >48 hours after hospitalization; healthcare associated if it occurred within 48 hours of hospitalization in patients with extensive contact with the healthcare system (such as nursing home residence, organ transplantation, hemodialysis dependence, presence of an indwelling intravascular catheter, or surgery within the previous 30 days), or the patients had been transferred from another hospital or long-term care facility; or community acquired if it occurred ≤48 hours of admission in patients without extensive healthcare contact. In the case of secondary bacteremia, a primary focus of infection was defined according to the criteria of the Centers for Disease Control and Prevention (*17*).

Antimicrobial drug therapy in the preceding 30 days was documented through a review of medical records. Previous antimicrobial drug therapy was defined as the receipt of an oral or parenteral antimicrobial agent for ≥72 hours within the preceding 2 months.

Identification of Isolates and Clonality

Bacteria colonies suspected of being *C. difficile* on the basis of characteristic odor, typical morphologic features, and Gram staining results were phenotypically identified by using standard methods (*18*) and the API 32A system (bioMérieux, Las Halles, France). These organisms were further confirmed to the species level by using partial 16S rRNA gene sequence analysis. Two primers were used: PS13 5′-GGAGGCAGCAGTGGGGAATA-3′ and PS14 5′-TGACGGGCGGTGTGTACAAG-3′, as described (*19*). The partial sequences obtained (529 bp) were compared with published sequences in the GenBank database by using the BLASTN algorithm (www.ncbi.nlm.nih.gov/blast). Molecular typing of these isolates by pulsed-field gel electrophoresis (PFGE) using *Sma*I (New England Biolabs, Beverly, MA, USA)–digested DNA and repetitive-element PCR typing (DiversiLab Kits for *C. difficile*; bioMérieux) were performed to identify the clonality of the isolates (*20*,*21*). For PFGE, gels were run for 22 h at 13°C by using a CHEF DR-III system (Bio-Rad Laboratories, Hercules, CA, USA) at 5.5 V/cm with initial and final pulse times of 5 s and 60 s, respectively (*20*). The production of *C. difficile* toxin A or B was detected by a qualitative enzyme immunoassay (Premier toxin A&B; Meridian Bioscience, Inc., Cincinnati, OH, USA).

Antimicrobial Drug Susceptibility Testing

MICs of 14 antimicrobial drugs (penicillin, piperacillin/tazobactam, ampicillin/sulbactam, cefmetazole, meropenem, imipenem, doripenem, ertapenem, metronidazole, vancomycin, teicoplanin, fusidic acid, clindamycin, and daptomycin) were determined by the agar dilution method described by the Clinical and Laboratory Standards Institute (*22*). MICs of tigecycline were measured by the broth microdilution method. The antimicrobial agents used for susceptibility testing were obtained from their corresponding manufacturers. The MIC breakpoints followed those recommended by the Clinical and Laboratory Standards Institute (*22*) or the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org).

Statistical Analysis

The results were analyzed with SPSS software for Windows, version 13.0 (SPSS, Chicago, IL, USA). Continuous variables were expressed as mean ± SD, and categorical variables were expressed as percentages of total number of specific patients analyzed. Categorical variables were compared by the Fisher exact test or χ^2 test, as appropriate. Continuous variables were compared by the Mann–Whitney U or Student *t* test. All tests for statistical significance were 2-tailed, and p values <0.05 were considered significant.

Twelve patients, 7 from National Taiwan University Hospital and 5 from National Cheng Kung University Hospital, with CDB during the period of the study were identified. Most (11) patients were identified after 2000. The partial 16S rRNA gene sequence analysis of 10 available isolates identified by conventional methods showed that they most closely matched *C. difficile* isolates (accession number FN545816.1; maximal identity: 100% [529/529]). Only 10 isolates were available, and the earliest isolate was obtained in 2005. Their genotyping patterns determined by the repetitive-element PCR and PFGE were different, indicating that no intrahospital or interhospital spread of *C. difficile* isolates occurred in the 2 hospitals.

The epidemiologic and clinical data of the 12 patients with *C. difficile* bacteremia are shown in Tables 1 and 2. Their mean ± SD age was 59.9 ± 22.1 years (range 12–87 years). Women (7 cases) outnumbered men. All patients had chronic medical illnesses, particularly diabetes mellitus (4) and cirrhosis of the liver (6). Eleven patients had no underlying or rapidly fatal diseases, according to the McCabe and Bearman classification, and the mean comorbidity score of Charlson et al. was 5.2 (SD 2.2). At the time of bacteremia onset, the mean Acute Physiology And Chronic Health Evaluation II Score or Simplified Acute Physiology Score II score was 25.4 (SD 0.9) or 50.0 (SD 25.2), respectively. Seven patients had critical illness defined as having at least 4 points of the Pittsburgh bacteremia score (*15*). Five patients were considered to have community-onset CDB, but all had a history of recent hospitalization or referral from a chronic care facility and thus all of their infections can be classified as healthcare associated. . The average length of hospital stay before CDB was 16.4 days. Primary bacteremia noted in 7 patients was the most common infection noted, followed by intraabdominal infections (4, 33%), bone and joint infections (1, 8.3%), and skin and soft-tissue infections (1, 8.3%).

When patients were first seen, their signs and symptoms included fever (9, 75%), abdominal pain (6, 50%), and leukocytosis, which was defined as a leukocyte count ≥12,000 cells/μL (7, 58.3%). Of note, 5 (41.7%) patients had gastrointestinal disorders or conditions at the time of bacteremia onset. Four (33.3%) had a documented episode of diarrhea before CDB, and 2 patients with diarrhea had endoscopically documented pseudomembranous colitis. Five (41.7%) patients had recent exposure to antimicrobial drugs. Six patients had concurrent bacteremia caused by pathogens other than *C. difficile* (Table 2).

Five (50%) of 10 available isolates showed a positive result for the *C. difficile* toxin assay. All isolates were susceptible to metronidazole and vancomycin (Table 3). Other antimicrobial agents demonstrated variable in vitro antibacterial activity against *C. difficile* isolates. In contrast to imipenem, ertapenem, and doripenem, meropenem showed excellent in vitro activity with a narrow MIC range (0.12 μg/mL–2 μg/mL). Of these isolates, 90% of 10 isolates were resistant to penicillin, and 30% were resistant to clindamycin. Tigecycline and daptomycin, 2 newly marketed drugs for which a recommended breakpoint for *C. difficile* has not yet been determined, show favorable antibacterial activity with low 90% MIC values, 0.06 μg/mL and 2 μg/mL, respectively.

Before microbiologic information was available, 6 patients received antimicrobial therapy, metronidazole (3 patients), vancomycin (2), or cefmetazole (1). Overall, only 1 of 8 patients definitively treated with metronidazole or vancomycin died; in contrast, all 4 patients treated by drugs other than metronidazole or vancomycin died (p = 0.01). Of 5 patients who died, 3 died directly of CDB. Seven patients survived and were discharged, and 1 patient was treated surgically for septic prosthetic hip arthritis. The survivors remained in the hospital for a mean of 28 days (range 12–47 days) after diagnosis of CDB.

Literature Review

Twenty reports of patients with CDB have been published in English in the literature since 1962 (*3*,*5*–*10*,*23*–*30*). The epidemiologic and clinical data are summarized in Table 2. Most (14) patients had polymicrobial bacteremia; concomitant gastrointestinal diseases or conditions of varying severity were noted in 15 patients. Excluding a patient

with unknown outcome, 10 of 19 patients died in the hospital. The results of stool toxin assay were available for 12 patients, and clostridial toxin A or B could be detected in 8 patients. Drug information was available for 16 patients. Seven patients (3 of whom died) were treated with metronidazole, and 7 were treated with vancomycin (3 of these patients died). The crude death rate for these published cases was similar to that in the present study (10/19, 52.6% vs. 5/12, 41.7%; p = 0.72).

Conclusions

A clear clinical picture of *C. difficile* bacteremia has been highlighted in this study: a rare extracolonic *C. difficile* infection with severe illness and high death rates in persons with chronic medical illness. However, the case-patients with CDB had several remarkable clinical characteristics, compared with those of patients with *C. difficile*–associated diarrhea. First, only 64% of 22 bacteremic *C. difficile* isolates had toxin A or B, the virulence factors of *C. difficile*–associated diarrhea. Second, bacteremic pa-

Table 1. Clinical manifestations, antimicrobial drug therapy, and outcome of 12 patients with *Clostridium difficile* bacteremia, Taiwan, 1989–2009*

Patient no.	Age, y/sex	Clinical signs and symptoms	Sources of bacteremia	Coexisting condition(s)	Copathogen(s)	Clostridial toxin assay result	Treatment/ outcome
Monomicrobial bacteremia							
1	69/F	Dead on arrival	Primary bacteremia	Liver cirrhosis	None	Positive	None/died
2	38/M	Abdominal pain	IAI (primary peritonitis)	Wilson disease	None	Negative	Cefmetazole for 22 d/died
3	65/F	Fever, abdominal pain	IAI (secondary peritonitis)	Perforated peptic ulcer with exploratory laparotomy	None	NA	Metronidazole† for 10 d/died
4	58/M	Fever, abdominal pain	IAI (primary peritonitis)	Liver cirrhosis	None	Negative	Metronidazole† for 12 d/survived
5	12/M	Fever, dyspnea	Primary bacteremia	Biliary atresia, liver transplantation	None	Negative	Piperacillin-tazobactam and vancomycin† for 15 d/survived
6	41/F	Fever, dyspnea	Primary bacteremia	Pulmonary fibrosis	None	Negative	Ceftazidime and gentamicin for 13 d; vancomycin† for 10 d/survived
Polymicrobial bacteremia							
7	45/M	Abdominal pain	Primary bacteremia	Liver cirrhosis	Coagulase-negative *Staphylococcus* spp.	Positive	Ceftriaxone for 3 d/died
8	83/M	Fever, bloody stool	Primary bacteremia	Gastrointestinal bleeding, hypovolemic shock	*Escherichia coli*	Negative	Imipenem for 1 d/died
9	87/F	Bloody stool	Primary bacteremia	Congestive heart failure, end-stage renal disease, pseudomembranous colitis	*Pseudomonas aeruginosa*, *Enterococcus faecium*, *E. coli*, ESBL–*Klebsiella oxytoca*	Positive	Vancomycin† and meropenem for 7 d/survived
10	80/F	Bloody stool	Primary bacteremia	Liver cirrhosis, pseudomembranous colitis	Coagulase-negative *Staphylococcus* spp.	Positive	Metronidazole† for 13 d/survived
11	66/F	Fever, lower gastrointestinal bleeding, abdominal pain	SSTI/septic arthritis	Femoral neck fracture (received total hip replacement with prosthetic infections), chronic kidney disease	*Enterobacter cloacae*	Negative	Debridement, cefepime and metronidazole† for 12 d/survived
12	75/F	Fever, chills, nausea, vomiting, abdominal pain	IAI (primary peritonitis)	Lymphoma, biliary tract infection	*K. pneumoniae*, *Clostridium perfringens*	NA	Cefepime for 10 d and metronidazole† for 7 d/survived

* IAI, intra-abdominal infection; NA, not available; SSTI, skin and soft tissue infection; ESBL, extended-spectrum β-lactamase.
†In vitro active against *C. difficile*.

tients did not commonly have diarrhea before or at admission to the hospital, and in patients with CDB, a history of recent antimicrobial drug exposure (a common predisposing factor of *C. difficile*–associated diarrhea) was rarely recognized.

Because *Clostridium* species are inhabitants of the gastrointestinal tract, they compromise the integrity of the gastrointestinal tract and may lead to translocation with bacteremia. This translocation is supported by the fact that 75% of 32 patients had certain gastrointestinal symptoms or disorders, which may predispose to CDB either by direct extension through perforation or by promoting translocation of the organism across the intestinal wall (*10*). These findings are consistent with the literature, in which *C. difficile* bacteremia is typically associated with underlying gastrointestinal pathology and frequently occurs as a mixed infection with other gut flora (*6*,*31*,*32*).

Clostridium species have been shown to enhance the pathogenicity of other bacteria in mixed infections (*10*), and even monomicrobial *Clostridium* bacteremia has been associated with a high death rate in 2 previous series (*10*,*33*). Half of the patients in the current series had polymicrobial bacteremia. The portal of entry of *Clostridium* species in cirrhotic cases is usually obscure, and the gastrointestinal tract is the most likely source (*34*,*35*). Translocation of intestinal bacteria is the major mechanism for the produc-

Table 2. Summary of clinical characteristics of 12 patients with *Clostridium difficile* bacteremia in the current series from Taiwan, 1989–2009, and of 20 additional cases published since 1962*

Characteristic	Total, n = 32	Reported cases, n = 20	Current series, n = 12	p value
Age, y, mean ± SD	51.4 ± 26.1	46.2 ± 27.5	59.9 ± 22.2	0.14
Elderly, age ≥60 y	18 (56.3)	11 (55.0)	7 (58.3)	1.0
Male	19 (59.4)	14 (70.0)	5 (41.7)	0.15
Place of acquisition				0.70
Community	12/28 (42.9)	5/16 (73.3)	7 (58.3)	
Hospital	16/28 (57.1)	11/16 (26.7)	5 (41.7)	
Calendar year range				0.001
1962–1990	8 (25)	8 (40.0)	0	
1991–2000	8 (25)	7 (35.0)	1 (8.3)	
After 2000	16 (50)	5 (25.0)	11 (91.7)	
Comorbidity				
Malignancy	9 (28.1)	6 (30.0)	3 (25.0)	1.0
Congestive heart failure	4 (12.5)	3 (15.0)	1 (8.3)	1.0
Immunosuppression	7 (21.9)	2 (10.0)	5 (41.7)	0.07
Chronic obstructive pulmonary disease	3 (9.4)	2 (10.0)	1 (8.3)	1.0
Chronic kidney disease	3 (9.4)	1 (5.0)	2 (16.7)	0.54
Liver cirrhosis	7 (21.9)	1 (5.0)	6 (50.0)	0.006
Cerebrovascular accident	2 (6.3)	1 (5.0)	1 (8.3)	1.0
Diabetes mellitus	4 (12.5)	0	4 (33.3)	0.014
Organ transplant	2 (6.3)	0	2 (16.6)	0.13
None	6 (18.8)	6 (30.0)	0	0.06
Clinical signs and symptoms				
Fever	19/27 (70.4)	10/15 (66.7)	9 (75.0)	0.70
Abdominal pain	14/29 (48.3)	8/17 (47.1)	6 (50.0)	1.0
Diarrhea	12/28 (42.9)	8/16 (50.0)	4 (33.3)	0.46
Gastrointestinal disease or condition	10/28 (35.7)	5/16 (31.3)	5 (41.7)	0.7
Gastrointestinal bleeding	5/27 (18.5)	0/15 (0.0)	5 (41.7)	0.01
Gastrointestinal perforation	3/28 (10.7)	2/16 (12.5)	1 (8.3)	1.0
Pseudomembranous colitis	5/27 (18.5)	3/15 (20.0)	2 (16.7)	1.0
Recent antimicrobial drug exposure	18/26 (69.2)	13/14 (92.9)	5 (41.7)	0.009
Sources of bacteremia				
Primary	17 (53.1)	10 (50.0)	7 (58.3)	0.73
Intraabdominal infection	11 (34.4)	7 (35.0)	4 (33.3)	1.0
Urosepsis	2 (6.3)	2 (10.0)	0	0.52
Skin and soft tissue infection	2 (6.3)	1 (5.0)	1 (8.3)	1.0
Bone and joint infection	1 (3.1)	0	1 (8.3)	0.38
Polymicrobial bacteremia	20 (62.5)	14 (70.0)	6 (50.0)	0.29
Positive clostridial toxin assay result	14/22 (63.6)	9/12 (75.0)	5/10 (50.0)	0.42
Crude death rate	15/31 (48.4)	10/19 (52.6)	5 (41.7)	0.72

*Values are no. patients (%) or no. patients/total no. patients evaluated (%), except for age. Patients may have >1 morbidity, clinical sign or symptom, and gastrointestinal disease or condition.

Table 3. In vitro antimicrobial drug susceptibilities of 10 bacteremic *Clostridium difficile* isolates, Taiwan, 1989–2009*

Antimicrobial agent	MIC, μg/mL			Resistance breakpoint, μg/mL	No. (%) resistant isolates
	Range	MIC_{50}	MIC_{90}		
Vancomycin†	0.5–1	0.5	1	≥2	0
Metronidazole	0.25–8	1	4	≥32	0
Clindamycin	0.03–>256	4	>256	≥8	3 (30)
Penicillin	0.25–8	4	4	≥2	9 (90)
Ampicillin/sulbactam	0.5–8	2	4	≥32/16	0
Cefmetazole	0.25–32	16	32	≥64	0
Meropenem	0.12–2	1	2	≥16	0
Imipenem	0.12–16	8	16	≥16	4 (40)
Ertapenem	0.06–16	8	8	≥16	0
Doripenem	0.12–8	4	4	NA	NA
Daptomycin	0.12–2	0.5	2	NA	NA
Tigecycline	0.03–0.12	0.03	0.06	NA	NA
Fusidic acid	1–8	1	2	NA	NA

*MIC_{50}, 50% MIC; MIC_{90}, 90% MIC, NA, not available.
†Vancomycin MIC breakpoint was recommended by the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org); otherwise by the Clinical and Laboratory Standards Institute (*22*).

tion of bloodstream infection (*34*). In our study, all patients had >1 preexisting illness, and most (75%) experienced gastrointestinal diseases or conditions. Substantial damage to normal mucosal barriers may provide a portal for anaerobes (*32*,*35*), which suggests that patients with gastrointestinal disorders, such as gastrointestinal bleeding and perforation or advanced liver failure, would be susceptible to *C. difficile* bacteremia. Primary bacteremia accounted for more than half (17, 53.1%) of the 32 cases of CDB. The likelihood of undiagnosed *C. difficile* colitis in these patients seems to be minimal given the absence of diarrhea and radiographic manifestation of colitis shown on computed tomographic scan. Subsequently, the resultant monomicrobial bacteremia was likely secondary to bacterial translocation in the setting of immunologic deficiency from an overwhelming preexisting illness (*2*,*6*,*31*,*36*).

Antimicrobial drug therapy for bacteremia and other extracolonic *C. difficile* infections varies greatly in the literature (*3*,*4*) because most infections have been polymicrobial in nature, and antimicrobial therapy has been directed at all organisms (*4*,*6*). Thus, optimal therapy for monomicrobial *C. difficile* bacteremia remains undefined. Intravenous vancomycin or metronidazole was frequently used in cases of bacteremia with well-described treatment regimens. Inappropriate antibacterial therapy for anaerobes, in general, appears to have serious consequences for patients (*32*,*35*,*37*). Based on in vitro antimicrobial drug susceptibility data, metronidazole may be the drug of choice for *C. difficile* bacteremia, although vancomycin was also effective in our patients. Clinical experiences in treating severe *C. difficile* infections were limited and mainly focused on *C. difficile*–associated diarrhea (*1*,*2*,*38*).

CDB, although uncommon, can be observed in persons with chronic underlying illness or coexisting gastrointestinal illnesses and is associated with high death rates. In the present study, a major finding was that the survival rate among patients who received appropriate antimicrobial therapy (either metronidazole or vancomycin) was higher than that among patients who received inappropriate antimicrobial drug therapy. Although multiple factorial conditions contributed to the high death rates among patients with CDB, insufficient therapeutic coverage for *C. difficile* may have contributed to the deaths of the patients. Early treatment of CDB with metronidazole or vancomycin may be helpful.

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Responses to Pandemic (H1N1) 2009, Australia

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In 2007, adults in Australia were interviewed about their willingness to comply with potential health interventions during a hypothetical influenza outbreak. After the first wave of pandemic (H1N1) 2009 in Australia, many of the same respondents were interviewed about behavior and protection measures they actually adopted. Of the original 1,155 respondents, follow-up interviews were conducted for 830 (71.9%). Overall, 20.4% of respondents in 2009 had recently experienced influenza-like illness, 77.7% perceived pandemic (H1N1) 2009 to be mild, and 77.8% reported low anxiety. Only 14.5% could correctly answer 4 questions about influenza virus transmission, symptoms, and infection control. Some reported increasing handwashing (46.6%) and covering coughs and sneezes (27.8%) to reduce transmission. Compared with intentions reported in 2007, stated compliance with quarantine or isolation measures in 2009 remained high. However, only respondents who perceived pandemic (H1N1) 2009 as serious or who had attained higher educational levels expressed intention to comply with social distancing measures.

The World Health Organization (WHO) declared a public health event of international importance on April 24, 2009, after recognition of a novel pandemic influenza virus strain, pH1N1, now called pandemic (H1N1) 2009 virus, which caused serious disease and deaths in Mexico and other parts of North America. This declaration triggered an immediate response in Australia; national pandemic plans were implemented, and the public was alerted to the risk and the activities that could keep them from contracting and spreading the infection.

Imported cases of pandemic (H1N1) 2009 were first identified in Australia on May 7, 2009, and within a month, local transmission had been identified in all 8 states and territories (*1*,*2*). By September 1, 2009, of Australia's population of 22 million, 154 had died and 4,440 had been hospitalized for pandemic (H1N1) 2009 (*3*). Within the first 2 months of the outbreak, the Australian Commonwealth instituted 3 management phases: delay, contain, and protect. Each phase required different messages to the public and healthcare workers (*1*,*2*,*4*). Ensuring consistent implementation through Australia's 3 government levels—national, state, and local—was challenging. The delay phase was aimed at preventing pandemic (H1N1) 2009 from arriving in Australia and focused attention on border control and communication with international travelers. However, after local transmission was recognized and the disease became established in Australia, the contain phase was implemented with an emphasis on identifying cases and tracing contacts. Those with confirmed pandemic (H1N1) 2009 infection and their contacts were actively managed by using isolation, home quarantine, antiviral medication, and enhanced infection control practices to reduce the spread of disease. Finally, when it became clear that pandemic (H1N1) 2009 infection in Australia was less severe than initially considered and that the workload was adversely affecting the provision of health services, the protect phase was implemented and the public health response was changed to early detection and management of infection in persons from recognized risk groups. The change in focus (from aggressively tracking new infections to treating all persons in Australia with influenza-like illness [ILI] to concentrating on those in high-risk groups) presented a major risk-communication challenge for health authorities.

The success of the pandemic management plan in Australia depends critically on public compliance with health measures (*5*,*6*). A study completed in 2007 found that a high proportion of respondents reported willingness to accept a range of public health measures (although the scenario provided in that study was a more severe pandemic) (*7*). In that study, 1,166 (58.0%) of 2,012 adults contacted

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participated in the survey. Nearly all (1,155) agreed to be available for future related research.

The 2009 pandemic provided a unique opportunity to conduct a follow-up study to compare respondents' previously reported willingness to adopt public health measures with their experiences during and after pandemic (H1N1) 2009 in Australia. We thus conducted a study during the protect phase, from August 20 through September 11, 2009, almost 4 months after the WHO declaration and 1 month after the peak of reported hospitalizations from the first wave of pandemic (H1N1) 2009 in Australia (*1*,*2*,*4*). We sought to identify the level of public knowledge concerning measures required to contain pandemic influenza spread, social impact of the pandemic wave, effectiveness of communication, compliance with control measures instituted by public health authorities, and relationships among these parameters.

Methods

Study Protocol and Participants

In the original 2007 study, the sample was selected in a randomized manner from printed telephone directories (for 2007) by using a quota system that ensured good representation from across the country. Eligible persons were ≥18 years of age, could converse in English, and provided verbal consent. Thus, in the current (2009) study, the youngest possible participant was 20 years of age.

Of the 1,155 participants in the 2007 study who had agreed to be involved in future research, 43 were excluded from the 2009 study because they had died, were unable to communicate, or had moved and were untraceable; 197 were not reachable at their recorded telephone number; and 85 refused to participate. Thus, 830 (71.9%) persons were successfully interviewed in the 2009 study. Ethics approval was obtained from the University of Newcastle's Human Research Ethics Committee (approval no. H-2009–0288).

In each study, data were collected by computer-assisted telephone interview, and an introductory letter was sent to households a week before telephone contact was attempted. Interviews took an average of 14 minutes to complete and were conducted on weekdays and Saturdays between 9:00 AM and 8:00 PM local time. The surveys were conducted by professional telephone interviewers who had extensive experience collecting public health data. A rigorous training program, which included dummy interviewing and written interviewing protocols, encouraged a consistent approach. As many as 10 attempts were made to contact each person in the database. The script for the compliance questions was identical to that used in 2007, but other questions were tailored to the new pandemic. In the survey, we chose to identify the novel influenza disease as "swine flu" because this was the term most commonly used in the media in Australia at the time of study and was accepted by the public.

Scope of Interview

Interviewers asked structured questions related to respondents' recent experience with pandemic (H1N1) 2009. Knowledge questions asked about pandemic (H1N1) 2009 transmission, its symptoms, and infection control; and 1 question gauged comprehension of the global situation. A variety of questions assessed anxiety, impact, behavior, compliance with public health advice, and access to and perceived success of communications. The interviews included closed- and open-ended questions. Questions regarding knowledge provided true or false response options; the situational awareness question was multiple choice with 3 options; questions about impact were answered on a scale of 4 levels (nil, little, quite, and extremely); and questions about perceptions encouraged open-ended answers that were subsequently coded. To assist implementation of a national vaccination program in Australia, we also investigated respondents' willingness to accept pandemic (H1N1) 2009 influenza vaccine and specific related concerns; these results are reported elsewhere (*8*).

Statistical Analyses

Analyses were conducted by using base SAS and SAS/STAT components of SAS 9.13 statistical software (SAS Institute Inc, Cary, NC, USA). Odds ratios and χ^2 tests were used to look for significant associations between sex, age group, perception of severity and educational status, and willingness to comply with public health interventions. A stepwise multivariate analysis that included variables of statistical significance was used. The sample was weighted to the age–sex distribution of the adult population of Australia by using June 2008 data projections (*9*).

Results

Demographics for the 830 respondents closely resembled those of the resident population of Australia during June 2008. Demographics for the 2009 study participants did not differ significantly from those of the 2007 study participants (*7*). Among the 2009 study population, 75.7% lived in urban areas, 20.8% in rural areas, and 3.3% in remote areas. Women (62.3%) and older age groups were moderately overrepresented. More information on the sample demographics is available from the earlier study report (*7*).

Knowledge

We asked 4 questions about knowledge of pandemic (H1N1) 2009 transmission, symptoms, and infection control measures: 1) almost everyone (99.4%, 825/830) knew that "handwashing and using a tissue to cover your mouth when coughing are practical ways of reducing the spread

of flu," 2) 17.2% (143/830) were unaware that "swine flu spreads very easily in the community," 3) 44.9% (373/830) incorrectly considered that "cough and rash are typical of swine flu," and 4) 30.2% (251/830) incorrectly reported that "swine flu never seriously affects people who have good health." Overall, only 14.5% (120/830) answered all 4 questions correctly, 48.9% (406/830) answered 3 correctly, and 30.8% (256/830) answered 2 correctly.

Pandemic (H1N1) 2009 situational awareness was investigated with a question aimed at determining how well the Australian public understood the extent of the outbreak. At the beginning of the study period, WHO had reported 177,457 confirmed cases from 170 countries and territories and noted that this understated the true number because testing had ceased in many countries (*10*). Only 56.5% (469/830) appreciated that there had been >100,000 cases around the world at the time of the study; 24.1% (200/830) answered that there had only "been ≈10,000 cases mainly affecting people in Mexico, the United States and the United Kingdom"; 8.1% (67/830) indicated that there had "been ≈10,000 swine flu cases mainly reported from Australia"; 11.2%, 93/830 reported that they did not know how many cases there had been; and 1 refused to answer.

Impact

Having experienced ILI ("fever, cough and tiredness") during the pandemic period was reported by 20.4% (169/830) of respondents; of these, 8.1% (67/830) had obtained a medical diagnosis but only 0.2% (2/830) had had their condition confirmed as pandemic (H1N1) 2009 by laboratory testing. The average duration of ILI was 9.2 days (median 6 days, interquartile range 4–10 days).

Most (77.7%, 645/830) respondents perceived pandemic (H1N1) 2009 as a mild disease or an only occasionally severe disease, 20.2% (168/830) considered it either mostly or always severe, and 2.0% (17/830) did not know. Most (77.8%, 646/830) reported being only a little or not concerned that they or a member of their family may become infected, 5.3% (44/830) were extremely concerned, and 16.9% (140/830) were quite concerned. In terms of risk perception, 25.4% (211/830) of respondents considered themselves to be in a group at risk for more severe illness or higher likelihood of infection. In terms of disruption as a result of public health containment measures enacted during the containment phase, most (94.5%, 784/830) respondents experienced no or only minor disruption, 4.0% (33/830) moderate disruption, 1.2% (10/830) major disruption, and 0.3% (3/830) were unsure of the effect these measures had on their lives.

Personal Protection

Respondents described specific behavioral changes that they had adopted to reduce the transmission of pandemic (H1N1) 2009. Increased handwashing was reported by 46.6% (387/830) and covering coughs and sneezes by 27.8% (231/830). Only 8.7% (72/830) had purchased masks, 6.0% (50/830) reported having worn a mask in public, 3.3% (27/830) said they had "purchased (not just been prescribed) an antiviral drug such as Tamiflu or Relenza," and 12.4% (103/830) indicated that they had "spent more time than usual cleaning the house."

Compliance with Public Health Containment Measures

The 2007 study used a scenario describing a future influenza pandemic as a potentially severe event. The interviewer then asked questions about willingness to comply with a range of public health containment measures. Although compliance with public health requests for self and community quarantine measures remained high during the 2009 survey, stated compliance with key social distancing activities significantly decreased (Table 1).

Compliance was analyzed by age group, gender, highest educational level achieved, experience of ILI in the past 3 months, stated degree of concern, performance on knowledge questions, and self-determined risk group. All factors were included in a multivariate logistical regression model; statistically significant findings are shown in Table 2.

Women were significantly more likely to agree to home quarantine if requested by public health officials. Those in the oldest age group (>61 years) and those who had experienced ILI were also more likely to agree to local quarantine, such as remaining within town limits when high influenza activity is evident. Perceptions of anxiety and level of education were associated with willingness to avoid public events; level of anxiety was also associated with willingness to avoid social gatherings and with wearing a mask to control influenza.

Communication

Slightly more than one third (288/830) of respondents reported that they had sought information on pandemic (H1N1) 2009 during the first pandemic wave in Australia. The most common sources were general practitioners (19.3%, 160/830), other healthcare workers (9.5%, 79/830), and government websites (13.3%, 110/830). The public health department was contacted by 4.8% (40/830), and the national health hotline was used by only 3.1% (26/830). Most (61.6%, 511/830) respondents reported not "actively searching for news on swine flu in the media," although 7.8% (65/830) sought a daily update.

Most (69.3%, 575/830) respondents thought that "health authorities had provided sufficient information on swine flu," 18.1% (150/830, including 12 who replied that they had not seen or heard any information) reported that there had not been enough information, 9.9% (82/830) reported that there had been too much information, and 2.8% (23/830) were

Table 1. Adults' reported willingness to comply with public health authority requests with regard to influenza pandemic, Australia*

Public health request	% (no.) willing to comply† 2007, n = 1,166	2009, n = 830	OR (95% CI)	p value
Home quarantine for 1 wk if exposed	97.5 (1,137)	96.0 (797)	1.62 (0.95–2.80)	0.059
Local quarantine of an affected area	95.2 (1,103)‡	94.6 (785)	1.13 (0.74–1.72)	0.555
Avoid public events for 1 mo	98.3 (1,146)	82.8 (687)	11.93 (7.35–20.28)	<0.001
Avoid social gatherings for 1 mo	97.2 (1,133)	62.7 (520)	20.47 (14.01–30.67)	<0.001
Wear a surgical mask in public	95.1 (1,109)	72.4 (601)	7.41 (5.42–10.25)	<0.001

*OR, odds ratio; CI, confidence interval.
†2007 responses were to hypothetical pandemic; 2009 responses were to actual pandemic (H1N1) 2009.
‡Because responses were not collected from 7 respondents, n = 1,159.

unsure. To assess whether the media campaign had been effective, we asked the 818 who had received media information whether it had changed any of their behavior. More than half the respondents (53.9%, 441/818) reported having "paid more attention to covering coughs and sneezes," 47.1% (385/818) had "increased the frequency of handwashing," and 43.8% (358/818) had "stayed at home when sick to reduce spreading the disease."

During the protect phase, which was declared on June 17, 2009, the Australian Commonwealth Government conducted a national information campaign (paid television, radio, and print advertisements). To determine the effect of this media campaign, which had been conducted 2 months after the onset of the emergency and after local and state messages had been broadcast, we asked participants whether they had seen or heard any of the advertisements and whether these had specifically prompted any change in their behavior. We found that 19.5% (162/830) of respondents neither heard nor saw any of these media messages, 14.3% (119/830) had noticed them every day, 44.1% (366/830) noticed them about once a week, 19.2% (159/830) noticed them about once a month, and 2.9% (24/830) were unsure how often they had noticed them. Of those who had seen advertisements, 88.2% (568/644) said that the information had little or no effect on their behavior. Multivariate analysis indicated that awareness of the information provided in the Commonwealth's promotional campaign was not associated with willingness to comply with public health containment measures. When we asked whether "health authorities should post hygiene messages at bus terminals, train stations and airports,

Table 2. Predicted compliance with public health authority requests with regard to influenza pandemic, Australia, 2009*

Variable	Home quarantine for 1 wk if exposed OR (95% CI)	p value	Local quarantine of an affected area OR (95% CI)	p value	Avoid public events for 1 mo OR (95% CI)	p value	Avoid social gatherings for 1 mo OR (95% CI)	p value	Wear a surgical mask in public OR (95% CI)	p value
Sex										
F	2.34 (1.21–4.53)	0.012								
M	1.00									
Age range, y										
20–40	0.30 (0.10–0.89)	0.030	0.29 (0.12–0.74)	0.010	0.98 (0.58–1.65)	0.930			0.56 (0.37–0.87)	0.009
41–60	0.37 (0.12–1.12)	0.078	0.34 (0.13–0.87)	0.024	0.57 (0.35–0.95)	0.031			0.58 (0.37–0.90)	0.014
≥61	1.00		1.00		1.00				1.00	
Personally experienced ILI during pandemic										
Yes			6.94 (2.10–22.95)	0.001					1.70 (1.09–2.64)	0.019
No			1.00						1.00	
Concerned										
Quite/ extremely					4.72 (2.60–8.54)	<0.001	2.63 (1.75–3.95)	<0.001	3.31 (2.12–5.18)	<0.001
A little					2.82 (1.86–4.29)	<0.001	1.49 (1.09–2.06)	0.014	2.85 (2.00–4.04)	<0.001
Not					1.00		1.00		1.00	
Highest education level										
Tertiary†					1.82 (1.23–2.68)	0.003			1.88 (1.37–2.59)	0.001
Other					1.00				1.00	

*Multivariate logistic regression analysis for 830 respondents, sample weighted to the age and sex distribution of the population of Australia (*9*). Only significant results are shown. OR, odds ratio; CI, confidence interval; ILI, influenza-like illness.
†University or professional qualifications.

such as avoiding travel when sick, and covering coughs and sneezes," 95.4% (792/830) of respondents answered affirmatively.

Discussion

It is widely accepted that the first wave of the 2009 influenza pandemic in Australia resulted mostly in a relatively mild disease with little of the forecasted social and economic consequences factored into prepandemic planning or relayed in prepandemic media messages (*1*,*2*,*4*,*11*). However, valuable lessons can be learned from the management of this pandemic and applied in the future. Public perceptions formed during the pandemic wave may influence future responses to public health disasters and must be considered when preparing future risk communication strategies.

Despite the considerable media attention to the pandemic, a high proportion of adults in Australia poorly understood the fundamental aspects of transmission, symptoms, and impact. Most persons did not actively seek information on pandemic (H1N1) 2009, but when they did seek information, they were most likely to turn to a general practitioner or other healthcare worker or a government website rather than use the national health hotline that was set up to ease the pressure on health professionals. This finding is in contrast with use of the UK call center, "NHS Direct," which experienced a heavy load of calls (*12*). There is clearly a need to improve basic health literacy through educational initiatives in schools, public health awareness campaigns, and other creative methods, and to more effectively channel enquiries away from those working on the front lines during emergencies.

We found that stated willingness to comply with social distancing requests decreased significantly in 2009 compared with 2007. However, multivariate analysis indicated that acceptance of public health containment measures was statistically more likely among those experiencing a higher level of concern, an observation supported by other researchers (*13*). The reduced level of compliance reported in the 2009 study likely resulted from the perceived mildness of disease and may result in less cooperation during future pandemic waves or other health emergencies. However, translating risk perception into behavior change is challenging (*14*). In a world constantly threatened by emerging infectious diseases, promoting effective risk communication strategies that accurately inform the public and health professionals of appropriate behavior changes that can be made to mitigate personal and community risk is essential (*15*). In addition, interview responses suggested a need for a closer tailoring of risk communication mechanisms and messages to adequately inform person's responses under the stress of emergency conditions (*16*,*17*).

Respondents may have considered pandemic (H1N1) 2009 to be a mild disease because the perceived focus of health officials was on simple containment measures such as increased handwashing and covering sneezes rather than sophisticated approaches, which may have created an impression of a trivial threat. Perception of risk governs the level of response; thus, the appropriate risk level must be communicated, although difficult to achieve particularly in the early stages when epidemiologic data are lacking (*17*–*19*). During a health emergency such as a pandemic, all levels of the health system must ensure that consistent messages are relayed to the public and clearly explain the value of proposed interventions.

Because 20% of respondents reported that they had not seen any of the advertisements during the multimedia blitz and 88% of those who did see them claimed that the advertising had little or no effect on their behavior, we conclude that the intensive media promotion did not substantially influence infection control behavior. It is possible that those who were likely to change their behavior had already done so as a result of earlier messages delivered through media statements from local health officials. This possibility supports findings from the 2007 study that indicated that persons seem to place most trust in state or regional health representatives for their disaster health information rather than national identities; thus, greater emphasis on local messaging may be merited (*7*).

Compared with many parts of the world, Australia is relatively sparsely populated; the preponderance of the population live in large cities and towns on the Eastern Seaboard. We ensured that the study population included a representative cross-section of the adult population of Australia that closely matched the most recently available census information (*9*); persons from inland rural and remote areas were proportionally represented. Women and elderly persons were slightly overrepresented, in keeping with other national computer-assisted telephone interview populations in Australia. To some extent our analysis accounted for this overrepresentation by weighting to the latest population estimates of age and gender. Because the study design was based on telephone contact, only persons with a landline telephone were included in the study. In Australia, landline telephone coverage is generally high (*20*,*21*), but our sample likely underrepresents disadvantaged groups such as indigenous Australians, particularly those who may live in remote communities without landline coverage, and persons with lower incomes who cannot afford a telephone. In addition, children were not included in the survey, yet their knowledge and behaviors may prove valuable in educating adults on preparedness and response (*22*). Those who declined to be interviewed or were excluded because of language and comprehension problems are likely to be more difficult to reach with conventional communication methods, further emphasizing the value of novel and enhanced communication strategies appealing to a broader demographic spectrum.

The gap between what health officials require the public to do in a pandemic and what members of the public are prepared to do seems to be growing. Our findings suggest that discordance between what people say they will do and what they actually do is related to their perception of risk. The public should be equipped with the appropriate knowledge and skills to positively influence their attitudes and behavior during a future pandemic wave or communicable disease event and to enable them to better interpret broadcasted risk assessments. Such a literacy program would be useful for pandemic preparedness, generating appropriate reassurance or concern, and could potentially achieve broader health goals.

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Bat Coronaviruses and Experimental Infection of Bats, the Philippines

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Fifty-two bats captured during July 2008 in the Philippines were tested by reverse transcription–PCR to detect bat coronavirus (CoV) RNA. The overall prevalence of virus RNA was 55.8%. We found 2 groups of sequences that belonged to group 1 (genus *Alphacoronavirus*) and group 2 (genus *Betacoronavirus*) CoVs. Phylogenetic analysis of the RNA-dependent RNA polymerase gene showed that groups 1 and 2 CoVs were similar to Bat-CoV/China/A515/2005 (95% nt sequence identity) and Bat-CoV/HKU9–1/China/2007 (83% identity), respectively. To propagate group 2 CoVs obtained from a lesser dog-faced fruit bat (*Cynopterus brachyotis*), we administered intestine samples orally to Leschenault rousette bats (*Rousettus leschenaulti*) maintained in our laboratory. After virus replication in the bats was confirmed, an additional passage of the virus was made in Leschenault rousette bats, and bat pathogenesis was investigated. Fruit bats infected with virus did not show clinical signs of infection.

Severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) is a newly emerged zoonotic CoV that caused an international epidemic in 2003. Epidemiologic studies have demonstrated that the first human cases of SARS were caused by CoVs closely related to those found in Himalayan palm civets and raccoon dogs in wildlife markets (*1*). This finding accelerated surveys of CoVs specific for various animals in Southeast Asia to identify reservoirs for SARS-CoV. These survey findings suggested that palm civets and raccoon dogs are an intermediate host of, but not a primary reservoir for, SARS-CoV because of the low prevalence of SARS-like CoVs in these animals (*2*). Moreover, a large variety of novel CoVs in these surveys, including bat SARS–like CoVs, were detected in many bat species in the People's Republic of China and Hong Kong Special Administrative Region (*3–6*).

Phylogenetic analysis of bat CoVs and other known CoVs suggested that the progenitor of SARS-CoV and all other CoVs in other animal hosts originated in bats (*5*,*7*). Recently, bat CoVs in North and South America, Europe, and Africa were also reported (*8–12*). Although extensive bat surveys have been conducted, no infectious bat CoVs have been isolated from cell cultures, which hinders characterization of bat CoVs and evaluation of the risks posed by these viruses to public health.

In this study, we detected bat CoVs in the Philippines. We attempted to isolate bat CoVs and virus RNA from cell cultures and from Leschenault rousette bats (*Rousettus leschenaulti*) orally infected with intestinal tissues and contents from a lesser dog-faced fruit bat (*Cynopterus brachyotis*). After infection, clinical signs of infected bats were examined, and pathogenesis in bats was investigated.

Materials and Methods

Bat Collection

We obtained 52 bats of 6 species during July 2008 from Diliman and Los Baños, the Philippines, after receiving permission from the government. All captured bats were anesthetized with an intraperitoneal injection (15 mg/kg) of tiletamine and zolazepam (Virbac, Carros, France) and killed by cardiac exsanguination. The experiment was conducted in accordance with the Guidelines for the Care

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Extraction of RNA and Reverse Transcription

Virus RNA was extracted from samples obtained from field bats and from experimentally infected bats by using an SV Total RNA Isolation System Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Extracted RNA was eluted in 50 μL of RNase-free water. For cDNA synthesis, RNA (5 μL), a random hexamer, and a SuperScript III Kit (Invitrogen, Carlsbad, CA, USA) were used.

PCR and DNA Sequencing

All cDNA samples obtained from field bats and experimentally infected bats were tested by using conventional and nested PCR. On the basis of previous reports, we used a PCR and a pair of consensus primers specific for a highly conserved region of the RNA-dependent RNA polymerase (RdRp) gene (*13*).

Two microliters of cDNA was added to a 25-μL reaction mixture containing 2× GoTaq PCR Master Mix (Promega) and 0.2 μM of 5′-GGT TGGGACTATCCTAAGTGTGA-3′ (primer 1) and 5′-CCATCATCAGATAGAATCATCATA-3′ (primer 2). The PCR conditions were 2 min at 94°C; 35 cycles for 20 s at 94°C, 30 s at 50°C, and 30 s at 72°C; and 1 min at 72°C. PCR amplicons were gel purified by using NucleoSpin Extract II (Machrey-Nagel, Düren, Germany) and cloned by using a TOPO-TA pCR2.1 Cloning Kit (Invitrogen). Sequencing was performed in an ABI 3130 XL DNA analyzer (Applied Biosystems, Foster City, CA, USA).

On the basis of the sequences obtained, we designed new specific primer pairs for the Bat-CoV/Philippines/Diliman1552G1/2008 sequence (5′-TGATTT CTGCAATGATACTTGGTTC-3′ and 5′-ACTTGATGAT CTGTAACAACAATCG-3′) and for the Bat-CoV/Philippines/Diliman1525G2/2008 sequence (5′-TACAAC CTACGCTGCAACTC-3′ and 5′-ATGAGTGTGCACAA GTGCTTAG-3′). These primers were used as the inner primer set for the nested PCR after the first PCR was performed with primers 1 and 2. Aliquots (2 μL) of cDNA for primary amplification were added to 2× GoTaq Master Mix (Promega) and primers 1 and 2. Amplification was performed by using 15 cycles at conditions described above. Aliquots (2 μL) of primary amplification products were used for the second PCR with GoTaq Master Mix and the inner primers. The second PCR was performed by using 35 cycles at the conditions described above. PCR products were extracted from gels by using NucleoSpin Extract II and subjected to direct sequencing or TA cloning.

Bat Samples

Leschenault rousette bats were obtained from zoos in Japan. Seven bats were randomly selected for the experiments. In each experiment, 2 bats were placed in a negative-pressure isolator. One additional bat was kept in a separate isolator as a control. A sample of large intestine from a lesser dog-faced fruit bat (*C. brachyotis*) was homogenized in a sterile mortar. After low-speed centrifugation (2,000 × *g* for 10 min), the supernatant was used for oral infection. Experimentally infected bats were examined daily for clinical signs of infection. Fecal specimens were obtained from a clean translucent plastic sheet spread along the bottom of the cage. All bats were killed after being anesthetized with diethyl ether, and organs (liver, kidney, spleen, lung, brain, and intestine) and serum samples were obtained.

Detection of Virus mRNA in Bats

To determine membrane, nucleocapsid, nonstructural (Ns)7a, Ns7b, and Ns7c protein nucleotide sequences, we conducted PCR and DNA sequencing in the same manner as for determination of partial RdRp nucleotide sequence described above by using the HKU9-Leader42–64 primer (5′-CCGTTTCGTCTTGTACGAATCAC-3′) and the 3siteAd20T primer (5′-CTGATCTAGAGGTACCGGA TCCTTTTTTTTTTTTTTTTTTTT-3′). To detect virus mRNA, we conducted reverse transcription–PCR (RT-PCR) by using 2 primer sets: HKU9-Leader42–64 and N468–448r (5′-GTTACGTGTGCCCATGTCACC-3′) and HKU9-Leader42–64 and Ns7a440–420r (5′-CAAGCCA CAACAACATTAGG-3′).

Quantitative Real-Time RT-PCR

cDNA synthesis was performed by using 0.5 μL total RNA and the PrimeScript RT Reagent Kit (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. Virus RNA was quantified by using Power SYBR Green PCR Master Mix (Applied Biosystems) with 2 μL of reverse-transcribed cDNA. Quantitative real-time PCR was performed by using the Thermal Cycler Dice System (TaKaRa). The temperature program consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The primer pair for the real-time PCR was designed on the basis of the partial RdRp sequences of Bat-CoV/Philippines/Diliman1525G2/2008; primers used were 5′-TCCTAAGTGTGATAGAGCTATGCC-3′ and 5′-GTGCACACTCATTTGCTAACCG-3′. In each experiment, 10-fold serial dilutions of plasmid DNA containing the partial RdRp gene were tested in duplicate to establish a standard curve for calculating the relative amount of RNA in each sample. All samples were analyzed at least 3 times. To confirm the specificity of each PCR product, we conducted a melting curve analysis immediately after

the amplification phase of each PCR. The amount of RNA in each sample was expressed as the average value (copy number per weight [milligrams] of sample).

Results

Virus Detected

During July 2008, a total of 52 bats were obtained at 3 locations in Diliman and 1 location in Los Baños, the Philippines (Table 1). RT-PCRs for a 440-bp fragment in the RdRp gene of CoVs were performed for large intestine samples, including intestinal contents; 9 (17.3%) of 52 bats were positive. Differences in the 440-nt sequence in the RdRp region were determined after TA cloning of the 9 positive samples. Sequences indicated that the 2 groups of sequences obtained belonged to group 1 CoV (genus *Alphacoronavirus*) (n = 4) and group 2 CoV (genus *Betacoronavirus*) (n = 5).

A 440-bp consensus nt sequence of the group 1 CoV was obtained on the basis of alignment of 4 group 1 CoV sequences detected (>98% nt identity with each other) and deposited in GenBank as Bat-CoV/Philippines/Diliman1552G1/2008 (DNA Database of Japan [DDBJ] accession no. AB539080). BLAST (www.ncbi.nlm.nih.gov/BLAST) search findings of GenBank indicated that the partial RdRp sequence was most similar to that of Bat-CoV/China/A515/2005 (95% nt identity).

A 440-bp consensus nt sequence of group 2 CoVs was also obtained (>98% nt identity with each other) and deposited in GenBank as Bat-CoV/Philippines/Diliman1525G2/2008 (DDBJ accession no. AB539081). A BLAST search suggested that the partial RdRp sequence was novel but most similar to that of Bat-CoV/HKU9–1/China/2007 (83% nt identity). A phylogenetic tree was constructed with the partial RdRp-deduced amino acid sequence (120 aa) and available sequences of known CoVs (Figure 1). Data in the tree suggested that Bat-CoV/Philippines/Diliman1552G1/2008 belonged to group 1b CoVs and Bat-CoV/Philippines/Diliman1525G2/2008 belonged to group 2d to CoVs.

Specific and nested primer pairs for group 1b bat CoV and group 2d bat CoV sequences were designed, and nested PCR was performed by using cDNAs of all samples. Twenty additional amplicons (≈200-bp sequences) were obtained by using primers specific for group 2d bat CoVs. After direct sequencing or TA cloning, partial sequences of all amplicons obtained were found to be nearly identical to group 2d bat CoVs (>98% nt identity) and resulted in a total CoV prevalence of 55.8% (Table 1). All sequences of group 1b bat CoVs were obtained from insectivorous bats (4/7, 57.1%), and all sequences of group 2d bat CoVs were obtained from frugivorous bats (25/45, 55.6%).

Virus in Cell Cultures

Cytopathic effects were not observed in any of the cells (Vero E6, Vero, Hrt18, A549, fcwf-4, BKT-1, Tb-1 Lu, or primary kidney cells derived from Leschenault rousette bats) tested with bat intestinal specimens positive for both detected viruses by RT-PCR. Results of RT-PCR for cell lysates to detect viral replication also were negative.

Virus Propagation in Fruit Bats

To obtain bat CoVs from field samples, we administered virus orally to 2 Leschenault rousette bats maintained in the Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, University of Tokyo. The volume of intestine samples collected from insectivorous bats was less than that from fruit bats because of their body size, and all positive samples for the group 1b bat CoV genome were derived from small insectivorous bats. Oral infection was conducted only with samples positive for the group 2d bat CoV genome. A homogenized large intestine sample (60 mg) derived from a lesser dog-faced fruit bat, which contained 7.8×10^6 copies of viral genome, was given orally to 2 fruit bats (bats A and B). After confirmation that these bats showed

Table 1. Prevalence of coronavirus in bats, the Philippines

Sampling site	Common name (species)	No. intestine samples tested	No. positive (group 1)	No. positive (group 2)
Los Baños	Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	4	0	2
	Cave nectar bat (*Eonycteris spelaea*)	3	0	2
	Greater musky fruit bat (*Ptenochirus jagori*)	14	0	11
Diliman (site A)	Lesser dog-faced fruit bat (*C. brachyotis*)	1	0	1
	Cave nectar bat (*E. spelaea*)	1	0	1
	Greater musky fruit bat (*P. jagori*)	1	0	0
Diliman (site B)	Cave nectar bat (*E. spelaea)*	1	0	1
	Java pipistrelle bat (*Pipistrellus javanicas*)	3	0	0
	Lesser Asiatic yellow bat (*Scotphilus kuhlii*)	4	4	0
Diliman (site C)	Lesser dog-faced fruit bat (*C. brachyotis*)	18	0	6
	Greater musky fruit bat (*P. jagori*)	1	0	0
	Geoffroy rousette bat (*Rousettus amplexicaudatus*)	1	0	1
Total		52	4	25

no clinical signs of infection, they were killed 6 days after infection.

Virus genome was detected only in the small and large intestines of both bats by RT-PCR (Table 2). Virus was not detected in these intestine samples by cell cultures. Virus genome was detected by RT-PCR in fecal samples obtained during daily observations for clinical signs, and viral genome copy number was determined by real-time RT-PCR (Table 3). Virus copy number peaked on day 3. On day 4, a fecal sample was not collected because feces were not found on the bottom of the isolator.

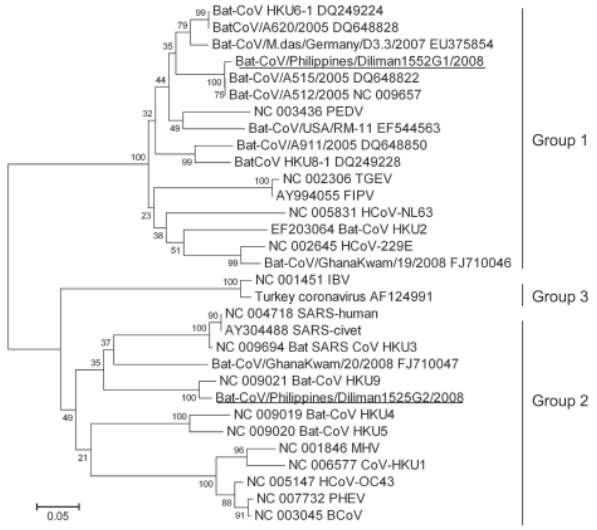

Figure 1. Phylogenetic tree based on deduced amino acid sequences of partial RNA-dependent RNA polymerase of coronaviruses (CoVs), the Philippines. The 2 new viruses detected in this study are underlined. Percentage of replicate trees in which the associated taxa clustered in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed by using the Poisson correction method and are shown as number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. The final dataset included 120 positions. Phylogenetic analyses were conducted in MEGA4 (*14*). Coronaviruses used for comparisons and their GenBank accession numbers are human coronavirus (HCoV) 229E (HCoV-229E) (NC_002645), porcine epidemic diarrhea virus (PEDV) (NC_003436), transmissible gastroenteritis virus (TGEV) (NC_002306), feline infectious peritonitis virus (FIPV) (AY994055), human coronavirus NL63 (HCoV-NL63) (NC_005831), bat-CoV/A512/2005 (NC_009657), bat-CoV/A515/2005 (DQ648822), bat-CoV/A620/2005 (DQ648828), bat-CoV/A911/2005 (DQ648850), bat-CoV/GhanaKwan/19/2008 (FJ710046), bat-CoV/GhanaKwan/20/2008 (FJ710047), bat-CoV/M.das/Germany/D3.3/2007 (EU375854), bat-CoV/USA/RM-11 (EF544563), bat-CoV HKU2 (EF203064), HKU4 (NC_009019), HKU5 (NC_009020), HKU6 (DQ249224), HKU8 (DQ249228), HKU9 (NC_009021), CoV-HKU1 (NC_006577), human coronavirus (HCoV-OC43) (NC_005147), murine hepatitis virus (MHV) (NC_001846), bovine coronavirus (BCoV) (NC_003045), porcine hemagglutinating encephalomyelitis virus (PHEV) (NC_007732), human severe acute respiratory syndrome coronavirus (SARS) (SARS-human) (NC_004718), civet SARS-like coronavirus (SARS-civet) (AY304488), bat-SARS-like coronavirus HKU3 (bat-SARS-CoV HKU3) (NC_009694), infectious bronchitis virus (IBV) (NC_001451), and turkey coronavirus (AF124991).

Virus mRNA in Experimentally Infected Bats

For murine hepatitis virus and several CoVs, an ≈70-bp leader sequence is added to the 5′ end of the transcription regulatory sequence of each nested mRNA during mRNA processing (*15*,*16*). For bat CoVHKU9–1, which was most similar to group 2d bat CoV, a complete genome sequence and putative transcription regulatory sequence of Bat-CoV HKU9 were predicted (*17*). On the basis of that report, primer HKU9-Leader42–64, including a leader sequence, was designed (Figure 2). The HKU9-Leader42–64 primer and 3siteAd20T primer, which included the oligo dT sequence, were used for PCR with RNA extracted from intestines of bats A and B. Amplicons were cloned, and partial genomic sequences of group 2d bat CoV membrane, nucleocapsid, Ns7a, Ns7b, and Ns7c genes were determined. These sequences were deposited in GenBank (DDBJ accession no. AB543561). A phylogenetic tree was also constructed with the deduced amino acid sequence (463 aa) of the complete N gene of group 2d bat CoV and available sequences of known CoVs. The tree showed the same topology as that constructed with deduced amino acid sequence of the partial RdRP gene. The N gene nucleotide sequence was most similar to that of Bat-CoV/HKU9–1/China/2007 (72% identity).

To confirm presence of transcribed virus mRNA in bats A and B (Table 2), RT-PCR specific for mRNA of the group 2d bat CoVs was conducted with HKU9-Leader42–64, N468–448r, and Ns7a440–420r primers. Virus mRNAs were detected in RNA extracted from the small intestines of bats A and B (Figure 3). All amplicons were sequenced and included the nucleotide sequence of the HKU9-Leader42–64 primer sequence at the 5′ end of the sequences obtained (Figure 2). These results suggest that virus mRNAs were transcribed in bats A and B.

Experimental Infection of Bats

To determine whether this 2d bat CoV was pathogenic, we experimentally infected 5 *R. leschenaulti* fruit bats. A 60-mg sample of the small intestine from bat A was given orally to 2 bats (bats C and D), and 500 μL of phosphate-buffered saline was given orally to 1 bat (bat E) as a control. These bats were killed 6 days after infection. Clinical signs were not observed in the experimentally infected bats. Virus genome amplification was not detected by RT-PCR in any samples (serum, brain, kidney, liver, lung, spleen, and feces). However, virus RNA was detected in the small intestine (Table 4). No pathologic changes were observed in the intestines or other organs.

Table 2. PCR results for bat coronavirus in fruit bats infected by using bat intestinal samples, the Philippines*

Bat	Assay	Liver	Kidney	Lung	Spleen	Brain	Small intestine	Large intestine	Serum
A	RT-PCR	–	–	–	–	–	–	+	–
	qRT-PCR	ND	ND	ND	ND	ND	1.25×10^6	3.53×10^6	ND
B	RT-PCR	–	–	–	–	–	+	+	–
	qRT-PCR	ND	ND	ND	ND	ND	1.47×10^6	1.50×10^6	–

*Bat intestinal samples containing 7.8×10^6 copies of coronavirus genome. Values are copies per milligram. RT-PCR, reverse transcription–PCR; –, virus RNA not detected; +, virus RNA detected; qRT-PCR, quantitative RT-PCR; ND, not done.

Table 3. Time course of detection of coronavirus viral genome by PCR in feces from 2 fruit bats, the Philippines*

	Days after infection					
Test	0	1	2	3	4	5
RT-PCR	–	–	–	+	ND	+
Quantitative RT-PCR	–	–	5.31×10^4	1.74×10^7	ND	1.5×10^6

*Values are copies per milligram. RT-PCR, reverse transcription–PCR; –, virus RNA not detected; +, virus RNA detected; ND, not done.

Because virus growth in fruit bats was weaker than virus growth in bats after primary infection with field samples, experimental conditions were changed. Samples (300 mg) from the small intestine of 2 bats (A and B) were given orally to 2 other bats (F and G), which were killed 3 days after infection. Six intestinal samples were obtained from each bat to determine site specificity of virus growth. The entire intestine (duodenum to the large intestine) was divided into 6 equal parts (1–6). Virus RNA was detected only by RT-nested PCR in the small and large intestines (Table 4), and no pathologic effect was detected in these bats. Virus RNA was detected in the lower region (parts 3–4 from bat F and parts 3–6 from bat G). However, we could not determine the specific site of virus replication.

Discussion

After the SARS epidemic in 2003, bats were identified as carriers of CoVs in China. Recently, bat CoVs have also been detected in several other regions, including Germany, North and South America, and Africa. In the current study, we confirmed the presence of 2 CoVs in bats in the Philippines. Our findings suggest that CoV circulation in bats is worldwide. Although only 52 bats were tested, CoV RNA was present in 55.8% of large intestine samples from these bats. Moreover, all bats tested seemed to be healthy. Thus, bats may be persistently infected carriers of CoVs. These data are consistent with results of previous reports of CoV detection in bats (*3*–*6*).

RNA of group 1b bat CoV was detected in 4 (57.1%) of 7 insectivorous bats. All bats positive for group 1b bat CoV RNA belonged to the same species, the Lesser Asiatic yellow bat (*Scotophilus kuhlii*). However, the partial RdRp sequence of the virus was most similar to that of Bat-CoV/People's Republic of China/A515/2005 (95% nt sequence identity), which was also detected in bats of the same species in the southern China on Hainan Island (*5*). The Lesser Asiatic yellow bat is distributed widely in eastern Asia in Philippines, Pakistan, Hainan Island, Taiwan, and Borneo (*18*). High similarities of sequences between group 1b bat CoV and Bat-CoV/China/A515/2005 suggest that these viruses are distributed widely in bats enzootic to eastern Asia.

Although group 1 bat CoV was detected in 1 species of insectivorous bats, group 2d bat CoV was detected in 4 species of frugivorous bats. Five of the 45 frugivorous bats were positive by RT-PCR, and an additional 20 were positive by RT-nested PCR (prevalence 55.6%). This finding suggests that replication of group 2d bat CoV in the intestine is low.

The complete N sequence of group 2d bat CoV suggests that it is a novel virus and most similar to that of Bat-CoV/

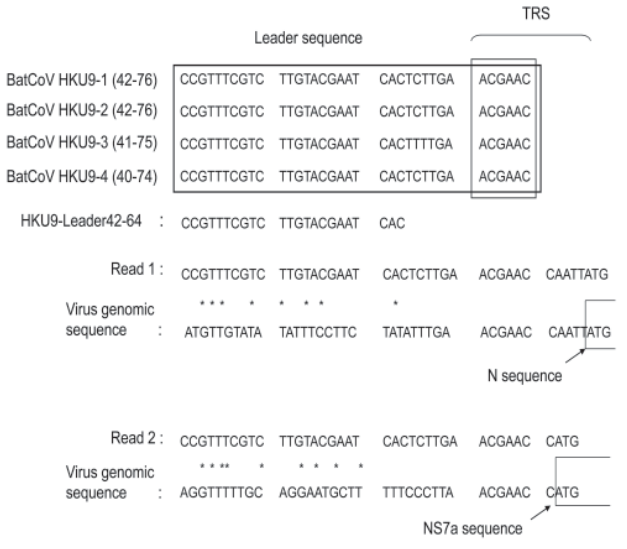

Figure 2. Comparison of mRNA sequences of bat coronavirus (BatCoV) with viral genomic sequences. Read 1 was obtained by using reverse transcription–PCR and HKU9-Leader42–64 and N468–448r primers. Read 2 was obtained by using HKU9-Leader42–64 and Ns7a440–420r primers. Asterisks indicate sequence identity for read and virus genome sequences. TRS, transcription regulatory sequence; N, nucleocapsid; NS, nonstructural.

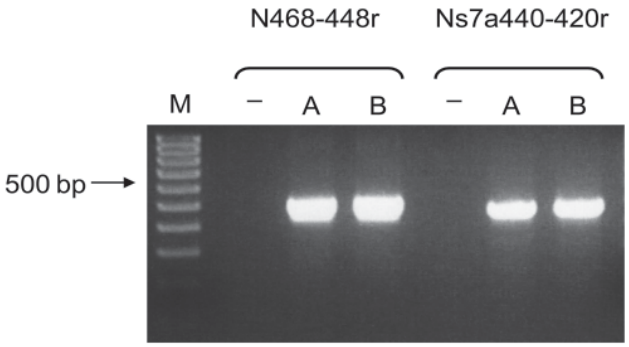

Figure 3. Bat coronavirus/Philippines/Dilliman1525G2/2008 mRNA in experimentally infected fruit bats, the Philippines. Reverse transcription–PCR results for small intestines of bats A and B. Lane M, 100-bp DNA ladder; lane –, nontemplate control.

HKU9–1/China/2007 (77% aa sequence identity). Woo et al. (*17*) detected Bat-CoV/HKU9–1/China/2007 and classified the viral nucleotide sequence as that of group 2d CoV. Our phylogenetic data (Figure 1) suggest that group 2d bat CoV and Bat-CoV/HKU9–1/China/2007 belong to the same group.

We attempted to isolate bat CoVs from several cell lines and primary cultured cells. However, virus replication was not observed, which is consistent with results of a previous report (*17*). No infectious bat CoV has been isolated from cell culture. In the current study, the amount of large intestine obtained per bat was ≤100 mg. Therefore, most samples were inadequate for virus isolation, especially virus-positive samples for group 1 bat CoVs from insectivorous bats.

To obtain sufficient tissue to isolate virus RNA, we attempted to infect fruit bats with bat CoV. Although we could not obtain bats of the species from which group 2d bat CoV was detected in the field survey, we obtained Leschenault rousette bats from zoos in Japan. In addition, Bat-CoV/HKU9–1/China/2007, which was most similar to group 2d bat CoVs by phylogenetic analysis, was identified in this species in Hong Kong (*17*). This finding indicates that fruit bats can be infected with this virus. No signs of clinical disease were observed after oral infection with an intestine sample derived from a lesser dog-faced fruit bat. However, virus RNA was detected in the small and large intestines (Table 2), and these intestinal samples contained more genome copies than input copies. Furthermore, virus RNA was amplified in fecal samples by real-time PCR, and viral mRNAs were detected in bats A and B (Figure 3). These findings indicate that group 2d bat CoVs can be orally transmitted to fruit bats and replicate in them.

Experimental infection was conducted in fruit bats by using tissues from virus-infected bats to determine virus pathogenicity. However, infected bats showed no signs of a pathologic effect, although low levels of virus RNAs were detected in the small and large intestines of these bats. These findings suggest that fruit bats can be infected with bat CoV without showing any signs of infection. However, compared with primary infection by field samples obtained from *C. brachyotis*, the level of viral genome amplification was low in experimental infection. This finding may have been caused by a difference in viral replication in bats of different species. In the field survey, partial nucleotide sequences of group 2d bat CoVs, were detected in 4 bat species. A high prevalence of virus RNA was observed in each bat species (Table 1). These findings suggest that the group 2d bat CoVs may infect fruit bats of many species. The oral infection study showed that CoV is easily transmitted across species. These results, and the fact that most reported bat CoV sequences have been detected in several bat species (*12*,*17*), imply that interspecies transmission in bats may be common.

Further investigation of bat CoV ecology is needed to better understand the risk for infection with this virus. Knowing this risk could help elucidate emergence of SARS. Although we demonstrated in vivo propagation of a bat CoV, a bat CoV culture system is needed to obtain additional information about this virus.

Table 4. Results of nested and quantitative RT-PCRs of cDNA from bat samples, the Philippines*

Bat	Liver	Kidney	Lung	Spleen	Brain	Small intestine				Large intestine		Serum
C	–	–	–	–	–	+ (ND)				–		–
D	–	+ (ND)	+ (ND)	+ (ND)	–	+ (6.57×10^4)†				–		+ (ND)
E	–	–	–	–	–	–				–		–
						Intestine section						
						1	2	3	4	5	6	
F	–	–	–	–	–	–	–	+ (ND)	+ (ND)	–	–	–
G	–	–	–	–	–	–	–	+ (ND)	+ (ND)	+ (ND)	+ (5.91×10^4)	–

*cDNA was synthesized from bat samples obtained after experimental infection (second passage of the group 2d coronavirus in Leschenault rousette bats). Values are copies per milligram. Virus load was quantified by real-time PCR. RT-PCR,reverse transcription–PCR; –, virus RNA not detected; +, virus RNA detected; ND, not detected by real-time PCR in RT-PCR–positive samples.
†Result of nested PCR.

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Multiyear Surveillance for Avian Influenza Virus in Waterfowl from Wintering Grounds, Texas Coast, USA

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We studied the prevalence of influenza A virus in wintering waterfowl from the Central Flyway on the Gulf Coast of Texas. Of 5,363 hunter-harvested migratory and resident waterfowl and wetland-associated game birds sampled during 3 consecutive hunting seasons (September–January 2006–07, 2007–08, and 2008–09), real-time reverse transcription–PCR detected influenza A matrix sequences in 8.5% of samples, H5 in 0.7%, and H7 in 0.6%. Virus isolation yielded 134 influenza A viruses, including N1–N9, H1–H7, H10, and H11 subtypes. Low-pathogenicity H7 subtype was isolated during January, September, and November 2007 and January 2008; low-pathogenicity H5 subtype was isolated during November and December 2007.

Wild waterfowl, primarily species in the orders Charadriiformes and Anseriformes (*1*), are natural reservoirs for type A influenza viruses. These viruses, which are occasionally transmitted to other species, including humans, poultry, and swine, result in subclinical to highly pathogenic diseases. Two subtypes (H5 and H7) have been most frequently associated with high pathogenicity in poultry and are of considerable interest to the poultry industry and to researchers who study avian influenza viruses (AIVs) (*2–4*). The migratory nature of many waterfowl species and the persistence of AIV in them present a potential vehicle for global dissemination of influenza viruses, as well as a constant source of viruses and genetic material for new pandemic strains. Preventing the introduction and adaptation of wild bird–origin AIVs to other susceptible species is an efficient strategy for minimizing the effects of AIV on global health and the global economy (*5*,*6*). Thus, surveillance in reservoir species is crucial for identifying viruses and gene pools with interspecies and intraspecies transmission potential.

In North America, migratory birds use 4 major flyways: Pacific, Central, Mississippi, and Atlantic (www.flyways.us). Three flyways (Pacific, Mississippi, and Atlantic) are well represented in the literature that addresses AIV surveillance (summarized in [*7*]); however, data are limited for the Central Flyway (*8–10*). Approximately 90% of waterfowl that use the Central flyway winter in Texas. Of these, ≈10 million ducks and geese winter in wetlands throughout the state, whereas 1–3 million ducks and >1 million geese winter along the Texas Gulf Coast (*11*). Before the implementation of surveillance programs to detect subtype H5N1 highly pathogenic AIV, few surveillance studies included migratory waterfowl on their wintering grounds or nonmigratory waterfowl during winter, particularly for the Texas–Louisiana Gulf Coast, where most studies were limited to just a few waterfowl species and limited by time of year and number of years studied (*8*,*9*,*12*,*13*). Although the US Interagency Strategic Plan for the Early Detection of Highly Pathogenic Avian Influenza H5N1 has extensively sampled waterfowl across all flyways, the program focuses on detection of subtype H5N1 virus;

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thus, only information pertaining to this subtype is publicly available (*14*). To understand the ecology, natural history, and evolution of influenza viruses, long-term surveillance studies are needed, particularly those that investigate waterfowl in understudied areas, such as wintering grounds. Long-term surveillance is even more important in areas where commercial poultry operations and migratory waterfowl stopover or wintering areas overlap (*15*).

We recently reported AIV prevalence, as determined by real-time reverse transcription–PCR (rRT-PCR) and virus isolation, from a multiyear surveillance project (September 2005–January 2009) of hunter-harvested waterfowl in the Texas mid–Gulf Coast region (*16*). We found little variation in overall AIV prevalence within or between seasons, except for 1 season (2007–08) when the overall prevalence was higher (*16*). The objectives of the current study were to 1) determine subtype diversity of AIV in both migratory and resident waterfowl populations (mostly ducks and geese) to which humans may be exposed and 2) compare prevalence and subtype diversity of AIV among species, according to age and sex, focusing on the Texas mid–Gulf Coast region during early fall and winter, which coincides with the regional hunting season.

Methods

Sample Collection and Analysis

During 2006–09, cloacal swab samples were collected from hunter-harvested waterfowl (*17*) and other wetland-associated game birds (*18*) during 3 consecutive hunting seasons: September 2006–January 2007 (season 1), September 2007–January 2008 (season 2), and September 2008–January 2009 (season 3) at 4 state wildlife management areas (WMAs) along the Gulf Coast of Texas: Justin Hurst WMA in Brazoria County, Mad Island WMA in Matagorda County, Guadalupe Delta WMA in Calhoun County, and Matagorda Island WMA in Calhoun County (Figure). Trained field personnel identified the species, sex, and age (when possible) of the bird on the basis of plumage (*19*). The bird's age was recorded as adult, if it was not the bird's hatch-year, and juvenile, if it was the bird's hatch-year. Waterfowl species and areas sampled reflected hunters' choices and personnel available to collect swabs on sampling days. Data from all 4 WMAs were combined for analysis.

All samples were collected, processed, and tested as previously described (*8*,*16*). Briefly, all samples (N = 5,363) were screened for AIV by AIV-matrix rRT-PCR, and virus isolation was performed on all 455 rRT-PCR–positive samples and 3,664 rRT-PCR–negative samples. All rRT-PCR–positive samples were screened for H5 and H7 subtypes by rRT-PCR by using the AgPath-ID One-Step RT-PCR Kit (Ambion, Inc., Austin, TX, USA) and an ABI 7500Fast Real-time PCR System (Applied Biosystems, Inc., Foster City, CA, USA) in a 25-μL final reaction volume. Primers and probes for the M and H5 (*20*,*21*) and H7 subtypes (*21*,*22*) were those previously described. All AIV isolates were submitted to the National Veterinary Services Laboratory (NVSL; Ames, IA, USA) for subtyping by hemagglutination (HA) and neuraminidase (NA) inhibition tests and screening for the presence of the N1 gene by rRT-PCR. Additionally, all H5 and H7 isolates were pathotyped at NVSL by analysis of the amino acid sequence at the HA protein cleavage site.

Statistical Analysis

We previously documented that prevalence estimates calculated on virus isolation following a positive AIV-matrix rRT-PCR provided results nearly identical to those obtained by performing both tests in parallel (*16*); for this reason, we calculated apparent prevalence by dividing the number of virus isolation–positive samples (after a positive rRT-PCR result) by the total number of samples collected and tested by rRT-PCR (*16*).

Pearson χ^2 analyses were used to evaluate differences in AIV-infected proportion by sex (drake vs. hen), age (adult vs. juvenile), species of waterfowl, and hunting season of collection (seasons 1, 2, 3). Fisher exact test was used instead of χ^2 when $\geq$1 cells were expected to have a frequency of $\leq$5. A p value <0.05 was considered significant. Wald 95% confidence intervals were calculated for all proportions of AIV infections (i.e., sex, age, species).

A multivariate main effects logistic regression model was also constructed to assess differences in AIV detection by using rRT-PCR by age, sex, and bird species. Spe-

Figure. Locations of state wildlife management areas where samples were collected from waterfowl for avian influenza virus surveillance, Texas mid–Gulf Coast, USA, September–January 2006–07, 2007–08, and 2008–09. Inset shows location of Texas (shaded).

cies were categorized as blue-winged teal, green-winged teal, gadwall, northern shoveler, or other species. We chose the 4 species-specific categories because they represented the largest numbers of tested birds. Sample records with missing rRT-PCR results or age, sex, or species data were removed from this analysis. We analyzed all data using Intercooled Stata version 9 (Stata Corp., College Station, TX, USA).

Results

Sampling Overview

A total of 5,363 cloacal swab samples were collected from 33 different potential host species, including a variety of waterfowl and other wetland-associated game birds (online Appendix Table 1, www.cdc.gov/EID/content/16/8/1224-appT1.htm; online Appendix Table 2, www.cdc.gov/EID/content/16/8/1224-appT2.htm; and online Appendix Table 3, www.cdc.gov/EID/content/16/8/1224-appT3.htm) during 3 consecutive hunting seasons (season 1: 2,171 birds; season 2: 2,424 birds; and season 3: 768 birds). Most samples (3,138 [58.5%]) were from teal (blue-winged [*Anas discors*] and green-winged [*A. crecca*]), followed by northern shovelers (*A. clypeata*; 703 [13.1%]), gadwall (*A. strepera*; 437 [8.2%]), and American wigeon (*A. americana*; 238 [4.4%]); the remaining samples (847 [15.8%]) were from a variety of other species (online Appendix Tables 1–3). Adults accounted for 2,759 (51.5%) samples; 1,504 (28.0%) were collected from juveniles, and 1,100 (20.5%) from birds of undetermined age. Additionally, 2,445 (45.6%) samples were from drakes, 2,262 (42.2%) from hens, and 656 (12.2%) from birds of undetermined sex.

Subtype Prevalences

Of 4,119 samples processed for virus isolation, influenza A viruses were isolated from 134. All 9 NA subtypes (N1–9) were isolated, whereas only 9 of the 16 different HA subtypes (H1–7, 10, and 11) were isolated. Thirty-two different HA and NA subtype combinations were identified (online Appendix Table 4, www.cdc.gov/EID/content/16/8/1224-appT4.htm), and for 8 isolates, either the HA (n = 7) or NA (n = 1) was not identified.

The most frequently identified HA subtypes during season 1 were H3 and H6 (8 [25.0%] and 9 [28.1%], respectively), whereas for season 2, H4 and H10 were predominant (26 [26.8%] and 17 [17.5%], respectively); the H4 subtype (4 [80.0%]) remained predominant in season 3. With respect to NA subtypes, N1 and N8 were most common in season 1 (8 [18.8%] and 10 [31.3%], respectively), whereas N6, N7, and N8 (19 [19.6%]; 16 [16.5%], and 19 [19.6%], respectively) were predominant in season 2, with N6 and N8 (2 [40.0%] each) remaining predominant in season 3. The most frequent HA and NA subtype combinations identified during season 1 were subtype H3N8 (n = 7) and H6N1 (n = 4) viruses, whereas H4N6 (n = 17), H3N8 (n = 9), and H10N7 (n = 9) viruses were the most common subtype combinations identified in season 2, and H4N6 (n = 2) and H4N8 (n = 2) were most common in season 3 (online Appendix Table 4).

H7 subtype was identified by rRT-PCR during all 3 hunting seasons (n = 2, 28, and 2, respectively). Additionally, H5 subtype was detected by rRT-PCR for all 3 seasons (n = 14, 21, and 2, respectively). Yet, H5 viruses were isolated only during season 2, whereas H7 viruses were isolated during all 3 hunting seasons (Tables 1, 2). All H5 and H7 viruses were determined to be low-pathogenicity AIVs by analysis of the amino acid sequence at the HA protein cleavage site.

Prevalence by Sex, Age, and Species

Apparent AIV prevalence did not differ significantly between hens and drakes by rRT-PCR or virus isolation during any of the 3 hunting seasons or all seasons combined (online Appendix Table 5, www.cdc.gov/EID/content/16/8/1224-appT5.htm). Prevalence as determined by rRT-PCR and virus isolation differed significantly between juvenile and adult birds during the 3 hunting seasons and for all seasons combined (Table 3). However, when data were analyzed on the basis of samples for which both sex and age of the birds were known, results differed significantly between adult drakes and hens according to rRT-PCR results during season 1 and between juvenile hens and drakes by virus isolation during season 3 and for all 3 seasons combined (online Appendix Table 6, www.cdc.gov/EID/content/16/8/1224-appT6.htm).

To determine whether a species effect existed for age differences, we assessed apparent AIV prevalence by age for species for which >100 samples from adult birds and >100 samples from juvenile birds were tested (i.e., blue-winged teal, green-winged teal, gadwall, and northern shoveler; Table 4; online Appendix Table 7, www.cdc.gov/EID/content/16/8/1224-appT7.htm; and online Appendix Table 8, www.cdc.gov/EID/content/16/8/1224-appT8.htm). When data from all 3 hunting seasons were combined, significantly more juvenile than adult birds were positive for AIV by virus isolation for 3 of the predominant host species analyzed (blue-winged teal, green-winged teal, and northern shoveler); no significant difference was observed for gadwall (online Appendix Tables 7, 8). However, apparent AIV prevalence by rRT-PCR was significantly higher only for juvenile blue-winged teal and northern shovelers (online Appendix Tables 7, 8). According to multivariate logistic regression, rRT-PCR results were associated with age and species but not with sex (Table 4).

Blue-winged teal and northern shovelers had the greatest diversity in subtypes, followed by green-winged

Table 1. Subtypes of avian influenza viruses isolated in the fall (September and November) from selected species during 3 consecutive hunting seasons, Texas mid–Gulf Coast, USA, 2006–07, 2007–08, and 2008–09

	Subtype (no. isolated)					
	September†			November		
Species*	2006	2007	2008	2006	2007	2008
Fulvous whistling duck (*Dendrocygna bicolor*)	–	–	–	H6N1	_	–
Mottled duck × mallard (*Anas fulvigula* × *A. platyrhynchos*)	–	–	–	–	H6N8	–
Mottled duck (*A. fulvigula*)	–	–	–	H6N5	–	–
Northern pintail (*A. acuta*)	–	–	–	–	H4N8	–
Northern shoveler (*A. clypeata*)	–	–	–	H2N9, H3N8, H4N2, H4N6, H4N8	H4N2, H5N2, H5N3, H6N2, H10N2, H11N9 (2)	H7N2
Teal, blue-winged (*A. discors*)	H1N1, H3N6, H3N8 (6)	H1N1 (2), H2N8, H3N4, H3N6, H3N8 (9), H4N1, H4N6 (17), H4N8 (6), H6N1, H7N1, H7N1/4, H7N7 (2), H10N7 (5)	H4N6, H4N8	H2N9, H4N2 H4N6, H4N8 H6N1 (3), H6N1/4, H6N5, H6N6, H6N8	H3N6, H5N2 (2), H5N3 (2), H7N4, H7N7 (3), H10N7, H11N9 (3)	H4N8
Teal, green-winged (*A. crecca*)	H6N2	H10N7	–	H1N1	H5N2, H7N1/4, H11N9	–

*Species selected by significance as determined by prevalence, uniqueness to the area, or native, nonmigratory species.
†Teal are the only species hunted during September on the Texas mid–Gulf coast.

teal (Tables 1, 2; online Appendix Tables 1–3). Nine HA (H1–7, 10, and 11) and all 9 NA (N1–9) subtypes were identified in blue-winged teal, 8 HA (H2–7, 10, and 11) and 6 NA (N2, N3, and N6–9) subtypes were identified in northern shovelers, whereas 6 HA (H1, 5–7, 10, and 11) and 6 NA (N1–4, N7, and N9) subtypes were identified in green-winged teal.

Discussion

The Texas Gulf Coast provides winter habitat for ≈2–3 million ducks and ≥1 million geese (*11*). In this region, migratory waterfowl intermingle with resident wild species such as the mottled duck, and are in close contact with poultry operations and humans, primarily hunters (*15*,*17*). Recently, we reported prevalence for the first multiyear study of AIV that covered waterfowl wintering grounds along the Texas Gulf Coast (*16*), a previously understudied area. Unlike results of previous studies, we found little to no variation in apparent AIV prevalence by month within wintering seasons (September–January) with the exception of rRT-PCR during December 2007–January 2008 and virus isolation during 2005–06 and 2006–07 (*16*). Additionally, AIV prevalence, as determined by rRT-PCR or virus isolation, varied little among the 4 consecutive hunting seasons studied (September–January 2005–06 through 2008–09), except for the 2007–08 season, during which overall AIV prevalence was higher than the other 3 seasons as determined by both rRT-PCR and virus isolation (*16*). Detection of AIV at low levels throughout the wintering season supports the contention that AIV can persist in wild-bird populations through continuous circulation in a proportion of the population (*1*). The low rate of virus isolation observed in the current study (29.9% of rRT-PCR–positive samples) is consistent with findings of other studies and is not surprising (*2*,*23*). Real-time RT-PCR is considered more sensitive than virus isolation, enabling the detection of genome fragments and viruses that do not grow in embryonated chicken eggs. Also, consistent with other surveillance studies, no differences were noted in AIV prevalence based on sex, and AIV was more prevalent in juvenile birds than in adults (*1*,*7*,*23*). The latter finding supports the assumption that immunologically immature (juvenile) birds are more susceptible to AIV than are mature (adult) birds (*24*,*25*).

Table 2. Subtypes of avian influenza viruses isolated in the winter (December–January) from selected species during 3 consecutive hunting seasons, Texas mid–Gulf Coast, USA, 2006–07, 2007–08, and 2008–09

	Subtype (no. isolated)					
	December			January		
Species*	2006	2007	2008	2007	2008	2009
Northern pintail (*Anas acuta*)	–	H10N3/7	H4N6	–	H10N3	–
Northern shoveler (*A. clypeata*)	–	H5N2, H6N2, H10N7	–	–	–	–
Teal, blue-winged (*A. discors*)	–	–	–	–	H10N3 (3)	–
Teal, green-winged (*A. crecca*)	H10N7, H11N3	–	–	H7N3	H7N3, H10N3 (2)	–

*Species selected by significance as determined by prevalence, uniqueness to the area, or native, nonmigratory species.

Table 3. Comparison of apparent prevalence of avian influenza virus in hunter-harvested waterfowl, Texas mid–Gulf Coast, USA, September–January 2006–07, 2007–08, and 2008–09*

Hunting season	Juvenile waterfowl†			Adult waterfowl†			p value	
	No. tested	rRT-PCR	VI	No. tested	rRT-PCR	VI	rRT-PCR	VI
2006–07	518	8.30 (5.92–10.68)	3.28 (1.75–4.82)	1,081	5.46 (4.10–6.81)	0.74 (0.23–1.25)	0.029	<0.001
2007–08	763	13.80 (11.30–16.20)	5.50 (3.89–7.12)	1,189	10.51 (8.77–12.20)	3.28 (2.27–4.29)	0.030	0.022
2008–09	222	8.56 (4.88–12.24)	1.80 (0.49–4.55)	489	4.70 (2.82–6.58)	0.20 (0.01–1.13)	0.043	0.035
Total‡	1,503	11.10 (9.52–12.69)	4.06 (3.06–5.06)	2,759	1.74 (1.25–2.23)	1.74 (1.25–2.23)	<0.001	<0.001

*rRT-PCR, real-time reverse transcription–PCR; VI, virus isolation.
†Values for rRT-PCR and VI are apparent prevalence, % (95% confidence interval).
‡Total = the 3 hunting seasons combined (September–January, 2006–07, 2007–08, and 2008–09).

The most commonly identified HA and NA subtype combinations during season 1 were H3N8 and H6N1; during season 2, H3N8 remained, but it was not detected during season 3. During season 2, H4N6 and H10N7, which have been reported on the Gulf Coast (*8*,*13*), were the predominant subtype combinations; H4N6 also was detected during season 3. The annual variations in AIV subtype prevalence observed in this study show the need for continued annual surveillance in domestic and migratory avian species, particularly in areas of high poultry and waterfowl density, such as the Texas Gulf Coast (*15*).

Outbreaks of H5 AIV have been documented previously in Texas. In 1993, an outbreak of H5N2 occurred in emus, in 2002 H5N3 was detected in chickens, and in 2004 highly pathogenic avian influenza virus (H5N2) was reported in a commercial poultry operation (*26*–*28*). We isolated subtype H5N2 and H5N3 viruses from apparently healthy free-roaming waterfowl only during season 2. Although no data are available on subtypes circulating in waterfowl on the Texas coast before the 3 outbreaks noted above, our data document the presence of these subtypes in migratory waterfowl near commercial poultry operations (*15*). Molecular characterization of the subtype H5N2 and H5N3 viruses we isolated should help clarify the relation between these viruses and those isolated from commercial species.

Table 4. Multivariate logistic regression model to identify variables associated with a positive real-time RT-PCR result, Texas mid–Gulf Coast, USA, 2006–07, 2007–08, and 2008–09*

Variable	Odds ratio (95% CI)	p value
Sex		
Drake	1.0†	
Hen	1.07 (0.859–1.320)	0.558
Age		
Adult	1.0†	
Juvenile	1.45 (1.17–1.81)	**0.001**
Species		
Other species	1.0†	
Gadwall	0.407 (0.120–0.825)	**0.013**
Northern shoveler	1.51 (0.987–2.320)	0.057
Blue-winged teal	2.18 (1.52–3.13)	**<0.001**
Green-winged teal	1.12 (0.742–1.680)	0.592

*Results for a total of 4,187 samples, collected during September–January for each season. RT-PCR, reverse transcription–PCR; CI, confidence interval. **Boldface** indicates significant result.
†Reference category.

Our isolation of AIVs from resident (nonmigratory) mottled ducks and mottled duck/mallard hybrids suggests AIV transmission on the wintering ground and is consistent with previous reports (*13*). Mallards interbreed with mottled ducks and are sister species phylogenetically (*29*). Before the isolation of H6 AIVs from a mottled duck/mallard hybrid in November 2006 and a mottled duck in November 2007, we isolated H6 subtypes from migratory teals and northern shovelers (September and November 2006 and 2007). Additional support for AIV transmission on wintering grounds included isolation of an H6 virus from a fulvous whistling duck, a species that breeds on the Texas–Louisiana coast and leaves during late summer to winter further south in Mexico; nearly all whistling ducks are gone by late January (*17*). Although circulation of AIVs within fulvous whistling ducks, mottled ducks, and mottled duck hybrids throughout the year cannot be ruled out, such circulation seems unlikely. Hanson et al. were unable to isolate AIVs from mottled ducks collected on the Texas Gulf Coast during August (*9*); additionally, we did not detect AIV by rRT-PCR in samples collected during June–August 2007 (n = 155, S. Rollo et al., unpub. data), which suggests that these viruses are not readily circulating in these resident populations during summer. Genetic characterization of these H6 isolates will help determine whether these isolates are related and help clarify the role of waterfowl wintering grounds in the transmission and perpetuation of AIVs in nature. Further studies focused on AIV prevalence and immune responses to AIV in these resident populations also are needed to clarify the maintenance and transmission of AIVs in the wintering grounds.

Before singling out a particular species on which to focus surveillance efforts, one must consider the technique used for subject selection (hunter-harvest vs. live-capture) as well as the area under study (e.g., breeding grounds vs.

wintering grounds; fresh water vs. salt water) and which populations are prevalent within the study areas. Mallards have become a primary species of interest not only because of their susceptibility to H5 and H7 subtypes but also because of their abundance and relative ease of capture (*2*,*17*,*23*,*30*–*32*). During our study, few mallard samples were collected because most Texas mallards winter in the playa lakes and sorghum fields of the Texas Panhandle with few (<4%) wintering along the Gulf Coast (*17*). Our data indicate that mallards, although appropriate focal species for AIV monitoring in some portions of North America, are not as suitable as blue-winged teal or northern shoveler in other regions, such as the Texas mid–Coast (*8*,*9*,*13*). In many studies that found mallards as a high-prevalence species for AIV infection, they were captured live for testing and dominated the samples (*2*,*23*). The few studies in which other species were more frequently sampled and tested positive for AIV were conducted on hunter-harvested waterfowl (*8*,*13*,*33*).

Our study supports the consensus that dabbling ducks are more likely than diving ducks to be positive for AIV; however, as others have documented, not all dabbling ducks are equally likely to be AIV positive (*2*,*23*,*33*). We found blue-winged teal to be the species with the highest prevalence, followed by northern shoveler and green-winged teal. Gadwalls, also a dabbling duck from which we collected substantial numbers of samples, were the least likely to test positive for AIV. Blue-winged teal are generally the first ducks to fly south in the fall, first arriving on wintering grounds in September, and the last to pass through Texas in late February–March on their return north (*17*). They also make exceedingly long flights compared with other dabbling ducks between feeding and resting areas during migrations (*17*). On the other hand, gadwalls are short-distance migrants and migrate later, generally beginning their southward migration in early September and their return north starting in February (*17*). The physiologic demands of long-distance migration can suppress the immune system (*34*); thus, blue-winged teal might be more susceptible to infection than some other dabbling ducks because of their long-distance migration. More extensive studies are needed incorporating more ecologic factors such as food resources, body mass, and immune status to more fully understand how AIV persists in nature and why the prevalence of AIV is higher in particular species.

Although our samples were not collected probabilistically (i.e., the samples reflect hunters' choices, as well as the relative abundance of each species), use of hunter-harvested waterfowl was convenient for obtaining large number of samples with which to estimate the prevalence of AIV subtypes carried by waterfowl in the Gulf Coast of Texas. In addition, because hunters have been identified as the human population most at risk for exposure to AIV (*35*) and antibodies to H11 subtype have been identified in hunters and wildlife professionals (*36*), continued monitoring of AIV in waterfowl and humans exposed to them should provide useful information about the prevalence and significance of wild animal-to-human transmission.

AIV surveillance studies over time in the same region are critical, particularly in understudied areas. Although studies in areas of low AIV prevalence are inconvenient because of the large sample sizes required to isolate substantial numbers of AIVs, such surveys are critical to gain more knowledge of the ecology of influenza viruses. Our data contribute temporal information about AIV prevalence and subtype diversity for a historically understudied area of North America, the waterfowl wintering grounds of the Texas Gulf Coast.

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Outbreak of *Corynebacterium pseudodiphtheriticum* Infection in Cystic Fibrosis Patients, France

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An increasing body of evidence indicates that nondiphtheria corynebacteria may be responsible for respiratory tract infections. We report an outbreak of *Corynebacterium pseudodiphtheriticum* infection in children with cystic fibrosis (CF). To identify 18 *C. pseudodiphtheriticum* strains isolated from 13 French children with CF, we used molecular methods (partial *rpoB* gene sequencing) and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Clinical symptoms were exhibited by 10 children (76.9%), including cough, rhinitis, and lung exacerbations. The results of MALDI-TOF identification matched perfectly with those obtained from molecular identification. Retrospective analysis of sputum specimens by using specific real-time PCR showed that ≈20% of children with CF were colonized with these bacteria, whereas children who did not have CF had negative test results. Our study reemphasizes the conclusion that correctly identifying bacteria at the species level facilitates detection of an outbreak of new or emerging infections in humans.

Cystic fibrosis (CF) is an autosomal recessive disease characterized by defective ion channels, resulting in multiorgan dysfunction, most notably affecting the respiratory tract. The alteration in pulmonary environment is associated with increased susceptibility to bacterial infections (*1*,*2*). These bacterial infections and the ensuing inflammation damage the airway epithelium and cause recurrent episodes of acute exacerbations, leading ultimately to respiratory failure. Respiratory infections account for 80%–90% of deaths of patients with CF (*2*). Recent advances in bacterial taxonomy and improved microbial identification methods have led to increasing recognition of the complexity of microbial ecology of the CF lung (*3*–*5*). Thus, infections of the lung in patients with CF are now considered as polymicrobial infections. In addition to well recognized CF pathogens (e.g., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Burkholderia cepacia* complex) numerous other opportunistic bacteria have been recently reported, such as *Stenotrophomonas maltophila*, *Achromobacter xylosoxydans*, and *Inquilinus limosus* and methicillin-resistant *S. aureus* and mucoid *P. aeruginosa* (*1*,*2*,*6*–*8*).

The first difficulty in studying infections in the lungs of patients with CF is that many bacteria present in the lung cannot be isolated from sputum either because of their fastidious growth requirements or because of the presence of other more common CF-related pathogens, including *P. aeruginosa*, *S. aureus*, *H. influenzae,* and *Branhamella catarrhalis*, that might ordinarily overgrow other bacteria in culture. Second, correct identification of bacteria in patients with CF remains challenging because phenotype variation is a common feature during chronic infection of the lung (*4*,*9*). Consequently, the list of bacteria that can be recovered from sputum specimens of patients with CF may be underestimated, and new or emerging bacteria that could be responsible for outbreaks in this population are not easily detected. Correct identification of these bacteria is not easily achieved.

Several studies have reported the use of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry as a powerful tool with good and reproducible results for rapid identification of clinical isolates in the microbiology laboratory (*10*) as well as for identifying

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nonfermenting gram-negative bacteria in patients with CF (*11*–*13*). This method is simple, rapid, easy to perform, inexpensive, and may ultimately replace routine phenotypic assays (*10*).

We report the clinical and microbiologic features of patients with CF who were infected or colonized by *C. pseudodiphtheriticum.* The index case-patient was a 9-year-old girl with fever and cough; a coryneform bacterium was isolated in pure culture from her sputum. After this first case, several other children with CF were found to be infected by coryneform bacteria; thus, we decided to investigate the possibility of an endemic transmission in this population. Isolated strains were identified by using existing phenotypic and molecular methods (*14*) as well as MALDI-TOF to decipher the relationship between these strains. Finally, a new real-time PCR with TaqMan probe (Applied Biosystems, Courtaboeuf, France) was developed and used in a retrospective analysis to detect these coryneform bacteria in our population with CF.

Methods

Sample Collection and Bacteriologic Culture

From August 2005 through June 2008, sputum samples, bronchoalveolar lavage samples, or both, were collected from patients with CF at 2 cystic fibrosis treatment centers (CFTCs), Timone Children's Hospital (patients <18 years of age; CFTC1) and Ste. Marguerite Hospital in Marseille (patients ≥18 years of age; CFTC2). Only samples that showed, by direct Gram staining, infrequent epithelial cells (<10 cells/field) and numerous polymorphonuclear cells (>25 cells/field) were further analyzed and processed according to current local guidelines (*4*). A portion of each sample was also frozen at –20°C for further study. Respiratory samples from patients who did not have CF (children admitted to the pediatric healthcare center and adults admitted to CFTC2) were also collected for control analysis. The *Corynebacterium* reference strains used in this study are listed in the Table. This study was approved by our local ethics committee (no. 07–011).

Phenotypic Identification

The positive bacilli from respiratory samples, identified by Gram stain, were investigated by metabolic tests, as oxidase and catalase activities, and by the use of Api (RAPID) Coryne Database 2.0 system (bioMérieux, Marcy-l'Etoile, France) (*15*). The antimicrobial drug susceptibility testing was performed by disk diffusion method on Mueller-Hinton agar with 5% sheep blood incubated for 24 h at 37°C.

Genotypic Identification and Sequence Analysis

Primers used in this study for amplification and sequencing the partial *rpoB* gene as well as PCR methods have been previously described (*16*). Multiple sequence alignment and percentage of similarities for the partial *rpoB* genes between the different species of corynebacteria were done by using the ClustalW program on the EMBL-EBI web server (www.ebi.ac.uk/clustalw). A phylogenic tree was generated by using the neighbor-joining method from MEGA 4.0 software (www.megasoftware.net). Kimura 2-parameter was used as a substitution model to construct the *rpoB* tree. Bootstrap replicates were performed to estimate the reliabilities of the nodes of the phylogenic tree obtained.

Bacterial Analysis by MALDI-TOF Mass Spectrometry

The strains were plated on Columbia agar with 5% sheep blood (COS) (bioMérieux) and incubated for 24 h at 37°C. One isolated colony from each strain was harvested and deposited on a target plate (Bruker Daltonics, Bremen, Germany) in 3 replicates. Two microliters of matrix solution (saturated α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, 2.5% trifluoroacetic acid) was then added and samples were processed in the MALDI-TOF mass spectrometry (337 nm) (Autoflex, Bruker Daltonics with the flex control software) (*10*). The profiles were compared and analyzed by Biotyper 2.0 (Bruker Daltonics) and finally a dendrogram of mass spectral data was constructed by us-

Table. Strains used to test the specificity of quantitative PCR and Ct obtained in a study of *Corynebacterium pseudodiphtheriticum* infection in CF patients, France, August 2005–June 2008*

Corynebacterium spp.	Reference	Ct
C. accolens	CIP104783T	38
C. afermentans subsp. afermentans	CIP103499T	–
C. afermentans subsp. lipophilum	CIP103500T	–
C. amycolatum	CIP103452T	–
C. coyleae	CIP104919T	–
C. diphteriae	CIP100721T	–
C. durum	CIP105490T	39
C. freneyi	CIP106767T	–
C. glucuronolyticum	CIP104577T	–
C. imitans	CIP105130T	–
C. jeikeium	Blood culture	–
C. macginleyi	CIP104099T	–
C. minutissimum	CIP100652T	–
C. mucifaciens	CIP105129T	–
C. propinquum	CIP103792T	23
C. pseudodiphtheriticum	CIP103420T	21
C. riegelii	CIP105310T	38
C. seminal	CIP104297T	–
C. singular	CIP105491T	–
C. striatum	CIP81.15T	–
C. ulcerans	CIP106504T	–
C. urealyticum	CIP103524T	–
C. xerosis	CIP100653T	–
C. aurimucosum	CCUG 47449T	–
C. fastidiosum	CIP103808	–

*CF, cystic fibrosis; Ct, cycle threshold; –, negative.

ing the instructor default setting. The Biotyper 2.0 program generates the tree depending on distance-based method and does not provide branch support values.

Real-time PCR

A new real-time PCR with a TaqMan probe (Applied Biosystems) that targets the *rpoB* gene of *C. pseudodiphtheriticum* has been developed and tested retrospectively in sputum samples that had previously been collected in a 1-year study from January through December 2006. Sputum samples from 4 groups of patients were analyzed: sputum samples from child (group 1) and adult (group 2) CF patients and sputum samples from non-CF children (group 3) and from non-CF adults (group 4). Primers and probe used were as follows: CorynPF (5′-GACGGYGCTTCCAACGAAGA-3′) and CorynPR (5′-CCGACGGAGATCGGGTGC-3′) and probe CorynPr (6FAM-TCTGTTGGCTAACTCCCGYCCAAA-TAMRA). Specificity of these primers and probe was verified in silico by using the BLAST program (www.ncbi.nlm.nih.gov/BLAST) as well as by using corynebacteria reference strains (Table). Sensitivity was assessed by using tenfold serial dilutions of a 0.5 MacFarland inoculum.

Results

Patients and Samples

Overall, 229 patients with CF were monitored from August 2005 through June 2008 in the 2 CFTCs in Marseille (118 children and 111 adults). During this period, 18 corynebacteria were isolated from respiratory samples of 13 children with CF (11.0%) but none from adults with CF ($p<0.001$). Details for the 13 patients are given in the online Appendix Table (www.cdc.gov/EID/content/16/8/1231-appT.htm). The mean age was 4.3 (0.3–16) years, and the sex ratio (M:F) was 0.6. Isolation of *C. pseudodiphtheriticum* was associated with clinical symptoms in 10 patients (76.9%), including cough, rhinitis, asthma crisis, and lung exacerbations (online Appendix Table). The culture of *C. pseudodiphtheriticum* from respiratory samples was pure in 6 cases (in 2 cases, patients had clinical symptoms). For 4 patients, a *Corynebacterium* isolate was obtained on >1 occasion (online Appendix Table). Six patients were treated, including 3 with β-lactams only, 1 with a combination of a β-lactam and cotrimoxazole, 1 with cotrimoxazole alone, and 1 with cotrimoxazole alone initially and then amoxicillin because no improvement was noticed and the isolate was resistant to cotrimoxazole.

Phenotypic and Molecular Identification of the Isolates

All corynebacteria were isolated from Columbia agar with 5% sheep blood. Colonies were white and nonhemolytic. They were all catalase positive and oxidase negative. The use of the ApiCoryne 2.0 system yielded identification of 16 *C. pseudodiphtheriticum* and 1 *C. propinquum*, with a confidence level 83%–99% (online Appendix Table). The remaining isolate was poorly identified as *Brevibacter* sp. with an uninterpretable pattern (online Appendix Table). All isolates were susceptible to β-lactams, vancomycin, rifampin, gentamicin, and doxycycline, whereas there was heterogeneity of susceptibility for erythromycin and cotrimoxazole (online Appendix Table). The partial *rpoB* gene sequencing provided an accurate identification for 18 isolates (A1 to M) with similarity ≥97% compared with reference strains (online Appendix Table). The results of the MALDI-TOF identification matched perfectly with the partial *rpoB* sequencing identification for all the isolates; mean score values were 1.8–2.5 (online Appendix Table). Figure 1 presents 2 trees built by using MALDI-TOF mass spectrometry (Figure 1, panel A) and by using partial *rpoB* gene sequences (Figure 1, panel B). Although comparison between these 2 trees was impossible because of the different methods used (i.e., Euclidean distance method for MALDI-TOF dendrogram and neighbor-joining method for phylogenetic tree), the 2 trees gave a similar clustering of the isolates (Figure 1).

Real-time PCR

Sensitivity of our new real-time PCR ranged from 5 CFU/mL to 10 CFU/mL; specificity for *C. pseudodiphtheriticum* was verified by testing *Corynebacterium* spp. reference strain cultures with *C. propinquum*, the most closely related species, which was also amplified. A low level of cross-amplification was also observed in 3 other species with cycle thresholds (Ct) >38 cycles, including *C. accolens*, *C. durum*, and *C. riegelii* (Table); this finding could not be considered clinically relevant. To estimate the prevalence of this bacterium in our CF population, we used the real-time PCR to test, retrospectively and blindly, all respiratory samples available from January through December 2006 from 146 patients with CF (n = 356 sputum samples; 86 children, group 1; and 60 adults, group 2) and from 56 patients without CF (n = 67 sputum samples; 18 children, group 3; and 38 adults, group 4). We found 24 PCR-positive sputum specimens (Ct <37) in 17 children (19.8%) and 3 adults (5%) in patients with CF (Figure 2). Among these 24 PCR-positive samples only 2 were culture positive (sample A1, online Appendix Table) ($p<0.0001$). Thus, 16 additional children and 3 adult patients with CF were eventually colonized with this bacterium. For the control group, although all samples were culture negative, we found 3 PCR-positive samples in 2 adult patients, who were followed up in CFTC2 for lung transplantation, and none from children (Figure 2). The 2 PCR-positive lung-transplant patients were hospitalized in the adult CFTC with 2 adult patients with CF during the same period. For these 2

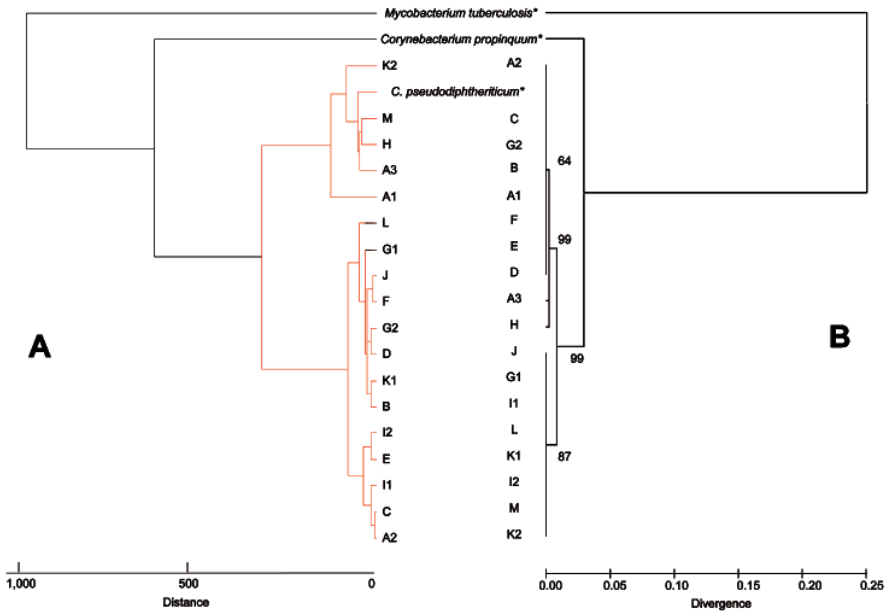

Figure 1. Phylogenetic tree showing the position of *Corynebacterium* spp. isolated in patients with cystic fibrosis based on comparisons of the mass spectra obtained with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (A) and of sequences of the partial RNA polymerase β-subunit gene *rpoB* (B). For MALDI-TOF, a tree was constructed with Biotyper 2.0 software (Bruker Daltonics, Bremen, Germany) using Euclidean distance. The *rpoB* tree was constructed by the neighbor-joining method and a maximum likelihood-based distance algorithm and numbers on branches indicate the bootstrap values derived from 500 replications. *Reference strains (*Mycobacterium tuberculosis* H37Rv, *C. propinquum* CIP103792T, *C. pseudodiphtheriticum* CIP103420T).

patients, cultures of sputum samples were polymicrobial, and findings were interpreted as normal flora. Finally, the number of PCR-positive children with CF was significantly higher than the number of either children without CF or adults with CF ($p = 0.03$ and $p = 0.01$, respectively; Figure 2). Conversely, this difference was not significant between adult patients with CF and adult patients without CF (Figure 2). Notably, the 18 children who did not have CF were seen by clinicians in the same hospital, but not in the same healthcare center, and were not in contact with CF children. Thus, the only risk factor found for being infected or colonized with *C. pseudodiphtheriticum* was to be monitored at the CF center.

Discussion

We report the isolation of *C. pseudodiphtheriticum* in children with CF who had respiratory disease, mainly cough and rhinitis. As reemphasized in our study, this group of organisms is poorly identified by current phenotypic methods that lack specificity and result in ambiguous or even erroneous identification. These bacteria are usually considered as part of the natural flora of the respiratory tract, skin, and mucous membranes (*17*) and are not reported to clinicians. Moreover, culture of bacteria from sputum samples of patients with CF is known to lack sensitivity, either because of the fastidious nature of several organisms or because of overgrowth by common bacteria such as mucoid *P. aeruginosa* (*4*,*5*). For these reasons, an outbreak in a specific population of patients such as patients with CF may easily go unnoticed.

Our study shows that correct identification of bacteria remains critical for detecting such a possibility and that surveillance of the circulation of bacteria within patients with CF should be addressed in the future so new or emerging pathogens can be detected. For this purpose, we eventually identified the isolates by using PCR amplification and sequencing of the *rpoB* gene (currently the standard method) (*16*) and compared the findings to those obtained with the MALDI-TOF mass spectrometry method. Interestingly, the bacteria identified were exactly the same with both methods. This result suggests MALDI-TOF may represent a rapid inexpensive alternative assay for identification of these bacteria at the species level as recently reported for routine identification of bacteria (*10*). Moreover, both methods were highly discriminatory and allowed us to demonstrate that patients were infected or colonized by *C. pseudodiphtheriticum*. The dendogram obtained with the MALDI-TOF technique for identification of *C. pseudodiphtheriticum* was in general agreement with that of a partial *rpoB* gene sequencing phylogenetic tree, but identification of the strains at the species level could be obtained within minutes. Further studies to evaluate the typing power of MALDI-TOF mass spectrometry to discriminate bacterial strains below the species level should be done. In addition, the correct identification of the bacteria was the critical step in designing a new tool, i.e., a specific real-time quantitative PCR, to investigate the presence of an outbreak in the CF population.

The importance of positive cultures for nondiphtheria corynebacteria obtained from clinical samples of patients with signs and symptoms should not be overlooked (*18*). Although nondiphtheria corynebacteria were historically considered as contaminants without clinical significance, an increasing body of evidence shows their pathogenicity,

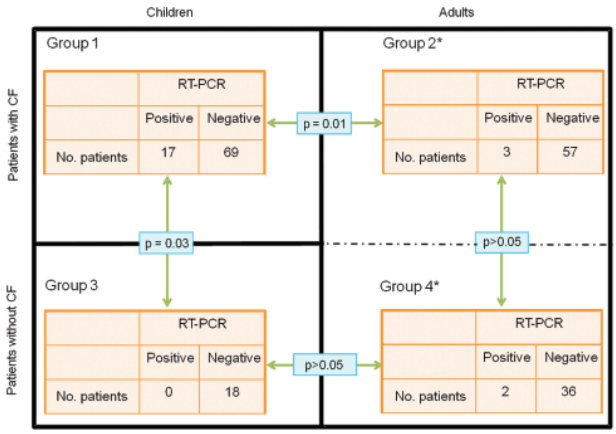

Figure 2. Results of real-time quantitative PCR specific for the *rpoB* gene for the detection of *Corynebacterium pseudodiphtheriticum* in sputum samples for the 4 groups of patients from 3 separate healthcare centers in Marseille, France, from January through December 2006. Black lines separate the different healthcare centers. Group 1, cystic fibrosis (CF) treatment center (CFTC) 1, with children with CF; group 2, CFTC2, with adults with CF; group 3, center with children without CF; group 4, patients without CF. *Patients from groups 2 and 4 hospitalized in the same healthcare center (CFTC2).

especially as a cause of nosocomial infection in hospitalized and immunocompromised patients (*19*). Among coryneform bacteria, *C. pseudodiphtheriticum* and *C. striatum* have been well documented as pathogens of the respiratory tract, leading to nosocomial and community-acquired pneumonia (*18*,*20*,*21*) as well as bronchitis, tracheitis, lung exacerbation, chronic obstructive lung disease, and lung abscesses (*22*–*29*).

In our study, we were initially surprised to isolate these bacteria in pure culture from sputa of patients with CF. About 70% of the patients had pulmonary symptoms, especially cough, and 6 (46.2%) case-patients required antimicrobial drug treatment. It is noteworthy that these clinical symptoms may be due either to coryneform toxins or to other respiratory pathogens, including viruses that were not investigated in this study. Surprisingly, 4 of 13 children had >1 isolate during the study period, which suggests that patients with CF become chronically colonized with *C. pseudodiphtheriticum*. Because of the difficulty in isolating these bacteria in respiratory samples, except for those that are in pure culture, their prevalence within the CF population may well be underestimated. This hypothesis was supported by the retrospective detection of DNA in additional sputum specimens from children with CF whose culture results were negative (≈20% of positive children) and adult patients by using our specific real-time PCR. We found that *C. pseudodiphtheriticum* was significantly associated with children with CF, which suggests transmission between patients with CF may have occurred in the CF healthcare center because none of the children without CF who were seen in a separate healthcare center (a different floor in the hospital) were PCR positive.

Patient-to-patient transmission could not be excluded and should be further investigated because 2 adult patients without CF who had PCR-positive specimens were detected in the same adult CFTC where they likely had contact with 2 PCR-positive CF adult patients. Such transmission has been recently demonstrated for *C. striatum* as a cause of nosocomial outbreak and respiratory colonization in patients with chronic obstructive pulmonary disease (*30*). Similarly, an outbreak of clonal multidrug-resistant strains of *C. striatum* as an emerging agent of pulmonary disease has been recently reported in Italy (*21*). Further epidemiologic studies are warranted to define the role of *C. pseudodiphtheriticum* transmission in the course of CF disease in other CF centers. Finally we believe that the implementation of isolation or segregation measures should be the rule in CF centers to reduce the risk of transmission of pathogens.

In conclusion, corynebacteria may colonize the respiratory tract of CF patients. Although the clinical importance of *C. pseudodiphtheriticum* in the complex setting of CF patients is less clear, we believe that this bacterium should be added in the list of new or emerging pathogens in these patients. Further clinical studies are needed to establish whether corynebacteria may contribute to the pathology of lung disease in CF patients.

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Dr Bittar is a postdoctoral researcher at Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, Unité Mixte de Recherche, Faculty of Medicine, Marseille. His research interests include detection and description of new or emerging pathogens in cystic fibrosis patients.

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White-Nose Syndrome Fungus (*Geomyces destructans*) in Bats, Europe

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White-nose syndrome is an emerging disease in North America that has caused substantial declines in hibernating bats. A recently identified fungus (*Geomyces destructans*) causes skin lesions that are characteristic of this disease. Typical signs of this infection were not observed in bats in North America before white-nose syndrome was detected. However, unconfirmed reports from Europe indicated white fungal growth on hibernating bats without associated deaths. To investigate these differences, hibernating bats were sampled in Germany, Switzerland, and Hungary to determine whether *G. destructans* is present in Europe. Microscopic observations, fungal culture, and genetic analyses of 43 samples from 23 bats indicated that 21 bats of 5 species in 3 countries were colonized by *G. destructans.* We hypothesize that *G. destructans* is present throughout Europe and that bats in Europe may be more immunologically or behaviorally resistant to *G. destructans* than their congeners in North America because they potentially coevolved with the fungus.

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White-nose syndrome (WNS) is a recently emerged wildlife disease in North America, which in 4 years has resulted in unprecedented deaths of hibernating bats in the northeastern United States (*1*–*3*), and is a widespread epizootic disease among bats. Although we have searched the literature describing observations of hibernating bats, we have been unable to find any similar historical accounts of white fungus growing on live hibernating bats in North America before the recent emergence of WNS.

In North America, WNS is known to affect 6 species of bats that use hibernation as their winter survival strategy: the big brown bat (*Eptesicus fuscus*), the eastern small-footed bat (*Myotis leibii*), the little brown bat (*M. lucifugus*), the northern long-eared bat (*M. septentrionalis*), the tricolored bat (*Perimyotis subflavus*), and the Indiana bat (*M. sodalis*) (*1*,*3*,*4*). Since its detection in February 2006 in a popular tourist cave near Albany, New York, USA, WNS has spread >1,300 km into Connecticut, Massachusetts, New Hampshire, New Jersey, Pennsylvania, Tennessee, Vermont, Virginia, and West Virginia in the United States and the provinces of Ontario and Quebec in Canada (*1*,*3*,*5*) in a pattern suggesting the spread of an infectious agent.

A recently discovered psychrophilic (cold-loving) fungus, *Geomyces destructans* (*6*), has consistently been isolated from bats that meet the pathologic criteria for WNS, including colonization of skin by fungal hyphae causing characteristic epidermal erosions and ulcers that can progress to invasion of underlying connective tissue (*2*,*7*). *G. destructans* is identified by its distinctive asymmetrically curved conidia and has a unique taxonomic position among other *Geomyces* spp. described to date (*6*). Its closest genetic relative is *G. pannorum*, a ubiquitous psychrophilic, keratinolytic fungus that has been isolated from a variety of sources and geographic regions, including soil and the fur of wild mammals in France (*8*), floors of trains and

ferryboats in Italy (*9*), boreal forests in Canada (*10*), and environmental samples from Arctic regions (*11*,*12*). *G. pannorum* var. *pannorum* has been reported as an unusual dermatophyte infecting fingernails and superficial skin of humans who have a history of close contact with soil and dust (*13*,*14*). However, *G. destructans* differs from other common soil fungi of North America in its ability to invade the living tissues of hibernating bats.

After WNS was described in North America (*1*), reports dating back to the early 1980s (*15*) described repeated observations of white fungal growth on muzzles of hibernating bats in Germany. However, these bats lacked the characteristics of WNS such as associated deaths. Moreover, fungus was not identified. In response to WNS in North America, researchers in Europe initiated a surveillance effort during the winter of 2008–09 for WNS-like fungal infections among hibernating populations of bats in Europe. *G. destructans* in Europe was previously reported in 1 hibernating bat that was sampled in France during March 2009 (*16*).

In this report, we describe results of a more extensive effort by scientists from 4 countries in Europe (Germany, United Kingdom, Hungary, and Switzerland) to obtain and analyze samples from hibernating bats with white patches on their faces or wing membranes. Our objectives were to identify the fungus colonizing such affected hibernating bats in Europe and to clarify its geographic distribution over a broad area of Europe.

Materials and Methods

During ongoing annual population surveys of caves and mines conducted by national nongovernmental organizations, hibernating bats with obvious fungal growth on their bodies (Figure 1, panel A) were opportunistically sampled in Germany, Switzerland, and Hungary; samples were also obtained from 2 dead bats from the same hibernaculum in the United Kingdom. Approximately 366 hibernacula were visited during mid-February–mid-April 2009: 336 in Germany, 20 in Hungary, and 10 in Switzerland. Two to 214 hibernating animals were observed at each site, with the exception of 2 sites in Germany, which harbored 2,000–7,000 animals at each site.

Samples were collected from live bats by using 2 methods. Touch imprints were obtained by holding adhesive tape against affected areas of skin or fur, or fur clippings were obtained from affected areas of bat muzzles. All species of bats in Europe are strictly protected under the Flora, Fauna, Habitat Guidelines of the European Union (92/43/EEC) (http://ec.europa.eu/environment/nature/legislation/habitatsdirective/index_en.htm) and The Agreement on the Conservation of Populations of European Bats (www.eurobats.org). We did not have permission to invasively sample or kill individual animals for histologic analysis to confirm skin infection by *G. destructans* (*7*). Samples were shipped to the Leibniz Institute of Zoo and Wildlife Research (IZW), Berlin, Germany, for further investigations.

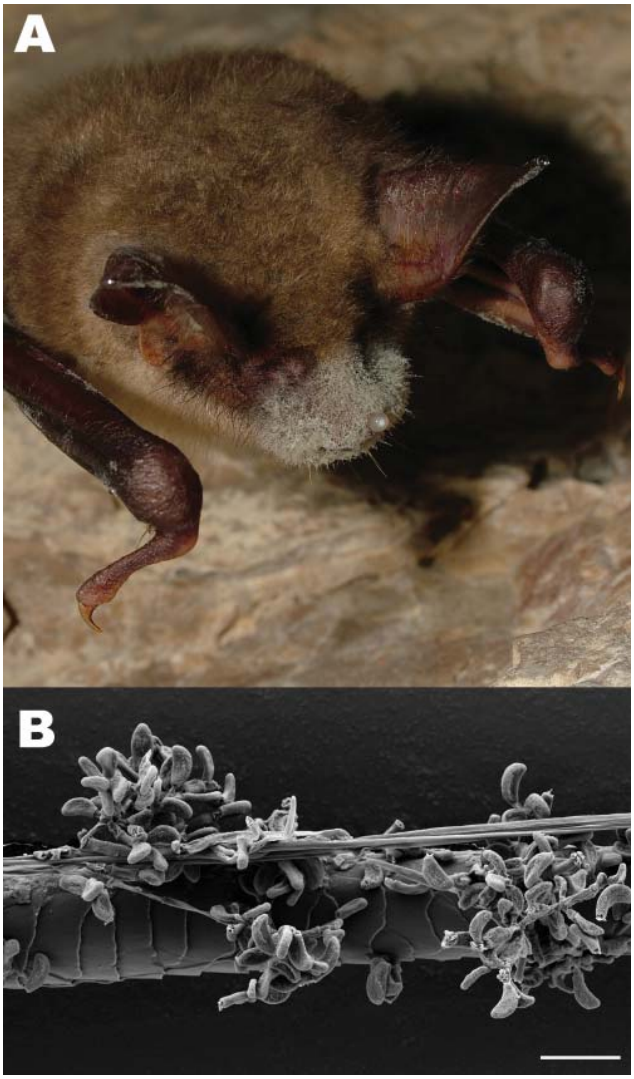

Figure 1. A) Greater mouse-eared bat (*Myotis myotis*) with white fungal growth around its muzzle, ears, and wing membranes (photograph provided by Tamás Görföl). B) Scanning electron micrograph of a bat hair colonized by *Geomyces destructans*. Scale bar = 10 µm.

Twenty adhesive tape samples were first screened by using light microscopy, and 21 hair samples were examined by using scanning electron microscopy for conidia characteristic of *G. destructans* (Figure 1, panel B). Two of the submitted samples (2 greater horseshoe bats from the United Kingdom) consisted of entire bat carcasses. Although the carcasses were examined externally for fungal growth on muzzle skin and hair, specimens were too decomposed to conduct internal pathologic examinations. Tape or hair samples from all bats were further investigated

by using direct PCR amplification of fungal rRNA gene internal transcribed spacer (ITS) region DNA (ITS1, 5.8S, and ITS2). Total nucleic acids were extracted from culture, tape, or hair samples by using PrepMan Ultra Reagent (Applied Biosystems, Darmstadt, Germany) following the manufacturer's instructions.

The rRNA gene ITS region DNA was amplified by using PCR with primers ITS4 and ITS5 (*17*) and GoTaq DNA polymerase (Promega, Madison, WI, USA). Cycling parameters were an initial 2-min denaturation at 98°C; followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. For fungal isolates, rRNA gene small subunit (SSU) DNA was amplified by using PCR with primers nu-SSU-0021–5′ (*17*) and nu-SSU-1750–3′ (*18*) as above, except the extension time was increased to 2 min. Sequencing primers were PCR primers; nu-SSU-0402–5′ (*18*), nu-SSU-1150–5′ (*17*), nu-SSU-0497–3′ (*18*), and nu-SSU-1184–3′ (*19*) were added for SSU. PCR products were sent to the Robert Koch Institute, Berlin, Germany, for direct sequencing.

Culture analyses of samples were performed at Munich University Hospital and IZW. After examining tape impressions by using light microscopy, we identified small areas with fungal conidia characteristic of *G. destructans* and excised them with a sterile scalpel blade. Half of the excised material was used for PCR; the remaining sample and samples of individual hairs with microscopic indication of *G. destructans* were immediately placed onto Sabouraud dextrose agar plates containing gentamicin and chloramphenicol and incubated at 4°C and 8°C. *G. destructans* isolates obtained during this study are maintained at IZW.

Results

We obtained and analyzed samples from live bats with obvious fungal growth on their bodies found between mid-February and the end of March 2009 at 11 sites (8 in Germany, 1 in Hungary, and 2 in Switzerland). Samples were also obtained from an additional bat in Germany in February 2008 and from 2 dead bats from a site in the United Kingdom in March 2009 (Tables 1, 2) All 12 hibernacula sampled contained 1–5 animals that exhibited obvious fungal growth. Forty-three samples were obtained from these 12 hibernacula and represented 23 adult bats of 6 species: 1 Brandt bat (*M. brandtii*), 3 pond bats (*M. dasycneme*), 1 Daubenton bat (*M. daubentonii*), 1 lesser mouse-eared bat (*M. oxygnathus*), 15 greater mouse-eared bats (*M. myotis*), and 2 greater horseshoe bats (*Rhinolophus ferrumequinum*).

After direct PCR amplification and DNA sequence analysis of fungal rRNA gene ITS regions, genetic signatures 100% identical with those from *G. destructans* type isolate NWHC 20631–21 (GenBank accession no. EU884921) were identified from 21 of 23 bats examined: 15/15 from Germany, 2/2 from Hungary, and 4/4 from Switzerland. Both bats from the United Kingdom were colonized by *Penicillium* sp. (Tables 1, 2). Fungi with conidia morphologically identical to those of *G. destructans* (Figure 1, panel B) as described by Gargas et al. (*6*) were isolated in axenic cultures from 8 of 23 bats examined: 3/15 from Germany, 1/2 from Hungary, and 4/4 from Switzerland) (Tables 1, 2; Figure 2).

Consistent with published descriptions for *G. destructans* (*6*), fungal colonies grew slowly and within 14 days attained diameters of 1.0 mm at 4°C and 4.0–5.0 mm at 8°C; no growth occurred at 25°C. The sensitivity of our method for isolating *G. destructans* from bat hair was comparable to published diagnostic sensitivity for culturing *G. destructans* from bat skin (*20*). Subsequent PCR/DNA sequencing analyses of the 8 isolates indicated that they all had rRNA gene ITS and SSU region DNA sequences identical to those of *G. destructans* type isolate NWHC 20631–21 (GenBank accession nos. EU884921 for ITS and FJ231098 for SSU).

Unlike other bats sampled in this study, the 2 greater horseshoe bats from the United Kingdom were found dead, and their nostrils were colonized by *Penicillium* sp. These bats did not fulfill the pathologic criteria for WNS (*7*) because fungal hyphae did not invade the epidermis but remained within the superficial layer of the epidermal stratum corneum. A more complete description of the postmortem analysis of the greater horseshoe bats has been reported (*21*). *G. destructans* was not isolated in culture, and its genetic signature was not identified by PCR and DNA sequencing of samples collected from greater horseshoe bats.

Table 1. Bats tested for *Geomyces destructans* by using microscopy, fungal culture, or PCR analysis, by country, Europe*

Species (common name)	No. positive/no. tested			
	Germany	Switzerland	Hungary	United Kingdom
Myotis myotis (greater mouse-eared bat)	10/10	4/4	1/1	–
M. dasycneme (pond bat)	3/3	–	–	–
M. daubentonii (Daubenton bat)	1/1	–	–	–
M. brandtii (Brandt bat)	1/1	–	–	–
M. oxygnathus (lesser mouse-eared bat)	–	–	1/1	–
Rhinolophus ferrumequinum (greater horseshoe bat)	–	–	–	0/2

*–, species not obtained in this country.

Table 2. Fungal culture and PCR results for 23 bats with evidence of fungal colonization tested by light or electron microscopy, Europe*

Country/ location no.†	Sample source	Species	Collection date	No./ hibernacula	PCR result	Culture result	GenBank accession no.	
							ITS	SSU rRNA
Germany/4	Hair 2	*Myotis dasycneme*	2008 Feb 25	10	+	+	GU350437	GU350442
Germany/8	Hair 7	*M. myotis*	2009 Mar 3	214	+	+	GU350436	GU350441
Germany/7	Tape 8	*M. myotis*	2009 Mar 7	57	+	+	GU999986	GU999983
Hungary/9	Hair 16	*M. myotis*	2009 Mar 29	64	+	+	GU350434	GU350439
Switzerland/10	Tape 10	*M. myotis*	2009 Apr 5	25	+	+	GU350433	GU350438
Switzerland/10	Tape 11	*M. myotis*	2009 Apr 5	25	+	+	GU999984	GU999981
Switzerland/11	Tape 12	*M. myotis*	2009 Apr 5	25	+	+	GU999985	GU999982
Switzerland/10	Tape 20	*M. myotis*	2009 Apr 11	25	+	+	GU350435	GU350440
Germany/1	Hair 1	*M. myotis*	2009 Feb 21	65	+	–	HM222616	–
Germany/6	Hair 20	*M. myotis*	2009 Mar 13	100	+	–	HM222617	–
Germany/2	Tape 1	*M. myotis*	2009 Feb 26	≈2,000	+	–	HM222618	–
Germany/2	Tape 2	*M. myotis*	2009 Feb 26	≈2,000	+	–	HM222619	–
Germany/8	Tape 5	*M. myotis*	2009 Mar 3	214	+	–	HM222620	–
Germany/8	Tape 6	*M. myotis*	2009 Mar 3	214	+	–	HM222621	–
Germany/7	Tape 9	*M. myotis*	2009 Mar 7	57	+	–	HM222622	–
Germany/6	Tape 16	*M. myotis*	2009 Mar 13	100	+	–	HM222623	–
Germany/8	Hair 6	*M. brandtii*	2009 Mar 3	214	+	–	HM222624	–
Germany/5	Hair 3	*M. dasycneme*	2009 Feb 28	29	+	–	HM222625	–
Germany/6	Tape 17	*M. dasycneme*	2009 Mar 13	100	+	–	HM222626	–
Germany/3	Hair 17	*M. daubentonii*	2009 Mar 5	≈7,000	+	–	HM222627	–
Hungary/9	Tape 13	*M. oxygnathus*	2009 Mar 29	64	+	–	HM222628	–
United Kingdom/12	Hair 10	*Rhinolophus ferrumequinum*	2009 Mar 11	558	–‡	–‡	HM222629	–
United Kingdom/12	Hair 11	*R. ferrumequinum*	2009 Mar 11	558	–‡	–‡	HM222630	–

*ITS, internal transcribed spacer; SSU, small subunit; tape, touch imprint with adhesive tape.
†Location number corresponds to a hibernation site in Figure 2.
‡Although samples were negative for *G. destructans*, they were positive for *Penicillium* sp. by PCR and culture.

Discussion

Laboratory analyses demonstrated that 5 species of the genus *Myotis* in Europe harbored *G. destructans*; male and female bats were equally affected. Despite laboratory confirmation that bats obtained in Germany, Switzerland, and Hungary were colonized by *G. destructans*, deaths were not observed at collection sites. Puechmaille et al. (*16*) reported a similar observation with a greater mouse-eared bat in France. Additionally, a lesser mouse-eared bat from Hungary with visible fungal infection during hibernation, from which *G. destructans* was isolated, was recaptured 5 months later (August 2009) and showed no external signs of fungal infection. On February 19, 2010, the same bat was again observed in the same hibernaculum without any visible sign of fungal growth. However, 7 other bats within that group of 55 animals displayed obvious fungal growth but were not sampled for this study.

In contrast, decreases in hibernating bat colonies infected by *G. destructans* in North America are often >90% (*2*,*3*), and mortality rates similar in magnitude would be difficult to miss among closely monitored winter populations of bats in Europe. Biologists in Germany and Switzerland have conducted annual censuses of bat hibernacula since the 1930s and 1950s, respectively. In Hungary, the largest hibernacula have been annually monitored since 1990. Similar death rates to those caused by WNS in hibernating bats in North America have never been documented in countries in Europe in which *G. destructans* has now been identified.

Although distribution of *G. destructans* in bats across Europe has not been exhaustively characterized, opportunistic sampling conducted as part of this study during the winter of 2008–09 demonstrated that the fungus was present on bats in 3 countries (Figure 2). The 2 most distant points from which bats colonized with *G. destructans* have been identified were separated by >1,300 km. Despite the observed distribution of *G. destructans* in Europe (Figure 2), the 5 bat species from which *G. destructans* was detected migrate average distances <100 km between their summer and winter roosting sites (*22*), indicating that the fungus is most likely spread as local bat populations emerge from hibernacula, disperse, and interact with populations within their dispersal range. Identification of bats colonized by *G. destructans* from such distant sites, in addition to the relatively homogenous distribution of the fungus among sites in Germany, suggests that *G. destructans* may be widespread in Europe.

Regardless of widespread occurrence of *G. destructans* among bat species in Europe (Figure 2), deaths of bats in Europe caused by WNS, similar to those caused by WNS in North America, have not been observed. Although no

bat species migrates between Europe and North America or is present on both continents (*23*,*24*), many species of the genus *Myotis* are infected by *G. destructans* on each continent. Although the mechanism(s) by which hibernating bats died because of infection with *G. destructans* in North America is not yet understood, bat species in Europe may exhibit greater resistance or respond differently to infection by this fungus than their counterparts in North America.

Before the emergence of WNS in North America, large aggregations of hibernating bats ranging from 1,000 to 50,000 animals were common in caves and mines of affected regions, and many hibernation sites in regions of North America still unaffected by WNS contained tens of thousands of bats during winter (some contain hundreds of thousands) (*25*). In contrast, aggregations of bats hibernating in caves and mines in Europe rarely exceed 1,000 animals. However, larger hibernating groups have been observed at a few natural sites, such as a cave in northern Germany with 13,000–18,000 bats (*26*) and human-made structures (e.g., Daubenton bats in bunkers and catacombs) (*24*). If host density plays a role in *G. destructans* transmission or deaths of bats, such as through increased disturbance of clustered bats, the bats in Europe may experience lower mortality rates because they form smaller hibernation groups composed of small clusters or individual bats. Apparent continental differences in susceptibility of hibernating bats to deaths associated with skin infection by *G. destructans* may indicate either circumstantial or evolved resistance in bats in Europe.

G. destructans has been detected in North America only in states and provinces where WNS has also been observed and in contiguous states. Recent emergence and spread of *G. destructans* with associated deaths of bats throughout hibernacula in the northeastern United States (*3*) may suggest ecologic release of an exotic pathogen into an uninfected ecosystem. Although this suggestion remains a hypothesis and how *G. destructans* may have been introduced to the United States is not known, initial documentation of WNS in a popular tourist cave near Albany, New York (*1*), suggests that a human vector could have been involved.

There are many examples of unintended introductions of fungal pathogens, particularly of those affecting plants and ectothermic animals with tissue temperatures permissive to fungal infection (*27*–*29*). One case with striking similarities is the panzootic chytrid fungus (*Batrachochytrium dendrobatidis*), which has caused global decreases among amphibian species (*30*). As with skin infection by *B. dendrobatidis* in amphibians, which can alter body electrolyte levels and lead to cardiac arrest (*31*), skin infection by *G. destructans* in hibernating bats may also kill by causing irreversible homeostatic imbalance because wing membranes play major roles in water balance, circulation, and thermoregulation of hibernating bats during winter (*32*,*33*).

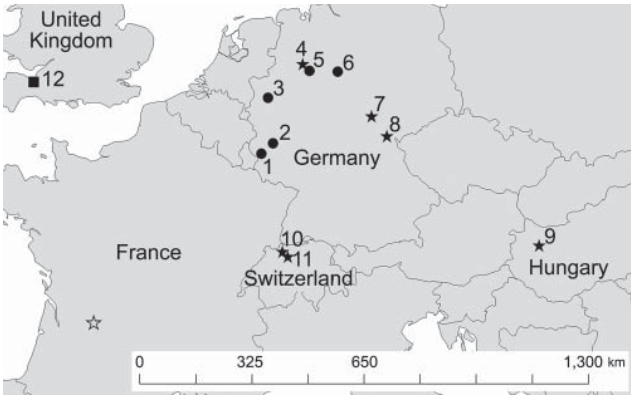

Figure 2. Locations in Europe of bats positive for *Geomyces destructans* by PCR alone (circles) or by PCR and culture (solid stars) and bats negative for *G. destructans* but positive for other fungi (square). Numbers for locations correspond to those in Table 2. Sites 7, 8, and 9 had additional bats that were positive for *G. destructans* only by PCR. Location of a bat positive for *G. destructans* in France (*16*) is indicated by an open star. Some sites had >1 bat species with evidence of colonization by *G. destructans*.

Bat species in Europe may be immunologically or behaviorally resistant to *G. destructans* because of having coevolved with the fungus. Additionally, microbial flora of bat skin or other abiotic surfaces in bat hibernacula in Europe may have also coevolved to incorporate *G. destructans* as a nonpathogenic component of the microbial community. Conversely, possible recent introduction of *G. destructans* into the United States, with subsequent infection of bat species in North America and ecosystems not infected with the fungus, provides a potential explanation for the devastating effects of WNS in North America. Although bats are reservoirs of various pathogens (*34*,*35*), research into the immune function of bats, particularly during hibernation, is just beginning.

In conclusion, nondetrimental colonization of bat species in Europe by *G. destructans* may be relatively common (Figure 2), and historical reports (*15*) suggest that such colonization of hibernating bats in Europe has occurred for several decades. In contrast to recent mass deaths associated with *G. destructans* skin infection, which is the hallmark of WNS in North America, bats in Europe appear to coexist with *G. destructans*. Studies to investigate mechanisms of pathogenesis, microbial ecology, and phylogeography of *G. destructans* will be essential for developing a comprehensive understanding of WNS. In particular, testing the hypotheses that bats in Europe are more resistant to fungal skin infection by *G. destructans*, that *G. destructans* was introduced from Europe to North America, and that environmental circumstances limit the pathogenicity of *G. destructans* in Europe seem warranted. Divergent manifestations of infection by *G. destructans* in bats in Europe and North America provide a unique opportunity to

address these research objectives with the ultimate goals of better understanding WNS and developing sound strategies to manage the devastating effects of this emerging wildlife disease in North America.

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Scavenging Ducks and Transmission of Highly Pathogenic Avian Influenza, Java, Indonesia

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In Java, Indonesia, during March 2007–March 2008, 96 farms with scavenging ducks that were not vaccinated against highly pathogenic avian influenza (HPAI) were monitored bimonthly. Bird-level (prevalence among individual birds) H5 seroprevalence was 2.6% for ducks and 0.5% for chickens in contact with ducks. At least 1 seropositive bird was detected during 19.5% and 2.0% of duck- and chicken-flock visits, respectively. Duck flocks were 12.4× more likely than chicken flocks to have seropositive birds. During 21.4% of farm visits, ≥1 sampled duck was H5 seropositive when all sampled in-contact chickens were seronegative. Subtype H5 virus was detected during 2.5% of duck-flock visits and 1.5% of chicken-flock visits. When deaths from HPAI infection occurred, H5 virus shedding occurred in apparently healthy birds on 68.8% of farms. Of 180 poultry deaths investigated, 43.9% were attributed to H5 virus. These longitudinal study results indicate that ducks are a source of infection for chickens and, potentially, for humans.

Since 1997, when highly pathogenic avian influenza (HPAI) subtype H5N1 outbreaks occurred in poultry in Hong Kong, People's Republic of China (*1*–*2*), the virus has caused epidemics in Asia, Europe, and Africa (*3*). In Indonesia, the first HPAI (H5N1) virus infections in poultry were officially announced in early 2004 (*4*); human cases have been reported since mid-2005 (*5*). Although extensive HPAI control efforts helped reduce the frequency of outbreaks in poultry (*6*), by 2009, subtype H5N1 virus had been detected in 31 of Indonesia's 33 provinces (*7*). In 2009, Indonesia had the highest incidence worldwide of human infections and deaths (*8*).

Waterfowl are the natural reservoir of avian influenza viruses (*9*), and experimental research indicates that ducks may play a role in the maintenance of HPAI (H5N1) viruses. Infected ducks may exhibit no clinical signs yet can excrete high concentrations of virus that are pathogenic to other poultry species (*10*–*13*). Possible risk factors for HPAI spread in Indonesia include duck movements, contacts between ducks and other poultry and animal species, poor poultry husbandry, inadequate handling of sick and dead ducks by flock owners, and poor awareness of control strategies among poultry farmers (*14*). However, no analytical study assessing risk factors for HPAI infection has been conducted in Indonesia.

In 2005, Indonesia's duck population was ≈34.3 million, of which 40% were on the island of Java, mainly on smallholder farms, i.e., backyard and small commercial farms (*14*). As in many other Asian countries, domestic ducks on smallholder farms in Indonesia are allowed to scavenge freely during the day around houses, in the villages, or in rice paddies; duck owners supply little or no feed (*15*). To assess the hypothesis that ducks contribute to the maintenance and transmission of avian influenza (H5N1) viruses, we conducted a longitudinal investigation describing temporal patterns of antibodies against HPAI (H5) and virus prevalence in unvaccinated scavenging ducks and chickens that have contact with these ducks (in-contact chickens) in Java, Indonesia.

Materials and Methods

Study Design

Ducks and in-contact chickens on 96 smallholder duck farms in 4 districts of Central Java were monitored once ev-

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ery 2 months over 13 months. Four districts were selected (Sleman, Magelang, Bantul, and Kulon Progo) because of their high abundance of duck farms and proximity to the Disease Investigation Center (DIC) in Wates, where field investigators were based and diagnostic work was conducted (Figure 1). Sample size calculations were based on DIC surveillance data collected in Central Java in 2006; 13 (4.7%) of 278 cloacal swabs from ducks were positive for H5 viral RNA on real-time reverse transcription–PCR (RT-PCR). On the basis of an expected true bird-level virus prevalence of 5%, a precision of the estimate of ±1.5 % and a 95% confidence interval (CI), a total of 811 ducks had to be sampled (*16*). We enrolled 96 duck farms in the study and sampled a total of 960 ducks (10 ducks per farm) and 480 in-contact chickens (5 chickens per farm) during each of 7 visits over 13 months (initial visit plus 6 bimonthly visits).

We used a multistage sampling strategy with stratification by district and a 3-level sampling process that involved villages, duck farms, and birds. A sampling frame was prepared by agriculture extension officers who listed all villages in the selected districts, including the total number of duck farms within each village. We selected 4 villages within each district using probability proportional to size sampling. Field veterinarians then prepared a second sampling frame containing the names of all duck farmers within the 16 villages selected and the number of ducks kept by each farmer. From this sampling frame, 6 duck farms per village were selected by using simple random sampling. Farms with <10 ducks were excluded (we wanted to sample 10 ducks per farm) as were farms with >700 ducks (which we considered to be large commercial farms). Random numbers for village and duck farm selection procedures were produced in STATA version 10.0 (StataCorp, College Station, TX, USA).

Data Collection and Diagnostic Tests

Four veterinarians from the DIC were trained in the use of data collection tools and interviewing techniques. Field visits were conducted once every 2 months from March 2007 through March 2008; duck owners were interviewed and swab and blood samples from birds were obtained during each visit. On the first visit, birds were selected for the study. The duck owner enclosed all ducks in a pen and selected the first 10 ducks that could be caught. If available, 5 chickens kept on the same farm were also selected in the same manner. Wing tags or leg bands were attached to each selected duck and chicken. Blood samples were collected from the wing vein of each bird, and an oropharyngeal swab and a cloacal swab were collected from each bird and placed into a single tube containing virus transport media (Universal Viral Transport 3mL; Becton Dickinson, Franklin Lakes, NJ, USA). Duck owners confirmed that none of the ducks and chickens sampled had been vaccinated against HPAI before the study and that none were vaccinated during the study.

Figure 1. Districts in Central Java, Indonesia, where ducks and in-contact chickens were monitored bimonthly for avian influenza (H5) during March 2007–March 2008.

Serum samples were tested for antibodies to avian influenza (H5) by using the hemagglutination inhibition (HI) test according to methods recommended by the World Organisation for Animal Health (OIE) (*17*). Antigen and control antiserum were supplied from Pusat Vetenerinaria Farma (Surabaya, Indonesia). The antigen was derived from an HPAI (H5N1) chicken isolate obtained in 2004 in Indonesia (A/chicken/Pare/East Java/2004). This antigen is commonly used for HI tests to detect antibodies to avian influenza (H5N1) at all veterinary diagnostic laboratories in Indonesia. A titer $\geq 2^4$ against 4 hemagglutinating units of antigen was considered positive (*17*). In accordance with the Australian Animal Health Laboratory protocol (*18*–*19*), RT-PCR was used to test the combined oropharyngeal and cloacal swabs of individual birds in pools of 5 for subtype H5 virus RNA. Sequencing was conducted on the H5 RT-PCR–positive samples to confirm the HPAI multiple basic amino acid motif at the cleavage site of the hemagglutinin gene and to determine whether the neuraminidase gene of the isolate belonged to the N1 subtype.

Investigations of Bird Deaths

Duck farmers involved in the study were asked to immediately report sickness or deaths of birds to the DIC. Compensation was paid to duck farmers to encourage reporting. Upon notification, veterinarians conducted an outbreak investigation at the reported farm by using a predesigned questionnaire. Clinical signs were recorded, and carcasses were collected for postmortem examination. Blood and swab samples from clinically normal birds from the same farm were obtained on the day of the investigation. Blood samples were tested for antibodies to avian

influenza (H5) as already discussed; swab samples from carcasses (combining lung, heart, liver, spleen, pancreas, and intestinal tissues) and from live birds were processed by virus isolation in embryonated eggs. Two passages of virus isolation were conducted, and allantoic fluid was tested for H5 antigen of avian influenza by using the HI test. An HPAI outbreak was defined as >1 bird dying within a few days of each other from HPAI (i.e., positive by subtype H5 virus isolation or RT-PCR).

Data Analyses

For both ducks and in-contact chickens, bird-level seroprevalence (proportion of study birds with antibodies to avian influenza [H5]) and flock-level seroprevalence (in which at least 1 study bird had antibodies) were calculated for each of the 7 sampling periods and pooled across the entire study period. Virus prevalence was calculated only at flock level (proportion of flock visits in which at least 1 pool of swab samples from the farm was positive for H5 RNA) for the entire study period. We accounted for the multistage sampling strategy in the data analyses by using survey commands in STATA version 10.0 (StataCorp); districts were treated as strata; villages were specified as primary, and farms as secondary, sampling units. For bimonthly bird-level prevalences, and for bird- and flock-level prevalences over the entire study period, sampling weights were the inverse of the product of the proportion of villages in the district that were sampled and the proportion of duck farms in the village that were sampled (*20*). The finite population correction factor for primary sampling units was the total number of villages in the district. Finite population correction accounted for reduction in variance associated with sampling without replacement (*21*). For bird-level seroprevalence calculations over the entire study period, we accounted for repeated measurements within the same birds by specifying the individual bird as the third level of sampling and incorporating the number of duck farms per village as the finite population correction factor for secondary sampling units. For the bimonthly flock-level seroprevalence, only primary sampling units with their finite population correction factor were specified in the analyses. Sampling weights for bimonthly flock-level seroprevalence were the inverse of the proportion of villages in the sampled district.

We used logistic regression models accounting for 3 levels of clustering (birds within farms within villages) to compare the odds of birds having titers positive for avian influenza (H5) between ducks and in-contact chickens. For flock-level comparisons, logistic regression models accounting for 2 levels of clustering (farms within villages) were used to compare the odds of flocks having at least 1 bird with antibodies to avian influenza (H5) between duck and in-contact chicken flocks, and for duck flocks between sampling months. Logistic regression accounting for 2 levels of clustering was also used to evaluate whether the odds of duck (or chicken) flocks being seropositive were independent of the results of the other species at the same farm and sampling. All logistic regression models also accounted for sampling weights and incorporated finite population correction. Adjusted Wald tests were used to assess the overall effect of sampling month. After fitting the logistic regression models taking the survey sampling design into account, we applied the F-adjusted mean residual goodness-of-fit test (*22*).

Results

From March 2007 through March 2008, a total of 8,993 serum and swab samples were collected from 6,705 clinically healthy ducks and 2,288 chickens during 670 farm visits (at 2 farm visits, all birds had been sold). During ≈80% of farm visits, chickens were present. Flock sizes for ducks and chickens averaged 53.7 and 8.5, respectively. Of all combined oropharyngeal and cloacal swab sets from individual birds, 8,900 were analyzed in pools of 5 by RT-PCR, and all serum samples were tested for antibodies to subtype H5 virus. In addition, during outbreak investigations, 174 sets of swabs from dead birds and 136 from apparently healthy live birds were collected from the outbreak farms.

Prevalence of Antibodies to Avian Influenza (H5)

Bird-level seroprevalences of subtype H5 antibody titers $\geq 2^4$ in clinically healthy birds for all bird samplings pooled over the entire study period were 2.6% (95% CI 1.8–3.5) for ducks and 0.5% (95% CI 0.0–0.9) for in-contact chickens. The odds of ducks being positive for avian influenza (H5) were 5.5× (95% CI 2.1–14.4) higher than for in-contact chickens. Flock-level seroprevalence of antibodies to avian influenza (H5) was 19.5% (95% CI 14.3–24.6) for ducks and 2.0% (95% CI 0.1–3.9) for in-contact chickens. The odds of duck flocks being seropositive were 12.4× (95% CI 3.9–40.1) higher than those for chicken flocks.

Figure 2 shows the H5 bird- and flock-level seroprevalences for ducks and in-contact chickens from the beginning of March 2007 through the end of March 2008. Duck flock–level prevalence varied over time from 5.9% to 24.7%. The odds of a duck flock being seropositive differed significantly by month ($p = 0.05$); odds were higher in July 2007 (odds ratio [OR] = 3.1; 95% CI 1.1–9.0), September 2007 (OR = 2.9; 95% CI 1.4–6.3), November 2007 (OR = 3.7; 95% CI 1.7–8.1) 2007, January 2008 (OR = 3.9; 95% CI 1.5–10.0), and March 2008 (OR = 5.3; 95% CI 1.9–14.7), relative to May 2007. Chicken flock-level seroprevalences remained <6.2% throughout the study.

At 21.4% of 501 farm visits, ≥1 study duck was seropositive for influenza (H5) when during the same farm

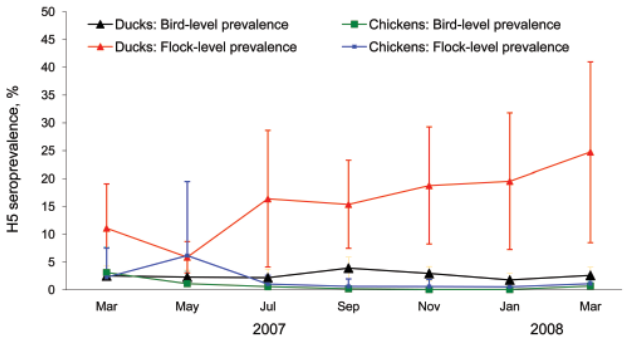

Figure 2. Bird- and flock-level seroprevalences of avian influenza (H5) in ducks and in-contact chickens monitored for infection, Central Java, Indonesia, March 2007–March 2008. Error bars indicate point-wise 95% confidence intervals. Flock-level seroprevalences are proportions of flocks where at least 1 bird had an antibody titer $\geq 2^4$ to H5 virus. Estimates are adjusted for the survey structure.

visits, all in-contact study chickens on these farms were seronegative (Table). Conversely, at only 1.4% of farm visits was ≥1 study chicken seropositive for avian influenza (H5), while all study ducks on the farm were seronegative. At flock level, seropositivity of ducks was not associated with seropositivity of chickens on the same farm (OR = 3.9, 95% CI 0.4–43.0). The goodness-of-fit statistics calculated after fitting the survey design–adjusted logistic regression models provided no evidence of lack of fit of any of the models (p>0.05).

HPAI (H5) Virus Prevalence in Clinically Healthy Birds

Birds on 25 (26%) of the 96 monitored farms tested positive for avian influenza (H5) virus RNA; on 20 farms, birds tested positive on 1 sampling occasion and, on 5 farms, on 2 different sampling occasions. On these 25 farms, 30 flocks (22 duck and 8 chicken flocks) tested positive for subtype H5 virus RNA. On 3 farms, both duck and chicken flocks tested positive for subtype H5 virus RNA at the same visit (6 flocks); otherwise, only 1 flock (either ducks or chickens) was positive for subtype H5 virus RNA at any 1 visit (19 duck and 5 chicken flocks). The flock prevalence of subtype H5 virus RNA (proportion of flock-visits during which at least 1 study bird was positive) in clinically healthy birds for all flock samplings pooled over the entire study period was 2.5% (95% CI 0.9–4.1) for ducks and 1.5% (95% CI 0.4–2.7) for chickens.

HPAI Outbreaks

Of 96 the study farms, 34 (35%) across all 4 districts had HPAI outbreaks during the study period (Figure 3). One farm had 3 outbreaks and 2 farms had 2 outbreaks in different months; each of the remaining 31 farms had 1 outbreak. The numbers of outbreaks increased substantially from 1 each in May and June 2007 to 7 each in July and September 2007.

On 16 of the 34 farms that experienced HPAI outbreaks, combined swab samples were collected from 136 clinically normal birds (109 ducks, 27 chickens) at the same time as samples from dead birds on the same farm. For 11 (69%) of 16 outbreak farms, HPAI (H5) virus was isolated from 37 (27%) of 136 clinically normal birds (28 ducks, 9 chickens).

Carcasses of 180 marked and unmarked birds (59 ducks, 121 chickens) were obtained; HPAI (H5N1) virus was isolated from 65 (10 ducks, 55 chickens). Another 14 birds (3 ducks, 11 chickens) had most likely died from HPAI (H5) infection; we based this determination on 1) sudden death with or without clinical signs of HPAI (such as lethargy; swelling or discoloration of combs, wattles, and legs; nasal discharge; coughing and sneezing; diarrhea; and lack of coordination) and 2) isolation of HPAI (H5) virus from other dead birds within the flock at the same time. Therefore, a total of 79 (44%) of 180 birds most likely died from HPAI infection.

Discussion

Scavenging duck farming has been proposed as an important contributor to HPAI in poultry flocks in Southeast Asia, predominantly on the basis of findings obtained through spatial analyses of national surveillance data of HPAI outbreaks (*23*–*25*). However, no field studies have investigated infection patterns over time in duck farming systems. Results of the current study indicate that scavenging ducks are a source of infection for other poultry and, possibly, for humans.

One explanation for the higher seroprevalence in ducks than in chickens is that HPAI (H5N1) virus circulated more successfully among ducks than among in-contact chickens; hence, ducks were more likely to harbor and transmit the virus. This could because of a higher risk for death among infected chickens, resulting in fewer surviving chickens with H5 antibodies, or to differences in scavenging behavior between ducks and chickens. Virulence of HPAI (H5N1) virus for ducks varies from inconsequential to highly lethal (*10*,*26*), and some of the 2003–04 Asian-lineage subtype H5N1 viruses can be shed by domestic ducks for up to 17

Table. Antibodies to avian influenza (H5) in ducks and in-contact chickens monitored on the same farms during 501 farm visits, Central Java, Indonesia, March 2007–March 2008

H5 serologic status of ducks*	H5 serologic status of in-contact chickens†	No. (%) farm visits
Positive	Positive	3 (0.6)
Positive	Negative	107 (21.4)
Negative	Positive	7 (1.4)
Negative	Negative	384 (76.6)

*Based on results from 10 ducks at each farm visit.
†Based on results from 5 in-contact chickens at each farm visit.

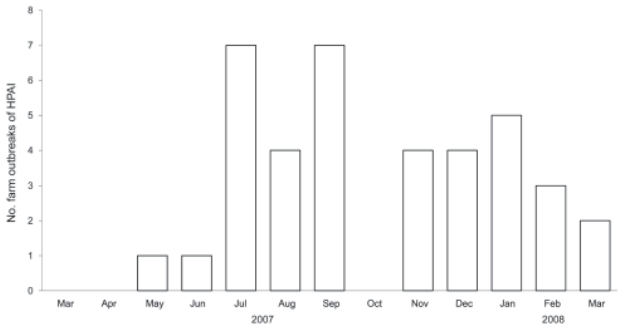

Figure 3. Number of highly pathogenic avian influenza (HPAI) (H5N1) outbreaks, by month, in 96 randomly selected smallholder duck farms, Central Java, Indonesia, March 2007–March 2008.

days postinfection (*11*,*12*). When viruses harbored by ducks are transmitted to gallinaceous species, such as chickens, severe clinical signs and high death rates can occur (*10*). However, for high-incidence HPAI outbreaks in chickens, virus excretion from infected ducks must be combined with an efficient reproductive number (R_0) to produce secondary cases in a susceptible chicken population. R_0 is influenced by the infectiousness of the agent causing the disease, the probability of transmission (determined by factors such as housing, mixing, and feeding practices), and the level of population immunity. During the 2004 HPAI (H5N1) epidemic in Thailand, R_0 estimates were lower for backyard chickens than for broilers and layers (*27*). Birds of the latter 2 groups are typically housed together; but backyard chickens usually have less contact with each other. On our study farms, ducks usually grazed together, behavior conducive to virus circulation between ducks; individual in-contact chickens scavenged more independently. Another possible explanation for the difference in seroprevalences between poultry species is that duck flocks were exposed to HPAI more frequently than were chickens. Duck flocks may graze in the same rice fields where other potentially infected domestic or wild birds may have grazed.

The higher flock-level seroprevalence in ducks than in chickens was probably not biased substantially on differences in the numbers of birds sampled (10 ducks, 5 chickens) at each farm visit. In flocks where no study birds were detected with avian influenza (H5) virus or antibody, the virus may have been in other ducks or chickens in the same flock, and therefore our flock-level prevalence estimates underestimated the true flock prevalences. Because we sampled more ducks than chickens in each study flock, the risk for nondetection of infection was higher for chickens. However, on the basis of sample size calculations for assessing freedom from disease, this bias is unlikely to explain the differences in flock prevalences of antibodies between ducks and chickens (*28*). For example, if the true seroprevalence was 3% in populations of 100 ducks and 100 chickens, the probability of detecting at least 1 seropositive bird from 10 sampled ducks is 0.27 and, from 5 sampled chickens, 0.16. This equates to an OR of 1.9, which is substantially lower than the observed difference in flock-level prevalences in which the odds of duck flocks being seropositive were 12.4× higher than that of chicken flocks.

HI tests in which horse erythrocytes were used to detect avian influenza antibodies in human serum were more sensitive than HI tests in which chicken erythrocytes were used (*29*). Because OIE does not recommend the horse erythrocyte method for HI tests on poultry serum samples, it is rarely used in poultry diagnostics, although some evidence supports a higher sensitivity in these species (*30*). We compared HI tests based on horse and chicken erythrocytes by using serum samples from 60 ducks experimentally infected with 2 of the HPAI (H5N1) field isolates from this study. These tests showed substantial agreement when results were categorized as positive ($\geq 2^4$) or negative ($\kappa = 0.74$, 95% CI 0.57–0.90), although some samples tested with horse erthrocytes had higher titers than when tested with chicken erythrocytes. Thus, seroprevalence estimates may have been similar to those reported here had the HI tests been conducted by using horse erthrocytes. We suggest that OIE review this issue and, if warranted, modify the recommended diagnostic methods.

Virus shedding was reported in apparently clinically healthy birds on nearly 11/16 outbreak farms. Despite a high risk for death in chickens and some deaths in ducks, other birds carrying the virus appeared to be unaffected, which indicates that host-specific characteristics of susceptibility might have varied among birds. Alternatively, some of these virus-positive clinically normal birds could have been sampled early in infection and had not yet developed clinical signs. However, a small number of chickens in the longitudinal study had antibodies to avian influenza (H5), providing further evidence that some chickens survive infection. These birds could have been infected with low pathogenicity avian influenza (LPAI) viruses. To our knowledge, the prevalence of LPAI in poultry in Indonesia is unknown. However, influenza (H5N1) viruses isolated from dead and live birds in our study were confirmed to be highly pathogenic. Further molecular characterization of these isolates is under way (*31*).

The frequency of HPAI outbreaks varied throughout the study period. Outbreaks increased in July 2007 (the beginning of the dry season), coinciding with an increased proportion of flocks with seropositive ducks. This increase suggests that HPAI (H5) virus was circulating among more duck flocks during this time and may be related to the practice of herding free-ranging ducks to scavenge on paddy fields postharvest as described for Thailand and Vietnam (*24*,*25*). Intermingling of ducks on paddy fields may al-

low extensive opportunities for virus release and exposure and contact with wild birds that also feed on leftover rice (*25*). However, the relationship between rice farming and HPAI outbreaks in Indonesia is likely to vary from those in Thailand and Vietnam because of different climatic conditions and rice farming calendars. Rice farming in Central Java is less seasonal, and rice paddies are smaller, often not separated by wide waterways, unlike in the Mekong Delta of Vietnam. Other factors, such as the long distance movement of duck flocks, may influence outbreak patterns in Indonesia. Further studies are needed on the management and movement of duck flocks, HPAI transmission pathways between different poultry species, and the association between rice harvest activities and increased HPAI outbreaks.

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West Nile Virus Range Expansion into British Columbia

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In 2009, an expansion of West Nile virus (WNV) into the Canadian province of British Columbia was detected. Two locally acquired cases of infection in humans and 3 cases of infection in horses were detected by ELISA and plaque-reduction neutralization tests. Ten positive mosquito pools were detected by reverse transcription–PCR. Most WNV activity in British Columbia in 2009 occurred in the hot and dry southern Okanagan Valley. Virus establishment and amplification in this region was likely facilitated by above average nightly temperatures and a rapid accumulation of degree-days in late summer. Estimated exposure dates for humans and initial detection of WNV-positive mosquitoes occurred concurrently with a late summer increase in *Culex tarsalis* mosquitoes (which spread western equine encephalitis) in the southern Okanagan Valley. The conditions present during this range expansion suggest that temperature and *Cx. tarsalis* mosquito abundance may be limiting factors for WNV transmission in this portion of the Pacific Northwest.

West Nile virus (WNV) is a vector-borne flavivirus that is transmitted in an enzootic cycle between birds by mosquitoes; incidental transmission to humans occurs during periods of intense amplification, typically in late summer in the Northern Hemisphere (*1*). WNV activity is inherently dependent on environmental and ecologic conditions that affect avian and vector populations because of the role these groups play in WNV transmission (*2*). Environmental factors such as temperature (*3*,*4*), precipitation (*4*), and drought (*5*), and ecologic conditions such as vector abundance (*6*) have been identified as possible determinants of WNV activity.

Canada represents the northernmost range of WNV in North America. The first positive WNV indicators appeared in Canada in 2001 when the virus was detected in birds and mosquitoes in Ontario (*7*). A total of 394 human cases occurred in Ontario and 20 in Quebec during 2002 (*7*). The virus quickly spread westward into the prairie provinces: 947 confirmed cases in Saskatchewan in 2003, of which 63 were West Nile neurologic syndrome (WNNS) (*8*), 144 in Manitoba (35 WNNS) (*9*), and 275 (48 WNNS) in Alberta (*10*). A second major outbreak occurred in Canada in 2007, a total of 1,456 (113 WNNS) cases were confirmed in Saskatchewan (8), 587 (72 WNNS) in Manitoba (*9*), and 318 (21 WNNS) in Alberta (*10*). Although mostWNV activity has occurred in the southern parts of the country, the virus has been detected as far north as Meadow Lake, Saskatchewan (54°8′N) (*11*).

Despite this widespread activity, no local WNV transmission was detected in Canada's westernmost province, British Columbia, during the WNV seasons (May to October) of 1999–2008 (*7*,*11*). The absence of WNV in British Columbia during this period puzzled provincial public health experts, who had been preparing for the virus's arrival since 2002; some speculated that British Columbia did not contain the prerequisite environmental and ecologic conditions essential for WNV activity. However, in August 2009, a long-delayed range expansion of WNV into British Columbia was confirmed; 2 locally acquired cases in humans, 10 positive mosquito pools, and 3 cases in horses were detected by provincial surveillance.

The official arrival of WNV in British Columbia puts to rest the question of whether the province can sustain within-season WNV activity. However, new questions have

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been raised relating to the mechanism of viral introduction, the environmental conditions that limited previous WNV activity in the province, the focus of WNV activity in the southern Okanagan Valley, and whether British Columbia can sustain activity between seasons. We examined spatial and temporal patterns of WNV activity in British Columbia in relation to mosquito abundance and temperature conditions present during the observed range expansion of 2009. Our goal was to identify potential determinants of WNV activity along this portion of British Columbia's northern and western ranges and to provide additional information regarding factors that facilitate the spread of WNV in North America.

Material and Methods

Study Area

The province of British Columbia is an ecologically, climatically, and geomorphologically diverse area covering 947,000 km^2 that contains a lengthy coastline, high mountain ranges, and a desert region (Figure 1). British Columbia has the most geological, climatic, and biological diversity in Canada (*12*). This province is dominated by vast regions of temperate forests in mountainous areas >1,000 m above sea level (*13*). Temperatures in the coastal regions of British Columbia are among the mildest in Canada; daily average temperatures are above freezing year-round (*14*). The coastal regions receive >1,100 mm of rain per year as moisture-laden air from the Pacific Ocean rises above the Coast Mountain Range, resulting in orographic precipitation. In contrast, the southern interior of the province is part of the semiarid steppe highlands ecoregion, which has near desert-like conditions including hot dry summers, cool winters, and average rainfall of 260 mm per year (*14*).

Provincial WNV Surveillance

The British Columbia Centre for Disease Control (BCCDC) and the BCCDC Public Health Microbiology and Reference Laboratory (PHMRL), in partnership with regional health authorities, municipalities, and regional governments, have conducted human surveillance, mosquito sampling, and dead corvid surveillance and testing since 2003. During the WNV seasons of 2003–2007, mosquito surveillance covered the southern extent of the province and extended as far north as 55°N latitude. However, in response to the prolonged absence of the virus, mosquito surveillance was reduced in 2008; mosquito traps were placed only at or below 50°N latitude (Table 1; Figure 1). An additional 16 traps were placed in the southern Okanagan Valley in 2009 as part of a research project to supplement the 91 traps operated by the province, effectively acting as targeted surveillance in this area. CDC light traps (Model 512; John W. Hock Company, Gainsville, FL, USA) baited

Figure 1. Select cities (lower case) in British Columbia, Canada, and regional health authorities (upper case). Each regional health authority undertakes West Nile Virus surveillance under the guidance and recommendations of the British Columbia Centre for Disease Control. The dashed oval encompasses the Okanagan Valley, which was the primary focal point of West Nile Virus activity in British Columbia during 2009. WA, Washington, USA; ID, Idaho, USA; MT, Montana, USA.

with dry ice were run 1 or 2 nights per week from June through September.

Collected mosquitoes were sent to the BCCDC PHMRL where they were sorted by gender, identified to the genus and/or species level, and pooled to a maximum of 50 mosquitoes per pool. All pools of female *Culex* spp. mosquitoes were homogenized, and RNA was extracted by using a QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). RNA extracts were subjected to an in-house–developed TaqMan real-time reverse transcription–PCR (RT-PCR) specific for the 3′ noncoding region and nonstructural protein 5′ of the WNV genome. Positive pools were then confirmed by using a second TaqMan real-time RT-PCR specific for the WNV envelope protein (*15*,*16*).

Passive dead corvid surveillance in British Columbia is conducted by regional health authorities and includes 1) online reporting of dead bird sightings by the public, and/or 2) collection of dead corvids, which are then submitted for testing at the British Columbia Ministry of Agriculture and Lands Animal Health Centre (AHC). Oropharyngeal swabs from dead birds are screened for WNV by using the VecTest (Microgenics Corporation, Fremont, CA, USA); RT-PCR was used as the confirmatory test on pooled tissues from suspected positive birds (*17*).

WNV infection is a reportable disease in British Columbia, and information about probable human cases is

communicated to the requesting physician and to public health officials; a case questionnaire is then administered to collect information on symptoms, travel history, and likely mode of transmission. Cases are classified as West Nile nonneurologic syndrome or WNNS according to the case definitions of the Public Health Agency of Canada (*7*). Cases are further categorized as probable or confirmed, depending on the level of specificity associated with the laboratory testing. All potential human case-patients are tested for WNV immunoglobulin M (IgM) and IgG by using ELISA (FOCUS Technologies, Cypress, CA, USA) and acute-phase and convalescent-phase serum samples; in-house hemagglutination inhibition (HI) tests are conducted when needed (*16*). Positive test results from the BCCDC are sent to the National Microbiology Laboratory in Winnipeg, Manitoba, Canada, for confirmatory plaque reduction neutralization testing.

Equine surveillance in British Columbia is passive. Positive equine cases are reported by local veterinarians to the AHC and the provincial chief veterinarian. WNV in horses is identified by using ELISA, serum neutralization, and/or plaque-reduction neutralization test. Horses suspected to have died of WNV are brought to the AHC for diagnostic necropsy. Although equine vaccinations are available in British Columbia, coverage is not widespread with the exception of horses that travel to the United States.

Temperature Analysis and Degree-Day Calculations

Development of WNV vectors and of the virus within an infected mosquito depends on temperature (*3*,*4*). Degree-day calculations use the product of temperature and time to estimate the cumulative energy required for an organism to develop (*18*). An estimated 109 degree-days are required for the completion of the extrinsic incubation period of WNV in *Cx. tarsalis* mosquitoes; the virus is unable to develop in this species at temperatures <14.3°C (*3*). We used the single-sign method (*19*) with a 14.3°C base to calculate the accumulated degree-days between January 1 and August 1 during 2003–2009 for select British Columbia communities. This method combines a 24-h sine wave with daily minimum and maximum thresholds to calculate the accumulated degree-days over 24 hours. The single-sine method provides the most accurate degree-day quantification when daily temperatures are below the minimum development threshold (*20*), and has been used in other WNV studies to estimate risk (*21*). Daily minimum temperature was evaluated for the southern Okanagan community of Osoyoos because it was the closest center to the focal point of WNV activity in British Columbia that also contains an official weather station. The daily minimum temperature for 2009 was compared with the 10-year average by using data from the Canadian National Weather Service, Environment Canada (*14*).

Results

Provincial and Regional WNV Activity

In early August 2009, serum samples from 2 residents of Kelowna (49°55′N, 119°30′W) were positive for WNV (Table 1; Figure 1). Travel histories indicated that neither person had been outside of interior British Columbia during the period of potential exposure and that each had recently traveled in the southern Okanagan Valley, which is 70–80 km south of Kelowna (Figure 1). During the same week, provincial surveillance detected a positive mosquito pool; 9 more were detected over the subsequent 2 weeks. All positive pools came from the southern Okanagan Valley and were located up to 35 km apart. Three WNV-positive horses were reported to the chief veterinarian and the AHC in early September: 2 from the southern Okanagan Valley and 1 from the more eastern Fraser Valley (Figure 1). None of the horses had traveled during their exposure period.

With the exception of British Columbia, WNV activity in Canada in 2009 (only 8 human cases nationwide) was among the lowest recorded (*7*). Washington, however, had its greatest WNV activity on record in 2009 (38 cases in humans and 73 cases in horses), up from previous highs of 3 cases in humans and 41 cases in horses or other mammals in 2008 (*22*).

Mosquito Abundance and Infection Rates

A total of 181,942 mosquitoes were collected in 2009 from 107 traps (Table 1). The most common mosquitoes in British Columbia are *Coquillettidia purturbans* and members of the genus *Aedes*. British Columbia is the only area in western Canada that has *Cx. tarsalis* and *Cx. pipiens* mosquitoes; the former are rare east of the Mississippi River, and the latter are absent in the prairie provinces (*23*). However, the abundance of these species is typically lower than in the prairie provinces of Saskatchewan and Manitoba, which experience the most intense WNV transmission in Canada (*8*,*9*). *Cx. pipiens* mosquito abundance in the Fraser Valley in 2009 increased relative to previous years; an average of 36.1 mosquitoes were caught per trap-night. An average of 33.1 *Cx. tarsalis* mosquitoes were caught per trap-night in the provincial interior, which was the highest abundance of this species reported in the previous 5 years (Table 1). This average from the southern Okanagan Valley includes data from 16 novel traps placed as part of a targeted research project. However, the average provincial count was still the highest since 2006 when these traps were excluded (Table 1). Peaks in the abundance of *Cx. tarsalis* mosquitoes have been observed previously in British Columbia in late June, but a second substantial increase in the abundance of this species was observed in early August 2009. Several locations in the

southern Okanagan Valley showed maximum nightly trap counts >800 *Cx. tarsalis* mosquitoes (Figure 2). The first WNV-positive mosquito pools were collected during this period of elevated *Cx. tarsalis* mosquito abundance; the estimated exposure period for both human cases also occurred at this time (Figure 2). *Cx. pipiens* mosquitoes consistently increased in the Fraser region throughout the summer; some traps caught up to 750 mosquitoes in a single night in 2009. Although the average trap catch of this species has been increasing continuously in this area since 2003, the abundance of WNV vectors in British Columbia remains much lower than in areas of Canada that have experienced large WNV outbreaks (*8*,*9*).

Cx. tarsalis was the only vector species in British Columbia that was positive for WNV in 2009. However, only *Cx. tarsalis* and *Cx. pipiens* mosquitoes are tested for WNV in British Columbia. Bias-corrected maximum-likelihood estimates (MLEs) of vector infection rates were calculated by using the Centers for Disease Control and Prevention's (CDC's) PooledInfRate Microsoft Excel add-on (*24*). The virus reached detectable levels in late July and peaked in the latter half of August (Table 2) with 2-week MLEs of mosquito infection rates reaching 4.97/1,000 (95% confidence interval [CI] 0.89–16.63) for the weeks of August 23, 2009, to Sepember 5, 2009 (Table 2). Low mosquito abundance has, however, limited the precision of these estimates. Minimum infection rates are larger than comparable MLEs from July 26–August 8 and smaller than MLEs thereafter (Table 2), indicating that >1 infected mosquito may be present per pool as is common when infection rates are high (*24*).

Bird Surveillance

A total of 6,681 corvids were tested for WNV during 2003–2009; none were positive (Table 1). The decreasing number of dead corvids reported since 2006 likely results from a combination of changes to regional surveillance strategies, decreases in the frequency of education campaigns, and changing public perception given the prolonged absence of the virus. We do not believe that the observed decrease resulted from a die-off of WNV-infected birds.

Climate

More degree-days were accumulated in 2009 for most locations in the province, including Osoyoos, than in any year since 2003–2004 (Table 3). Daily minimum temperatures in the winter and spring in Osoyoos in 2009 were below the 10-year average yet quickly increased in late May and early June and remained above the 10-year-average for much of the summer (Figure 3). The average minimum temperature in July 2009 in Osoyoos (15.5°C) was nearly a full degree higher than the 20-year average; average minimum temperatures in August (15.3°C) were the highest seen in 20 years (*14*). Maximum temperatures reached 34.9°C, 38.6°C, and 39.5°C in June, July, and August, respectively (*14*).

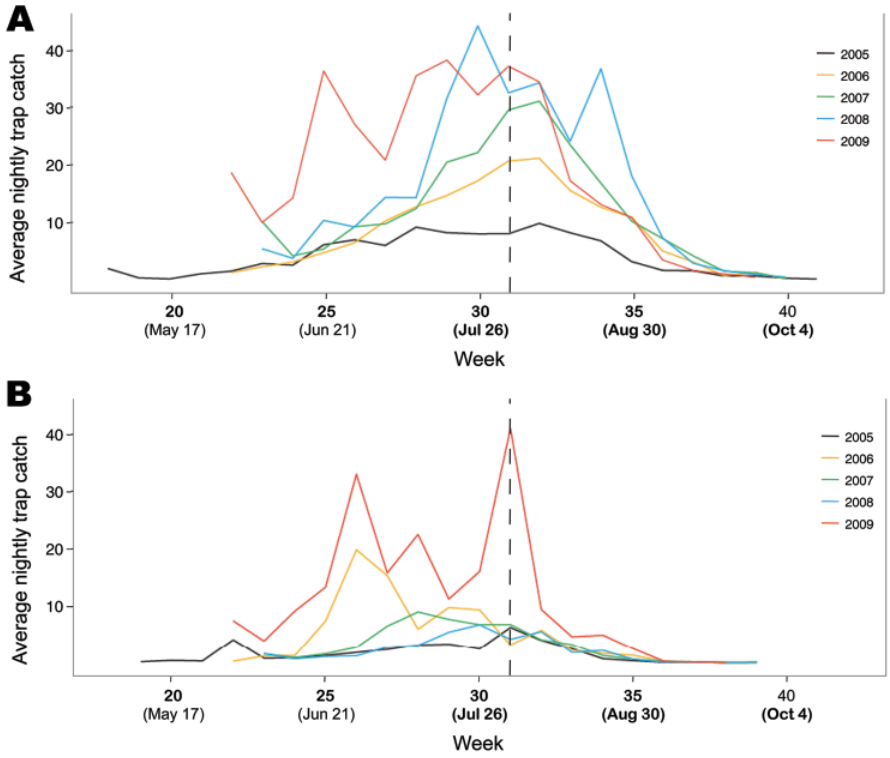

Figure 2. Nightly average catch for *Culex pipiens* (A) and *Cx. tarsalis* (B) mosquitoes across all trapping locations in British Columbia, Canada, during 2005–2009. Provincial vector surveillance data are aggregated by week beginning January 1, and the dates provided represent the first day of a given week. Vertical dashed line represents the estimated exposure date for human cases and the collection date for the first positive mosquito pools.

Table 1. Summary of British Columbia WNV surveillance activities during the WNV seasons of 2004–2009*

Surveillance type	2004	2005	2006	2007	2008	2009
Mosquito						
No. permanent trap locations	145	189	148	155	98	91
No. mosquitoes collected	52,657	198,228	394,047	242,215	202,460	181,942
Mosquito pools†	2,980	6,631	2,329	2,568	1,873	1,469
Provincial						
No. *Culex pipiens*	4.6	5.1	8.6	14.3	10.5	21.1
No. *Cx. tarsalis*	0.8	1.9	4.8	3.5	1.4	11.8
Interior‡						
No. *Cx. pipiens*	12.6	8.9	9.5	8.2	4.7	7.6 (9.3)
No. *Cx. tarsalis*	11.0	12.0	22.5	10.1	2.9	33.1 (14.3)
Fraser						
No. *Cx. pipiens*	10.5	13.0	22.3	32.5	33.7	36.1
No. *Cx. tarsalis*	6.9	4.9	7.7	7.1	7.0	15.1
Bird						
No. corvids sighted	1,292	740	605	562	458	355
No. corvids submitted	1,437	1,058	803	740	205	144
No. corvids positive	0	0	0	0	0	0
Human						
No. tested	481	755	239	805	530	340
No. positive§	0	0	0	19 (0)	0	3 (2)

*WNV, West Nile Virus.
†Values represent average trap catch. Provincial, average trap catch for all traps in British Columbia; Interior, average trap catch from the Interior Health Authority, which includes the southern Okanagan Valley (Figure 2); Fraser, average trap catch from the Fraser Health Authority (Figure 2).
‡Values represent average trap catch from all traps run in the southern Okanagan Valley in 2009, including 16 traps run in the area as part of a research project. Values within parentheses represent average trap catch without the research traps.
§No. human cases detected in British Columbia (no. cases acquired within British Columbia).

Discussion

The delayed establishment of WNV in British Columbia may stem from 1) limited or failed introduction of the virus from adjacent areas with WNV activity before 2009, and/or 2) previously unsuitable environmental or ecologic conditions that prevented establishment, persistence, and amplification of WNV to detectable levels. Although both factors have contributed to the delayed establishment of WNV in British Columbia, they should be clearly separated because virus introduction and persistence are unique events (*25*). The comparative role of these processes in explaining the prolonged absence of WNV in British Columbia is difficult to determine, but the timing and location of British Columbia's initial WNV activity do provide clues as to potential drivers of this range expansion.

WNV activity in British Columbia in 2009 was centered in the south-central part of the province in the southern Okanagan Valley (Figure 1). Provincial risk maps created by the BCCDC identified this area as having relatively high WNV risk for reasons other than its proximity to the United States. WNV activity is negatively associated with mountainous landscapes (*26*), and the Okanagan Valley is one of the few nonmountainous areas in southern British Columbia. Valleys may act as paths of least resistance for local vector- or reservoir-mediated introduction of the virus into British Columbia from Washington (*27*,*28*) or by migrating birds along the Pacific Flyway (*29*). The southern Okanagan Valley also contains abundant irrigated landscapes that are clustered along with human habitation near the rivers and lakes in the southern Okanagan Valley. This aggregation of favorable habitats brings vectors, reservoirs, and humans into close proximity and may facilitate virus amplification and transmission to humans and horses (*30*).

Not only does the southern Okanagan Valley contain favorable habitats, but it also has a climate that, unlike much of British Columbia, is favorable for WNV amplification and transmission. The southern Okanagan Valley is the hottest region in British Columbia during the summer months. Temperature is positively related to mosquito development rates and the frequency with which mosquitoes take blood meals (*31*). Rates of virus development within mosquito vectors also increase with temperature, and such relationships have consequences for disease transmission because failure of the virus to replicate before mosquito death can halt virus amplification (*3*).

Temperatures in 2009 were above average for much of British Columbia, including the southern Okanagan Valley. WNV outbreaks in the United States and Canada have occurred primarily during years with above-normal temperatures (*7*,*32*), and a recent case-crossover study (an epidemiologic study design in which each case serves as its own control, allowing comparison of exposure at the time of disease onset to exposure at another time point) of 16,298 WNV cases in the United States showed that a 5°C increase in mean maximum weekly temperature is

associated with a 32%–50% increase in WNV incidence (*4*). The first positive mosquito pool in the southern Okanagan Valley was detected ≈1 week after heavy rainfall and immediately after a period of extreme heat during which nightly temperatures were well above the 14.3°C limit for virus replication in *Cx. tarsalis* mosquitoes (*3*) (Figure 3). This rainfall likely increased the number of vector development sites; the ensuing period of high temperatures facilitated rapid mosquito development, virus amplification, and subsequent transmission in avian and mosquito populations.

The above-average abundance of *Cx. tarsalis* mosquitoes observed in 2009 is likely another key driver of the observed WNV range expansion (Table 1). *Cx. tarsalis* mosquitoes are bridge vectors that feed on birds and mammals (*33*). The elevated abundance of this species in 2009, especially the large peaks observed at the end of July and beginning of August (Figure 2), likely facilitated virus transmission from avian populations into humans and horses. However, *Cx. tarsalis* mosquitoes are much less common in British Columbia compared with other areas of Canada that experience large WNV outbreaks (8,9); this rarity may be 1 factor that has prevented past WNV activity in this region. Little is currently known about the ecology of *Cx. tarsalis* mosquitoes in the southern Okanagan Valley, and specific information is needed regarding the habitat preferences and overwintering practices of this species to enable more focused prevention efforts.

The detection of WNV in British Columbia in 2009 proves that the southern portion of the province contains the prerequisite environmental and ecologic conditions for within-season WNV amplification and transmission, at least in some years. What is less certain is whether the observed range expansions along the virus's northern limit will lead to yearly endemic activity or to rare instances of sporadic disease as is typical in Washington State. WNV can overwinter in adult mosquitoes (*34*), thereby increasing the probability of future virus transmission in areas that had positive WNV indicators in 2009. Historic trends in some areas of the United States show marked increases in outbreak severity in the year after WNV introduction (*32*). Furthermore, the presence of a WNV-positive horse in the Fraser Valley is concerning given its proximity to British Columbia's populated urban areas where *Cx. pipiens* mosquitoes are a consistent presence between June and August. Urban transmission of WNV in British Columbia in 2010 could lead to an increase in human cases and identifies a need for continued surveillance programs and appropriate prevention.

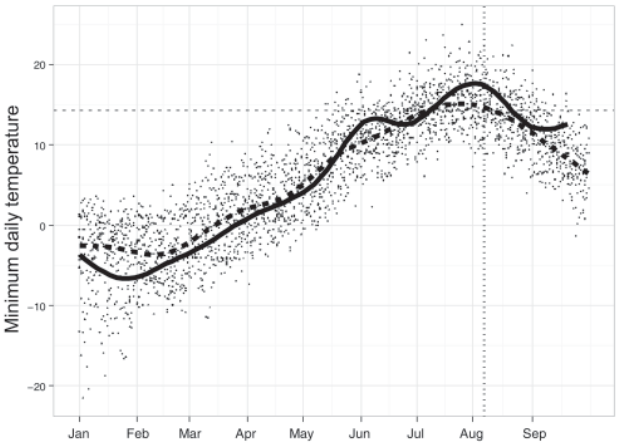

Figure 3. Minimum daily temperature for Osoyoos, British Columbia, Canada, January–September 2009. The solid line represents values observed in 2009; the dashed line represents the best-fit 10-year average. The horizontal dotted line at 14.3°C represents estimated temperature required for *Culex tarsalis* mosquito development and transmission (*3*). The vertical dashed line represents the estimated exposure date for human cases and the collection date for the first positive mosquito pools.

Outbreaks in human populations do require specific sequential weather conditions that may not be met in 2010 despite predictions for an El Niño year (*35*). In addition, the historically low abundance of key WNV vectors in British Columbia may limit WNV transmission in this region, and a return to provincial norms for *Cx. tarsalis* mosquito abundance could disrupt WNV transmission in the rural areas of the southern Okanagan Valley. St. Louis encephalitis virus, an arbovirus that shares vectors and reservoirs with WNV, was detected in southern British Columbia in mammals and humans in 1968 (*36*). Yet St. Louis encephalitis virus has caused no locally acquired human cases since, which indicates that arboviruses can circulate endemically in animal populations in the area without resulting in human cases.

Table 2. MLEs and MIRs of 2-week infection rates in *Culex tarsalis* mosquitoes, South Okanagan Valley, British Columbia, 2009*

Week	Total no. individuals	No. pools	No. positive pools	MLE (95% CI)	MIR
Jun 28–Jul 11	1,542	52	0	0.00	0.00
Jul 12–Jul 25	670	30	0	0.00	0.00
Jul 26–Aug 8	3,376	98	4	1.21 (0.39–2.92)	1.85
Aug 9–Aug 22	959	67	4	4.49 (1.45–10.95)	4.17
Aug 23–Sep 5	424	37	2	4.97 (0.89–16.63)	4.71
Sep 6–Sep 19	4	1	0	0.00	0.00

*MLE, maximum-likelihood estimate; MIR, minimum infectious rate estimate; CI, confidence interval. Estimates are calculated by using the software PooledInfRate from Centers for Disease Control and Prevention (*24*) and represent number of positive mosquitoes per 1,000 tested. Values within parentheses represent 95% confidence intervals.

Table 3. Cumulative degree-days for communities in British Columbia between, January 1–August 31, 2003–2009*

Year	Community							
	Cranbrook	Creston	Osoyoos	Kamloops	Abbotsford	Vancouver	Victoria	Prince George
2003	599	770	962	820	485	408	375	283
2004	479	668	993	880	562	485	425	361
2005	409	581	850	738	481	386	357	275
2006	542	700	851	821	469	366	347	333
2007	561	757	859	738	417	344	311	273
2008	475	611	811	729	387	312	202	265
2009	477	661	919	860	518	422	365	343

*Degree-days are calculated by using the single-sine method (*19)* with a 14.3°C base (3). See Figure 1 for location of select communities.

In summary, the introduction and within-season amplification of WNV in 2009 represent a long-delayed range expansion. Although reasons for the delay remain unknown, we hypothesize that WNV activity in Washington State in 2009 provided, for the first time, a sufficient nearby source of WNV for northward introduction of the virus into British Columbia through cross-border mountain valleys. This introduction likely combined with uniquely warm nightly temperatures and elevated numbers of *Cx. tarsalis* mosquitoes in the southern Okanagan Valley; this combination of factors presented a convergence of favorable events that facilitated establishment and amplification in mosquito and avian populations. WNV activity levels in British Columbia in 2010 will provide valuable insight into the nature of WNV expansion and transmission along British Columbia's northern and western borders. The presence of WNV activity in 2010, despite a return to normal temperatures and vector abundance, would suggest that ineffective virus introduction may be responsible for the prolonged absence of WNV in the province. Conversely, a return to normal temperatures and vector abundance combined with a lack of WNV activity in 2010 would suggest that environmental and ecologic conditions in this part of the Pacific Northwest are typically unsuitable for yearly WNV establishment, amplification, and transmission. Regardless, surveillance and ongoing consideration of appropriate prevention strategies are required to lessen the possibility of future WNV transmission to human populations in the region.

Members of the British Columbia WNV Surveillance Team are Lucy Beck, Victoria Bowes, Elizabeth Brodkin, Steve Chong, Ken Christian, Dalton Cross, Murray Fyfe, Roland Guasparini, Paul Hasselback, Randy Heilbron, Mira Leslie, James Lu, Craig Nowakowski, Robert Parker, Tim Shum, Kevin Touchet, and Eric Young.

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Pandemic (H1N1) 2009 Surveillance for Severe Illness, New York, New York, USA, April–July 2009

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On April 23, 2009, the New York City Department of Health and Mental Hygiene (DOHMH) was notified of a school outbreak of respiratory illness; 2 days later the infection was identified as pandemic (H1N1) 2009. This was the first major outbreak of the illness in the United States. To guide decisions on the public health response, the DOHMH used active hospital-based surveillance and then enhanced passive reporting to collect data on demographics, risk conditions, and clinical severity. This surveillance identified 996 hospitalized patients with confirmed or probable pandemic (H1N1) 2009 virus infection from April 24 to July 7; fifty percent lived in high-poverty neighborhoods. Nearly half were <18 years of age. Surveillance data were critical in guiding the DOHMH response. The DOHMH experience during this outbreak illustrates the need for the capacity to rapidly expand and modify surveillance to adapt to changing conditions.

On April 23, 2009, a nurse from a high school in New York City (NYC) called the Department of Health and Mental Hygiene (DOHMH) to report an outbreak of respiratory illness (*1*). The cause of the outbreak was rapidly confirmed to be influenza A pandemic (H1N1) 2009 virus. This outbreak was detected just a few days after initial reports of mild disease caused by pandemic (H1N1) 2009 virus in California and Texas (*2*,*3*) and at the same time as an outbreak of severe respiratory disease associated with pandemic (H1N1) 2009 virus in Mexico (*4*). Information about the clinical severity and transmission characteristics of this new influenza virus was limited. Given preliminary media reports about the Mexican outbreak and concern that NYC might also experience widespread severe disease, DOHMH launched a large-scale public health response.

Before the spring of 2009, DOHMH routine surveillance systems for influenza included 1) syndromic surveillance for medication sales, school absenteeism, and emergency department visits for influenza-like illness (ILI) (*5*,*6*); 2) electronic laboratory reporting of confirmed cases from commercial and hospital laboratories; 3) active surveillance of all NYC virology laboratories to determine the weekly number of specimens submitted for influenza testing and the percentage of those positive; 4) typing samples of influenza isolates obtained from patients in NYC hospitals at the DOHMH Public Health Laboratory (PHL); 5) enhanced passive surveillance for pediatric influenza deaths; 6) monitoring trends in influenza and pneumonia-related mortality through the DOHMH Vital Registry; and 7) monitoring outpatient ILI through the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) Influenza-like Illness Surveillance Network (*7*), a sentinel network through which providers reported weekly on the proportion of ILI in their practices during influenza season. The DOHMH had also created a plan for local response to a potential influenza pandemic, including enhanced surveillance to guide public health officials in determining how to prioritize use of antiviral agents and vaccines (*8*). Surveillance data could also inform community control measures, such as school closures. Proposed surveillance strategies in this plan focused on mechanisms for monitoring trends in hospitalizations and deaths, but not necessarily for trying to count every severe case. Methods were also proposed for obtaining more detailed clinical and epidemiologic data for a sample of cases.

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The DOHMH also has an incident command system (ICS), an agency-wide structure for addressing and responding to emergencies that is different from the usual DOHMH structure. Divided into 10 sections, the ICS is led by an incident commander who reports directly to the Commissioner of Health (*9*). All DOHMH employees are assigned to a section within the ICS and can be called on to assist their section upon activation of the system. In a public health emergency, the Surveillance and Epidemiology Section establishes and conducts surveillance to assess the illness and deaths associated with the event and conducts any needed epidemiologic studies to guide the public health response. ICS activation provides surge capacity by increasing the workforce available to conduct surveillance or epidemiologic activities beyond the staff members who are normally responsible for the specific disease or public health issues involved in the emergency.

We describe some of the surveillance methods used in the investigation of pandemic (H1N1) 2009 in NYC from April to July 2009. DOHMH investigated the high school outbreak (*1*,*10*), and set up an enhanced citywide surveillance system to track the scope and severity of infections. The agency also prioritized identification and diagnostic testing of patients with severe or fatal cases of ILI in hospitals or clusters of those with ILI in schools and other congregate settings; this surveillance was essential because evidence of severe pandemic (H1N1) 2009 would have prompted more aggressive public health control measures. In addition, because surveillance of cases in hospitalized patients, and particularly of fatal cases, was an important part of this investigation, we provide an overview of epidemiologic findings among hospitalized patients.

Methods

On Saturday, April 25, 2009, when preliminary laboratory results suggested a likely pandemic (H1N1) 2009 outbreak at high school A, the DOHMH activated its ICS and initially mobilized >200 staff members for a large-scale public health response (later adding additional staff). From April 25 through May 8, the agency also expanded its hours of operation to 7 days a week from 9:00 AM to 9:00 PM; staff worked in shifts to cover the extended hours.

The ICS was deactivated on May 8, since minimal evidence existed of community circulation of pandemic (H1N1) 2009. By mid-May, however, DOHMH noticed an increase in ILI, especially in schoolchildren. On May 17, 2009, the first NYC death from pandemic (H1N1) 2009 virus occurred. In response to these developments and to increasing reports of hospitalized case-patients, DOHMH reactivated its ICS on May 19. This second activation continued until July 7, 2009.

Enhanced Citywide Surveillance

Active Surveillance for Critically Ill Case-Patients

Starting April 26, the DOHMH conducted active citywide surveillance in hospital intensive care units (ICUs) for severe, unexplained, febrile respiratory illnesses (defined as a temperature ≥100.4°F (≥38°C) and pneumonia, acute respiratory distress syndrome, or respiratory distress (as diagnosed by clinicians) with no known cause. DOHMH staff contacted all 57 NYC hospitals with medical or pediatric ICUs daily by telephone and queried the clinician in charge of the ICU that day to determine the number of patients with conditions that met the surveillance definition. Active ICU surveillance was discontinued on May 8 since few cases of severe illness were being identified**.**

Enhanced Passive Surveillance for Hospitalized Case-Patients with Noncritical Illness

To ascertain the number of hospitalized patients with pandemic (H1N1) 2009 outside of the ICU setting, DOHMH relied on enhanced passive surveillance. Providers were notified of reporting requirements through the NYC Health Alert Network, which sends faxes and email alerts to 29,000 clinicians and healthcare institutions in NYC, and through daily conference calls with all NYC acute care facilities. DOHMH set up a dedicated NYC telephone access line to triage provider calls. Providers were initially asked to report any hospitalized patients outside of the ICU setting with severe, unexplained, febrile respiratory illnesses (as defined above). However, because of the increasing number of calls and limited staff and laboratory testing capacity at the NYC PHL, beginning on May 12, providers were asked to only report non-ICU cases of severe, febrile respiratory illness if initial test results were positive for influenza A virus by enzyme immunoassay, PCR, direct fluorescent antibody test, or virus culture at the hospital laboratory. However, DOHMH continued to accept reports on all patients with febrile respiratory illness who were in the ICU or were receiving ventilation, regardless of influenza testing status.

Active Laboratory Surveillance

During the week after the recognition of pandemic (H1N1) 2009 in NYC (April 25–30), DOHMH actively collected specimens from laboratories chosen to be geographically representative of the city to determine whether evidence existed of community circulation of the pandemic virus that was not associated with the outbreak at the high school. Five sentinel laboratories were selected and asked to submit 1–3 influenza A virus–positive specimens from the previous 2 days to the NYC PHL to test for pandemic (H1N1) 2009 virus.

Case-Patient Interviews

During the first 3 weeks of the outbreak, DOHMH staff attempted telephone interviews of all patients (or their proxies) who had confirmed pandemic (H1N1) 2009 for demographic, epidemiologic, and clinical information. Providers of care for hospitalized case-patients were also interviewed to gather information about patient demographics, underlying conditions, and clinical course of illness. Once community circulation in NYC was established, DOHMH stopped interviewing patients about possible risk of exposure (e.g., travel to Mexico, school attendance).

Surveillance for Deaths

To track pandemic (H1N1) 2009–related deaths, DOHMH asked that hospitals report any fatal cases of unexplained, acute, febrile respiratory illness to DOHMH and to the Office of the Chief Medical Examiner (OCME). The OCME collected specimens and performed autopsies on any patient whose death was preceded by a sudden, unexplained, febrile respiratory illness, as well as for all pediatric patients who died with clinically compatible illness in which there was a positive influenza test result, a sudden unexplained death thought to be due to a natural cause, or death of a child from an unknown febrile respiratory illness. If no testing results for pandemic (H1N1) 2009 virus were available from the hospital, OCME collected a postmortem nasopharyngeal swab specimen for influenza diagnostic testing at PHL. In addition, the dataset of patients who tested positive for pandemic (H1N1) 2009 virus was matched weekly with the NYC Vital Records database of recent deaths in NYC to ensure that no pandemic (H1N1) 2009 deaths were missed.

Laboratory Methods

DOHMH physicians screened reported potential cases to determine if they met testing criteria, and if so, nasopharyngeal specimens were requested. Recognizing the need to prioritize PHL resources for hospitalized patients and those with fatal cases, DOHMH specifically requested that clinicians not test patients with mild ILI unless the patient was part of a reported cluster in a school, jail, nursing home, or other congregate setting.

Specimens were initially tested for influenza A or B viruses, and then, if positive for influenza A, were further tested for seasonal influenza A virus (H1N1 or H3N1) by using the QIAamp Viral RNA manual extraction method (QIAGEN, Valencia, CA, USA) and real-time reverse transcription–PCR by using the Cepheid SmartCycler (Cepheid, Sunnyvale, CA, USA). Initially, specimens that were positive for influenza A virus, but not seasonal influenza A virus subtypes H1N1 or H3N1, and suspected to be pandemic (H1N1) 2009 virus were sent to CDC for confirmation. Then, on May 11, the PHL started to perform the CDC Influenza Virus Real-time reverse transcription–PCR detection and characterization panel for pandemic (H1N1) 2009 virus on all nonseasonal influenza A specimens by using a high-throughput system including an automated extraction system and ABI7500 Fast-Dx (Life Technologies, Carlsbad, CA, USA). Beginning on May 20, PHL performed the same CDC assay on all influenza specimens by using the same high throughput system.

A confirmed case of pandemic (H1N1) 2009 was defined as a person who had a specimen that was PCR positive for pandemic (H1N1) 2009 virus. A probable case was defined as a patient with nonsubtypeable influenza A virus infection for whom confirmatory testing was not conducted. Confirmatory influenza testing was performed at PHL, CDC, or the New York State Wadsworth Center Laboratory.

Analytic Methods

We analyzed surveillance data to describe NYC residents who were hospitalized with pandemic (H1N1) 2009 in NYC from the start of the first ICS activation to the end of the second activation (April 24–July 7). We also calculated pandemic (H1N1) 2009 rates by dividing the number of confirmed and probable cases among hospitalized patients by NYC population counts from the US Census 2000. We examined rates by demographic characteristics of hospitalized patients and performed direct age-adjustment by using weights based on US Census 2000 (*11*).

Additionally, patient poverty level was assessed by linking ZIP code of residence with income and population data from the US Census 2000. We defined neighborhoods using the United Hospital Fund (UHF) designation, which aggregates adjoining ZIP codes to create 42 NYC neighborhoods (*12*). We then created a neighborhood poverty variable by categorizing UHF neighborhoods into tertiles (low-, medium-, and high-poverty neighborhoods) based on the percentage of residents living <200% of the federal poverty level, according to the US Census 2000, and calculated rates for confirmed and probable pandemic (H1N1) 2009 cases by UHF neighborhood poverty status. Poverty data were available for 993 of the 996 persons who were hospitalized with confirmed or probable pandemic (H1N1) 2009.

For all analyses, significance was determined at $p<0.05$. All statistical analyses were conducted by using SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Hospitalizations

During April 24–May 7, corresponding to the first ICS activation, 15 patients with confirmed or probable cases of pandemic (H1N1) 2009 were hospitalized (median stay was 1 day). At that time, most cases were linked to the high school influenza A outbreak and only 2 case-patients

reported travel to Mexico. No deaths had been reported. Since few cases of severe illness had occurred, the ICS was deactivated, and staff who would normally be involved in communicable disease outbreak investigations continued to monitor pandemic (H1N1) 2009 activity.

By July 7, the end of the second ICS activation, 996 patients had been hospitalized. The distribution of 996 hospitalized case-patients (929 confirmed and 67 probable) over time, including the increased incidence in late May, can be seen in Figure 1. From April 24 through July 7, the estimated age-adjusted rate of confirmed and probable pandemic (H1N1) 2009 hospitalizations was 12.3/100,000 NYC residents (95% confidence interval [CI] 11.8–13.4). The rate among patients ≤4 years of age (40.9/100,000, 95% CI 35.6-46.3) was almost 7× that among those ≥65 years of age (6.0/100,000, 95% CI 4.5–7.7) (Table). The estimated age-adjusted rate of pandemic (H1N1) 2009 hospitalized patients in high-poverty neighborhoods (18.4/100,000, 95% CI 16.8–20.1) was significantly higher than that in low-poverty neighborhoods (8.9/100,000, 95% CI 7.6–10.4) (Table; Figure 2).

Deaths

The first NYC death occurred on May 17. Additional information about NYC pandemic (H1N1) 2009 deaths has been published elsewhere (*13*).

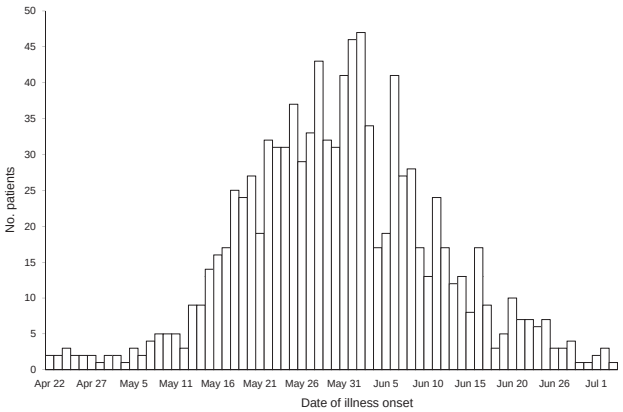

Figure 1. Hospitalized patients with confirmed or probable pandemic (H1N1) 2009, by date of onset, New York, New York, USA, April 24–July 7, 2009. Onset date was missing for 98 patients with confirmed pandemic (H1N1) 2009 and 16 with probable pandemic (H1N1) 2009. Surveillance data as of August 25, 2009.

Discussion

The experience of NYC with pandemic (H1N1) 2009 demonstrates the need for flexibility in surveillance approaches and ongoing modification of surveillance methods to best respond to a changing public health emergency. Although DOHMH had not planned to do such intensive active and enhanced surveillance during an influenza pan-

Table. Demographic characteristics of patients hospitalized with confirmed or probable pandemic (H1N1) 2009, New York, NY, USA, April 24–July 7, 2009*

Characteristic	No. (%) patients with confirmed or probable pandemic (H1N1) 2009	% All residents of city†	Crude rate/100,000 residents (95% CI)	Age-adjusted rate/ 100,000 residents (95% CI)‡
Total	996		12.3 (11.5–13.1)	12.6 (11.8-13.4)
Age group, y				
0–4	224 (22)	6.7	40.9 (35.6–46.3)	–
<2	128 (13)	2.7	58.1 (48.0–68.1)	–
2–4	96 (10)	4.0	29.4 (23.8–35.9)	–
5–24	297 (30)	27.4	13.3 (11.8–14.8)	–
5–17	197 (20)	17.4	13.9 (12.0–15.9)	–
18-24	100 (10)	10.0	12.3 (9.9–14.7)	–
25–64	419 (42)	54.0	9.6 (8.6–10.5)	–
25–49	245 (25)	39.5	7.6 (6.7–8.6)	–
50–64	174 (17)	14.5	14.8 (12.6–16.9)	–
≥65	56 (6)	14.5	6.0 (4.5–7.7)	–
Sex§				
M	468 (47)	47.4	12.2 (11.1–13.3)	12.5 (11.4–13.7)
F	526 (53)	52.6	12.3 (11.3–13.4)	12.9 (11.9–14.1)
Poverty status§¶				
High-poverty area	498 (50)	32.7	18.8 (17.1–20.4)	18.4 (16.8–20.1)
Medium-poverty area	323 (33)	40.9	9.7 (8.7–10.8)	10.1 (9.1–11.3)
Low-poverty area	172 (17)	26.4	8.0 (6.8–9.2)	8.9 (7.6–10.4)

*CI, confidence interval.
†Percentage of all residents of the city in each category. Estimates based on 2000 US Census data (*11*).
‡Direct age standardization was performed by using weights based on 2000 US Census data (*11*). Median age for hospitalized patients was 23 y and for all city residents was 34 y.
§2 persons were missing sex data; 3 persons were missing poverty data.
¶Neighborhood poverty was based on the tertiles of percentage of residents living <200% of the federal poverty level according to the 2000 US Census (*11*).

demic (*9*), active case-based surveillance was initially implemented because little was known about the severity of this novel strain of H1N1, and public health officials were concerned on the basis of initial media reports from Mexico. To learn more about the severity of illness, DOHMH focused on surveillance of hospitalized cases and deaths. Surveillance and reporting requirements were modified when it became clear that circulation of pandemic (H1N1) 2009 was citywide, but surveillance for deaths and hospitalized cases continued to help officials assess the severity and at-risk groups for pandemic (H1N1) 2009, and the resulting information helped inform DOHMH planning and response to this new virus.

Approximately half of hospitalized patients lived in a high-poverty neighborhood; this association between poverty and severe illness has been reported for seasonal influenza (*14*). Our finding of the association between young age and severe illness is consistent with other studies (*15–20*), and the greater proportion of persons 0–17 years of age in low-income neighborhoods in NYC (*21*) may contribute to this distribution. Other possible explanations include higher attack rates among residents living in crowded housing, or that residents with known risk conditions in high-poverty neighborhoods may be less likely to receive early treatment or prophylaxis, given that the proportion of people without personal doctors is higher in high-poverty areas relative to low-poverty areas (20% vs. 11%) (*21*). In addition, the proportion of uninsured persons in low-income areas (18%) is higher than the proportion in high-income areas (9%), according to NYC's Community Health Survey from 2008 (*21*). Future studies should assess poverty status and its relationship to severe influenza illness.

Our analysis had several limitations. By limiting testing to those patients who had positive influenza A test results (unless patients were in the ICU), our surveillance approach systematically undercounted hospitalized patients with pandemic (H1N1) 2009. Although this enabled us to monitor hospitalization trends, we most likely do not have a complete count of cases. Published studies have found a wide range of results for the sensitivity of rapid influenza testing for the pandemic (H1N1) 2009 strain (17%–70%) (*22–25*). Applying the published range of sensitivities to our results would suggest that the true number of hospitalized patients in NYC ranged from 1,400 to 6,000, which is 1.5–7.0× higher than those for cases detected and confirmed. Also, because of the limited amount of data collected on all patients, we were unable to examine variables at the individual level; such data (for example, having a primary care physician and insurance status) may have modified the findings regarding the relationship between poverty and severe illness. Lastly, demographic and economic information was from 2000, and changes may have occurred.

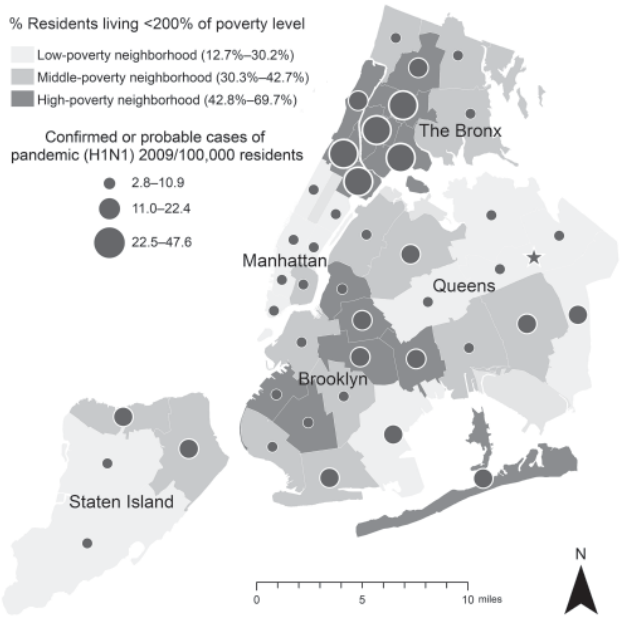

Figure 2. Age-adjusted rates of hospitalization for confirmed or probable pandemic (H1N1) 2009, by neighborhood poverty level, New York, New York, USA, April 24–July 7, 2009. Direct age standardization was performed by using weights from the 2000 US Census (*11*). Of 996 total patients, 993 had complete poverty data available. Star represents location of high school A.

Surveillance data from the spring outbreak informed NYC planning and response to pandemic (H1N1) 2009 during the 2009–10 fall and winter influenza season. Because young children represented a large proportion of hospitalized cases and because of the role children likely play in transmission, NYC created a school-based vaccination program for elementary and middle schoolchildren and vaccinated all children who had parental consent. In the fall of 2009, >60 influenza diagnostic and treatment community based centers were established for persons with ILI who did not have a primary care physician; an advice hotline, staffed by nurses, was created to answer questions and help connect NYC residents to care. Antiviral medications were made available to those who could not afford them, and points of distribution provided the vaccine free of charge to New Yorkers, initially targeting those who had risk factors for severe pandemic (H1N1) 2009 as identified in NYC and elsewhere (*15–20*,*26*,*27*).

DOHMH has continued to use emergency room and outpatient syndromic surveillance systems to follow trends in influenza-like activity citywide. We also requested passive reporting of influenza hospitalizations by all city hospitals and collected some data on clinical status and risk factors. Finally, to more effectively monitor the clinical and epidemiologic characteristic of pandemic (H1N1) 2009

during the fall and winter seasons, we established a sentinel hospital surveillance program at 5 sites where active surveillance and influenza testing were conducted on any patient with fever and respiratory syndromes. Collection of isolates from sentinel hospitals and active laboratory surveillance also allowed circulating influenza subtypes, as well as antiviral resistance, to be monitored. Surveillance guided and informed the NYC response to pandemic (H1N1) 2009, and this experience will help NYC plan a response to future epidemics.

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Correlation of Pandemic (H1N1) 2009 Viral Load with Disease Severity and Prolonged Viral Shedding in Children

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Learning Objectives

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

- Identify the most common clinical manifestations associated with pandemic (H1N1) 2009 infection.
- Recognize different diagnostic tests for pandemic (H1N1) 2009 infection.
- Identify independent predictors of viral shedding in pandemic (H1N1) 2009 infection, including impact of age and comorbidities.

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Pandemic (H1N1) 2009 virus causes severe illness, including pneumonia, which leads to hospitalization and even death. To characterize the kinetic changes in viral load and identify factors of influence, we analyzed variables that could potentially influence the viral shedding time in a hospital-based cohort of 1,052 patients. Viral load was inversely correlated with number of days after the onset of fever and was maintained at a high level over the first 3 days. Patients with pneumonia had higher viral loads than those with bronchitis or upper respiratory tract infection. Median viral shedding time after the onset of symptoms was 9 days. Patients <13 years of age had a longer median viral shedding time than those ≥13 years of age (11 days vs. 7 days). These results suggest that younger children may require a longer isolation period and that patients with pneumonia may require treatment that is more aggressive than standard therapy for pandemic (H1N1) 2009 virus.

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The influenza A pandemic (H1N1) 2009 virus, initially identified in Mexico and the United States in April 2009, has been reported in >213 countries (*1*). Severe ill-

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ness and death can occur in humans infected with this virus, particularly young persons and pregnant women (*2*–*5*). Recent data from the United States showed that almost half of hospitalized case-patients were children <18 years of age and suggested that antiviral drugs were beneficial in these patients, especially when initiated early (*6*). This finding implies that the successful control of viral replication by using antiviral drugs is associated with a good clinical outcome.

Because viral replication is necessary for disease pathogenesis in other influenza infections (*7*,*8*), information on the correlation between viral load and the clinical spectrum of illnesses among persons infected with pandemic (H1N1) 2009 virus is emerging. However, viral replication patterns and the effect of antiviral drugs on viral load have not been adequately studied.

We undertook the present study to characterize the kinetic changes in viral load and shedding in a hospital-based cohort by real-time reverse transcription–PCR (RT-PCR) and to analyze the factors that influence the rate of viral RNA clearance. A correlation between the virologic profile and the clinical features of pandemic (H1N1) 2009 virus–infected patients would provide essential information for epidemiologic control and clinical management in terms of antiviral therapy and infection control approaches.

Materials and Methods

Study Design and Study Subjects

The outbreak of pandemic (H1N1) 2009 in Taiwan began in July 2009. Our hospital, a 2,600-bed medical center that had experienced an outbreak of severe acute respiratory syndrome in 2003 (*9*–*12*), reorganized the severe acute respiratory syndrome research team to study pandemic (H1N1) 2009 virus in Taiwan. We established fever clinics (also called flu-like illness clinics) after a seriously ill child died of a pandemic (H1N1) 2009 virus infection at the beginning of this outbreak. Throat (tonsillopharyngeal) swab specimens from patients with influenza-like symptoms were obtained for the diagnosis of pandemic (H1N1) 2009 virus infection by differential and quantitative reverse RT-PCR or virus culture and RT-PCR. Patients confirmed as infected with pandemic (H1N1) 2009 virus received oseltamivir treatment for 5 days in a dosage based on body weight, according to the manufacturer's recommendations.

Respiratory specimens were serially collected during the period of hospitalization or upon outpatient follow-up after informed consent was obtained. We collected the following clinical data: demographic characteristics, disease severity, coexisting conditions, fever-onset time, time to initiate antiviral treatment, and duration of fever after antiviral treatment. For the classification of disease severity, pneumonia was defined by the presence of patchy alveolar opacities on chest radiographs, and meningoencephalitis was diagnosed on the basis of presence of brain swelling with leptomeningeal enhancement seen on magnetic resonance imaging. This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital.

Virus Culture

Virus culture was performed by using a Madin-Darby canine kidney cell line obtained from American Type Culture Collection (Manassas, VA, USA). Supernatants from infected cultures were mixed with equal volumes of 1% guinea pig erythrocyte suspension and incubated for 1 h at 4°C. Culture cells with supernatants found positive by hemagglutination test were subjected to a direct fluorescent antibody assay with fluorescein-conjugated monoclonal antibodies against influenza viruses A and B and Evans blue dye (Oxoid, Ely, UK) to differentiate between influenza A and B viruses. Influenza A viruses were further differentiated from novel or seasonal influenza A viruses by real-time RT-PCR, as described below.

Differentiation and Quantification of Pandemic (H1N1) 2009 Virus

We extracted total nucleic acids from throat swab specimens or the supernatant of positive virus cultures with the Roche MagNA Pure Compact System (Roche Molecular Diagnostics, Mannheim, Germany) by using the manufacturer's external lysis protocol and extraction reagents (Total Nucleic Acid Isolation Kit; Roche Molecular Diagnostics) to yield 100 μL of elutes. All PCRs were performed by using the standard real-time RT-PCR procedure of the Centers for Disease Control and Prevention for swine (H1N1) influenza (*13*). Briefly, 20 μL of RT-PCR mixture containing 1× Universal PCR Master Mix, 1× MultiScribe and RNase Inhibitor, 100 nmol/L TaqMan probe, and 5 μL extracted RNA, or water for no template controls, was subjected to RT-PCR in the presence of 250 nmol/L swine influenza nucleoprotein gene-specific primers or internal control (RNase P) primers. The reactions were performed and analyzed in a ABI PRISM 7000 sequence Detection System (Applied Biosystems, Branchburg, NJ, USA) under the following conditions: 30 min at 48ºC and 10 min at 95ºC, followed by 40 cycles of 30 s at 95ºC and 1 min at 60ºC.

For the quantitative assay, a reference standard was prepared by using the pUC57 vector (GeneDireX, Las Vegas, NV, USA) containing the corresponding specific viral sequence. Tenfold dilutions equivalent to 1 to 1.0×10^7 copies per reaction were prepared to generate calibration curves and to be run in parallel with the test samples. The limit of detection of this real-time RT-PCR was 1 copy/mL. The viral load in each sample was calculated and corrected by using the threshold cycle value of the internal control (RNase P) (*14*).

Statistical Analysis

The correlation between the viral load and the number of days after the onset of symptoms was analyzed by using the Spearman rho correlation. Comparisons of the viral load among groups with different disease severity were analyzed by using Tukey post hoc multiple comparison after analysis of variance. The duration of viral shedding was calculated by using the Kaplan-Meier method and tested by the log-rank test. For multivariate analysis of the duration of viral shedding, we used the Cox proportional hazards model. All variables such as age, gender, disease severity, coexisting conditions, viral load from the first throat swab specimen, and time to initiate antiviral treatment were treated as categorized data in the multivariate analysis. The median value was taken as the cutoff value between the different groups in continuous variables such as age and viral load in the days after the onset of symptoms.

Results

Demographic Data for the Patients Infected with Pandemic (H1N1) 2009

From August 1 through September 30, 2009, a total of 1,052 patients were analyzed for novel influenza (H1N1) infection by RT-PCR, and pandemic (H1N1) 2009 virus infections were confirmed in 602 patients. In the 425 samples analyzed by virus culture and RT-PCR, 222 were RT-PCR positive and 204 were culture positive. Of the 204 culture-positive samples, 192 (94%) were RT-PCR positive; of the 222 RT-PCR–positive samples, 194 (87%) were culture positive. The RT-PCR method is a quantitative assay and was quicker and more sensitive than virus culture.

After 21 patients who had received oseltamivir therapy before RT-PCR diagnosis were excluded, 581 patients were monitored for involvement of other organs beyond the upper respiratory tract. Of the 581 patients, the median age was 10.1 years (range 0.38–78.8 years) (Table 1). Twenty (3.4%) patients had a severe illness, including pneumonia and meningoencephalitis. Most patients (502, 86.4%) visited the hospital for diagnosis of pandemic (H1N1) 2009 virus infection within 2 days of the onset of fever. The viral load from the first throat swab specimen was >4 $\log_{10}$ copies/mL in 278 (47.8%) patients. No deaths were found in this cohort study, but 1 patient died of severe pneumonia and cerebral hemorrhage before the start of the study.

Table 1. Demographic data for 581 patients with RT-PCR–confirmed pandemic (H1N1) 2009 virus infection, Taiwan*

Demographic variable	No. (%) patients†
Sex, M/F	337/244
Age, y	
Median	10.1
Range	0.38–78.8
<4	49 (8.4)
≥4 to <7	87 (15.0)
≥7 to <13	256 (44.1)
≥13 to <18	130 (22.4)
≥18 to <50	54 (9.3)
≥50	5 (0.9)
Days after onset of fever	
0 (no fever)	8 (1.4)
1	354 (60.9)
2	148 (25.5)
3	49 (8.4)
4	12 (2.1)
5	7 (1.2)
≥6	3 (0.5)
Disease severity	
URI†	518 (89.2)
Bronchitis	43 (7.4)
Pneumonia	17 (2.9)
Meningoencephalitis	3 (0.5)
Virus load from first throat swab, $\log_{10}$ copies/mL	
<1.0	6 (1.0)
1.0– 2.0	47 (8.1)
>2.0 –3.0	80 (13.8)
>3.0 –4.0	170 (29.3)
>4.0–5.0	190 (32.7)
>5.0– 6.0	83 (14.3)
>6.0	5 (0.9)

*RT-PCR, reverse transcription–PCR; URI, upper respiratory tract infection.
†Except as indicated.

Viral Load and Days after the Onset of Fever

Most pandemic (H1N1) 2009–infected patients visited the hospital to receive treatment for fever; the mean time to visit the hospital after the onset of fever was 1.57 days. Eight patients infected with pandemic (H1N1) 2009 virus sought treatment without fever. The mean viral load was significantly lower in afebrile patients than in febrile patients (2.61 vs. 3.82 $\log_{10}$ copies/mL, p = 0.004; Figure 1, panel A). The viral load was maintained at a high level during the first 3 days and was inversely correlated to the days after the onset of fever (Figure 1, panel B; Spearman correlation, R = –0.21, p<0.001).

Higher Viral Loads in Patients with Pneumonia

We analyzed whether the viral load was correlated with age or disease severity. The viral load in patients of all ages was not significantly different (Figure 2, panel A). We found that patients with pneumonia had significantly higher mean viral loads (4.92 $\log_{10}$ copies/mL; 95% confidence interval [CI] 4.30–5.54; n = 17) than in those with upper respiratory tract infection (3.77 $\log_{10}$ copies/mL, 95% CI 3.67–3.87; n = 518, p<0.001) or with bronchitis (3.74 $\log_{10}$ copies/mL, 95% CI 3.43–4.05; n = 43, p = 0.002; Figure 2, panel B).

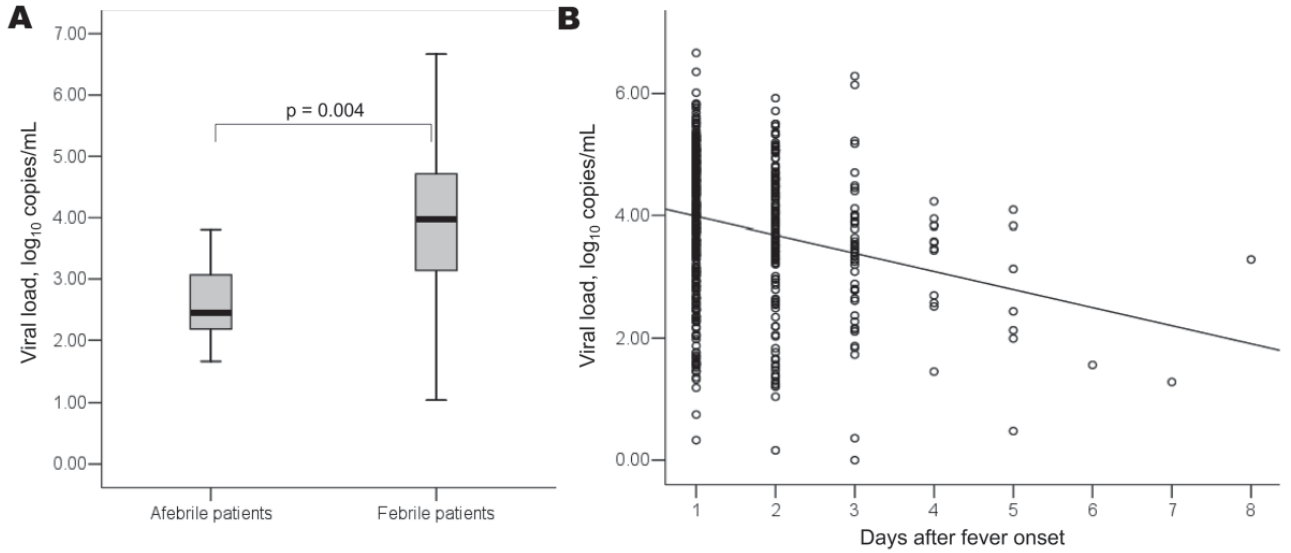

Figure 1. A) Viral loads in afebrile and febrile patients infected with pandemic (H1N1) 2009 virus, Taiwan, before they received oseltamivir treatment. B) Correlation of the virus load with number of days after the onset of fever in febrile patients. Median, quartiles, and range are shown. Circles indicate individual values.

Prolonged Viral Shedding Time after Oseltamivir Treatment

Sixty patients agreed to have serial measurements taken of viral shedding after oseltamivir treatment by quantitative RT-PCR analysis of the number of viral RNA copies. Specimen collection was started from the day a participant agreed to join the study and continued in 2– 3-day intervals until viral RNA was undetectable. The results showed that the median viral shedding time was 9 days (95% CI 7.37–10.63) after the onset of symptoms, and 65% of patients had detectable influenza viral RNA for >7 days after the onset of symptoms (Figure 3, panel A). One patient with severe pneumonia had prolonged viral shedding for >27 days. The mean viral load in the throat quickly decreased from 4.13 $\log_{10}$ copies/mL to 1.15 $\log_{10}$ copies/mL from days 0–2 to days 6–8, respectively, after the onset of symptoms (Figure 3, panel B).

Prolonged Period of Viral Shedding in Patients <13 Years of Age

We evaluated the factors that could affect the rate of viral shedding, including age, gender, coexisting conditions, disease severity, viral load from the first throat swab specimen, and time from onset of symptoms to start of

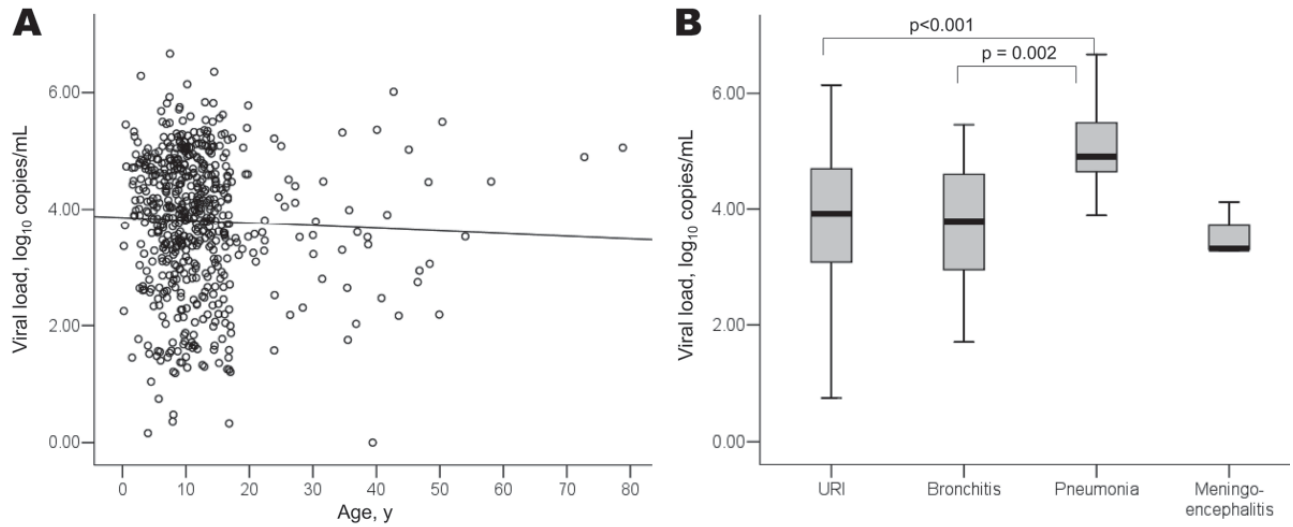

Figure 2. Viral load before treatment in relation to age (A) and disease severity (B) in patients infected with pandemic (H1N1) 2009 virus, Taiwan. Circles indicate individual values. URI, upper respiratory tract infection. Median, quartiles, and range are shown.

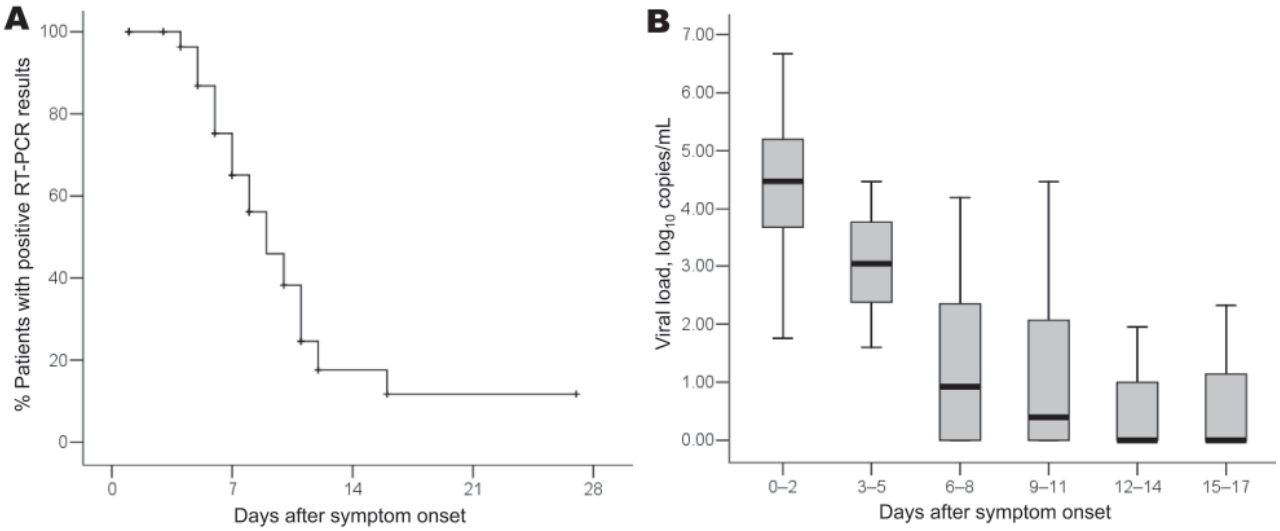

Figure 3. A) Percentage of patients with positive reverse transcription–PCR (RT-PCR) results and B) viral load in throat swabs from 60 pandemic (H1N1) 2009 virus–infected patients treated with oseltamivir, according to the number of days after the onset of symptoms, Taiwan. Median, quartiles, and range are shown.

treatment. By using multivariate analysis, we found that only age was associated with a prolonged period of viral shedding (Table 2). To compare age differences, 13 years was chosen as the median cutoff age between the younger and older groups. We found that patients <13 years of age (n = 31) had a median viral shedding time of 11 days (95% CI 10.19–11.81) after the onset of symptoms; patients ages ≥13 years (n = 29) had a shorter median viral shedding time of 7 days (95% CI 5.85–8.15) after the onset of symptoms (p<0.001; Figure 4). Among patients <13 years of age, 89% had detectable influenza viral RNA for >7 days after the onset of symptoms, and 36% had detectable influenza viral RNA for >14 days (Figure 4).

The clinical characteristics and associated coexisting conditions for patients <13 and ≥13 years of age are shown in Table 3. No significant differences were found between the 2 groups in gender, disease severity, coexisting conditions, viral load from the first throat swab, time to initiate antiviral treatment, and duration of fever after antiviral treatment. Most of the patients in each group were afebrile within 24 h after the initiation of antiviral therapy (74.2% vs. 89.7%). Of the 60 patients, 14 (23.3%) had a coexisting condition, including 8 (25.8%) <13 years of age and 6 (20.7%) ≥13 years. Among patients ≥13 years, 1 patient had 3 underlying medical conditions (diabetes, hypertension, and congestive heart failure), and another patient had 2 conditions (hypertension and cerebrovascular disease). Asthma was the most common condition in patients <13 years of age (22.6%).

Discussion

Pandemic (H1N1) 2009 virus has provided an opportunity to examine the virus-host interaction of an emerging infection. The present study demonstrated that viral load was maintained at a high level during the febrile period and was inversely correlated to the days after the onset of fever. Moreover, pandemic (H1N1) 2009 virus–infected patients with pneumonia had a higher viral load than those with bronchitis or upper respiratory tract infection. The higher viral load may be a reflection of disease severity or im-

Table 2. Factors associated with duration of viral shedding in 60 patients with pandemic (H1N1) 2009 virus infection, Taiwan*

Variable	Viral RNA detected 7 d after symptom onset, %	p value (UVA)	HR (95% CI)	p value (MVA)
Age (<13 vs. ≥13 y)	88.7 vs. 38.1	<0.001	4.99 (2.34–10.66)	<0.001
Sex (M vs. F)	59.6 vs. 71.5	0.650	1.18 (0.57–2.44)	0.658
Disease severity (mild vs. severe)†	60.8 vs. 77.9	0.247	1.38 (0.56–3.45)	0.487
Time to initiate antiviral treatment (≤48 h vs. >48 h)	60.6 vs. 81.8	0.176	2.56 (0.88–7.69)	0.085
Viral load from the first throat swab (<4.35 vs. ≥4.35 log_{10} copies/mL)	64.7 vs. 55.8	0.745	1.68 (0.64–4.37	0.292
Coexisting conditions (no vs. yes)	70.7 vs. 45.5	0.402	1.85 (0.78–4.39)	0.137

*UVA, univariate analysis; MVA, multivariate analysis; HR, hazard ratio; CI, confidence interval.
†Severe illness included pneumonia and meningoencephalitis; mild illness included upper respiratory tract infection and bronchitis.

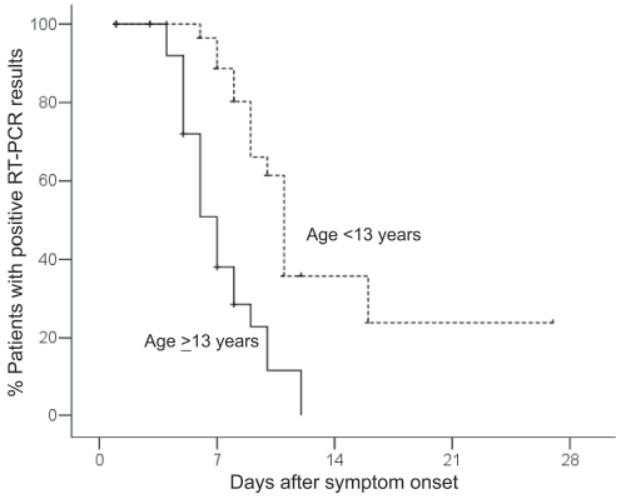

Figure 4. Kaplan-Meier estimates of patients with positive reverse transcription–PCR (RT-PCR) results for pandemic (H1N1) 2009 infection, by age group, Taiwan.

paired host immunity, requiring immediate attention and aggressive treatment.

As described in previous reports (*2*,*5*,*6*), infection with the pandemic (H1N1) 2009 virus caused severe illness, including pneumonia, requiring hospitalization, and even death. More severe respiratory diseases and increased mortality have been observed in young patients (*5*,*6*). These phenomena may be partly attributed to the lack of a cross-reactive antibody response to pandemic (H1N1) 2009 virus after vaccination with recent seasonal influenza vaccines (*15*). However, apart from the cross-reactive antibody response, the level of virus load is also important in predicting disease severity in influenza infections (*7*,*8*,*16*). Further investigation to correlate the virus load with the immune response in patients infected with pandemic (H1N1) 2009 virus is necessary to clarify the pathogenesis of the disease and deaths it causes.

Studies of seasonal influenza A epidemics have demonstrated that early antiviral treatment is more effective in reducing virus load when compared with no treatment (*17*,*18*). Viral clearance is also associated with resolution of symptoms (*16*,*19*). A recent study indicated that oseltamivir-treated patients showed a greater rate of virus load reduction in nasopharyngeal aspirates than did nontreated patients when the treatment was initiated ≤2 days after the onset of symptoms (*20*). In the present study, after oseltamivir treatment, the mean virus load detected by RT-PCR decreased quickly from 4.13 $\log_{10}$ copies/mL to 1.15 $\log_{10}$ copies/mL, thus showing the therapeutic efficacy of oselta-

Table 3. Characteristics of 60 patients who were infected with pandemic (H1N1) 2009 virus, by age group, Taiwan*

Characteristic	No. (%) patients		p value†
	Age <13 years, n = 31	Age ≥13 years, n = 29	
Sex, M/F	14/17	15/14	0.611
Disease severity			
URI/bronchitis	22 (71.0)	22 (75.9)	0.859‡
Pneumonia	9 (29.0)	5 (17.2)	
Meningoencephalitis	0	2 (6.9)	
Duration of fever after antiviral treatment, h			
<24	23 (74.2)	26 (89.7)	0.122§
24–48	5 (16.1)	2 (6.9)	
>48	3 (9.7)	1 (3.4)	
Time to initiate antiviral treatment, h			
≤48	25 (80.6)	24 (82.8)	0.833
>48	6 (19.4)	5 (17.2)	
Viral load from the first throat swab, $\log_{10}$ copies/mL			
<4.35	16 (51.6)	14 (48.3)	0.796
≥4.35	15 (48.4)	15 (51.7)	
Coexisting condition			
Any 1 condition	8 (25.8)	6 (20.7)	0.640
Asthma	7	0	
Ventricular septal defect	1	0	
Hypertension	0	5	
Cerebrovascular disease	0	1	
Congestive heart failure	0	1	
Diabetes	0	1	
Pregnancy	0	1	

*URI, upper respiratory tract infection.
†p values were calculated by χ^2 test.
‡The URI/bronchitis group was compared with the combined group of pneumonia and meningoencephalitis.
§Fever lasting <24 h after antiviral treatment vs. fever lasting ≥24 h after antiviral treatment.

mivir treatment. Additional controlled trials may be necessary to clarify the clinical benefit of antiviral treatments.

Prolonged viral RNA detection has been observed in the presence of coexisting conditions, and is associated with a longer duration of illness and hospitalization in elderly patients (13.5 vs. 7.0 days) (*17*). A more prolonged period of viral shedding was found in immunosuppressed patients such as those undergoing hematopoietic stem cell transplantation (*21*); moreover, antiviral therapy substantially decreased the duration of seasonal influenza viral shedding (*22*,*23*). Children shed seasonal influenza virus for a longer period than did adults (*24*). Similar to these reports, in our study we demonstrated that the duration of viral shedding time was notably longer in patients <13 years of age than in older patients. However, why children have a longer period of viral shedding is unknown. Delayed cell-mediated immunity in children responding to a novel virus may explain, in part, their longer viral shedding time.

The median duration of time to virus detection in seasonal influenza infections is typically 7–8 days after the onset of illness, but viral shedding for up to 21 days has been reported (*24*). A recent study in China reported that the median time of pandemic (H1N1) 2009 viral shedding after the onset of symptoms, according to the results of RT-PCR, was 6 days (*25*); this finding is similar to the results of a study conducted in Singapore (*26*). However, in our study, the median shedding time was 9 days after the onset of symptoms. The longer duration of viral shedding found in our study may have been due to the younger median age (10.1 years) of participants in our study compared to those in the other studies in China (23.4 years) and Singapore (26 years). Because all of our patients received oseltamivir treatment, we could not determine the actual effect of the antiviral therapy on infection caused by the novel pandemic virus. Further studies that compare patients who have received oseltamivir therapy with those who have not may be necessary to assess the effect of antiviral treatment on viral shedding and clinical outcomes.

In conclusion, the results of our study indicate that virus load was high in the febrile period and in patients with pneumonia. Children <13 years of age had a significantly longer viral shedding period than did children ≥13 years, even after oseltamivir therapy. These results suggest that younger children may require a longer isolation period and that patients with pneumonia may require more aggressive treatment for infection with pandemic (H1N1) 2009 virus.

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Pearl Kendrick, Grace Eldering, and the Pertussis Vaccine

Carolyn G. Shapiro-Shapin

In light of the reemergence of pertussis (whooping cough), the pioneering research of Pearl Kendrick and Grace Eldering is worth revisiting. In the 1930s, working in the Michigan Department of Health laboratory in Grand Rapids, Michigan, USA, they began researching a pertussis vaccine. Their research offers an instructive case study of the creative public health research performed in state health department laboratories during the interwar years. State department of health laboratory directors actively promoted research by supporting advanced education; making facilities and funding available for individual projects; and, when possible, procuring new facilities. Using Michigan Department of Health resources and local and federal funding, Kendrick and Eldering developed standardized diagnostic tools; modified and improved extant vaccines; conducted the first successful, large-scale, controlled clinical trial of pertussis vaccine; and participated in international efforts to standardize and disseminate the vaccine. Their model may again offer a promising avenue for groundbreaking research.

Photo credit: Michigan Women's Hall of Fame (www.michiganwomenshalloffame.org)

Figure. Pearl Kendrick (left) and Grace Eldering.

In light of the reemergence of pertussis (whooping cough), the pioneering pertussis vaccine research conducted by Drs Pearl Kendrick and Grace Eldering (Figure) at the Michigan Department of Health laboratory is worth revisiting. Their pertussis research offers a model that would be useful today.

Although scientists had developed vaccines to control many infectious diseases including smallpox, typhoid fever, diphtheria, and tetanus by the 1920s, whooping cough proved a more difficult puzzle. French researchers Bordet and Gengou described *Bordetella pertussis* as the causative agent of whooping cough in 1906 (*1*). In the 1920s, pharmaceutical companies in the United States offered many pertussis and mixed-serum pertussis vaccines designed to both treat and prevent whooping cough, but none proved effective (*2*). In 1931, the American Medical Association's Council on Pharmacy and Chemistry found no "evidence even for the presumptive value of stock or commercial vaccines" because "the pertussis vaccines seem to have absolutely no influence [as a preventive], and after the disease is thoroughly established even freshly prepared vaccines seem useless" (*3*).

By the 1920s, pertussis had claimed the lives of ≈6,000 US children each year, more than did each of the childhood scourges of diphtheria, scarlet fever, and measles (*4*). Thorvald Madsen of the Danish Serum Institute in Copenhagen spurred further pertussis research when he announced that his vaccine prepared from freshly isolated *B. pertussis* cultures offered some protection in his Faroe Islands studies in the 1920s (5). English scientists P. H. Leslie and A. D. Gardner described 4 antigenic groups or phases for *B. pertussis* and highlighted the importance of selecting appropriate cultures for vaccine production in 1931 (*6*). Illinois pediatrician Louis Sauer and his assistant Leonora Hambrecht conducted smaller scale tests of their effective vaccine (*4*).

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Still, the disease remained a killer (*7*). In 1932, when Kendrick and Eldering began their research at the Michigan Department of Health laboratory in Grand Rapids, Michigan, USA, many questions remained unanswered.

Starting in the mid-19th century, public health leaders across the nation developed city and state departments of health. When bacteriology was introduced in the late 19th century, these health departments gradually shifted their mission from promoting general sanitation to public health efforts that targeted the specific vectors of disease and focused on laboratory diagnosis (*8*–*10*). By 1915, most major cities and all states had invested in laboratory facilities dedicated to bacteriologic analysis, biologics production, and (in many) fundamental research (*10*,*11*). After World War I, state health department laboratory directors expanded their laboratory divisions with funds newly available from the federal government, the American Public Health Association (APHA), and the Rockefeller Foundation (*9*,*10*). For Charles Chapin, Superintendent of Health for Providence, Rhode Island, however, most of these laboratories had not reached their potential because of limited funds and personnel. "On the whole," he noted, "investigation of the sources of diseases has not attained very brilliant results in the hands of most state health departments, as their energies have been largely forced into other channels whether they wished it or not" (*10*).

To staff their growing departments and stay within their limited budgets, laboratory directors often sought out talented female scientists. Pearl Kendrick, from her days as a student and teacher in upstate New York, was recognized by her teachers as being "a first class student, thorough, accurate and rapid" (*12*). While teaching, she continued her own education, studying bacteriology at Columbia University under Hans Zinsser during the summer of 1917 (*12*). After Kendrick had worked for 2 years as an assistant at the New York State Department of Health laboratories, C.C. (Cy) Young, director of the Bureau of Laboratories for the Michigan Department of Health, recruited her. Young assured Kendrick that "I'm sure that we can make it interesting for you and there is every chance for advancement" (*12*). In 1926, Young assigned Kendrick to direct the health department's newly opened Grand Rapids branch. Young provided his employees funding and time to pursue advanced education. In 1932, Kendrick earned a Doctor of Science degree in bacteriology from Johns Hopkins University. Young's strategy of pursuing talent, supporting advanced education, and funding research paid dividends. By the late 1920s, Young's Bureau of Laboratories in Lansing and Grand Rapids had established a national reputation for bacteriologic research.

Grace Eldering, Kendrick's laboratory partner, hailed from eastern Montana. Eldering studied at the University of Montana in Missoula and taught English and biology at Hysham High School for a year after graduation before answering the Michigan Bureau of Laboratories' call for additional staff. In the fall of 1928, Eldering traveled to Lansing to take advantage of this opportunity. Within 6 months, she was hired into the department to do routine bacteriologic analysis. Eldering would earn her Doctor of Science degree from Johns Hopkins University in 1942 (*13*).

In 1932, Kendrick brought Eldering to Grand Rapids, and, when a virulent strain of *B. pertussis* infected the children of Grand Rapids that year, Kendrick and Eldering began the whooping cough research project. The state laboratory gave Kendrick and Eldering the freedom to conduct their research after all of the laboratory's routine water and milk analyses were completed. They developed and improved methods for growing the pertussis bacillus, inactivating it, and creating a safe vaccine (*14*,*15*). In addition, they were pioneers in the field, designing and directing the first large-scale controlled clinical trial for pertussis vaccine. In the pages of Reader's Digest, Paul DeKruif, a Grand Rapids native and author of the best seller Microbe Hunters, celebrated their study as one of the "greatest field tests in microbe-hunting history" (*16*).

To pursue their groundbreaking research, Kendrick and Eldering brought together a diverse coalition of local and state public health departments, physicians, citizens' groups, women's groups, and parent–teacher associations that would provide organizational support and funding. By building relationships with local physicians and the Grand Rapids Health Department, Kendrick and Eldering ensured a steady supply of cough plates containing *B. pertussis* samples. When city physician A.H. Edwards notified them of pertussis infections in the community, Kendrick and Eldering sped off to visit families hit hard by the economic downturn and to collect samples. As Grace Eldering noted in an interview, they "learned about pertussis and the Depression at the same time" (*15*). In addition, the city health department aided their research by allowing its network of public and private nurses to collect cough plates for the pertussis research.

During the first stage of their research, Kendrick and Eldering modified the then-standard Bordet-Gengou growth medium; the result was a growth medium that fostered more rapid and profuse growth of *B. pertussis* colonies and that could therefore be used as a routine diagnostic tool (*15*). On November 1, 1932, Kendrick's laboratory began offering a cough plate diagnostic service to local doctors. In addition to aiding the doctors, the rapid-growth plates enabled Kendrick and Eldering to determine that during the first 3 weeks of infection, a child's cough contained enough active pertussis bacilli to infect his or her peers; that most children were noninfectious by week 4; and that after 5 weeks, 90% of the children posed no risk to others (*17*).

Proper quarantine length could now be determined scientifically. Before these studies, whooping cough quarantines varied from 2 to 4 weeks, depending on locale. The Grand Rapids Health Department adopted Kendrick and Eldering's quarantine recommendations, which required physicians to report the disease; ordered the health department to place warning placards on homes; and enforced a 35-day isolation period or, with 2 consecutive negative cough plates, release by day 28 after onset of symptoms (*18*). As Pearl Kendrick noted in 1934, these "regulations have crystallized out of our bacteriological studies and are now under test as part of the Grand Rapids Communicable Disease Regulations" (*19*). In 1935, Kendrick reported that the "cough plate technic [sic] has become a routine procedure in the laboratory"; that year, Kendrick's Michigan Department of Health laboratory in Grand Rapids examined 4,515 cough plates for *B. pertussis* (*20*).

After several months of producing autogenous vaccines for local physicians to use as treatment and preventive, Kendrick appealed to state laboratories director Cy Young for permission to develop a more general vaccine. "Rather than handl[ing] each request on the basis of an autogenous vaccine," Kendrick explained to Young on February 4, 1933, "we can more efficiently make a supply from several local pertussis strains." She then asked: "May we do this on an experimental basis—supplying these few pediatricians who are the type to cooperate as to records [?]" (*12*). Young supported her efforts. In a handwritten note dated February 21, 1933, he told Kendrick: "Go ahead and do all you can with pertussis if it amuses you" (*12*).

Kendrick and Eldering performed carefully controlled animal studies of vaccines, using the general methods of Madsen, Sauer, and Hambrecht to design a vaccine that was safer and more potent (*15*). They inactivated the pertussis bacilli with thimerosol at cold room temperature for ≥1 week and conducted numerous sterility and safety tests (including injecting the vaccine into their own arms to test for safety) (*12*,*14*,*15*). After these vaccines were declared safe, they were distributed to local physicians who, in return for the serum, supported Kendrick and Eldering's laboratory work by spreading the news of the vaccine to area medical personnel and encouraging wider use of diagnostic cough plates (*12*).

Recruiting study participants and gathering financial support for a wide-scale vaccine trial required concerted community outreach efforts (*18*,*20*). In a 1958 retrospective on their field studies, Grace Eldering noted that "among the many who contributed to the success of the program were the parents and their children who accepted the requirements for test and control groups in the field trials. This acceptance was basic, and laid a foundation in the community upon which other studies could be built" (*21*).

In the 1930s, there were no accepted standards and few established models for conducting field studies, a problem made clear in failed experiments with human participants, including the Brodie-Park and Kolmer polio trials in the 1930s (*22*). Many researchers used orphans or institutionalized children for their research, noting that by participating, these children were repaying a debt to society (*23*). Instead of relying on these vulnerable populations, Kendrick and Eldering built outreach networks during the early stages of their research. The Kent County Welfare Relief Commission aided these efforts by collecting statistics on the prevalence of whooping cough and the number of children who had received "a treatment to prevent whooping cough" in their 1935 vaccination survey. This study of vaccination of preschool-aged children and the careful records of the city health department's nursing districts enabled Kendrick and Eldering to select controls matched for age, sex, and district (*12*).

Private physicians joined school physicians and city health officials in administering the series of 4 or 5 shots at the vaccination clinics held in primary schools around the city, federally funded nursery schools, and at City Hall (*12*,*24*). For each child in the study, city health department nurses completed a vaccine inoculation form, a home visit slip, an exposure record, and a case history; the researchers matched each inoculated child with a control selected from the Kent County Welfare Relief Commission study. At 3–4-month intervals, the nurses visited children in both groups and collected information about exposures; checked patients for the bacillus with cough plates; and when needed, obtained case histories for exposures and illness (*14*). The 1934–1935 field trial involved 1,592 (712 vaccinated and 880 control) children. In their 1935 report to the APHA, Kendrick noted that only 4 of the 712 vaccinated children had whooping cough, and then only mild cases, but 45 of the 880 unvaccinated controls (90% of those exposed) contracted the disease and suffered its full ravages. Despite the 89% efficacy rate found in the trial, they cautioned against the "danger of giving [the numbers] too much weight in the face of the relatively small number of whooping cough cases" (*14*).

Before the large-scale federal financing of science that emerged after World War II, scientists were often forced to cobble together funds from private and public sources (*25*). Kendrick and Eldering conducted their research on a shoestring budget. The Michigan Department of Health allowed them to use laboratory facilities after hours for their early pertussis research, and for the first 2 years of their studies, a total of $1,250 arrived from private citizens, the Grand Rapids City Commission, and the National Research Council. Later, and only after their vaccine showed progress, did they receive additional funding for staffing and research from the Federal Emergency Relief Administration

(a New Deal Agency) and from the Michigan Department of Health (*15*). When, in early 1936, the whooping cough vaccine project's funds again ran low, Kendrick invited Eleanor Roosevelt to visit their laboratory. Roosevelt helped secure the funds needed to add several Works Progress Administration workers to Kendrick and Eldering's staff. In 1938, the Works Progress Administration furnished additional clerical staff, and the APHA helped defray the cost of statistical analysis (*26*). Funding from all sources for the study amounted to $181,695.60 (*12*). Later, the National Institutes of Health would fund additional pertussis studies, and the Michigan Department of Health would continue funding public health research into the 1980s.

That Kendrick and Eldering crafted a well-controlled trial is revealed in their successful defense of their research against the skepticism of public health leaders. Soon after they announced their vaccine results, James Doull, a prominent Cleveland epidemiologist, reported that children received no protection from his whooping cough vaccine. The APHA subcommittee on whooping cough, which included both Kendrick and Doull, evaluated the divergent results of the 2 studies. Unable to explain the difference in results, committee members then enlisted Wade Hampton Frost, a Johns Hopkins University epidemiologist and head of the APHA, to review the work. Although predisposed to find fault with Kendrick's work because of his belief that few studies could meet strict standards of control (*12*), Frost journeyed twice to Michigan to inspect Kendrick's findings and, in the end, supported her work. Frost noted to Kendrick, "I think it may be assumed, not as a conclusion but merely as a working hypothesis, that your data when finally analyzed are likely to show some protection in the vaccinated group. Therefore, without accepting this as a conclusion, I think it is proper to make plans for further work based on this presumption, and I would suggest two additional projects" (*12*). Had Frost not died shortly thereafter, this productive collaboration might have continued (*27*,*28*).

Encouraged by the results of the 1936 trials, parents in Grand Rapids flocked to enroll their children in Kendrick and Eldering's 1938 follow-up study in which children were given smaller doses of the pertussis vaccine, administered in 3 injections. This new regimen was found to be as effective as the 4 injections given in the original study (*29*). On the basis of this study, the Michigan Department of Health Biologic Products Division began mass-producing the pertussis vaccine for children in Michigan in 1938, and, by 1940, pertussis vaccine was widely distributed across the nation.

In 1943, the American Academy of Pediatrics approved the vaccine for routine use; a year later, the American Medical Association recommended its use (*30*). The nation's whooping cough incidence and death rates would drop dramatically. In 1934, the whooping cough incidence in the United States was 209 cases/100,000 residents, and the death rate was 5.9/100,000. By 1948, routine use of the vaccine reduced the incidence to 51 cases/100,000 residents and the death rate to <1/100,000. After 1960, incidence was <10 cases/100,000 residents (*31*).

In the early 1940s, Kendrick's Michigan Department of Health laboratory participated actively in APHA Pertussis Study Group studies designed to standardize the pertussis vaccine (*28*). At this juncture, the public health community used adherence to a manufacturing process as the standard measure of their vaccine's safety and efficiency, despite the fact that methods of inactivating the bacillus and manufacturing the vaccine varied widely. Before advocating wider dissemination of the vaccine, the APHA Pertussis Study Group worked closely with Kendrick and pharmaceutical companies to develop measurable standards and verifiable tests that could be applied to the end product regardless of the manufacturing process used. As Kendrick noted on March 16, 1942, to W.A. Feirer of Sharpe and Dohme, "May I repeat that in relation to the work of your committee on standards, it seems to me that the problem of first importance is to attempt to reach some degree of uniformity in judging the concentration of the organisms in the product. This does not mean necessarily that the same method of standardization be used by all manufacturers. It does mean that it should be possible to check their labeled concentrations within an accepted range of variation, by a single method" (*13*). Using APHA and pharmaceutical company funding, Kendrick and Eldering developed an opacity standard by adjusting "the turbidity of a suspension of Pyrex glass particles to be equivalent to that of a specified number of bacteria of an aged vaccine determined by direct count" (*32*,*33*). In 1946, the United States adopted this standard; in 1958, the World Health Organization designated it as the international standard.

Although the American medical community readily adopted Kendrick and Eldering's whooping cough vaccine, the editor of the British Medical Journal expressed more skepticism, arguing that none of the American studies used proper control groups and that their own trials had shown such vaccines to be ineffective (*34*). David Evans of the British Medical Research Council (MRC) and J.S. Wilson of the London School of Hygiene did not share the British Medical Journal's concerns; indeed, they turned to Pearl Kendrick to assist with the MRC's next series of studies. Kendrick not only supplied the British with American serum to compare with the British vaccines and assisted in designing their study but also, with MRC funding, tested the potency of the vaccines, by using a mouse protection assay developed in the Grand Rapids laboratory, before the vaccines were used in the MRC field trials (*13*,*35*). In the 1950s, the World Health Organization and the Whooping

Cough Immunization Committee of the MRC funded Kendrick's trip to England so that she could review the MRC's pertussis vaccine field trials (*35*).

Over the course of their careers, Kendrick and Eldering published >60 articles in a wide variety of journals, including the American Journal of Public Health, the Journal of Infectious Diseases, the American Journal of Hygiene, the Journal of Bacteriology, the Journal of Pediatrics, and the Journal of Laboratory and Clinical Medicine; they received frequent requests for reprints (*13*). Kendrick's thick correspondence files make clear that they shared their vaccines, plates, cultures, and research with scientists around the world and hosted many international visitors in their laboratory. Kendrick traveled the world, often as a consultant for the World Health Organization, helping to establish vaccine programs in Mexico, eastern Europe, and Central and South America. In 1962, she served as part of an exchange delegation on immunology to the Soviet Union (*13*,*35*).

Kendrick and Eldering participated actively in the inner circles of the international bacteriology and public health communities. Indeed, they were well known in scientific circles for their gracious hospitality at the dinner parties and picnics they hosted at their Grand Rapids home (*13*). Still, they did not seek the traditional rewards of fame, despite the many opportunities offered later in life. Indeed, they actively shunned publicity, turning down opportunities to appear on the Today Show as so much attention was, in the words of Grace Eldering, "embarrassing" (*36*).

Shortly after Kendrick's death, Dean Richard Remington, writing in the University of Michigan's School of Public Health newsletter, noted:

> A life saved by prevention cannot even be identified. Who are the men and women living today who would be dead from whooping cough had it not been for Pearl Kendrick's vaccine? We can conclude with reasonable certainty that several hundred thousand of them are now leading productive lives, in this country alone. But who are they? Name one. You can't do it and neither can I.…The accomplishments of disease prevention are statistical and epidemiological. Where's the news value, the human interest in that? … But a public service orientation can provide more than ample compensation. Dr. Kendrick never became rich and, outside a relatively small circle of informed friends and colleagues, never became famous. All she did was save hundreds of thousands of lives at modest cost. Secure knowledge of that fact is the very best reward (*37*).

In recent years, state department of health laboratories have lost personnel and much of their research funding (*38*). Kendrick and Eldering's model of enlisting the support and resources of the local, state, and national communities may once again offer a promising avenue for conducting groundbreaking research.

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Dr Shapiro-Shapin is a professor of history at Grand Valley State University, Allendale, Michigan. She is currently writing a book titled Quieting the Cough: Pearl Kendrick, Grace Eldering, and the Standardization of Public Health in the Interwar Years.

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etymologia

Bordetella pertussis

[bor″-də-tel′ə pər-tus′is]

Named for Belgian bacteriologist Jules Bordet, members of the genus *Bordetella* are small, gram-negative, aerobic coccobacilli that infect the respiratory epithelium in mammals. In 1906, Drs Bordet and Octave Gengou succeeded in isolating and cultivating the bacterium, later called *Bordetella pertussis* (from Latin per, intensive, and *tussis*, cough), which causes whooping cough, a deadly disease in young children. For this work and his pioneering immunologic studies, Dr Bordet was awarded the Nobel Prize in Physiology or Medicine in 1919.

Source: Bordet J, Gengou O. Le microbe de la coqueluche. Ann Inst Pasteur (Paris). 1906;20:731–41. http://nobelprize.org; Dorland's illustrated medical dictionary, 31st edition. Philadelphia: Saunders Elsevier; 2007.

Duck Hunters' Perceptions of Risk for Avian Influenza, Georgia, USA

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To determine duck hunters' risk for highly pathogenic avian influenza, we surveyed duck hunters in Georgia, USA, during 2007–2008, about their knowledge, attitudes, and practices. We found they engage in several practices that could expose them to the virus. Exposures and awareness were highest for those who had hunted >10 years.

Introduction of highly pathogenic avian influenza virus (H5N1) (HPAI) could have several devastating effects in the United States. Illness and death caused by HPAI have been reported for humans, waterfowl, and other animals (*1*). In 2009, the estimated population of ducks susceptible to HPAI in the traditional survey area of North America was 42 million (*2*). Domestic poultry are also susceptible to HPAI (*1*). The retail equivalent of the broiler industry (which accounts for most commercial chicken production) in the United States was $44 billion in 2008; in 2007, Georgia led the country by producing 16% of all broilers (*3*).

Waterfowl and shorebirds are natural reservoirs of influenza A viruses (*4*). Antibodies to avian influenza virus (H11N9) have been detected in 2 of 68 Iowa Department of Natural Resources employees and in 1 of 39 Iowa duck hunters (*5*). These 3 men had 27, 30, and 31 years of experience, respectively, possibly indicating time- or behavior-dependent associations with exposure. We therefore sought to gain a better understanding of the knowledge, attitudes, and hunting practices of duck hunters and to better characterize their potential for exposure to influenza virus while hunting North American waterfowl. We hypothesized that the recent focus on the potential for introduction of HPAI into a North American migratory bird flyway (*6*) may increase hunter awareness of this virus.

The Study

From November 17, 2007, through March 27, 2008, a convenience sample of 192 participants across the state of Georgia, USA, were surveyed in person (online Technical Appendix, www.cdc.gov/EID/content/16/8/1279-Techapp.pdf). Participants included 61 active duck hunters in a wildlife management area and 131 members of Ducks Unlimited. Duck hunters at the wildlife management area were asked to complete a survey as they finished a morning of hunting. Ducks Unlimited members were approached at several of their banquets around Georgia and were asked to complete a survey if they were active duck hunters.

Analyses of survey data were conducted by using SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA). Results across study groups were compared by using *t* tests, Mann-Whitney tests, and prevalence odds ratios (PORs). Statistical results were determined to be significant at $p \leq 0.05$. This study was approved by the Georgia Department of Community Health Institutional Review Board.

To determine differences between those who were and were not members of Ducks Unlimited, we evaluated results from wildlife management area participants separately. A total of 37 (61%) wildlife management area participants reported that they were currently, or had been within the past 5 years, a member of Ducks Unlimited. Compared with nonmembers, members hunted more often per season—an average of 9.1× (95% confidence interval [CI] 2.0–16.2, p = 0.012) more than nonmembers. In addition, Ducks Unlimited members were 2.8× (95% CI 1.1–7.4, p = 0.033) more likely to have >10 years of hunting experience. Because Ducks Unlimited members did not differ significantly from nonmembers with regard to any other knowledge, attitude, or practice variable, we combined results of the wildlife management area survey with those of the Ducks Unlimited member survey.

In terms of hunting patterns and practices (Tables 1, 2), most (68%) hunters reported having hunted outside Georgia in the past 5 years. The 5 most common states visited for duck hunting outside of Georgia—from most to least common—were Arkansas, Alabama, Mississippi, Louisiana, and North Dakota. Experienced hunters (those with >10 years of hunting experience) reported hunting an average of 3.2 days more per season than those who had been hunting ≤10 years (p = 0.03). Experienced hunters were also significantly more likely to hunt outside of Georgia (POR 1.92, 95% CI 1.02–3.60, p = 0.042).

Almost all (91%) hunters reported having had direct contact with water while hunting. Experienced hunters were significantly more likely to report having submerged their head in water during a hunt (POR 2.76, 95% CI 1.50–5.10, p = 0.001). Most (87%) hunters processed their harvested ducks themselves, and 84% did not wear gloves while doing so. However, most (88%) somewhat limited their postharvest exposures by leaving most of the bird intact and

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Table 1. Experience and hunting practices among surveyed duck hunters, Georgia, USA, November 17, 2007–March 27, 2008

Hunting practice	All hunters, no. (%)		≤10 y, no. (%)		>10 y, no. (%)		Prevalence OR (95% CI)*	p value
	Yes	No	Yes	No	Yes	No		
Hunted outside Georgia in past 5 y, n = 190	130 (68)	60 (32)	64 (62)	39 (38)	66 (76)	21 (24)	1.92 (1.02–3.60)	0.042
Have direct contact with water, n = 190	173 (91)	17 (9)	91 (88)	12 (12)	82 (94)	5 (6)	2.16 (0.73–6.40)	0.156
Submerge head, n = 190	68 (36)	122 (64)	26 (25)	77 (75)	42 (48)	45 (52)	2.76 (1.50–5.10)	0.001
Always or occasionally use dog while hunting, n = 192	146 (76)	46 (24)	73 (70)	32 (30)	73 (84)	13 (16)	2.29 (1.13–4.63)	0.020
Process harvested duck (dress out), n = 190	165 (87)	25 (13)	88 (85)	15 (15)	77 (89)	10 (11)	1.31 (0.56–3.09)	0.533
Pluck and gut duck	73 (51)	69 (49)	34 (45)	41 (55)	39 (58)	28 (42)	1.68 (0.86–3.27)	0.125
Cut off breast muscle only	138 (88)	18 (12)	74 (87)	11 (13)	64 (90)	7 (10)	1.36 (0.50–3.71)	0.549
Wear gloves while dressing out, n = 163	26 (16)	137 (84)	15 (17)	71 (83)	11 (14)	66 (86)	0.79 (0.34–1.84)	0.583
Take ducks to taxidermist, n = 190	113 (59)	77 (41)	50 (49)	53 (51)	63 (72)	24 (28)	2.78 (1.51–5.11)	0.001
Share harvested ducks, n = 188	118 (63)	70 (37)	63 (62)	38 (38)	55 (63)	32 (37)	1.04 (0.57–1.88)	0.905
Dress ducks before sharing, n = 115	62 (54)	53 (46)	36 (60)	24 (40)	26 (47)	29 (53)	0.60 (0.29–1.25)	0.171
Consume meat from harvested ducks, n = 187	176 (94)	11 (6)	93 (93)	7 (7)	83 (95)	4 (5)	1.56 (0.44–5.53)	0.486
Know that others consume meat from harvest, n = 188	144 (77)	44 (23)	74 (73)	27 (27)	70 (80)	17 (20)	1.50 (0.75–2.99)	0.246

*Referent group for calculation is duck hunters who reported ≤10 y of experience duck hunting. OR, odds ratio; CI, confidence interval.

simply cutting the breast meat from the carcass; only 51% reported completely dressing out the duck by plucking and gutting the carcass. Awareness of HPAI infection, or bird flu, was common among duck hunters (86%), but knowledge of the signs and symptoms in infected humans was not (23%). Only 6 (3%) respondents said that they would stop hunting if HPAI were found in US duck populations, and 36 (19%) would stop duck hunting if the virus were found in the state of Georgia.

Experienced hunters were nearly 3× more likely than less experienced hunters to have heard of HPAI (POR = 2.72, 95% CI 1.09–6.78, p = 0.027). However, experienced hunters who reported this virus as a personal concern said that they were not more likely to change their hunting practices if it were found in the United States or Georgia. Unlike the experienced hunters, less experienced hunters who reported concern about HPAI were 7.5× more likely to stop hunting if the virus were found in ducks in Georgia (95% CI 2.08–27.02, p = 0.001); those who were concerned about their own risk for illness through contact with sick birds were 4.8× more likely to stop hunting if HPAI were found in ducks in Georgia (95% CI 1.70–13.59, p = 0.002).

Conclusions

If HPAI were to become established in duck populations in North America, risk for human exposure to the virus through hunting could be substantial. In Georgia, each of the ≈12,000 active duck hunters (*7*) and ≈19,000 members of Ducks Unlimited potentially has contact with influenza-infected ducks and their water environments while hunting. By processing an influenza-infected duck, a hunter may be exposed to virus-laden nasal and/or fecal excretions in addition to blood, tissues, and other body fluids (*8*). Most hunters process harvested ducks themselves and do not use gloves. In the Republic of Azerbaijan, defeathering waterfowl infected with HPAI was associated with 8 confirmed cases of human illness and 5 deaths (*9*), but worldwide, no reports of HPAI infections among waterfowl hunters have been documented.

Influenza A viruses can persist in water for extended periods (*10*) and on clothing for several hours (*11*). Most hunters have direct contact with water during a hunt, but experienced hunters are more likely to have their head submerged. Although hunters could be exposed to virus during contact with contaminated water and by aerosols generated

Table 2. Duck hunter experience and exposure to avian influenza (H5N1), Georgia, USA, November 17, 2007–March 27, 2008

Hunters	Years of hunting, no. (%) hunters, n = 189				Exposure, mean (range)		
	1	2–5	6–10	>10	Hunts/y, n = 191*	Harvests/y, n = 188†	Ducks sent to taxidermist in past 5 y, n = 108‡
All	9 (5)	42 (22)	51 (27)	87 (46)	14.8 (1–60)	26.2 (1–240)	5.6 (1–60)
Hunted ≤10 y	9 (9)	42 (41)	51 (50)	0	13.4 (1–60)	24.3 (1–240)	5.6 (1–60)
Hunted >10 y	0	0	0	87 (100)	16.5 (2–60)	28.3 (2–200)	5.6 (1–30)

*Differences in mean values, calculated by Mann-Whitney U test, were –3.2 (p = 0.03).
†Differences in mean values, calculated by Mann-Whitney U test, were –4.0 (p = 0.08).
‡Differences in mean values, calculated by Mann-Whitney U test, were 0.01 (p = 0.03).

when water- or carcass-contaminated clothing are removed, no data are available to realistically evaluate this possibility.

To minimize duck hunter exposure to HPAI, we recommend more use of personal protection, such as gloves, while processing harvested ducks. If this virus were found in North America, increased efforts to educate duck hunters on the potential severity of illness resulting from HPAI and the potential for exposure during hunting might decrease risky hunting practices.

If HPAI were found in United States or Georgia duck populations, most duck hunters indicated that they would not stop hunting. These findings suggest that hunting practices and attitudes among the subpopulation of experienced hunters may contribute to an increased risk for avian influenza infection.

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Amblyomma imitator Ticks as Vectors of *Rickettsia rickettsii*, Mexico

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Real-time PCR of *Amblyomma imitator* tick egg masses obtained in Nuevo Leon State, Mexico, identified a *Rickettsia* species. Sequence analyses of 17-kD common antigen and outer membrane protein A and B gene fragments showed to it to be *R. rickettsii*, which suggested a potential new vector for this bacterium.

Rickettsia rickettsii is a gram-negative, obligate, intracellular bacterium and the cause of Rocky Mountain spotted fever (RMSF). In Mexico, its transmission has been attributed to *Rhipicephalus sanguineus* and *Amblyomma cajennense* ticks.

Amblyomma imitator has close affinity with *A. cajennense* and was formerly confused with this species. These ticks' distributional range extends from southern Texas, southward through Mexico (where they are widely sympatric with *A. cajennense* ticks) into Central America (*1*). In this study, we isolated and characterized *R. rickettsii* from *A. imitator* from Mexico by using molecular methods.

The Study

Males, females, and nymphs of 5 tick species (*A. imitator*, *Rhipicephalus microplus*, *Dermacentor variabilis*, *Rh. sanguineus*, and *Ixodes* species) were collected in Nuevo Leon State, Mexico (24°50′N, 100°4′W) in 2007 (females, males, and nymphs) by using dry ice traps (*1*). Ticks were identified by using morphologic keys as defined by Kohls (*2*) and Keirans and Durden (*3*) and maintained in the laboratory at the University of Texas Medical Branch (Galveston, TX, USA) according to methods described by Brossard and Wikel (*4*). The colonies were maintained at 22°C, under a 14-hour light/10-hour dark photoperiod. Ticks were held in 16-mL glass vials (Wheaton Glass, Millville, NJ, USA) with a mesh top over a supersaturated solution of potassium nitrate. Larvae and nymphs obtained blood meals from mice, and adults were fed on pathogen-free rabbits. Only the *A. imitator* colony was successfully established.

Real-time PCR analysis of eggs laid by 1 full generation of laboratory-reared females identified rickettsial DNA in 2 egg masses. DNA from 5 egg masses laid by different females was extracted by using a DNeasy Kit (QIAGEN, Valencia, CA, USA). Real-time PCR was performed by using *Rickettsia* spp.–specific primers CS5A and CS6 (*5*) for amplification of a 150-bp fragment of the citrate synthase gene in an iCycler thermocycler (Bio-Rad, Hercules, CA, USA) as described (*5*). *R. australis* DNA and water served as the positive and negative controls, respectively, and serial dilutions of a plasmid that contained the *R. prowazekii* citrate synthase gene were used as standards.

We selected egg masses for isolation of the rickettsial agent in Vero cells by using shell vials as described (*6*). The shell vials were incubated at 34°C and monitored daily by Diff-Quik staining (Dade International Inc., Miami, FL, USA) for rickettsiae. Slides that contained ≥4 rickettsiae were considered positive. The monolayer from the corresponding shell vial was removed manually, placed in a T-25 flask containing Vero cells (in Dulbecco minimal essential medium containing 3% heat-inactivated bovine calf serum). Samples were then placed in 150-cm^2 flasks containing Vero cells for propagation of the agent.

For characterization of isolates, partial sequences of *Rickettsia*-specific genes were amplified and analyzed. Nested PCR was performed by using primers 17K3 and 17K5 (*5*) for the first reaction and 17KD1 and 17kD2 (*7*) for the second reaction of amplification of 17-kD common antigen (*htrA*) gene fragments. For amplification of an outer membrane protein B (*ompB*) gene fragment, primers 120-M59 and 120–807 were used (*8*). Seminested PCR with primers 120-M59 and 120–807 for the first reaction and 120–607 and 120–807 for the second reaction was performed for 1 of the samples (*8*). An *ompA* fragment was amplified by using primers Rr190.70F and Rr190.602R (*9*). For 1 of the samples, a seminested PCR was necessary; primers Rr190.70F and Rr190.701R (*10*) were used

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for the first reaction and Rr190.70F and Rr190.602R for the second reaction.

Fragments were cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA, USA), and plasmids from selected clones were sequenced ≥3× by using universal primers M13F and M13R. Nucleotide sequences were edited with SeqMan (www.dnastar.com/t-sub-products-lasergene-seqmanpro.aspx) and used for BLAST analysis (*11*). Amplification of the *ompB* fragment was achieved for only 1 of the samples. This finding was probably the result of a mutation at the site of primer annealing because amplification of another region of the gene was possible.

Analysis of sequences obtained from the *htrA* gene fragments (434 bp) amplified from both samples showed 99% identity with spotted fever group *Rickettsia* sequences, including the *R. rickettsii* sequence from a fatal case of RMSF in southwestern Mexico, Yucatan State (GenBank accession no. DQ176856.1) (*12*), with only a 1-nt difference. Analysis of the partial sequence of *ompB* (856 bp) and *ompA* (533 bp) genes showed 100% identity with *R. rickettsii* Sheila Smith strain (CP000848.1). Sequences obtained in this study were submitted to GenBank under accession nos. GU723476 and GU723477 for *htrA* fragments, GU723478 and GU723479 for *ompA* fragments, and GU723475 for the *ompB* fragment.

For detection of rickettsiae in *A. imitator* ticks, salivary glands, midguts, and ovaries were dissected from unfed adult ticks and fixed in modified Ito fixative (*13*), postfixed in 1% osmium tetroxide for 1 h, stained en bloc with 2% aqueous uranyl acetate for 20 min at 60°C, dehydrated in ethanol, and embedded in epoxy resin (Poly/Bed 812). Ultrathin sections were cut with an Ultracut S Ultramicrotome (Reichert, Vienna, Austria), placed on copper grids, and stained with lead citrate. Ticks were tested for *Rickettsia* spp. by nested PCR with primers 17K3/17K5 and 17KD1/17KD2, and sections of organs from PCR-positive samples were examined in a Philips CM-100 electron microscope (FEI, Hillsboro, OR, USA) at 60 kV.

Single rickettsial cells were found in highly vacuolated cytoplasm of midgut epithelial cells of 1 male tick (no. 5) (Figure, panel A). Cells had typical ultrastructure for gram-negative bacteria and were surrounded by 2 trilaminar membranes (Figure, panel B): an inner cytoplasmic membrane and an outer cell wall membrane. Their size was 0.6 × 0.2 μm. Rickettsial organisms were not visualized in either salivary gland or ovaries probably because of a low number of rickettsiae, as suggested by the fact that detection of rickettsial DNA by PCR required a nested PCR.

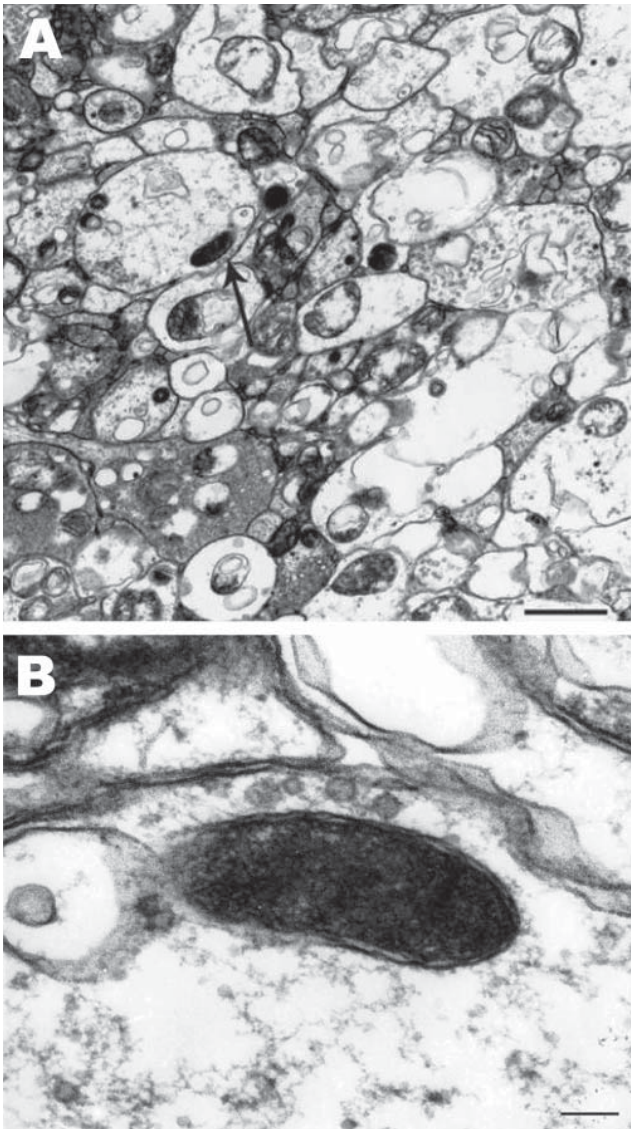

Figure. *Rickettsia rickettsii* (arrow) in a midgut cell of an *Amblyomma imitator* tick (A). The trilaminar cell wall is separated from the cell membrane by the periplasmic space (B). Scale bars = 0.1 μm.

Conclusions

This study demonstrated that *R. rickettsii* was present in egg masses of *A. imitator* ticks. Because eggs were laid by field-collected, unfed, adult ticks that were fed in the laboratory on pathogen-free rabbits for 1 full generation, *R. rickettsii* in eggs documents their transovarial transmission by naturally infected ticks and suggests a role for this tick species in the maintenance of *R. rickettsii* in nature.

Because hosts of *A. imitator* ticks are various species of birds and mammals (*3*), the notable finding of this study is the potential participation of *A. imitator* ticks in a zoonotic cycle of *R. rickettsii*. According to Kohls (*2*), RMSF transmission studies performed with supposed *A. cajennense* ticks by Parker et al. (*14*) were actually performed with *A. imitator* ticks, which suggests that this species of tick could be a vector of *R. rickettsii*. On the basis of the results of our study and the aggressive nature of the tick

for humans, we suggest that *A. imitator* ticks are a potential vector or at least involved in maintenance of *R. rickettsii* in nature in Mexico.

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Bat Rabies in Massachusetts, USA, 1985–2009

Xingtai Wang, Alfred DeMaria, Sandra Smole, Catherine M. Brown, and Linda Han

To investigate rabies in Massachusetts, we analyzed bat rabies test results before and after introduction of raccoon variant rabies and after release of revised 1999 US Advisory Committee on Immunization Practices recommendations for rabies postexposure prophylaxis. Bat submissions were associated with level of rabies awareness and specific postexposure recommendations.

For the past 20 years, bat-associated rabies virus variants have accounted for most human rabies infections acquired in the United States. Most infections were associated with reports of minimal, if any, direct contact with a bat, which suggested that superficial or unrecognized bat bites may transmit infection (*1*–*5*). In 1999, the Advisory Committee on Immunization Practices (ACIP) updated their human rabies prevention guidelines to include consideration of rabies postexposure prophylaxis (PEP) in some circumstances without recognized direct bat contact (*6*). Since the first rabid bat was reported in Massachusetts in 1961, testing of rabid animals has been conducted at the state public health laboratory (*7*). Raccoon rabies virus variant (RRV) was first detected in Massachusetts in 1992 and had spread throughout most of the state by 1996.

We reviewed bat rabies data for Massachusetts during 1985–2009. We analyzed the effect of RRV introduction on specimen submission, the impact of the 1999 ACIP guidelines on specimen submission, and differences in the likelihood of rabies infection among bats with different submission characteristics.

The Study

From 1985 through 2009, a total of 10,257 bats were submitted to the Massachusetts Department of Public Health for rabies testing: 8,850 big brown bats (*Eptesicus fuscus*, 86.3%), 1,074 little brown bats (*Myotis lucifugus*, 10.5%), 94 Keen long-eared bats (*Myotis keenii*, 0.9%), 48 red bats (*Lasiurus blossevillii*, 0.5%), 17 hoary bats (*Lasiurus cinereus*, 0.2%), 17 silver-haired bats (*Lasionycteris noctivagans*, 0.2%), 1 zoo-submitted Seychelles fruit bat (*Pteropus seychellensis*), and 156 (1.5%) unspeciated bats.

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The proportion of rabies-positive bats by species was 5.0% (443/8,850) big brown bats, 3.6% (39/1,074) little brown bats, 23.5% (4/17) hoary bats, 8.3% (4/48) red bats, 5.9% (1/17) silver-haired bats, and 3.2% (3/94) Keen long-eared bats; 751 (7.3%) of 10,257 bats were not suitable for rabies testing. Among all rabies-positive bats, 89.3% were big brown bats, 8.0% were little brown bats, and 2.7% were of less frequently submitted species. During 2005–2008 (Figure 1), submissions of little brown bat sharply increased then decreased, although the proportion that was rabies positive (2%–4%) remained stable.

The average annual number of bat submissions increased significantly from 103 during 1985–1991 to 302 during 1992–1998 and to 675 during 1999–2009 ($p<0.0001$) (Figure 2). The average annual number of confirmed rabid bats increased from 7 to 19 to 28 for those periods, and the proportion of bats positive for rabies decreased from 6.9% (50/720) to 6.4% (135/2,113) to 4.2% (311/7,424). The proportion of rabid bats was significantly lower during 1999–2009 ($p<0.05$).

Among 961 testable bats submitted before RRV introduction, 76 (7.9%) were positive for rabies compared with 420 (4.9%) of 8,545 bats submitted after RRV introduction. No positive association was identified between RRV introduction and proportion of bats positive for rabies, even when adjusting for potential confounders such as bat species (big brown and little brown vs. other pooled species), reason for bat submission (human and pet exposure, human exposure only, and pet exposure only vs. undefined), and time of submission (1985–1991 and 1992–1998 vs. 1999–2009). Limited RRV strain typing results performed on 52 rabies-positive bats showed that all bats were infected with non-RRV (X. Wang et al., unpub. data).

Before publication of the 1999 ACIP recommendations, the most common reason for testing bats was pet

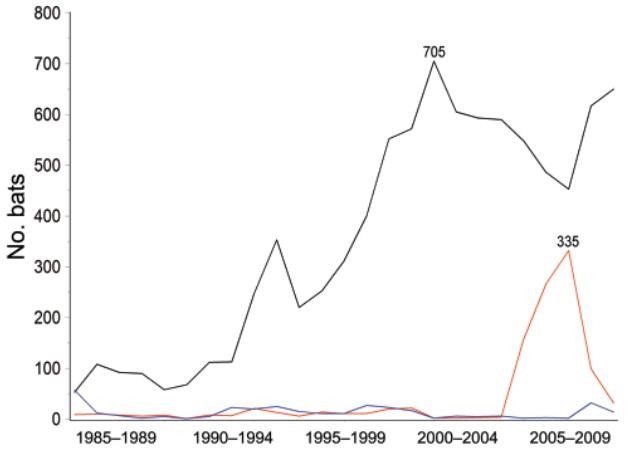

Figure 1. Bats submitted for rabies testing in Massachusetts, USA, 1985–2009. Black line indicates *Eptesicus fuscus*, red line indicates *Myotis lucifugus*, and blue line indicates other pooled bats.

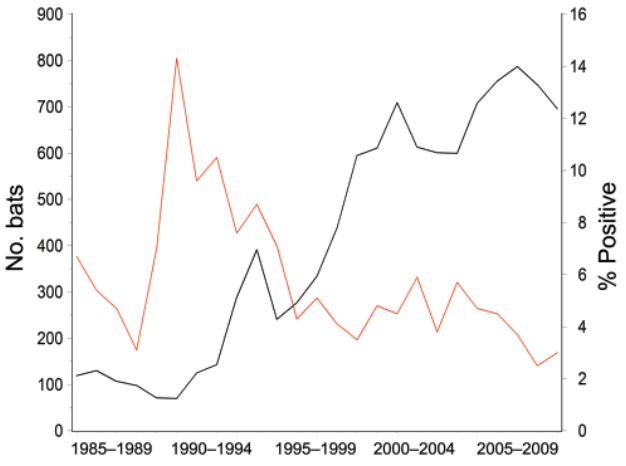

Figure 2. Rabies in bats in Massachusetts, USA, 1985–2009. Black line indicates number of bats submitted and red line indicates percentage of bats positive for rabies.

exposure only, which accounted for 50.3% of submissions during 1985–1991 and 43.5% during 1992–1998 (Table 1). After 1999, the most common reason for bat testing was human exposure, accounting for 72.0% of submissions during 1999–2009. Although the number of bat submissions because of human exposure increased with time, the rabies-positive proportion of these bats decreased from 10.3% (17/165) to 5.8% (51/885) to 3.8% (204/5,343) during the 3 periods. No significant differences in rabies positivity among bats submitted for different reasons were found during 1999–2009 (Table 2).

Clinical signs were reported for 2,291 (24%) of 9,537 bats submitted since 1992 (Table 2). The most common signs were death (1,206, 52.6%), disorientation (514, 22.4%), lethargy (358, 15.6%), or aggressiveness (275, 12.0%). Bats described as having aggression, ataxia, disorientation, or lethargy were significantly more likely to have rabies than were bats with no reported signs ($p<0.05$). Bats found dead were no more likely to have rabies than were bats reported alive before submission.

Conclusions

The increase in bat submissions for rabies testing after 1992 correlated with RRV introduction and associated statewide enhancement of rabies surveillance and awareness generated by arrival of raccoon rabies. However, in contrast to other animal species in which RRV introduction resulted in an increase in identification of rabies (*8*), the proportion of bats with rabies was constant in the periods before (1985–1991) and after (1992–1998) RRV introduction. These findings are supported by limited laboratory typing data in rabid bats, which showed no evidence that RRV plays a role in bat infection. This finding is consistent with reports that insectivorous bat rabies virus variants circulate separately from terrestrial viral variants (*9*,*10*). Although incidents of bat rabies virus variant spillover into terrestrial mammals are documented (*11*), spillover of terrestrial variants into bats has yet to be reported.

After release of the ACIP guidelines, the number of annual bat submissions for rabies testing doubled relative to the previous period, and the proportion of rabies-positive bats decreased (6.5% vs. 4.2%). Between 1985–1991 and 1999–2009, the proportion of bats submitted on the basis of human exposure (human exposure alone and human and pet exposure) increased from 22.9% to 72.0%. This finding was likely caused by adherence to the ACIP recommendations and increased awareness of rabies in bats.

In examining the effect of various bat characteristics on the likelihood of rabies infection, we found that signs of central nervous system involvement, including aggression, ataxia, disorientation, or lethargy, were associated with rabies. However, use of reported bat behavior and appearance in assessing the risk for rabies is not feasible (*8*).

Big brown bats were submitted in the highest numbers, and had the highest rabies positivity. Increases in little brown bat submissions began 1 year before identification of bat white-nose syndrome in upstate New York in 2006 (*12*). This increase in little brown bat submissions was not associated with rabies positivity or with bats found dead as a reason for submission.

The role of rabies laboratory testing and public health follow-up is reflected in part by the number of costly courses of PEP potentially averted or discontinued. During 1999–2009, a total of 4,766 Massachusetts residents were exposed to bats that were negative for rabies virus. With each course of PEP costing an estimated $2,376 in biologics alone in 1998 (*13*), and without considering costs associated with medical evaluation and vaccine administration, this cost amounts to $10–20 million in healthcare savings in Massachusetts in 1999–2009. A recent study of PEP recommendations for potentially unrecognized bat exposures suggests that the rate of human rabies associated with such exposures was only 1/2.7 billion person-years, and medical costs of such exposures could be up to 2 billion Canadian dollars (*14*). Analyses such as this have already prompted

Table 1. Reasons for bat submissions for rabies testing, Massachusetts, USA, 1985–2009

Reason	No. (%) submissions		
	1985–1991	1992–1998	1999–2009
Human and pet exposure	5 (0.7)	250 (11.8)	1,004 (13.5)
Human exposure only	160 (22.2)	635 (30.1)	4,339 (58.5)
Pet exposure only	362 (50.3)	918 (43.5)	1,295 (17.4)
Sickness and other reasons	193 (26.8)	310 (14.7)	786 (10.6)
Total	720	2,113	7,424

Table 2. Characteristics of bats submitted for rabies testing, Massachusetts, USA, 1985–2009*

Characteristic	No. rabid/no. tested (%)		
	1985–1991	1992–1998	1999–2009
Total no. bats	50/720 (6.9)	135/2,113 (6.4)	311/7,424 (4.2)
Bat species			
Big brown (*Eptesicus fuscus*)	47/581 (8.1)	126/1,898 (6.6)	270/6,371 (4.2)
Hoary (*Lasiurus cinereus*)	0/5	4/12 (33.3)	NS
Little brown (*Myotis lucifugus*)	1/50 (2.0)	2/83 (2.4)	36/941(3.8)
Keen long-eared (*M. keenii*)	2/9 (22.2)	1/54 (1.9)	0/31
Red (*L. blossevillii*)	0/8	2/20 (10.0)	2/20 (10.0)
Seychelles fruit (*Pteropus seychellensis*)	NS	0/1	NS
Silver-haired (*Lasionycteris noctivagans*)	NS	0/4	1/13 (7.7)
Unidentified	0/67	0/41	2/48 (4.2)
Reasons for bat test			
Human and pet exposure	0/5	16/250 (6.4)	40/1,004 (4.0)
Human exposure	17/160 (10.6)	35/635 (5.5)	164/4,339 (3.8)
Pet exposure	21/362 (5.8)	50/918 (5.5)	67/1,295 (5.2)
Sick and other reasons	12/193 (6.2)	34/310 (11.0)	40/786 (5.1)
Signs			
Aggression			
Unobserved	NC	118/2,033 (5.8)	291/7,229 (4.0)
Yes	NC	17/80 (21.3)	20/196 (10.3)
Ataxia			
Unobserved	NC	128/2,087 (6.1)	307/7,392 (4.2)
Yes	NC	7/26 (26.9)	4/32 (12.5)
Death			
Unobserved	NC	114/1,765 (6.5)	284/6,566 (4.3)
Yes	NC	21/348 (6.0)	27/858 (3.2)
Disorientation			
Unobserved	NC	113/1,985 (5.7)	278/7,038 (4.0)
Yes	NC	22/128 (17.2)	33/386 (8.6)
Lethargy			
Unobserved	NC	119/2,034 (5.9)	275/7,145 (3.9)
Yes	NC	16/79 (20.3)	36/279 (12.9)
Paralysis			
Unobserved	NC	135/2,105 (6.4)	304/7,394 (4.1)
Yes	NC	0/8	7/30 (23.7)
Salivation			
Unobserved	NC	135/2,111 (6.4)	309/7,409 (4.2)
Yes	NC	0/2	2/15 (13.3)
Seizures			
Unobserved	NC	135/2,107 (6.4)	309/7,416 (4.2)
Yes	NC	0/6	2/8 (25.0)

*NS, no specimen; unobserved, none or unknown; NC, not collected.

changes in rabies PEP recommendations in Canada to specify direct exposure to a bat (*15*). PEP recommendations in the United States are based on national guidelines and include considerations of PEP for cryptic bat exposures. Current practice is in place pending reconsideration of and changes to these guidelines.

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Lyme Borreliosis, Po River Valley, Italy

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We aimed to determine the presence of *Ixodes ricinus* ticks in heavily populated areas of the Po River Valley after report of a Lyme disease case. Eighteen percent of ticks examined from 3 locations were positive for Lyme disease borreliae. Lyme disease was diagnosed for 3 workers at risk for tick bite.

Lyme disease, caused by *Borrelia burgdorferi* sensu lato (*1*), is endemic to various areas of Italy (*2*). The main vector of Lyme disease in Italy is the hard tick (*Ixodes ricinus*), a species widespread in mountain regions populated by wild ungulates (*2*). Residents of these areas and forestry workers are at risk for Lyme disease (*3*). Heavily populated flat regions are not considered as risk areas. For example, *I. ricinus* ticks have never been reported in the flat areas of the Po River Valley in the Lombardy region, one of the most important industrial districts in Europe and an area of intensive agriculture and livestock breeding. Human population density is high; >6 million persons reside in Milano and surrounding counties. In areas of Italy to which *I. ricinus* ticks are known to be endemic, physicians have appropriate awareness of the risks from tick bite and Lyme disease; outside these areas, awareness probably is not adequate.

In late spring 2008, a forestry worker at a natural park west of Milano in the Po River Valley was treated for cutaneous mycosis on the basis of an erythematous rash on an arm. In August 2008, this patient described this skin alteration to one of us (C.B.). Subsequent clinical examination and serologic analyses led to diagnosis of Lyme disease. Because of this case, we investigated different areas of the park for ticks; collected ticks were screened by PCR for Lyme borreliae. We conducted a retrospective analysis of forestry workers in the area; 2 workers reporting the appearance of erythematous rash in the previous months underwent serologic analyses.

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The Study

During May–August 2009, ticks at different stages of development were collected by dragging. Ticks were collected in rural or suburban areas of the municipalities of Somma Lombardo, Lonate Pozzolo, Magenta, and Pavia (Figure). These sites are located along the Ticino River, which crosses the counties of Varese, Novara, Milano, and Pavia.

Of 1,094 ticks collected, 576 were larvae, 507 nymphs, and 11 adults (7 males). The samples were stored in 96% ethanol and later identified according to standard taxonomic keys (*4*). All ticks were *I. ricinus*. In a subsample of 240 nymphs of the 507 collected, each nymph was broken apart with a sterile needle and then subjected to DNA extraction by using the IllustraTissue & Cells Genomic Prep Mini Spin Kit (GE Healthcare, Little Chalfont, UK). The quality of the extracted DNA was checked by PCR for the *I. ricinus* tick mitochondrial 12S rRNA gene (*5*). Positive amplification was found for most (234/240) nymphs examined. A subset of the amplified 12S rRNA genes (20/234) were sequenced by using ABI technology (Applied Biosystems, Foster City, CA, USA), and the sequences obtained confirmed the specimens as *I. ricinus*. PCR screening for *B. burgdorferi* sensu lato was performed on DNA from the 234 nymphs by using primers BBLD5′ and BBLD3′ for 16S rRNA (*6*). Positive samples were examined by using a nested PCR protocol (*7*) for the 23S–5S rRNA spacer region of *B. burgdorferi* sensu lato. In addition, all 11 adults and pools of 10 larvae from Somma Lombardo, Lonate Pozzolo, Magenta (collection sites A, B, and C in Figure) were screened for *B. burgdorferi* sensu lato by using the same procedure.

B. burgdorferi sensu lato was detected in 42 (18%) of the 234 nymphs analyzed (Figure). One of the 7 adult males was positive; none of the 4 adult females and none of the pools of larvae were positive. The PCR products obtained from the 42 positive nymphs and from the adult male were sequenced by using ABI technology (Applied Biosystems), and the sequences were searched for homology using BLAST on the National Center for Biotechnology Information nonredundant database (www.ncbi.nlm.nih.gov/BLAST). 16S rRNA sequences confirmed identification as *B. burgdorferii* sensu lato, whereas rRNA spacer sequences showed the highest scores for *B. afzelii* (36/43) and *B. lusitaniae* (7/43) (Figure). Six rRNA spacer sequences representing the entire variability were deposited in the European Molecular Biology Laboratory database (FN658703–FN658708), then aligned with homologous sequences of *Borrelia* species by using MUSCLE (*8*). Neigh-

[1]These authors contributed equally to this article.

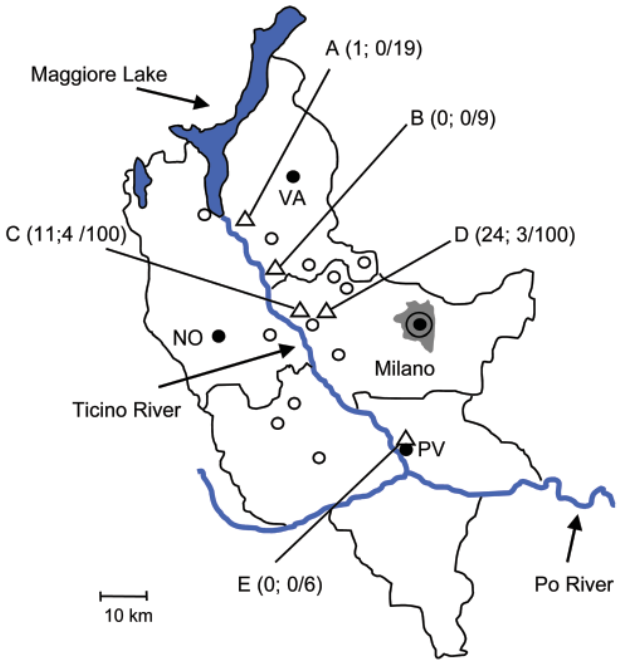

Figure. Collection sites (triangles A–E) of *Ixodes ricinus* ticks in the counties of Milano, Pavia, and Varese, Po River Valley, Italy, 2008. Ticks were collected in rural or suburban areas of the municipalities of Somma Lombardo (collection site A), Lonate Pozzolo (B), Magenta (C, D), and Pavia (E). The 3 numbers in parentheses for each collection site indicate number of tick nymphs positive for *Borrelia afzelii,* number of nymphs positive for *B. lusitaniae,* and number of nymphs examined by PCR. The adult specimen positive for *B. afzelii* was collected at site D. Empty circles indicate towns with 10,000–50,000 residents; black circles indicate towns with >50,000 residents. NO, Novara; PV, Pavia; VA, Varese. Milano residents = 4 million persons.

bor-joining phylogenetic analysis, using SeaView 4.2 (*9*), confirmed placements of the obtained sequences into the clusters of *B. afzelii* and *B. lusitaniae* (data not shown). The relative prevalence of the 2 species of borreliae that we detected differs from those reported in other studies (*10*), probably because of environmental conditions, particularly the presence and relative abundance of the different reservoir hosts (*11*).

The 3 forestry workers who reported having had cutaneous erythema, 2 of whom noted a tick bite, underwent physical examination, then ELISA and Western blot analysis following the recommended stepwise protocol for the serodiagnosis of Lyme disease (*12*). Western blot, performed by using the recomBlot Borrelia IgG kit (Mikrogen Diagnostik, Neuried, Germany), according to the manufacturer's instructions, confirmed the positive results of the ELISA. The 3 patients then received the recommended therapy (*13*).

Conclusions

We found evidence of the Lyme disease vector *I. ricinus* ticks in the Po River Valley in the Lombardy region of Italy and of ticks from 3 locations that harbored Lyme disease borreliae. In addition, we detected evidence for *B. burgdorferi* sensu lato infection in 3 persons at risk for tick bite who work in the area. One location from which we collected *I. ricinus* ticks (location E) is in the suburban area of Pavia, a densely populated town. The risk of contracting Lyme disease in Italy is thus not limited to mountains and wild areas but extends to the plains, such as the Po River Valley, and possibly reaches suburban areas. The characteristics of the territory of the sampled area, although in heavily populated counties, are ecologically compatible with the presence of *I. ricinus* ticks because of the woods and bushes, corridors of vegetation connecting the plains and the river banks to mountain areas, and microrodents. In addition, the area along the Ticino River that includes collection locations C and D (where most tick specimens were sampled) is populated by roe deer (*Capreolus capreolus*), whose role as a major host for *I. ricinus* ticks is well known (*2*). These ungulates were introduced into this area 2 decades ago (*14*).

The Lyme disease case initially diagnosed as a mycosis and the 2 undiagnosed cases among forestry workers in the area west of Milano suggest that awareness of risks associated with tick bite probably is not adequate among physicians in the region. Moreover, before our investigation, visitors of the wild areas along the River Ticino were not adequately informed about the presence of ticks. Our report provides a basis for supplying proper information to health institutions and physicians in the area, as well for helping park administrators adopt proper precautions.

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Pandemic (H1N1) 2009 Surveillance in Marginalized Populations, Tijuana, Mexico

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To detect early cases of pandemic (H1N1) 2009 infection, in 2009 we surveyed 303 persons from marginalized populations of drug users, sex workers, and homeless persons in Tijuana, Mexico. Six confirmed cases of pandemic (H1N1) 2009 were detected, and the use of rapid, mobile influenza testing was demonstrated.

The first declared influenza pandemic in 40 years likely originated in March 2009 in La Gloria, Veracruz, a small rural town in southern Mexico (*1*). The virus responsible for the outbreak, identified as a novel influenza A (H1N1) virus, now referred to as pandemic (H1N1) 2009, spread quickly. By the end of 2009, ≈70,000 cases were confirmed and 944 deaths were recorded in Mexico (*2*), and >600,000 cases were reported worldwide (*3*).

When pandemic (H1N1) 2009 first emerged there was concern that Mexico–US border cities, which have served as corridors for binational transmission of infectious diseases (*4*), might become overwhelmed by the disease before national surveillance resources could be mobilized. Of particular concern was the fact that traditional hospital-based disease surveillance would miss the emergence of pandemic (H1N1) 2009 in the dense, highly mobile (*5*), marginalized border populations. Furthermore, the poorest and most underserved border populations, composed of the homeless, commercial sex workers, and those with alcohol and drug abuse problems, have disproportionately high levels of diseases such as HIV (*6*) and tuberculosis (*7*), illnesses suspected of increasing vulnerability to acquiring and dying of pandemic (H1N1) 2009 (*8*,*9*). We report the results of an enhanced surveillance effort to detect early pandemic (H1N1) 2009 cases and assess perceptions of the pandemic and risk factors associated with acquiring the disease in marginalized populations in Tijuana, Mexico.

The Study

This surveillance project was a collaboration between Mexican public health officials, nongovernment organizations, and the University of California, San Diego (UCSD). The project was approved by the institutional review boards of Tijuana General Hospital and UCSD.

Our binational research team of US and Mexican researchers has been conducting prospective studies of populations at high risk for infectious diseases in the most vulnerable communities of Tijuana and Ciudad Juarez, Mexico, since 2004 (*10*,*11*). More than 2,500 drug users, sex workers, and homeless persons have been recruited to participate in those studies. From May 1 through November 20, 2009, we conducted active and passive influenza surveillance within our existing marginalized study populations in Tijuana. We used mobile outreach units and community clinics in the city's Zona Roja (red light zone) as magnet sites (Figure).

Active surveillance included contacting all known current and previous study participants, parking mobile health units in study zones (Figure), and having outreach workers refer persons who reported feeling unwell for any reason. Passive recruitment, conducted by word-of-mouth advertising, was open to all persons in communities served by our mobile and fixed-site clinics.

Persons seen by the surveillance team had their oral temperatures recorded and completed brief surveys documenting demographics, influenza risk factors, and clinical symptoms. Persons meeting inclusion criteria for influenza-like illness based on early Centers for Disease Control and Prevention guidelines (*12*) were considered pandemic (H1N1) 2009 suspected case-patients. Inclusion criteria were a subjective or objective fever (oral temperature >100°F [37.8°C]) and 1 of the following signs or symp-

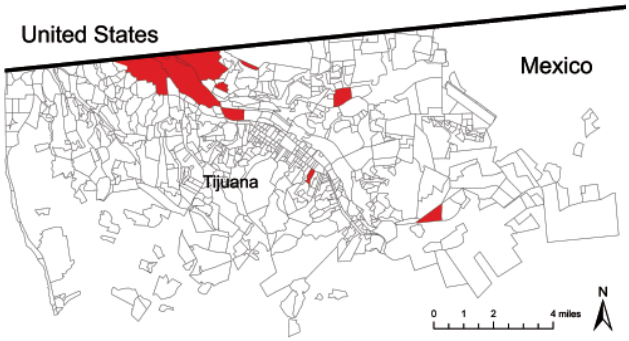

Figure. Red shaded areas indicate *colonias* of Tijuana, Mexico where pandemic (H1N1) 2009 virus screening took place, May 1–November 20, 2009.

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toms: cough, sore throat, severe headache, diarrhea, vomiting, or severe muscle/body aches and fatigue.

Persons with influenza-like illness completed an in-depth survey and underwent rapid influenza testing. Participants were compensated $5 US. In-depth surveys included questions on symptom duration, treatment choices, and pandemic (H1N1) 2009 knowledge.

Enrolled pandemic (H1N1) 2009 suspected case-patients provided a nasopharyngeal swab for rapid influenza testing with the BinaxNOW assay (Inverness Medical, Waltham, MA, USA). Suspected case-patients with positive rapid test results for type A influenza were considered probable pandemic (H1N1) 2009 case-patients and provided another nasopharyngeal swab for influenza A strain confirmation and characterization. Persons with positive test results were transported to Tijuana General Hospital for evaluation and treatment. Suspected case-patients with negative rapid influenza test results were not evaluated further.

Nasal swab specimens were cultured on MDCK cells within 48 hours of field collection. Positive cultures were detected by cytopathic effect and by hemabsorbtion. Viral isolates were characterized by hemagglutinin and neuraminidase type by using immunofluorescent staining (ViroStat, Portland, ME, USA and eEnzyme, Gaithersburg, MD, USA) and confirmed to be pandemic (H1N1) 2009 by real-time PCR (*13*).

From May 1 through November 20, 2009, a total of 303 persons were screened. Forty-three (15%) met inclusion criteria as pandemic (H1N1) 2009 suspected case-patients and were tested with the rapid influenza test. Six suspected case-patients (14%) had rapid test results positive for influenza A, and all 6 were confirmed to have influenza A pandemic (H1N1) 2009 virus. One patient had a rapid test result positive for influenza B. Pandemic (H1N1) 2009 cases were not related and were detected at a rate of ≈1/month during July–November.

Median age of screened persons was 35 years (interquartile range 29–43 years), and 62% were men (Table). Median oral temperature of patients with confirmed pandemic (H1N1) 2009 was 37.9°C (IRQ 37–38.3). Six (86%) of 7 patients with influenza reported a cough and 5 (71%) reported sore throat or severe joint/muscle ache. Less than 10% of screened persons not identified as influenza suspected case-patients had these symptoms. No patients with confirmed influenza reported vomiting or diarrhea.

All patients with confirmed pandemic (H1N1) 2009 received antiviral drug treatment within 24 hours of their positive rapid influenza test result; no pandemic (H1N1) 2009 related deaths were reported by completion of the study. One person with a negative rapid test result who was severely ill during the survey died within days of being referred to Tijuana General Hospital but was diagnosed with end-stage tuberculosis after death, not influenza.

Awareness was high; 88% of screened persons (n = 264/300) who answered the question about prior knowledge reported hearing about the pandemic (H1N1) 2009 epidemic in Mexico before the time of screening. More than 80% heard about the epidemic from television broadcasts. All pandemic (H1N1) 2009 suspected case-patients (n = 43) reported that they would get a pandemic (H1N1) 2009 vaccine if available; most suspected case-patients (71%) preferred to be vaccinated in a hospital.

Conclusions

Of ≈1,200 persons and their families who were contacted directly during the course of this survey, 303 self-identified as "feeling unwell" during May 1–November 20, 2009. Six persons had confirmed pandemic (H1N1) 2009 virus, but given that the rapid influenza test used for screening in this study has only moderate sensitivity (*14*), this is likely an underestimate of cases. Confirmed cases represented ≈0.7% of the 826 pandemic (H1N1) 2009 cases recorded in Tijuana during April–November 2009 (*15*), suggesting that isolation of these populations from the general population might have been protective.

All case-patients were treated with antiviral medication within 24 hours of a rapid influenza A positive test result, but access to antiviral drugs was through nontraditional means requiring many levels of negotiation by study staff and medical professionals in Tijuana that would not have been practical if more cases had been detected. The antiviral medication, oseltamivir, although plentiful in the United States, was only available in limited quantities in Tijuana, indicating that there are major hurdles to overcome in binational distribution of medical resources in an early pandemic setting.

Although rapid influenza tests are relatively insensitive, especially for pandemic (H1N1) 2009 (*14*), these tests can identify persons for PCR confirmation in an efficient and cost-effective fashion for early detection of cases. If pandemic (H1N1) 2009 were to develop more extensively in this population, changing inclusion criteria to objective fever (>100°F [37.8°C]) plus sore throat and/or cough would likely be effective for identifying pandemic (H1N1) 2009 cases with greater precision. Despite the limited health, financial resources, and unstable housing in our study population, it appears that television broadcasts were effective in distributing early messages about pandemic (H1N1) 2009. This enhanced surveillance survey, planned and executed safely and efficiently within 4–6 weeks of the initial pandemic (H1N1) 2009 cases in Mexico, demonstrated the potential of binational academic/public health collaborations to respond to emerging health threats in Mexico/US border regions in real time.

Table. Univariate comparisons of 303 persons screened for pandemic (H1N1) 2009 virus in Tijuana, Mexico, May 1–November 20, 2009*

Variable	Recruits not meeting inclusion criteria, n = 260	Suspected case-patients negative for influenza A/B,† n = 36	Suspected case-patients positive for influenza A/B,† n = 7
Age, y	35 (29–43)	34.5 (31.5–42.5)	28 (6–48)
Male sex	162 (63.3)	20 (55.6)	3 (42.9)
Heard about "swine flu" epidemic before screening	228 (87.7)	30 (90.9)	6 (85.7)
Subjective fever	14 (5.4)	13 (37.1)	7 (100.0)
Objective fever, oral temperature, °C	36.5 (36.2–36.8)	37.1 (36.6–37.5)	37.9 (37.0–38.3)
Cough	27 (10.4)	15 (42.9)	6 (85.7)
Sore throat	16 (6.2)	18 (51.4)	5 (71.4)
Severe headache	40 (15.4)	23 (65.7)	4 (57.1)
Joint aches	16 (6.2)	16 (47.1)	5 (71.4)
Muscle aches	18 (6.9)	18 (52.9)	5 (71.4)
Severe fatigue	23 (8.9)	17 (48.6)	5 (71.4)
Diarrhea	12 (4.6)	9 (25.7)	0
Vomiting	10 (3.9)	5 (14.3)	0
Traveled outside Tijuana since symptoms started	10 (3.9)	7 (20.0)	3 (42.9)
Contact with possible "swine flu" case in past 2 wk	6 (2.3)	11 (31.4)	4 (57.1)
Ever received influenza vaccination	13 (5.0)	5 (14.3)	6 (85.7)
Heard about "swine flu" from friends	74 (32.5)	11 (37.9)	5 (83.3)
Heard about "swine flu" from family	22 (9.6)	7 (24.1)	4 (66.7)
Heard about "swine flu" from co-workers	14 (6.1)	7 (24.1)	1 (16.7)
Heard about "swine flu" from newspaper	33 (14.5)	3 (10.3)	2 (33.3)
Heard about "swine flu" from radio	80 (35.1)	12 (41.4)	6 (100.0)
Heard about "swine flu" from television	182 (79.8)	23 (79.3)	6 (100.0)
>3 alcoholic drinks/d‡		5 (14.7)	0
Injection drug user‡		16 (45.7)	0
In jail 2 weeks before onset of symptoms‡		2 (5.7)	0
Would be vaccinated for "swine flu"‡		36 (100.0)	7 (100.0)
Prefer to be vaccinated at hospital‡		25 (71.4)	5 (71.4)
Prefer to be vaccinated at clinic‡		1 (2.9)	0
Prefer to be vaccinated at PrevenCasa/Casita‡		5 (14.3)	3 (42.9)
Prefer to be vaccinated at mobile clinic ‡		0	0

*Values are no. (%) except for age and objective fever, which are median (interquartile range).
†By rapid influenza testing.
‡Only pandemic (H1N1) 2009 suspected case-patients (n = 43) were asked these questions.

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Novel *Mycobacterium tuberculosis* Complex Pathogen, *M. mungi*

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Seven outbreaks involving increasing numbers of banded mongoose troops and high death rates have been documented. We identified a *Mycobacterium tuberculosis* complex pathogen, *M. mungi* sp. nov., as the causative agent among banded mongooses that live near humans in Chobe District, Botswana. Host spectrum and transmission dynamics remain unknown.

A previously unidentified *Mycobacterium tuberculosis* complex pathogen has emerged in banded mongooses (*Mungos mungo*) in Botswana; we named the pathogen mongoose bacillus, or *M. mungi* sp. nov. This pathogen causes high mortality rates among banded mongooses that live in close association with humans because these animals live in human-made structures and scavenge human waste, including feces.

Banded mongooses are social, fossorial, viverids that feed on invertebrates and small mammals including subterranean species (*1*). We initially identified tuberculosis (TB) disease in banded mongooses in 2000. The outbreak appeared to spread as a point-source infection between mongoose troops living in close association with humans and human waste; infection spread through towns and the associated national park (*2*). During 2000–2010, a total of 7 outbreaks occurred (increasing in duration), mongoose troop involvement increased, and the spatial and temporal connection between cases decreased. Infected mongoose troops are now widely identified across the landscape, including protected areas and urban centers (Figure 1), and high mortality rates threaten the survival of smaller troops. In this study area of Chobe District, Botswana, TB has been identified in only humans and mongooses. Strain assessment of human TB has not been conducted; the full host spectrum and transmission dynamics of this pathogen, currently unknown, are the focus of our ongoing research.

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The Study

During 2000–2010 in Chobe District, Botswana, we performed 38 necropsies on macroscopically TB-positive mongooses, of which 18 were further evaluated and TB was confirmed by histopathologic examination. An in-depth histologic evaluation was performed on a subsample of 8 of these animals from the 2008 outbreak. The most striking feature identified in the sick mongooses was anorexia, followed by nasal distortion and, less commonly, erosions of the nasal planum with involvement of the hard palate. For 7 of the 8 TB-positive animals examined intensively, macroscopic lesions were noted on the nasal planum. Histologic examination detected unequivocal TB lesions in the skin of the nose and the anterior nasal mucosa. Our findings suggested entry of the organism through erosions on the nasal planum, perhaps in association with abrasions, which might occur during foraging. Such lesions were present in the hairless parts of the nose tip of most TB-infected mongooses. Furthermore, granulomatous inflammation and mycobacterial organisms were found in the dermis of the skin directly below these erosions. Inflammation and organisms were present in some cases in the nasal mucosa, but erosion was not found. Thus, organisms could not have been in the lumen of the nasal cavity. This finding is consistent with pneumonic TB being present in only a few advanced cases of disseminated TB. This pattern was consistent among all animals examined postmortem during the study period. Histologically, the TB pneumonia was determined to be hematogenous rather than bronchogenous (i.e., by inhalation); thus, no evidence for aerosol transmission was found. Rather, pathogen invasion

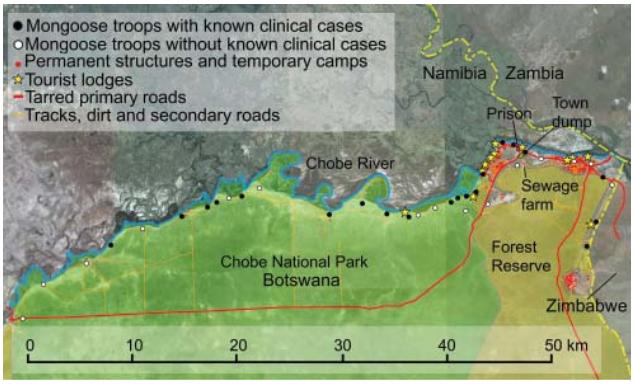

Figure 1. Locations of infected and unaffected banded mongoose troops and human infrastructural development, Chobe District, Botswana.

appears to have occurred through the nasal planum of the mongoose, and hematogenous or lymphatic spread through the body was a strikingly unique feature of this particular *M. tuberculosis* complex organism.

Samples from histologically positive mongooses were positive by PCR for the MPB70 target, IS*6110* element, and 16S rDNA, indicating that the infective organism was a member of the *M. tuberculosis* complex (*3*,*4*). Samples were further evaluated with an *M. tuberculosis* complex–specific multiplex PCR (*5*), which provided distinct results, differing clearly from those for other members of the *M. tuberculosis* complex (Table 1).

The *gyrB* gene (encoding for gyrase B) sequence, used to identify *M. tuberculosis* complex member–specific sequence single-nucleotide polymorphisms (SNPs) (*6*), identified the position of the organism as being situated between dassie bacillus and *M. africanum* subtype 1(a) and showed no detectable new SNPs (Figure 2, panel A). Amplification of RD701 and RD702 and lack of SNPs in *rpoB* and *hsp65* genes demonstrated that this organism was not a member of the *M. africanum* subtype 1(a) sublineage (*6*,*11*) (Figure 2, panel A).

Three markers were evaluated to definitively exclude the organism from being dassie bacillus: N-RD25^{das} deletion, RD1^{das} deletion, and SNP 389 in the gene Rv0911 (*6*). The N-RD25^{das} amplification gave the right product for the presence of a deletion in this region, and further sequencing confirmed that it contained a deletion in the same position as N-RD25^{das}; however, sequencing of Rv0911 showed no SNP at position 389, indicating that this organism was not dassie bacillus. As a final test, we amplified the RD1^{das} region but were unable to amplify a product from the mongoose isolates. We redesigned primers to amplify a smaller region of different diagnostic sizes (248 bp when RD1^{das} is deleted and 318 bp when RD1 is intact) but still had no amplification. This finding indicates that the RD1 region is deleted in this organism but that the deletion is larger than that of the dassie bacillus.

We then used spoligotyping analysis (*12*) to further evaluate mongoose samples and identified a unique spoligotype pattern with no known matches in the international spoligotyping database SpolDB4 (*9*) or the *M. bovis*–specific spoligotype database (www.Mbovis.org) (Figure 2, panel B). This pattern was constant during 2000–2009 in different mongoose troops and locations (online Technical Appendix, www.cdc.gov/EID/content/16/8/1296-Techapp.pdf). This unique spoligotyping pattern will enable identification of *M. mungi* in future TB surveillance programs.

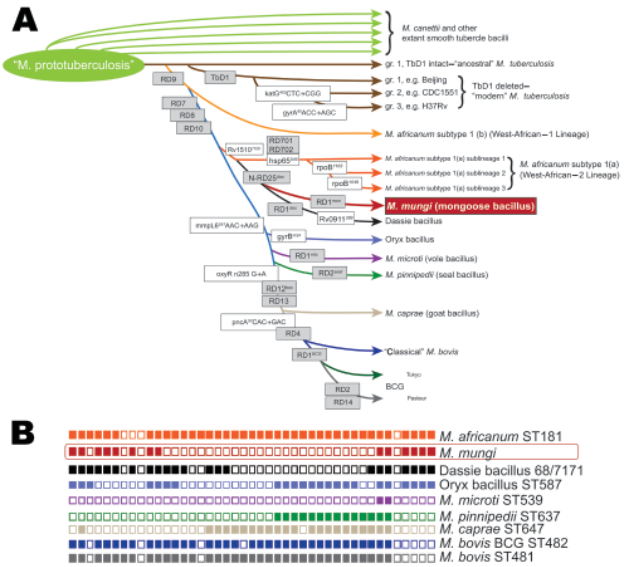

Figure 2. A) Schematic of the phylogenetic relationships among *Mycobacterium tuberculosis* complex species, including newly discovered *M. mungi,* based on the presence or absence of regions of difference (gray boxes) as well as specific single-nucleotide polymorphisms (white boxes), modified from (*7*). B) Spoligotype of *M. mungi* compared with representative spoligotypes from other *M. tuberculosis* complex species (*8*–*10*).

For these same isolates, the full set of 24 mycobacterial interspersed repetitive unit–variable number tandem repeats (*13*) identified a pattern that was unique compared with others in the international database at www.miru-vntrplus.org (Table 2). Our examination also included dassie

Table 1. Genomic regions of difference of *Mycobacterium tuberculosis* complex members compared with *M. mungi**

Mycobacterium species	Region of difference					
	RD1^{BCG}	RD4	RD9	RD12	RD1^{mic}	RD2^{seal}
M. canettii	P	P	P	A	P	P
M. tuberculosis	P	P	P	P	P	P
M. africanum	P	P	A	P	P	A
M. microti	P	P	A	P	A	P
M. pinnipedii	P	P	A	P	P	A
M. caprae	P	P	A	A	P	P
M. bovis	P	A	A	A	P	P
M. bovis BCG	A	A	A	A	P	P
M. mungi	P	P	A	P	P	P

*Determined by PCR amplification (*5*). RD, region of difference; RD1^{BCG}, *M. bovis* BCG–specific RD1; RD1^{mic}, *M. microti*–specific RD1; RD2^{seal}, *M. pinnipedii*–specific RD2; P, present; A, absent.

Table 2. Comparison of full 24-set MIRU-VNTR of selected *Mycobacterium mungi* isolates*

Sample no.	1883	6601B	6606A	6600B	6875	–	8163/02	24	287/99	7739/01	5358/99	8490/00	H37Rv
Year isolated	2000	2002	2002	2004	2008	–	–	–	–	–	–	–	–
Species	*M. mungi*	*M. mungi*	*M. mungi*	*M. mungi*	*M. mungi*	DB	*M. a.*	OB	*M. microti*	*M. p.*	*M. caprae*	*M. bovis*	*M. t.*
MIRU 02	2	2	2	2	2	2	2	2	2	2	2	2	2
VNTR 0424/ Mtub04	3	3	3	3	3	2	4	2	3	3	4	2	2
VNTR 0577/ ETR-C	3	3	3	3	3	5	5	5	5	4	5	5	4
MIRU 04/ ETR-D	3	3	3	3	3	3	2	3	4	5	3	4	2
MIRU 40	1	1	1	1	1	2	2	2	2	2	2	2	1
MIRU 10	5	5	5	5	5	7	7	7	5	6	6	2	3
MIRU 16	3	3	3	3	3	3	4	4	6	4	2	4	2
VNTR 1955/ Mtub21	3	3	3	3	3	3	4	3	3	4	3	3	2
MIRU 20	2	2	2	2	2	2	2	2	1	2	2	2	2
VNTR 2163b/ QUB11b	0	No	No	No	No	7	5	No	6	9	4	4	5
VNTR 2165/ ETR-A	6	6	No	6	6	6	6	3	9	9	5	4	3
VNTR 2347/ Mtub29	3	3	3	3	3	3	3	3	3	3	3	3	4
VNTR 2401/ Mtub30	4	4	4	4	4	3	4	4	4	4	4	4	2
VNTR 2461/ ETR-B	4	4	4	4	4	4	4	2	3	3	3	3	3
MIRU 23	4	4	4	4	4	4	4	4	4	4	4	4	6
MIRU 24	2	2	2	2	3	2	2	1	2	2	2	1	1
MIRU 26	4	4	3	3	4	5	4	4	2	2	4	3	3
MIRU 27	3	3	3	3	3	4	3	3	2	2	3	3	3
VNTR 3171/ Mtub34	3	3	3	3	3	3	3	3	3	3	2	3	3
MIRU 31/ ETR-E	8/9	8/9	8/9	8/9	8/9	5	5	4	1	3	5	3	3
VNTR 3690/ Mtub39	2	No	No	No	No	5	4	4	3	3	1	2	5
VNTR 4052/ QUB26	No	No	No	No	No	4	6	2	9	7	3	5	5
VNTR 4156/ QUB4156	No	No	No	No	No	3	3	3	3	0	3	1	2
MIRU 39	2	2	2	2	2	2	2	2	2	2	2	2	2

*MIRU, mycobacterial interspersed repetitive unit; VNTR, variable number tandem repeats; DB, Dassie bacillus; *M. a., M. africanum*; OB, Oryx bacillus; *M. p., M. pinnipedii*; *M. t., M. tuberculosis*; –, not applicable.; no, no amplification. Gray shading highlights differences between the *Mycobacterium tuberculosis* complex species and *M. mungi* as well as variation within *M. mungi* samples evaluated. DB data supplied by S. Parsons. OB data supplied by P. Supply. *M. africanum* West African 2 strain 8163/02 (ST181), *M. microti* strain 287/99 (ST539), *M. pinnipedii* strain 7739/01, *M. caprae* strain 5358/99 (ST647), *M. bovis* strain 8490/00 (ST482), and *M. tuberculosis* strain H37Rv data from www.miru-vntrplus.org.

bacillus, which had not been previously analyzed. Evidence of multiple *M. mungi* substrains circulating between years and within social groups (6601B and 6600B) in the same outbreak year (Table 2) suggests complexity in *M. mungi* transmission and potential evolution of the organism over the past decade.

Conclusions

This newly identified mycobacterial pathogen has many unique ecologic characteristics that set it apart from other members of the *M. tuberculosis* complex. First, it causes high numbers of deaths of banded mongooses, threatening local extinction of smaller social groups. Second, rather than having a primary respiratory transmission route with direct transmission between individuals, as is characteristic of other *M. tuberculosis* complex species, *M. mungi* appears to infect banded mongooses by means of a nonrespiratory route through the nasal planum, suggestive of environmental transmission. Third, the time from clinical presentation to death for affected mongooses is generally short (2–3 months) compared with that for other *M. tuberculosis* complex pathogens (more chronic infection, can take years to progress to death). Acute illness and high mortality rates, as seen in banded mongooses with *M. mun-*

gi infection, have been associated with extremely isolated human communities newly exposed to TB (*14*).

Conventional laboratory culture, biochemical testing, and a limited molecular evaluation were insufficient for differentiating *M. mungi* from *M. tuberculosis (2).* Organism differentiation required an extensive suite of additional molecular assessments not available at that time, thus underscoring the difficulty of diagnosing *M. tuberculosis* complex agents correctly and the inability of most national health laboratories to do so. The fact that new host-adapted *M. tuberculosis* complex species continue to be identified illustrates the diversity within the *M. tuberculosis* complex and stresses the need for sensitive techniques for species differentiation. The identification of this previously unknown pathogen within the *M. tuberculosis* complex identifies new concerns for human and animal health and illustrates the continuing scope of the threat posed by TB pathogens.

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Quarantine Methods and Prevention of Secondary Outbreak of Pandemic (H1N1) 2009

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During the 2009 influenza (H1N1) pandemic, some countries used quarantine for containment or mitigation. Of 152 quarantined university students we studied, risk for illness was higher for students quarantined in a room with a person with a confirmed case; we found no difference between students quarantined in double or single rooms.

Pandemic (H1N1) 2009 influenza emerged in Mexico in March 2009 and by June 10 had rapidly spread to 74 countries (*1*,*2*). Nonpharmaceutical interventions for pandemic influenza at the community level were recommended by the World Health Organization before and during the pandemic (*3*,*4*). One such nonpharmaceutical intervention was quarantine of contacts of persons with confirmed cases. A key question in closed settings (e.g., military barracks) was how to prevent a secondary outbreak of influenza among those quarantined.

The first identified case of pandemic (H1N1) 2009 in mainland People's Republic of China was imported from the United States and reported on May 11, 2009 (*5*). On May 29, the Chinese Ministry of Health required that each confirmed case-patient and each contact be isolated and quarantined in 1 separate room to contain transmission of the virus (*6*). Containment efforts appeared to successfully prevent community spread until mid-August (*7*). The Chinese Ministry of Health adjusted the quarantine guidelines on August 20 and permitted incomplete quarantine of contacts (e.g., quarantining >1 contact in 1 room) (*8*). We conducted an observational study of a pandemic (H1N1) 2009 outbreak among students of a university in northern China in September 2009. Our goal was to compare the effectiveness of different quarantine methods for preventing a secondary outbreak among the persons in quarantine.

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The Study

During August 31–September 12, 2009, an outbreak of pandemic (H1N1) 2009 occurred among students of a university in northern China. On August 31, pandemic (H1N1) 2009 was laboratory confirmed in 6 students who had fever and acute upper respiratory symptoms. A subsequent investigation found that all of the confirmed case-patients, along with 27 other students, had traveled by train from Shanghai to the university on the afternoon of August 28. One of the students (the index case-patient) had a cough during the trip on August 27. Another 5 students also had fever and influenza-like symptoms and visited the school medical services for treatment during August 28–30. When the outbreak was identified, a total of 202 contacts (average age 21 years, range 19–23 years) were traced and immediately quarantined in a separate dormitory on September 1. Eighty-nine rooms (each with 1 toilet) and 9 apartments (each with 2 bedrooms and 1 toilet) were occupied. One or 2 contacts were assigned to each bedroom. Other control measures, such as ventilating and disinfecting each room, wearing masks, and washing hands, were strictly implemented in accordance with guidance provided by the Chinese Ministry of Health (*8*).

Oropharyngeal swabs from all contacts were collected and tested on the first day of quarantine (September 1). Virologic laboratory testing subsequently indicated that 39 contacts were positive for pandemic (H1N1) 2009. Among the 163 virus-negative contacts, 11 had fever (≥38°C) or influenza-like symptoms at the beginning of the quarantine; the other 152 had no fever or influenza-like symptoms on September 1.

Among the 152 virus-negative contacts who had fever or influenza-like symptoms, 20 were exposed to a virus-positive contact during quarantine; 19 shared a bedroom and a toilet with a virus-positive contact; and 1 shared a toilet but not a bedroom with a virus-positive contact. The other 132 were not exposed to any virus-positive contacts during quarantine, including 6 persons housed in single rooms and 126 sharing double rooms.

The temperature and symptoms of contacts were recorded 3× per day (8:00 AM, 2:00 PM, and 6:00 PM). We defined a suspected case as a virus-negative contact with sudden onset of fever ≥38°C and at least 1 of the following symptoms: cough, sore throat, runny nose, shortness of breath, headache, body aches, fatigue, vomiting or diarrhea, and absence of other diagnoses. Students with suspected pandemic (H1N1) 2009 infection who had high fever (≥38.5°C) or severe respiratory symptoms (bad cough or dyspnea) were transferred to hospital for treatment; others who had fever <38.5°C or mild influenza-like symptoms were treated in the quarantine building. Because of a short-

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age of resources, laboratory testing of suspected case-patients in quarantine was not performed after the first day.

Data were analyzed by using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). χ^2 test and Fisher exact test were used to compare the attack rates of suspected cases between the groups under different quarantine arrangements. A p value <0.05 was considered significant.

During the quarantine, 14 suspected cases were identified among 152 virus-negative contacts, but none had high fever (≥38.5°C) or severe respiratory symptoms. Of 19 initially virus-negative contacts sharing a room with a virus-positive contact, 5 suspected cases were identified; onset of symptoms occurred during September 2–8. Also, of 126 initially virus-negative contacts who shared a room with a virus-negative contact, 9 suspected cases were identified; onset of symptoms occurred during September 2–7 (Table, Figure). The attack rate of suspected cases differed significantly between the 2 groups (p = 0.02, 2-tailed Fisher exact test).

Among 132 initially virus-negative contacts, who were not exposed to a virus-positive contact during the quarantine, no suspected cases were identified among the 6 students in single rooms, but 9 students with suspected infection were identified among 126 students in double rooms; onset of symptoms occurred during September 2–7 (Table; Figure). Attack rates for suspected cases between the virus-negative contacts in single rooms and those in double rooms did not differ significantly (p = 1.00, 2-tailed Fisher exact test). All students with suspected infection had normal temperature with no influenza-like symptoms after September 9, and all quarantined persons were released by September 12.

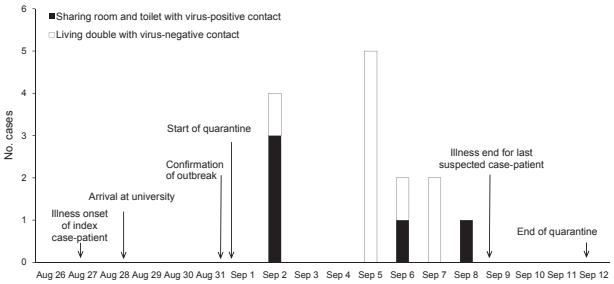

Figure. Number of new suspected cases of pandemic (H1N1) 2009 infection per day among 152 quarantined persons who were virus-negative at the start of quarantine during an outbreak in northern People's Republic of China, 2009.

Conclusions

In our study, the attack rate of suspected cases among pandemic (H1N1) 2009 virus–negative contacts increased significantly when persons were quarantined in the same room or used the same bathroom as a virus-positive contact (p = 0.02, 2-tailed Fisher exact test). Nevertheless, quarantining each virus-negative contact in 1 room did not significantly decrease the attack rate of suspected cases among virus-negative contacts in comparison with quarantining 2 persons in 1 room. Our results support the effectiveness of quarantine in preventing a secondary outbreak of pandemic (H1N1) 2009 among contacts of confirmed cases. Our results also support quarantining 2 virus-negative contacts in 1 room in situations where a large number of contacts have been traced but space is limited. Whether our findings support quarantining >2 contacts in 1 room deserves further study.

During the quarantine, every room was routinely disinfected by university staff 1 × per day. Compliance of all contacts with regulations governing personal protection and hygiene was good. Also, staff were assigned to supervise the behavior of contacts in quarantine. These control measures did not contribute to the differences of the attack rate of suspected cases between the different cohorts in this study.

Our study is limited in that virologic laboratory confirmation of suspected cases was not available. However, no other respiratory infections were known to be circulating at that time in the student population, and pandemic (H1N1) 2009 is probably the most likely explanation for influenza-like illness in recent contacts of laboratory-con-

Table. Evaluation of suspected cases of pandemic (H1N1) 2009 among 152 quarantined persons who were virus-negative at the start of quarantine during an outbreak in northern People's Republic of China, 2009

Group	Total no.	No. suspected	No. uninfected	p value*
Exposed to virus-positive contacts				
Sharing same room and toilet	19	5	14	0.02†
Sharing same toilet and different room	1	0	1	
Not exposed to virus-positive contacts				
Quarantined 1 to a room	6	0	6	1.00‡
Quarantined 2 to a room	126	9	117	
Total	152	14	138	

*2-tailed Fisher exact test.
†p value for the comparison between attack rates of suspected cases among the virus-negative contacts comparing those sharing the same room and toilet with a virus-positive contact and those sharing the same room and toilet with a virus-negative contact.
‡p value for the comparison between attack rates of suspected cases among the virus-negative contacts not exposed to the virus-positive contacts compared with those quarantined in single rooms and those in double rooms.

firmed cases. Lack of laboratory testing also means that we may have underestimated the attack rate during quarantine; some secondary infections may have been associated with asymptomatic or subclinical disease.

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Picornavirus Salivirus/Klassevirus in Children with Diarrhea, China

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To learn more about salivirus/klassevirus, we tested feces of children with diarrhea in China during 2008–2009. We isolated the virus from 9/216 diarrhea samples and 0/96 control samples. The nearly full polyprotein of 1 isolate, SH1, showed 95% identity with a salivirus from Nigeria, indicating widespread distribution and association with diarrhea.

Diarrhea causes ≈2 million deaths each year (*1*), primarily among young children in developing countries (*1*,*2*). The causative agents for ≈40% of cases remain unknown (*2*–*4*).

Studies have documented an association between Aichi viruses and gastroenteritis (*5*,*6*). Recent studies have documented human infections with the salivirus/klassevirus-related Aichi virus (*7*–*9*) that were associated with diarrhea (*9*). The previously unknown picornavirus klassevirus has recently been recently detected in fecal samples from persons with diarrhea in the United States and Australia and in sewage in Spain (*7*,*8*). Closely related saliviruses have been identified in fecal samples from persons in Nigeria, Tunisia, and Nepal and have been statistically associated with diarrhea in Nepal (*9*).

Klassevirus/salivirus is genomically organized similar to other picornaviruses and most closely related to *Aichi virus* in the genus *Kobuvirus* (*5*–*7*). The family *Picornaviridae* is highly diverse and contains small, nonenveloped viruses with a single-stranded positive-sense RNA genome that encodes a single polyprotein; it consists of 12 genera and 2 possibly new genera (*7*), a subset of which can infect and cause disease in humans.

To our knowledge, there have been no reports of infection with this virus in the People's Republic of China. Therefore, to extend these initial findings, we tested for this newly characterized virus in fecal samples from children with diarrhea in China and sequenced the nearly full genome of 1 isolate, SH1.

The Study

During April 2008–March 2009, a total of 216 fecal samples were collected from children, 2–6 years of age, who were hospitalized with diarrhea in Shanghai Children's Hospital, China. A total of 96 children, 3–5 years of age, from 2 childcare centers in Shanghai City were included as healthy controls.

Samples were suspended to 10% (wt/vol) in phosphate-buffered saline (0.01 M, pH 7.4), and total RNA was extracted from 200 μL of the suspension by using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Viral RNA was dissolved in 30 μL RNase-free water and stored at –80°C.

To understand the possible association between salivirus/klassevirus and diarrhea, we conducted prevalence studies by using nested reverse transcription–PCR (RT-PCR). We used a nested set of PCR primers (SAL-L1, 5′-CCCTGCAACCATTACGCTTA-3′; SAL-R1, 5′-CA CACCAACCTTACCCCACC-3′; SAL-L2, 5′-ATTGAGT GGTGCAT(C)GTGTTG-3′; SAL-R2, 5′-ACAAGCCGG AAGACGACTAC-3′) to amplify a 414-bp fragment located in the 5′ untranslated region (UTR). The expected size DNA bands were excised from an agarose gel, purified with the AxyPrep DNA gel extraction kit (Axygen, Union City, CA, USA), cloned into pMD-18T vector (TaKaRa, Shiga, Japan), and sequenced on an Applied Biosystems 3730 DNA Analyzer (Invitrogen). Of 216 samples, 9 (4.2%) were positive for the newly described picornavirus; ages of the children were 2 years (n = 1), 3 years (n = 3), 4 years (n = 2), 5 years (n = 1), and 6 years (n = 2). Sequence analysis, based on the 414-bp sequences, showed that these 9 sequences shared 98.3%–99.8% identity with each other, suggesting that they could be considered members of the same virus species. The sequences shared 94.7%–97.3% sequence identities with GenBank isolates nos. GQ253930 (klassevirus 1, Australia), GQ184145 (human klassevirus 1, USA), and GQ179640 (salivirus, Nigeria). The 9 salivirus/klassevirus–positive samples were further investigated for Aichi virus, parechovirus, norovirus, sapovirus, rotavirus, astrovirus, and cosavirus by using RT-PCR with the primers previously described (*10*–*13*). Results indicated that 1 sample, for which the 518-bp–specific fragment was sequenced, was also positive for human parechovirus. No salivirus/klassevirus was detected in samples from the 96 healthy controls. The Fisher exact test showed a significant ($p = 0.03$) association between salivirus/klassevirus detection and diarrhea.

The complete genomic sequence of strain SH1 was then determined by using 10 sets of specific oligo-

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nucleotide primers designed on the complete genome of GQ184145, GQ253930, and GQ179640. The nearly full-genome genome of this virus strain was 7,798 nt and contained an open reading frame (ORF) with a length of 7,107 nt, encoding a putative polyprotein precursor of 2,369 aa. This ORF is preceded by a 5′ UTR at least 624 nt long and followed by a 3′ UTR at least 67 nt long. Phylogenetic analysis using the more variable P1 region of SH1 and 45 representative picornaviruses (including 3 salivirus/klassevirus strain) confirmed the close relationship of this strain with strains from other continents; it was most closely related to a human klassevirus from the United States (GQ184145) (Figure).

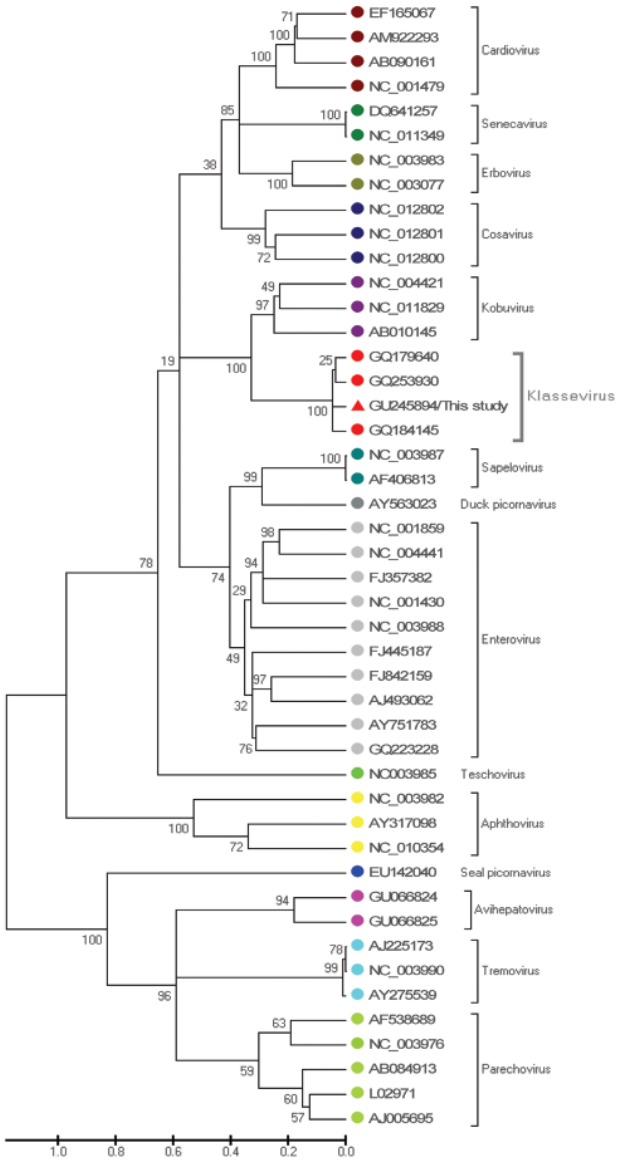

Figure. Phylogenetic analysis of the more variable P1 region of the salivirus/klassevirus isolated from fecal samples of 9 (4.2%) of 216 children with diarrhea in the People's Republic of China, April 2008–March 2009, and 45 representative strains. Phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates by using MEGA4.0 software (www.megasoftware.net). Bootstrap values are indicated at each branching point. The isolate SH1 is marked with a triangle. Scale bar indicates estimated phylogenetic divergence.

The nearly full genome of SH1 has been submitted to GenBank under accession no. GU245894. The 9 partial 414-bp sequences of salivirus/klassevirus are deposited in GenBank under accession nos. GU376738–GU376746.

Conclusions

Our finding of salivirus/klassevirus in fecal samples of children with diarrhea in China is consistent with Li et al.'s report of this virus' association with diarrhea (9). This finding, plus the identity with the Nigeria reference strain, support widespread distribution of this newly characterized virus species and its association with diarrhea**.**

Dr Shan works at Shanghai Jiao Tong University and is interested in discovering novel viruses from biological samples by using metagenomic methods.

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Human *Rickettsia heilongjiangensis* Infection, Japan

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A case of *Rickettsia heilongjiangensis* infection in Japan was identified in a 35-year-old man who had rash, fever, and eschars. Serum contained *R. heilongjiangensis* antibodies, and eschars contained *R. heilongjiangensis* DNA. *R. heilongjiangensis* was also isolated from ticks in the suspected geographic area of infection.

Spotted fever group (SFG) rickettsiosis is the most prevalent arthropod-borne infectious disease in Japan (*1*). Before publication of a 1984 report about Japanese spotted fever (JSF) caused by *Rickettsia japonica*, scrub typhus caused by *Orientia tsutsugamushi* had been known as the sole rickettsiosis in Japan (*1*). Although many SFG *Rickettsia* species (*R. japonica*, *R. helvetica*, *R. tamurae*, *R. asiatica*, and other related *Rickettsia* spp.) were known, only *R. japonica* had been isolated or detected by PCR from Japanese SFG rickettsiosis patients (*1*–*3*). *R. japonica* was found in *Dermacentor taiwanensis*, *Haemaphysalis cornigera*, *H. flava*, *H. formonensis*, *H. hystricis*, *H. longicornis*, and *Ixodes ovatus* ticks, and *R. helvetica* in *H. japonica*, *I. columnae*, *I. monospinosus*, *I. ovatus*, *I. pavlovskyi*, *I. persulcatus*, and *I. turdus* ticks (*3*,*4*). Cases of SFG rickettsiosis caused by *R. heilongjiangensis*, showing mild rash associated with fever and an eschar, have been reported in the Russian Far East and the People's Republic of China (*5*–*8*). In Russia and China, *R. heilongjiangensis* was isolated from *H. concinna* and *D. sylvarum* ticks (*6*,*7*). Highly related *Rickettsia* spp. were detected from *H. longicornis* ticks by PCR in South Korea (*9*). In this study, we confirmed a human case of *R. heilongjiangensis* infection in Japan. We also isolated *R. heilongjiangensis* from *H. concinna* ticks, a probable transmission vector, in the suspected geographic area of infection.

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The Study

A 35-year-old man had chills and malaise on July 29, 2008 (day 0). On day 3, the patient became febrile (39.3°C). On day 5, a physician recognized the rash and prescribed oral minocycline (200 mg/d). On day 6, the patient was hospitalized because of constant fever and a whole body rash of unknown cause. At that time, laboratory data showed leukocyte count 7.2×10^9 cells/L, thrombocyte count 275×10^9 cells/L, aspartate aminotransferase 129 U/L, alanine aminotransferase 98 IU/L, and C-reactive protein 3.5 mg/dL. Biopsies were performed on eschars 1 and 2 (5–8 mm diameter) with erythema (≈20 mm diameter), above the left scapula and on the right lower back. During hospitalization, the patient received minocycline, 200 mg/day, intravenously. DNA was extracted from skin biopsy specimens by using a commercial kit according to the manufacturer's instructions (Gentra Puregene; QIAGEN, Valencia, CA, USA). PCR was performed by using primers of 3 rickettsial genes: outer membrane protein A (*ompA*; primers Rr190.70p and Rr190.602n) (*10*), citrate synthase (*gltA*; primers Cs2d and CsEndr) (*6*), genus *Rickettsia*–specific outer membrane (17-kDa antigen gene; primers R1 and R2) (*11*), and primers for *O. tsutsugamushi*, as reported previously (*12*).

Although many cases of *R. heilongjiangensis* infection show a single eschar as a result of a tick bite, *ompA*, *gltA*, and 17-kDa antigen genes were detected by PCR (but not with *O. tsutsugamushi*–specific primers) in both eschar specimens. Amplicons were sequenced and analyzed phylogenetically (Figure 1). The 491-bp fragment of *ompA* from eschar 1 (GenBank accession no. AB473995) demonstrated 99.8% and 97.1% nucleotide homology with *R. heilongjiangensis* strain HLJ-054 and *R. japonica* strain YM, respectively. The 1,250-bp fragment of *gltA* of eschar 1 (accession no. AB473991) demonstrated 99.9%, 99.8%, and 96.8% nucleotide homology with the *R. heilongjiangensis* strain HLJ-054, *R. japonica* strain YM, and *R. helvetica* strain C9P9, respectively. The 392-bp fragment of the 17-kDa antigen gene of eschar 1 (accession no. AB473987) demonstrated 100.0% and 99.2% nucleotide homology with *R. heilongjiangensis* strain HLJ-054 and *R. japonica* strain YM, respectively. Blood specimens were negative for rickettsial antigens by PCR, possibly because they were collected after minocycline treatment. Three serial blood samples were tested serologically by immunoperoxidase assays against rickettsial antigens: *R. japonica* strain YH;

[1]These authors contributed equally to this article.

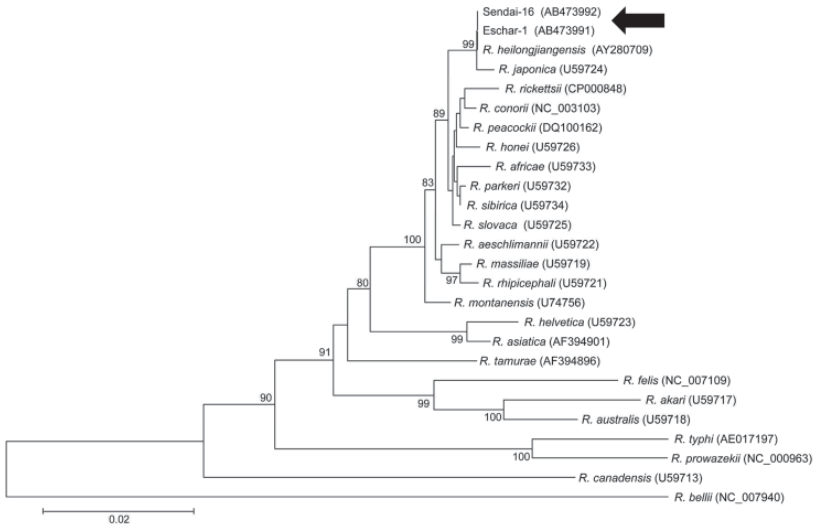

Figure 1. Phylogenetic analysis of citrate synthase (*gltA*) sequences of *Rickettsia* spp. Sequences were aligned by using MEGA4 software (www.megasoftware.net). Neighbor-joining phylogenetic tree construction and bootstrap analyses were performed according to the Kimura 2-parameter distances method. Pairwise alignments and multiple alignments were performed with an open gap penalty of 15 and a gap extension penalty of 6.66. The percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test (1,000 replicates) was calculated. Phylogenetic branches were supported by bootstrap values of >80%. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion). Scale bar indicates the percentage of sequence divergence. Arrow indicates eschar specimens.

O. tsutsugamushi Karp, Kato, Gilliam, Kawasaki, Kuroki, and Shimokoshi strains; and *R. heilongjiangensis* strain CH8–1 (*13*). The *R. heilongjiangensis* strain CH8-1 used in our analysis was isolated from *H. concinna* ticks collected in Inner Mongolia, China, as an unknown SFG *Rickettsia* species (*3*). Strain CH8–1 was identified as *R. heilongjiangensis* by DNA analysis in this study (*ompA*, accession no. AB473813; *gltA*, AB473812; and 17-kDa antigen genes, AB473811). *R. japonica* and *R. heilongjiangensis* antibody titers were substantially elevated from day 6 to day 16. Titers against *R. heilongjiangensis* were 2–4 times higher than titers against *R. japonica* on day 16 (Table).

An interview with the patient after the laboratory diagnosis of *R. heilongjiangensis* infection revealed more information about the context of the infection. He resided in an urban area of Sendai, Miyagi Prefecture, Japan (Figure 2). For 2 weeks before onset of symptoms, his outdoor activity was limited to daily walking with a companion dog along a river near his residence. The suspected area where he may have become infected through a tick bite was investigated in September 2008. We captured and examined 72 *Haemaphysalis* spp. ticks (52 *H. longicornis*, 15 *H. concinna*, 4 *H. flava*, and 1 *H. megaspinosa*) and 7 rodents (4 *Rattus norvegicus* and 3 *Microtus montebelli*) for investigation of SFG *Rickettsia* spp. Tick and rodent spleens were homogenized and subjected to isolation studies with L929 cells in shell vial (*3*), and detection of *Rickettsia* DNA by PCR was performed in parallel as previously described. Of the 72 tick samples, 3 *H. concinna* nymphs yielded *Rickettsia* isolates and a DNA fragment of *Rickettsia*, which was detected by PCR. Sequences of 3 isolates and amplicons were identical to those from the patient's specimens (Figure 1, tick-derived isolates assigned Sendai-16, 29, 32; Sendai-16: *ompA*, *gltA*, and 17-kDa antigen gene accession nos. AB473996, AB473992, and AB473988, respectively; Sendai-29: *ompA*, *gltA*, and 17-kDa antigen gene accession nos. AB473997, AB473993, and AB473989, respectively; and Sendai-32: *ompA*, *gltA*, and 17-kDa antigen gene accession nos. AB473998, AB473994, and AB473990, respectively). PCR-detectable rickettsial agents were not isolated from the rodents; however, 3 of the 4 *R. norvegicus* specimens had high antibody titers to *R. heilongjiangensis*.

To date, most cases of SFG rickettsiosis have been reported as JSF in the western regions of Honshu Island, Japan (*1*,*2*). *R. japonica* was isolated from ixodids in the area where JSF is endemic, and *R. helvetica* from the entire country of Japan (*3*). Moreover, only *R. japonica* has been isolated from patients with SFG rickettsiosis (*1*,*2*). A case-patient with JSF demonstrated serologic evidence of SFG rickettsiosis caused by agents other than *R. japonica*; however, those agents have not been defined (*R. helvetica* in Fukui) (*14*). In 2007, another case of JSF was detected serologically by using only *R. japonica* antigen in Aomori Prefecture, the northernmost prefecture of Honshu Island (*15*). However, *R. japonica* has not been detected in this area (*3*). These results suggest that some cases of SFG rickettsiosis in Japan may have been caused by SFG *Rickettsia* species other than *R. japonica*.

The case reported in this article occurred in an urban area of Sendai in the northern section of Honshu Island (Figure 2). Scrub typhus caused by *O. tsutsugamushi* oc-

Table. Antibody titers to spotted fever group rickettsiae in patient's serum samples, Sendai, Japan, 2008*

Days after symptom onset	Antibody titers (IgG/IgM)		
	Rickettsia japonica	*R. heilongiangensis*	*Orientia tsutsugamushi*
6	<10/<10	<10/<10	<10/<10
16	40/160	160/320	<10/<10
23	320/640	320/640	<10/<10

*Ig, immunoglobulin.

Figure 2. Distribution of reported Japanese spotted fever cases in Japan (≈2008). Prefectures in which Japanese spotted fever cases were reported up to 2008 are shown in black; Fukui, Aomori, and Miyagi prefectures are shown in gray. The map was drawn by using data on reported infectious diseases in Japan (http://idsc.nih.go.jp/idwr/pdf-j.html).

curs in this area with 2 seasonal peaks: from early spring to early summer, and from early fall to early winter (*1*). Serologic and microbiologic data ruled out scrub typhus in the present case. *R. heilongjiangensis* infection has been reported in the summer in the disease-endemic area of the Eurasian continent. Notably, the present case occurred in midsummer.

Conclusions

R. japonica has been the only known causative agent of SFG rickettsiosis in Japan, possibly because of limited availability of laboratory test systems. Further studies are needed to define the prevalence of SFG rickettsiosis caused by *Rickettsia* species other than *R. japonica*.

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Pandemic (H1N1) 2009 Vaccination and Class Suspensions after Outbreaks, Taipei City, Taiwan

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In Taipei City, class suspensions were implemented beginning September 1, 2009 when transmission of pandemic (H1N1) 2009 infection was suspected. The uptake rate of pandemic (H1N1) 2009 vaccination (starting on November 16, 2009) among students 7–18 years of age was 74.7%. Outbreaks were mitigated after late November 2009.

As of April 25, 2010, >214 countries have reported laboratory-confirmed cases of pandemic (H1N1) 2009, including >17,919 deaths. Preparedness measures such as having substantial antiviral drug stockpiles for treatment and chemoprophylaxis and implementation of vaccination programs are considered crucial for effective control of the pandemic (*1*,*2*). In many countries, vaccination programs for pandemic (H1N1) 2009 were implemented by the end of 2009.

The US Centers for Disease Control and Prevention (CDC) recommends that nonpharmaceutical interventions should be implemented to reduce influenza transmission between persons after an outbreak and before vaccination programs begin (*3*). During the 1918–19 and 2009 influenza pandemics, class suspension and school closures, either reactively following outbreaks or proactively at a regional level, were implemented by many countries (*4*–*8*). CDC guidance suggests that during an influenza outbreak, policymakers should weigh the advantages and disadvantages of school closures before making a decision (*9*).

The Study

In Taiwan, persons were confirmed to have pandemic (H1N1) 2009 infection if they had an acute febrile respiratory illness with an epidemiologic link and a positive test result for pandemic (H1N1) 2009. From June 1, 2009, through January 29, 2010, a total of 3,159 patients having documented pandemic (H1N1) 2009 infections were reported to the Taiwan Centers for Disease Control (Taiwan CDC) (*10*). Of these patients, 887 (28.1%) were hospitalized and 39 (4.4%) died (Figure 1, panel A). In Taipei City, 117 hospitalized patients infected with pandemic (H1N1) 2009 virus were reported, and 4 patients (3.4%) died during the same period. Patients <18 years of age accounted for 51.9% and 47.9% of all hospitalized patients in Taiwan and Taipei City, respectively (Figure 1, panel A) (*10*).

In Taiwan, a 2-3-5 intervention policy for class suspension was implemented beginning September 1 (week 35 of 2009) for all students <18 years of age when transmission of influenza-like illness or influenza A/B virus infection (identified by positive rapid antigen test [RAT] for influenza A/B) was suspected (*11*). Three commercial kits were available: QuickVue Influenza A+B (Quidel Corporation, San Diego, CA, USA), BD Directogen EZ Flu A+B (Becton Dickinson, Sparks, MD, USA), and BinaxNOW Influenza A&B (BinaxNOW, Portland, ME, USA) (*11*). Under the 2-3-5 policy, any class was suspended if >2 students were noted to have a positive RAT result or confirmed pandemic (H1N1) 2009 within 3 days. Students from suspended classes were asked to stay home for at least 5 days before returning to school. In addition, students' temperatures were checked at the entrance of each school during the pandemic; if any student's temperature was ≥38°C, the student was suspended.

Two pandemic (H1N1) 2009 vaccines were available: Focetria (Novartis, Basel, Switzerland, available since November 1, 2009) and AdimFlu-S (Adimmune Corporation, Taichung, Taiwan, available since November 16, 2009). Vaccination of front-line healthcare personnel began November 2, 2009; infants >6 months and <1 year received vaccine beginning November 11, 2009. Beginning November 16, 2009, pregnant women, preschool children 1–6 years of age and students 7–12 years of age were vaccinated. Students 13–15 years of age (since November 23, 2009) and 16–18 years of age (since November 30, 2009) (Figure 1, panel B), and persons with major illness or injury were vaccinated simultaneously (*10*). For children 6 months to 9 years of age, a second dose of vaccine was recommended with an interval of at least 3 weeks (*10*). For this age group, coverage rate of the vaccine in this report included those who had received at least 1 dose of vaccine. Immunization with pandemic (H1N1) 2009 vaccines was free and voluntary.

By January 29, 2010, less than half (5.6 million) of the planned doses (12 million) of pandemic (H1N1) 2009 vaccine had been administered. The overall coverage of the vaccine in Taiwan (population ≈23 million) was ≈24.3%; the rate was 21.8% for Taipei City (population 2.6 million). Coverage rates of pandemic (H1N1) 2009 vaccine for dif-

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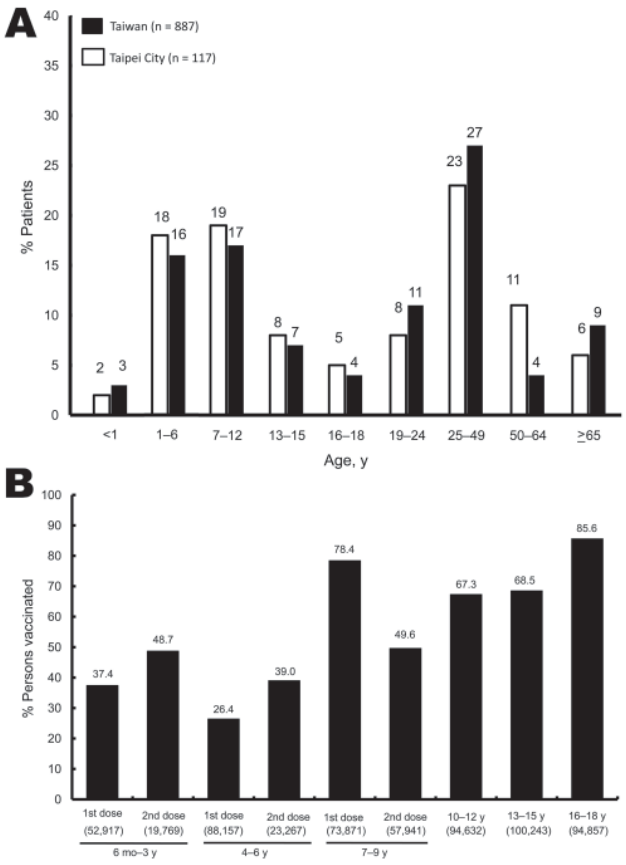

Figure 1. A) Incidence of hospitalization among patients infected with pandemic (H1N1) 2009 in each age group in Taipei City and throughout Taiwan as of week 4, 2010. B) Pandemic (H1N1) 2009 vaccine coverage among persons ≤18 years of age by age group in Taipei City as of week 4, 2010 (from November 11, 2009, to January 29, 2010). Numbers within parentheses indicate the number of persons within each age group who were recommended for vaccination.

ferent age groups as of January 29, 2010, in Taipei City are shown in Figure 1, panel B. Overall, the coverage rate of the vaccine for school students 7–18 years of age was 271,460/363,603 (74.7%) as of January 29, 2010. The coverage rate for younger children (≤6 years of age) and persons 19–24 years of age was 30.3% (first dose) and 3.1%, respectively (*10*).

As of January 29, 2010, a total of 1,708 classes in Taipei City's elementary/primary, junior, and senior high schools had been suspended (Figure 2). The peak number of class suspensions (without school closures) occurred during September and November 2009, and no class was suspended after week 4 of 2010 (*10*).

In Taipei City, a total of 171 hospitalized patients infected with influenza viruses were identified from June 1, 2009, through January 29, 2010. These included 117 hospitalized patients infected with pandemic (H1N1) 2009 (Figure 2) (*11*). The pandemic (H1N1) 2009–related death rates were 1.54 and 1.78 per 1 million population in Taipei City and Taiwan, respectively; the death rate for Taiwan was the third lowest among the 32 members and observers of the Organization for Economic Cooperation and Development (*12*). Three waves of influenza were identified with activities that paralleled the intensity of class suspensions (Figure 2).

Conclusions

Although only about one fifth of the population in Taipei City had received pandemic (H1N1) 2009 vaccination, the number of hospitalized patients with pandemic (H1N1) 2009 declined remarkably after mid-November to December 2009; no cases were reported after January 29, 2010. The rationale of the 2-3-5 intervention policy in Taiwan was based on the incubation period of seasonal influenza. If influenza developed in 2 students in the same class within 3 days, it was anticipated that the virus had already been spread within the class. Because influenza virus shedding begins 24 hours before illness onset, we assumed that a 5-day observation period should detect all infected classmates.

The classroom structure in Taiwan's middle schools and high schools is different from western countries and referred to as a "platoon" system. A group of students are placed together in a specific homeroom with a core teacher who also provides counseling to students and performs administrative work. Other teachers who specialize in different subjects move from class to class for teaching. The core teacher and administrative officials can audit off-school activities of each student through information technology.

Class suspensions or school closures alone may not be able to quell an epidemic, but these nonpharmaceutical interventions may be able to provide additional time to prepare for vaccination (*5*–*8*). Children**,** especially older students in middle or high school, play a primary role in transmission of influenza within schools, families, and communities and should be a key target group for vaccination (*11*,*13*,*14*). In our study, the number of class suspensions also decreased concurrently with the declining trend of hospitalized patients with pandemic (H1N1) 2009. Implementation of a vaccination policy for students, which began in mid-November (week 47), resulted in a remarkable decline of the third wave of pandemic (H1N1) 2009 two weeks later (week 49). This scenario suggests that the high vaccine coverage rate among students 6–18 years of age, as well as the 2-3-5 intervention policy, might have contributed to the rapid mitigation and subsequent cessation of the outbreak.

The results of our study demonstrate a more effective mitigation strategy to control influenza outbreaks during the wait for vaccines. Citywide class suspensions in Taipei

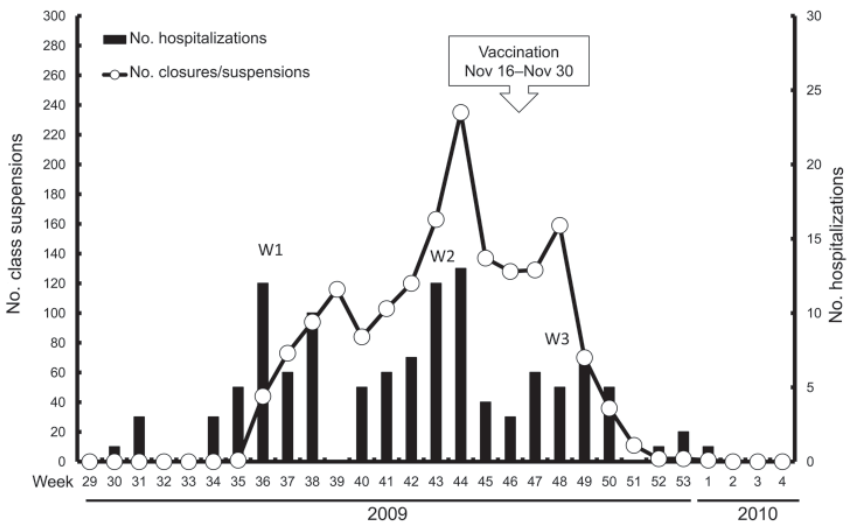

Figure 2. Weekly number of class suspensions (including nursery schools, kindergartens, elementary/primary schools, and junior and senior high schools) and new hospitalized patients caused by pandemic (H1N1) 2009, confirmed by real-time reverse transcription–PCR in Taipei City, Taiwan, from week 29 in 2009 to week 4 in 2010. W1–3, 3 waves of pandemic (H1N1) 2009 outbreaks. See text for details of the vaccination program for pandemic (H1N1) 2009 for school children 7–18 years of age.

City and the high uptake rate of vaccination among students may have had a combined effect in ending the influenza outbreaks.

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Pandemic (H1N1) 2009 Virus and Down Syndrome Patients

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We compared prevalence of hospitalization, endotracheal intubation, and death among case-patients with and without Down syndrome during pandemic (H1N1) 2009 in Mexico. Likelihoods of hospitalization, intubation, and death were 16-fold, 8-fold, and 335-fold greater, respectively, for patients with Down syndrome. Vaccination and early antiviral drug treatment are recommended during such epidemics.

Down syndrome is the most common chromosomal abnormality in persons worldwide; prevalence is ≈1/1,000 live births (*1*). It is characterized by a variety of dysmorphic features, congenital malformations, and other health problems. Several risk factors for influenza also occur in Down syndrome patients (*2*): congenital heart disease in one half of patients (*3*), childhood obesity or excess weight in the majority (*4*), and a 8× higher prevalence of type 1 diabetes than in an age-matched control population (*5*). In addition, specific alterations in immune response are frequently present, including leukopenia, chemotactic defects (*6*), decreased immunoglobulin G_4 levels (*7*), and T- and B-cell abnormalities (*7*,*8*) with reduced B lymphocytes (*9*). Patients with Down syndrome have an increased need for hospitalization because of lower respiratory tract disease caused by respiratory syncytial virus (*10*) and other respiratory infections (*11*) as well as reduced access to healthcare (*12*). An increased proportion of patients with Down syndrome have pneumonia as cause of death (*13*). However, respiratory infections have not been associated with congenital heart disease (*14*). Although persons with Down syndrome are likely at increased risk for complications, these patients are not explicitly listed in groups that should receive priority vaccination or for early treatment of influenza. The objective of the study was to determine whether Down syndrome was associated with adverse outcomes in cases of influenza-like illness (ILI) and severe acute respiratory illness (SARI) during the first months of the outbreak of influenza A pandemic (H1N1) 2009 virus (*15*).

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The Study

In the ILI/SARI database of the Mexican Ministry of Health, we identified all persons for whom Down syndrome (World Health Organization's International Statistical Classification of Disease and Health-related Problems, 10th revision [ICD-10] codes Q-90.0 to Q-90.9) was cited among their coexisting conditions or among the causes of death from May 2009 through October 2009. In Mexico, influenza is a disease of mandatory notification, and the database includes all cases reported from 597 sentinel health units, including outpatient clinics as well as hospitals from all 32 states. Notification includes obtaining information for each patient on such factors as hospitalization, antiviral drug treatment, and complications, and we compared selected characteristics of patients with and without Down syndrome. ILI is defined as fever (≥38ºC) with cough and headache and 1 additional respiratory or digestive symptom. The definition of SARI adds difficulty breathing or acute respiratory failure to the ILI definition.

We also reviewed the hospital database from the National Institute of Respiratory Diseases (INER) for associations among Down syndrome, hospitalizations, and adverse outcomes. INER is a national referral center for respiratory diseases that cares mainly for patients from metropolitan Mexico City who lack health insurance. For analysis, we included all patients with discharge diagnoses of pneumonia and influenza (ICD-10 codes J09–J18).

We compared the prevalence of hospitalization, intubation, and death among patients with Down syndrome and among the remaining patients. As estimators of association, we used odds ratios (ORs) with 95% confidence intervals (CIs), and to adjust for additional variables such as age or sex, we used logistic regression. We also described medians and interquartile ranges (IQRs) for selected variables and compared age and number of coexisting conditions by the standard *t* test for independent groups.

The ILI/SARI database had 214,902 reported cases during the pandemic; mean patient age was 23 years (SD 17 years); 52% of these patients were female, 83% were outpatients, 9% were hospitalized, and the remaining 8% did not have a record of hospitalization. Among the reported cases, 45,772 patients had a record of coexisting conditions, mainly diabetes and asthma. We found 60 (0.03%) patients with reported ILI/SARI who also had a diagnosis of Down syndrome. Patients with and without Down syndrome had a similar median time from onset of signs and symptoms to hospitalization (2 days, IQR 1–4 days), and the frequency of symptoms reported was similar.

Treatment with oseltamivir was provided for most hospitalized patients (for 92.0% with Down syndrome and 85.2% without Down syndrome). Persons with Down syndrome were younger on average than the remainder of patients (median age 8.5 years, IQR 3–26 years, vs. 20 years, IQR 9–34 years, respectively). In Mexico, seasonal influenza vaccination is recommended for children <3 years of age, but only 24% of patients with Down syndrome within this age range were reported to have received the vaccine, compared with 33% patients without Down syndrome (OR 0.5, 95% CI 0.1–2.4, p>0.05).

According to the ILI/SARI database, patients with Down syndrome had an increased risk for hospitalization, endotracheal intubation, and death compared with patients without Down syndrome. Hospitalization was reported for 61.7 % of patients with Down syndrome compared with 9.2% of patients without Down syndrome (crude OR 15.9, 95% CI 9.5–26.8; age-adjusted OR 21.2, 95% CI 12.4–36.4). Endotracheal intubation of patients for whom information was available was reported for 18.2% of case-patients with Down syndrome and 2.6% of those without Down syndrome (crude OR 8.2, 95% CI 3.4–19.9); 23.3% of those with Down syndrome died vs. 0.1% of those without Down syndrome (crude OR 335, 95% CI 181–619, age-adjusted OR 521, 95% CI, 274–991).

From January 2000 through June 25, 2009, a total of 42,298 admissions to INER were registered; patients had a mean age of 42 years, (SD 23 years), and 53% of these were male. Fifty-nine patients had a diagnosis of Down syndrome (0.14%), and 12 of these had a diagnosis of pneumonia or influenza (20.3%) vs. 6.8% of the remaining population (OR 3.5, 95% CI 1.7–6.5, p<0.05). Patients with Down syndrome had an increased risk for in-hospital death (age- and gender-adjusted OR 4.6, 95% CI 2.1–9.7, p<0.05). Although patients with Down syndrome were younger (mean age 15.2 years vs. 41.6 years, p<0.001), they were more likely to have a disease of the cardiovascular system (29% vs. 16%, OR 2.1 95% CI 1.2–3.7, p = 0.01), a congenital malformation of the cardiovascular system (19% vs. 0.5%, OR 47, 95% CI 24–93, p<0.001), and more coexisting conditions per patient (3.3 from 4 possible vs. 2.1, p<0.001).

Conclusions

Persons with Down syndrome often manifest a variety of immune defects and several risk factors for adverse outcomes for pandemic (H1N1) 2009, including obesity, diabetes, and cardiovascular diseases. Down syndrome patients are not explicitly listed in the groups of patients at increased risk for influenza, so early antiviral drug treatment and priority vaccination are prescribed for Down syndrome patients only if a high-risk health condition is found, for example, a cardiovascular disease or if persons with Down syndrome are considered to have a cognitive disorder (*2*). These factors may lead to inconsistent healthcare for those affected with Down syndrome and to missed opportunities for prevention and early treatment of ILI.

Patients with Down syndrome and ILI/SARI reported to the Mexican Ministry of Health had an increased risk for hospitalization, endotracheal intubation, and death. Consistent with the national database, in a referral respiratory center, patients with Down syndrome were more likely to have a diagnosis of pneumonia or influenza and to die during hospitalization.

The Ministry of Health database lacks detailed clinical information, and surveillance is based on sentinel health units chosen to be representative of the whole Mexican health system and that would provide information of good quality. The main risk factors for influenza are routinely requested and reported. However, the INER database includes diagnoses after hospital discharge but solely from 1 referral hospital. Yet both sources confirmed an increased risk for hospitalization and death for patients with Down syndrome and ILI. Patients with Down syndrome should be vaccinated against the seasonal influenza viruses and the influenza A pandemic (H1N1) 2009 virus. Early treatment of Down syndrome patients for ILI should be promoted by health systems and Down syndrome organizations.

Dr Pérez-Padilla is a pulmonologist at the National Institute of Respiratory Diseases of Mexico. His main research interests have been quality control of pulmonary laboratories, health effects of biomass smoke inhalation, the effects of altitude on respiratory diseases, and since April 2009, influenza.

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Household Effects of School Closure during Pandemic (H1N1) 2009, Pennsylvania, USA

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To determine the effects of school closure, we surveyed 214 households after a 1-week elementary school closure because of pandemic (H1N1) 2009. Students spent 77% of the closure days at home, 69% of students visited at least 1 other location, and 79% of households reported that adults missed no days of work to watch children.

Some studies have suggested that school-age children are influential in the ongoing transmission of influenza (*1*,*2*). Closing schools may potentially reduce the spread of influenza (*3*,*4*). In mid-May 2009, an elementary school (kindergarten-4th grade) in a semirural area of Pennsylvania closed for 1 week after an abrupt increase in absenteeism due to influenza-like illness (ILI) and the confirmation of influenza A pandemic (H1N1) 2009 virus infection in 1 student. Other schools in the district remained open. From May 26 through June 2, 2009, investigators from the Pennsylvania Department of Health and the Centers for Disease Control and Prevention surveyed households with students at the school by telephone to assess influenza symptoms, childcare arrangements, movements of affected children during the school closure period, and household demographics and socioeconomic status. This study did not address the transmission effects, but assessed the potential disruption to households resulting from school closure.

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The Investigation

The survey was considered a public health response. School administrators provided contact information for households with children attending the school. Investigators asked to speak to an adult in the household. If an adult was available and consented, the survey was administered. For each day of school closure, respondents were asked for the following information: where the student spent most of the day; whether the student went elsewhere (prompted by specific venues), who watched the student; and whether the person watching the student missed work. Questions were asked regarding the oldest student if multiple children attended the school.

Respondents were also asked, for each household member, whether the person had symptoms of ILI (defined as fever with cough and/or sore throat) between May 1, 2009, and the time of the survey. Children were defined as persons <18 years of age, and those ≥18 years of age were considered adults. The online Technical Appendix (www.cdc.gov/EID/content/16/8/1315-Techapp.pdf) describes the process followed to calculate variables used in the analysis.

The locations where students spent most of the day and other venues visited were tabulated. Significant differences in venues visited by students with and without ILI were determined by using the Fisher exact test. We computed unadjusted and adjusted odds ratios (ORs) for the following characteristics versus whether the household reported missing ≥1 workdays: whether the oldest student reported ILI (repeated for whether any adult, any student at the closed school, or any child in the household reported ILI), whether the household had a single child, whether the household had just 1 adult, whether all adults in the household worked outside the home, and whether household income was above the median (online Technical Appendix). Adjusted ORs were computed in a logistic regression model for variables that had unadjusted ORs significant at $p<0.10$ by the Fisher exact test.

Surveys were completed for 214 (59%) of 364 households (59%), and accounted for 269 (59%) of the 456 students enrolled at the school. Table 1 shows the demographics of surveyed households. Most households had at least 2 adults, at least 2 wage earners, and ≥2 children. Households with incomes ≥$60,000 were at or above the median income. Because some of the oldest students spent days in multiple locations during the 5 days of school closure, we calculated the number of student-days at each venue (number of students at each type of venue multiplied by the number of days spent there). Home was the primary location during the school closure for 77% of the

[1]Members of the Pennsylvania H1N1 Working Group are listed at the end of this article.

Table 1. Demographic variables of households affected by school closure during pandemic (H1N1) 2009, Pennsylvania, USA*

Variable	No. (%) households†
No. adults (≥18 y)	
1	25 (11.7)
2	157 (73.4)
>2	32 (15.0)
No. children (<18 y)	
1	44 (20.6)
2	92 (43.0)
3	53 (24.8)
>3	25 (11.7)
Households with ≥1 adult with ILI	34 (15.9)
Households with ≥1 child with ILI	88 (41.1)
Households with the oldest student with ILI	67 (31.3)
Household income (US$)	
0–29,999	27 (12.6)
30,000-59,999	65 (30.4)
60,000–89,999	51 (23.8)
≥90,000	42 (19.6)
Don't know/refused/missing	29 (13.6)
No. wage earners	
1	64 (29.9)
2	135 (63.1)
≥3	12 (5.6)
Don't know/refused/missing	3(1.4)
Time adult in household missed work to watch oldest student, d	
0	168 (78.5)
1	13 (6.1)
2	7 (3.3)
3	4 (1.9)
4	4 (1.9)
5	18 (8.4)
% Adults in household who work	
33	5 (2.3)
40	1 (0.5)
50	44 (20.6)
67	16 (7.5)
75	3 (1.4)
100	142 (66.4)
Don't know/refused/missing	3 (1.4)

*ILI, influenza-like illness.
†Categories are mutually exclusive and exhaustive, but percentages may not sum to 100% due to rounding.

student-days (online Technical Appendix Figure 1). The next most common location was another family member's home.

Sixty-nine percent of students visited other venues during school closure (online Technical Appendix Figure 2). Those reported as having ILI were more likely to have visited a healthcare provider than those without ILI ($p<0.01$), but no other statistically significant differences were found in terms of venues visited between those with ILI and those without ILI. Seventy-nine percent of households reported zero missed workdays (Table 1); of the remaining households in which work was missed, ≈40% missed work during all 5 days of school closure.

The only household characteristics for which the OR for missing any workdays was significantly different from 1 at $p<0.10$ were single child, all adults work, and household income is greater than or equal to median income (Table 2). When adjusted ORs were calculated, household income greater than or equal to median was significant at $p<0.05$, but because income data were only available for 184 households (vs. 214 for the other factors), the sample on which the adjusted ORs were calculated was somewhat different. All adults in the household working was significantly associated with household income greater than or equal to the median ($p<0.01$).

Conclusions

Estimating the economic effects of school closure can provide useful information to aid in estimating whether it is likely to achieve the intended goals. Households that reported missed work incurred costs, even if those costs were only in terms of lost vacation or sick time.

The data show that most of the oldest students spent the days of school closure at home. However, most students left the home at least once during the closure period to visit routine venues (stores, locations of sports events or practices, restaurants). Few differences were found for reported ILI (with the obvious exception that students with ILI had significantly more visits to healthcare providers). These latter 2 findings are similar to those found in a 2006 study of an influenza B–related school closure in North Carolina, USA (*5*). This behavior, particularly by students who reported ILI, may increase the risk for onward transmission. A survey of 2 school districts in Kentucky that experienced a seasonal influenza–related school closure also found that students engaged in many activities outside the home (*6*), as did a survey of households affected by pandemic (H1N1) 2009 school closure in Australia (*7*).

In our study, only 22% of households reported missing any work to watch the students, fewer than during the

Table 2. Predictors of households reporting days of work missed to watch children during school closure for pandemic (H1N1) 2009, Pennsylvania, USA*†

Variable	OR	Adjusted OR‡
Oldest student with ILI	1.22	
Any student with ILI	1.20	
Any child with ILI	1.16	
Any adult with ILI	1.67	
Single adult	1.50	
Single child	2.02§	2.02§
All adults work	2.35¶	2.08
Household income above median income	2.62¶	2.31¶

*OR, odds ratio; ILI, influenza-like illness.
†When a household had >1 child attending the school that was closed, we asked about time taken from work to watch the oldest child.
‡Adjusted OR estimated by logistic regression.
§$p<0.10$.
¶$p<0.05$.

closure in Australia (*7*). However, in ≈40% of households in which work was missed, an adult missed work for all 5 days of closure, indicating a relatively large effect on those households (Table 1). A limitation is that the question regarding missed work was narrowly worded (online Technical Appendix) and did not explore whether an adult missed work for other reasons. As shown in Table 2, adult ILI was not significantly associated with missing work. Some adults with ILI may have stayed at home to watch students but determined that they would have stayed home because of their own illness had the school not been closed and answered "no." In the Kentucky school closure situation, 29% of households had working adults who provided childcare. In 16% of households, adults missed work and lost pay (*6*). Closures for >1 week may result in more households that report missing work days. The factors "all adults working" and "having a household income equal to or greater than the median" were associated with missed workdays, as were fewer children (other children in the home may have made it possible for some households to avoid having an adult miss work to watch students whose school was closed).

These findings add to the body of literature on the effects of school closure on households. They can be used by decision makers, as well as parents, to assess the potential social disruption of school closure in the context of future influenza outbreaks.

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Imported Human Rabies, the Philippines and Finland, 2007

To the Editor: Rabies is a fatal zoonotic infection of the central nervous system caused by viruses of the genus *Lyssavirus* (*1*). Because person-to-person transmission is rare (*2*), public health action requirements in the countries where imported human rabies is seen are limited. However, communication with persons in places of exposure to provide information that could lead to public health action may not be easy.

On August 28, 2007, a 45-year-old man from the Philippines who worked on a passenger ship used for pleasure voyages was admitted to a hospital in Helsinki, Finland, because of difficulty swallowing since August 23 (Figure). He suspected rabies because he had been bitten by a dog, allegedly owned by a neighbor in the Philippines, in June. When he called home on August 27, he found out that the suspected dog was still alive.

Results of a physical examination, which included detailed neurologic tests, were unremarkable except for a subfebrile axillary temperature. Basic laboratory test results were within reference limits. Cerebrospinal fluid (CSF) contained marginally increased levels of leukocytes, erythrocytes, lactate, and proteins. Results of bacterial staining and culture of CSF were negative. Brain magnetic resonance imaging and an electroencephalogram showed no signs of encephalitis.

On August 29, the patient became febrile and disoriented, and samples (saliva, throat swab, and CSF) were obtained for rabies testing at the Finnish Food Safety Authority. A reverse transcription–PCR (RT-PCR) result for a saliva sample was positive for rabies virus RNA glycoprotein–RNA polymerase intergenic region (*3*); CSF and throat swab samples showed negative results. The virus strain (GenBank accession no. GQ856149) was closely related to genotype 1 strains isolated from dogs in the Philippines (GenBank accession nos. AB116577 and AB116578). Saliva and throat swab samples were positive by real-time RT-PCR also performed at the Institute of Tropical Medicine, Hamburg, Germany. The patient died 12 days after onset of symptoms (September 4).

The deceased patient was transported to the Philippines in an impervious body bag placed in a zinc coffin according to instructions from the Philippine consulate and the Agreement on the Transfer of Corpses (*4*). Because there are no rabies-vaccinated embalmers in Finland, embalming was not performed.

The diagnosis was conveyed to the patient's family in the Philippines by relatives in Europe and the United States. These relatives also informed public health authorities in Finland that the dog considered to be the source of rabies was alive and had caused anxiety and accusations in the home town of the deceased. Suspected transmission from a dog still alive 7 weeks after the incident and anxiety among the population prompted a detailed investigation by the National Public Health Institute (Helsinki, Finland) and the Research Institute for Tropical Medicine (RITM; Manila, the Philippines).

On September 6, a veterinary and medical team from RITM conducted an investigation in the home town of the patient. The suspected dog, a 2-year-old mongrel, had not been vaccinated against rabies but showed no clinical signs. The family indicated that the bite in June had probably occurred in the evening, making identification of the dog difficult. With the owner's consent, the dog was humanely killed and tested for rabies at the RITM Rabies and Special Pathogens Laboratory. The cadaver was treated with 70% formalin before it was buried. Results of a fluorescent antibody test for rabies were negative.

Relatives of the patient received rabies prophylaxis in an animal bite clinic in the Philippines on September 2 because of suspected exposure. The local government had conducted dog rabies vaccination in the area. An information campaign of lectures about rabies in the affected area was initiated, and the need for dog vaccination and stray dog control was emphasized.

An investigation of the passenger ship on which the patient worked showed that he had shared a cigarette with his cabin mate and they had drunk from the same bottle. The cabin mate was advised to receive prophylaxis. Attempts to contact the ship and shipping company in the United States were not successful. Investigation in Finland identified 33 healthcare-associated contacts before virus isolation was attempted. These persons received postexposure prophylaxis. Guidelines regarding those exposed to a rabies patient were then updated. This case highlights the need to increase awareness of rabies infection among healthcare workers.

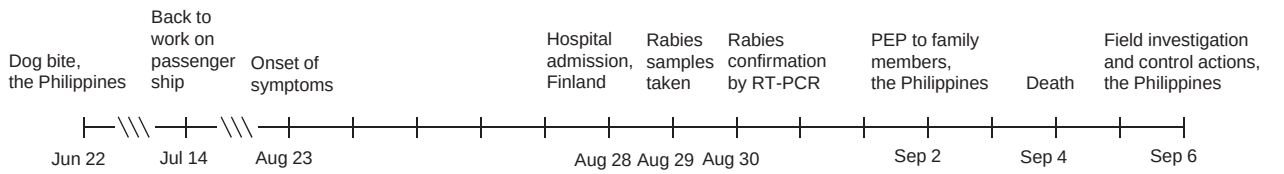

Figure. Time sequence of rabies case in a 45-year-old man from the Philippines who had been bitten by a dog, June–September 2007. RT-PCR, reverse transcription–PCR; PEP, postexposure prophylaxis.

Rapid collaboration between public health authorities in the Philippines and Finland led to appropriate action at the site of origin of the rabies case within a few days. In a country in which rabies is not endemic, diagnosing rabies and implementing control measures in healthcare settings are often difficult because of limited experience with this disease. The last human rabies case in Finland was diagnosed in 1985, when a bat researcher died after being bitten by bats abroad and in Finland (5). For imported cases, patient history may be incomplete, but use of RT-PCR for saliva can provide a rapid confirmation of the diagnosis. To support risk assessment and decision making, better definition of the roles of public health authorities regarding a mandate or responsibility to acquire information concerning international ships is needed.

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Eye-Opening Approach to Norovirus Surveillance

To the Editor: It is said that a picture is worth a thousand words. The Figure illustrates this axiom and provides several new insights into the spread of norovirus infections. These infections are assumed to greatly affect society, but little is known about the prevalence of the disease in the community. Samples sent to laboratories usually originate from hospitalized persons and thus give a good view of the situation in healthcare settings. We suspect, however, that these numbers do not depict the true prevalence of norovirus infections in society. We therefore present a new approach to estimate the number of cases and spread of norovirus infections in the community.

We plotted the number of queries for *vomit* (asterisks denote any prefix or suffix) submitted to the search engine on a medical website in Sweden (www.vardguiden.se). This number was normalized to account for the increasing use of the website over time and aggregated by week, starting with week 40 in 2005. We also plotted the number of norovirus findings per week from 16 regional laboratories, as recorded by the Swedish Institute for Infectious Disease Control.

For the time series on Web search queries and laboratory findings (Figure), we fitted harmonic functions on the half-year with no or little activity, defining baselines for each series (*1*,*2*). By performing this procedure, we can identify the onset of each activity that is assumed to occur when the level rises above the 99% prediction interval of the baseline. The week this increase occurs is shown in the Figure. The Figure also contains the number of media articles on winter vomiting disease provided by a search engine for news in Sweden (www.eniro.se/nyhetssok). By analyzing the figure and investigating the statistical outcomes, we glimpse the prevalence of norovirus infections in society, as estimated by the search pattern.

We found 3 striking insights. First, the onset of vomiting in the community precedes the onset of confirmed norovirus infections in healthcare settings. In 3 of the 4 full seasons investigated, this precedence was 1–4 weeks. Second, the curve for the Web queries shows much sharper increases and decreases than does the curve on the number of reported norovirus findings. Third, neither search behavior nor reporting of positive tests is driven by media for the winter vomiting disease (confirmed by a linear regression).

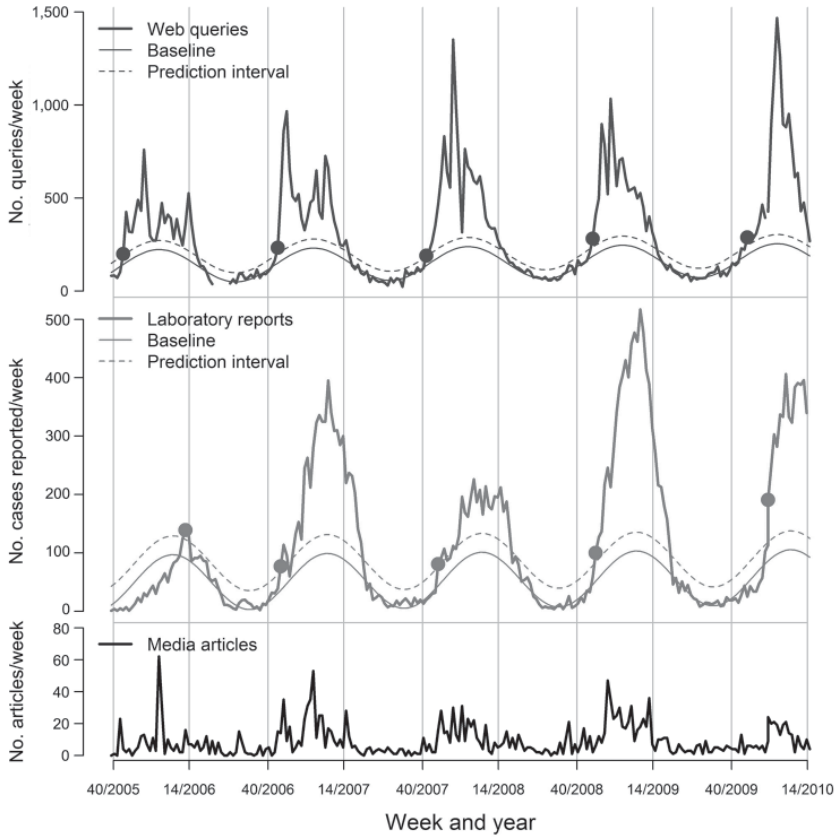

Figure. Number of queries for *vomit* submitted to a medical Web site (A), number of laboratory-verified norovirus samples (B), with baselines and 99% prediction intervals, and number of media articles about winter vomiting disease (C) in Sweden, 2005–2010. A color version of this figure is available online (www.cdc.gov/EID/content/16/8/1319-F.htm).

In the 2005–06 season, the laboratory reporting raised above the defined prediction interval in week 13, much later than the Web queries. This season had no new variants of norovirus genotype GII.4. This season still showed community infections, even though few reports came from institutions. For the current season (2009–10), the interval between onset of Web queries and onset of norovirus infections in hospitals (week 46 and week 1, respectively) was 8 weeks. In comparison with previous seasons, this delay could mean a low total number of reported cases. However, in late December, a new variant of GII.4 affected healthcare settings in southern Sweden with increasing norovirus infections, while the rest of the country still showed relatively low virus activity.

Other pathogens such as rotavirus, *Salmonella* spp., *Staphylococcus aureus*, and *Bacillus cereus* can cause vomiting. Usually in Sweden, rotavirus infections peak in late winter, and bacterial diseases have a minor incidence compared with norovirus. In our opinion, these other pathogens would not interfere with the interpretation of the results.

In our routine surveillance of Web queries, we also include other query terms, such as diarrhea and stomach flu. However, searches for vomiting show the most distinct pattern, and vomiting is the most pronounced symptom of a norovirus infection.

The use of harmonic functions for describing baseline Web searches and laboratory reporting is a simple model, especially because the parameters are estimated by using the half-year with the least activity. Nonetheless, it is a direct approach, and we believe that the method still captures the time of onsets well.

Web queries indicate the presence of norovirus infections in communities. Predictions of the onset of the norovirus laboratory reporting should also be possible, but further studies are needed to confirm that theory. Web queries have previously been correlated with influenza (*3*–*7*) and have been explored retrospectively for listeriosis (*8*), *Salmonella* spp. (*9*), West Nile virus, and respiratory syncytial virus (*10*). With the Web queries, we get an additional surveillance system for the time of the year when few norovirus tests are conducted. In addition, knowing more about the impact of norovirus in the community means that we could provide more adequate information and advocate wiser measures for prevention and control.

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Possible Recurrent Pandemic (H1N1) 2009 Infection, Israel

To the Editor: We report 2 cases of possible recurrent laboratory-confirmed infection with pandemic (H1N1) 2009 virus in Israel. Patient 1, a 24-year-old man, had Noonan syndrome (*1*,*2*). He was hospitalized on August 10, 2009, because of high-grade fever and cough. At admission, a nasopharyngeal specimen was collected for pandemic (H1N1) 2009 virus real-time reverse transcription–PCR (RT-PCR) (ABI 7500; Applied Biosystems, Foster City, CA, USA) for the pandemic hemagglutinin gene; a validated in-house protocol developed at Israel Central Virology Laboratory was used, as previously described (*3*). Briefly, the in-house assay was validated against the assay for pandemic (H1N1) 2009 virus developed by the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA). The in-house assay was as sensitive as the CDC assay; however, the in-house primers and probes were more specific for detecting pandemic (H1N1) 2009 virus with 105% amplification efficiency of viral RNA that was logarithmically serially diluted. In addition, of 100 samples tested side by side with the in-house and CDC assays, 75 samples were positive by both assays, and 25 were negative by both assays; thus, the sensitivity and specificity of the in-house assay were 100%.

The patient was not treated with neuraminidase inhibitors and did not require supportive treatment; after 1 day of hospitalization, he was discharged with a diagnosis of upper respiratory tract infection. The laboratory subsequently reported the RT-PCR as positive for pandemic (H1N1) 2009 virus. On November 22, the man was hospitalized again for dyspnea and fever. The RT-PCR result from a nasopharyngeal sample collected at admission was positive. Hemagglutination-inhibition assay demonstrated a high titer (320) of serum antibody against pandemic (H1N1) 2009 virus in a blood sample taken at admission. The patient took oseltamivir for 5 days, and his condition markedly improved. Result of a repeat RT-PCR at discharge was negative.

An identical neuraminidase gene sequence was detected during both illness episodes (August and November). The specimens were also tested with an experimental RT-PCR assay for rapid detection of the oseltamivir resistance mutation H275Y on the pandemic neuraminidase gene (*4*). For specimens collected during both episodes, the virus was oseltamivir sensitive.

Patient 2, a 13.5-year-old boy, had severe cerebral palsy. On July 27, 2009, high-grade fever with dyspnea developed. He was treated as an outpatient for 5 days with oseltamivir and clinically improved. However, on August 11, he had fever with respiratory distress and was hospitalized. RT-PCR for pandemic (H1N1) 2009 virus was positive on August 14. A second course of oseltamivir was administered for 10 days with the dosage adjusted for age and doubled from that of the previous regimen. Further testing with the experimental rapid RT-PCR indicated the viral strain had the oseltamivir resistance mutation. On September 14, RT-PCR was negative.

On December 11, the boy was again hospitalized because of respiratory distress and high-grade fever. On December 14, RT-PCR was positive for pandemic (H1N1) 2009 virus, and a 5-day regimen of oseltamivir was started. Another specimen taken the same day was negative. A high serum antibody titer (320) to pandemic (H1N1) 2009 virus was measured by hemagglutination-inhibition assay on December 16; no oseltamivir resistance mutation was found. Additional laboratory testing included a complete panel for respiratory viruses, which

was negative for human metapneumovirus; respiratory syncytial virus; adenovirus; seasonal influenza virus types A and B; and parainfluenza virus types 1, 2, and 3.

These 2 cases of possible recurrent pandemic (H1N1) 2009 infection demonstrated a wide interval between illness episodes. Neither patient had accompanying immunodeficiency, and both had antibody titers far beyond the accepted seroprotective threshold for influenza (*5*), albeit ineffective. These titers probably resulted from primary infection rather than from subclinical exposure, which manifests itself as a lower titer by order of magnitude (*6*,*7*).

Virus clearance was not laboratory confirmed for patient 1 after the first episode because no samples were taken after hospital discharge. Patient 2 had both positive and negative RT-PCRs for pandemic (H1N1) 2009 virus (Table) from samples collected the same day during the second hospitalization, which also may disprove reinfection. The positive result could be explained by laboratory contamination during the RT-PCR processing that indicated a false-positive result. However, contamination is unlikely because each run of the RT-PCR was routinely accompanied by runs of negative controls (that contain water) to rule out such contamination. Nonanalytic factors such as specimen misidentification also are unlikely because the central virology laboratory, which is the national reference center, has an ISO-9000 qualification from the Standards Institution of Israel (www.sii.org.il/20-en/SII_EN.aspx). Furthermore, no other respiratory virus was found by laboratory testing at that time. The patient was infected with an oseltamivir-resistant pandemic (H1N1) 2009 virus during the first illness episode and with an oseltamivir-sensitive virus during the second episode and had 2 RT-PCRs with negative results between the episodes.

The novel pandemic influenza virus may be able to reinfect certain chronically ill persons. Caregivers should be aware of this trait when considering the differential diagnosis of influenza-like illness in a patient with a documented, and even treated, pandemic (H1N1) 2009 infection.

Table. Real-time RT-PCR for pandemic (H1N1) 2009 virus and results of experimental assay* for oseltamivir resistance mutation H275Y, Israel, 2009†

	Patient 1		Patient 2	
Date	RT-PCR	Oseltamivir resistance/sensitivity	RT-PCR	Oseltamivir resistance/sensitivity
Aug 10	Positive	Sensitive	–	–
Aug 14	–	–	Positive	Resistant
Aug 19	–	–	Positive	Resistant
Sep 1	–	–	Positive	Resistant
Sep 14	–	–	Negative	–
Nov 22	Positive	Sensitive	Negative	–
Nov 29	Negative	–	–	–
Dec 14	–	–	Positive	Sensitive
Dec 14	–	–	Negative	–
Dec 17	–	–	Negative	–
Dec 21	–	–	Negative	–

*Source: (*4*).
†RT-PCR, reverse transcription–PCR; –, not tested.

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Novel *Chlamydia*-like Organisms as Cause of Bovine Abortions, UK

To the Editor: Despite the worldwide economic impact of, and welfare issues associated with, infectious bovine abortifacients, as well as potential zoonotic threats to human health, accurate diagnosis of the causes of abortion is uncommon (*1*). This poor diagnosis could be explained in part by the lack of identification of infectious abortifacient agents.

Although *Chlamydophila abortus* is a known etiologic agent of ruminant abortion, several novel species of *Chlamydia*-like organisms have recently emerged as putative ruminant abortifacients. *Waddlia chondrophila* was isolated from the brain and nervous tissue of an aborted bovine fetus in Germany (*2*), and *Parachlamydia acanthamoebae* and other unidentified *Chlamydia*-like species were identified in 18.3% of bovine placenta samples in Switzerland (*3*,*4*). Given the paucity of information about the causes of infectious bovine abortion and the high prevalence of *Chlamydia*-like organisms in the samples from Switzerland, we attempted to determine whether such organisms can be detected in bovine fetal tissues in the United Kingdom.

Pooled tissue samples comprising brain, heart, and/or placenta (depending on availability) were obtained from bovine fetuses submitted for diagnosis to the Scottish Agricultural College Disease Surveillance Centre, Dumfries, Scotland, UK, during 2008. Tissue pools were homogenized by using a Precellys bead mill homogenizer (Bertin Technologies, Ann Arbor, MI, USA), and DNA was extracted by using the Wizard Genomic DNA Purification Kit (Promega, Southampton, UK) according to manufacturer's instructions. A pan-Chlamydiales PCR for the 16S rDNA sequence was performed by using forward primer 16S FOR2 (5′-CGT GGA TGA GGC ATG CAA GTC GA-3′) and reverse primer 16S REV2 (5′-CAA TCT CTC AAT CCG CCT AGA CGT CTT AG-3′) to generate amplicons of ≈260 bp (*5*). Negative-control reactions contained DNA-free water instead of extracted DNA. PCR products were purified (QIAquick PCR Purification Kit; QIAGEN, Crawley, UK) before direct sequencing by using the PCR primers and dideoxy chain termination/cycle sequencing on an ABI 3730XL DNA sequencer (MWG Operon, Ebersberg, Germany).

After the initial PCR, 22 (26.5%) of the 83 fetal samples tested were Chlamydiales positive. Serologic, bacteriologic, and histopathologic examination of fetal tissues identified no other infectious abortifacient agents in the Chlamydiales-positive samples. Sequence information was successfully obtained for 15 of these 22 samples with forward and reverse primers; sequences ranged from 140 bp to 194 bp (European Molecular Biology Laboratory/GenBank accession nos. GQ919016–GQ919030). These 15 short sequences were carefully aligned to a representative set of 22 similar Chlamydiales 16S rDNA sequences, identified by a BLAST (www.ncbi.nlm.nih.gov/BLAST) similarity search of the European Molecular Biology Laboratory/GenBank database, plus alignment of an outgroup of 7 non-Chlamydiales sequences. A Bayesian phylogenetic tree (Markov Chain Monte Carlo settings: 2 runs of 625,000 generations; burn-in of 125,000 generations; trees sampled every 100 generations) was then estimated with a general time reversible + Γ nucleotide substitution model by using the MrBayes program (*6*) launched from the TOPALi v2 package (*7*).

Despite the short sequence length of the 15 samples, the tree was well resolved with the Chlamydiales sequences and formed 3 clusters (*Chlamydiaceae*, *Rhabdochlamydiaceae*/*Simkaniaceae*, and *Parachlamydiaceae*/*Waddliaceae*/*Criblamydiaceae*) (Figure). Two of these sequence clusters represented 10 and 5 of the samples, whereas no samples were represented in the cluster containing the *Chlamydiaceae*, which includes *C. abortus*. Most (10/15) sequences were found in the cluster containing the *Parachlamydiaceae*. This finding agrees with those of the aborted bovine placenta studies in Switzerland (*3*,*4*) and provides further evidence

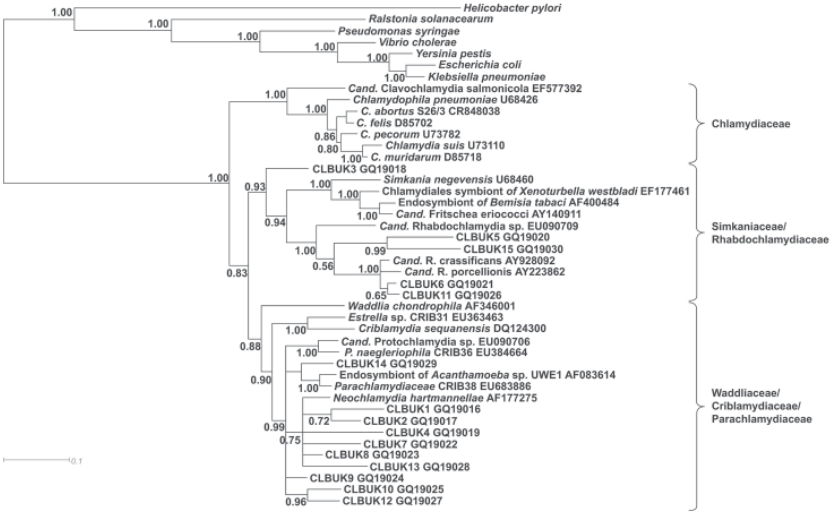

Figure. Bayesian phylogenetic tree demonstrating the relationship of 15 isolated organisms from the older chlamydiales samples to known chlamydial species. Cand., *Candidatus*; *R.*, *Rhabdochlamydia*; *P.*, *Protochlamydia*.

that *Parachlamydia*-like species may play a substantial role in bovine abortion in mainland Europe and the United Kingdom. Four of the remaining 5 samples clustered with members of the family *Rhabdochlamydiaceae*; the fifth sequence (CLBUK3), although present in the same *Rhabdochlamydiaceae*/*Simkaniaceae* cluster, appeared to be more distinct from other family members.

The identification of these organisms in such a large percentage of the bovine fetal tissue samples tested may indicate a role for these organisms in undiagnosed bovine abortions in the United Kingdom and Europe and may be a zoonotic source of infection for humans. Indeed, considerable evidence supports a role for *Parachlamydia* spp. in human pneumonia, whereas *Rhabdochlamydia* spp. is a suspected cause (*8*). In addition, evidence suggests that *P. acanthamoebae* crosses the human placenta to the unborn fetus (*9*). Also, the presence of both parachlamydial and rhabdochlamydial DNA in the lung secretions of hospitalized premature human neonates recently correlated with increased medical interventions and increased duration of hospital stay (*10*).

We demonstrate the presence of *Parachlamydiaceae* and *Rhabdochlamydiaceae* species in bovine abortions in the United Kingdom. Given the zoonotic potential and the economic and welfare impacts of bovine abortion on the agricultural sector, further studies are required to understand the incidence and pathogenic roles of these organisms in both humans and animals. These studies should include broader molecular epidemiologic studies, as well as detailed histologic/immunohistochemical investigations and organism recovery through culture of infected placental and fetal tissues.

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West Nile Virus Knowledge among Hispanics, San Diego County, California, USA, 2006

To the Editor: West Nile virus (WNV), spread by infected mosquitoes, is a serious public health threat throughout the United States and can cause life-altering and even fatal disease (*1*). In San Diego County, California, the human infection rate was 0.18 per 100,000 persons during 2003–2006 (5 cases, 1 locally acquired) and then increased to 0.52 and 1.17 per 100,000 persons in 2007 and 2008, respectively, despite few changes in surveillance activities (*2*). Community-based mosquito control programs, adoption of personal protective behavior (PPB), and education are the most effective ways to prevent human WNV infection because no specific antiviral drug treatment or vaccine exists (*1*,*3*). Although WNV-associated illness has occurred in all racial and ethnic groups, Hispanics are potentially at risk because of language and cultural barriers to obtaining information regarding WNV prevention (*4*). San Diego County Department of

Environmental Health's education and outreach efforts include airing public service announcements in English and Spanish on television and radio and posting information on a website and social networking sites.

In 2006 we administered a survey to assess knowledge, attitudes, and practices (KAPs) regarding WNV among Hispanics in San Diego County. According to the US Census Bureau, the county is home to >900,000 Hispanics, of whom ≈41% are foreign born (*5*). A multistage cluster sampling scheme was used to identify Hispanics >18 years of age in 3 county regions (northern, central, and southern) and has been described elsewhere (*6*). Interviewers went door to door and, for each selected household, asked to speak to a Hispanic member of the household >18 years of age who was the most knowledgeable person about residents' health.

We examined KAPs regarding WNV by using 8 questions that were part of a larger survey assessing Hispanics' KAPs regarding several health-related issues, including topics such as influenza and lead poisoning. We used 4 questions to assess knowledge, 1 to assess attitude, and 3 to assess practices or adoption of PPB. For example, we asked respondents "What precautions, if any, have you taken to protect yourself and/or your family against West Nile virus?" The interviewer then read a list of possible responses (e.g., removed areas of standing water, used insect repellent with DEET [N, N-diethyl-meta-toluamide]) from which the interviewee indicated yes or no. Multiple responses were allowed. Questions were based on those used in previous studies and in border-region focus groups and were modified according to input from local experts and pilot testing. Spanish translation and back translation were conducted separately by 2 translators.

Interviewers completed 226 surveys, which represented 53.8% of all houses approached and 69.5% of 325 households in which a person answered the door. Respondents' mean age was 41 years (range 18–87 years), 79.2% were foreign born, 85.8% completed the survey in Spanish, and 65.9% were women. Overall, 149 (66.2%) of the 226 respondents were aware of WNV; key demographic covariates differed, including greater awareness among English speakers, respondents living in the United States >5 years, and respondents completing >12 years of education (Table). News media (e.g., television, radio, newspaper) were the most frequent sources cited (93.2%) for WNV knowledge, followed by doctor or healthcare professionals (12.2%). Of the respondents who had heard of

Table. Awareness of West Nile virus and adoption of PPB among survey respondents, San Diego County, California, USA, 2006*

Characteristic	WNV awareness, no. (%), n = 226	p value†	PPB use, no. (%), n = 149	p value†
Overall	149 (66.2)	–	62 (41.6)	–
Age, y‡§		0.026		0.188
18–29	37 (55.2)		14 (38.9)	
30–44	52 (65.0)		26 (50.0)	
45–64	40 (83.3)		17 (42.5)	
≥65	20 (66.7)		5 (23.8)	
Education, y		0.048		0.926
≤12	121 (64.7)		50 (41.3)	
>12	26 (81.3)		11 (42.3)	
Preferred language for interview		0.004		0.249
Spanish	120 (62.2)		47 (39.2)	
English	29 (90.6)		15 (51.7)	
Country of birth		0.158		0.104
United States	36 (76.6)		19 (54.3)	
Other	113 (63.5)		43 (37.7)	
Years in United States		0.004		0.183
≤5	9 (34.6)		5 (55.6)	
>5	104 (68.4)		38 (36.2)	
Gender		0.602		0.042
M	49 (63.6)		15 (30.6)	
F	100 (67.6)		47 (47.0)	
Region¶		0.018		0.151
Northern	20 (48.8)		5 (25.0)	
Central	34 (64.2)		11 (31.4)	
Southern	95 (72.5)		46 (48.9)	

*WNV, West Nile virus; PPB, personal protective behavior.
†Proportions were compared by using the χ^2 test.
‡Awareness: 18–29 vs. 45–64, p = 0.003; 30–44 vs. 45–64, p = 0.064; 45–64 vs. ≥65, p = 0.089; other comparisons are nonsignificant.
§Adoption of PPB: 30–44 vs. ≥65, p = 0.045; other comparisons are nonsignificant.
¶Awareness: northern region vs. southern region 3, p = 0.033; other comparisons are nonsignificant.

WNV, 87.9% knew it was transmitted by infected mosquitoes. More than 75% of respondents described their level of concern regarding WNV as "not at all" or "somewhat."

Among the 149 respondents who were aware of WNV, 62 (41.6%) adopted PPBs to protect themselves or their families; more women than men adopted PPBs (Table). The most frequent PPB cited was the removal of standing water around the home (58.1%), followed by use of repellent with DEET (48.4%), and repairing broken windows or screens (43.5%).

We found lower awareness of WNV among San Diego County Hispanics (66.2%) than previously reported for predominantly non-Hispanic populations (range 77.2%–99.0%) (*7*–*9*). One survey reported that 41% of 17 Spanish-speaking respondents were aware of WNV (*9*). We also identified women as the primary source of PPB adoption among Hispanic households and a potential target population for interventions. Previous studies examining KAPs regarding WNV included small numbers of Hispanics and thus were unable to identify this subgroup for targeted interventions.

The finding of low awareness, concern, and PPB adoption may have 2 possible explanations. First, the observations may be appropriate given the low incidence of WNV in San Diego County and Mexico. At the time the survey was conducted, only 1 locally acquired case of WNV infection among humans had been reported in San Diego County; through 2006, WNV was rarely reported among humans in Mexico (*10*). Second, the low levels of awareness, concern, and PPB adoption may simply reflect the priority of WNV prevention compared with other basic necessities and health risks among the largely immigrant survey population.

Differences in awareness, concern, and practices among Hispanics by age, education, gender, language, years living in United States, and region of San Diego County indicate that varied educational tactics are needed to inform this population. Most educational efforts for Hispanics are simple translations of material into Spanish, which are likely not sufficient to reach this heterogeneous population.

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Crimean-Congo Hemorrhagic Fever in Man, Republic of Georgia, 2009

To the Editor: Crimean-Congo hemorrhagic fever (CCHF) virus is widely distributed in the southwestern regions of the former Soviet Union, the Balkans, the Middle East, western People's Republic of China, and Africa (*1*). Public health officials in the Republic of Georgia have long suspected that CCHF occurs in this country, but laboratory confirmation by using molecular diagnostic techniques has not been possible there until recently.

CCHF virus is primarily transmitted by ticks, but other modes of transmission have been described (*2*). This virus infects humans mainly by the bite of adult *Hyalomma* spp. ticks. Infected sheep and cattle have also been implicated in transmission (*3*).

Contact with highly infectious blood from patients has also led to several nosocomial hospital outbreaks, which resulted in the deaths of medical personnel (*4*,*5*). It is estimated that exposure to CCHF virus leads to symptoms in 1 of 5 patients exposed to this virus (*6*). Mortality rates up to 30% have been reported (*7*).

Virus can be isolated from blood of acutely ill patients by cell cultures or by passage through suckling mice. Antigen-detection ELISA is useful for diagnosis, particularly for severe cases (*2*). PCRs may provide additional sensitivity with no loss of specificity. Antibodies are detectable by a variety of methods and generally appear within 5–14 days of disease onset and coincide with clinical improvement. ELISA detection of immunoglobulin M is an established diagnostic method (*2*,*3*). Ribavirin may be effective for treatment of patients with severe CCHF; in vitro, animal, and clinical experience with this drug support its use (*8*). No human or veterinary vaccines against CCHF are currently recommended (none are licensed in the United States). We report a patient in Georgia with CCHF.

The patient was a 30-year-old man who lived in suburban Tbilisi, Georgia. Fever and sore throat without distinguishing characteristics developed in the patient. After 7 days of symptoms, gastrointestinal bleeding, melena, and hematemesis developed. He was admitted to the First City Hospital in Tbilisi, Georgia, on August 25, 2009. He reported frequent fishing in rural areas. The patient lived in a private house on the outskirts of the city that had a yard and vegetation. No specific rodent exposures were noted, and no other travel was reported.

Because his symptoms increased in severity, the patient was transferred to the Ghudushauri National Medical Center in Tbilisi on August 28, 2009. At this time, the patient had a temperature of 38.0°C–38.5°C, decreased consciousness, and hemorrhages primarily on the chest and medial surfaces of the upper extremities (Figure). Prominent hepatomegaly and moderate splenomegaly were observed. Laboratory tests showed pancytopenia with severe thrombocytopenia (thrombocyte count 4.0×10^9 cells/L, erythrocyte count 3.34×10^{12} cells/L, leukocyte count 2.92×10^9 cells/L). Neutropenia was also observed (neutrophil count 788 cells/mm^3), but hematuria was not observed. Creatinine level was within the reference range. Levels of liver transaminases were increased (alanine aminotransferase 3 U/L, aspartate aminotransferase 1,550 U/L). His bilirubin level was 80 mmol/L (direct bilirubin 41 mmol/L). Chest radiograph showed hemorrhagic alveolitis, and gastroduodenoscopy showed erosive duodenitis. The patient began receiving mechanical ventilation at the time of transfer. CCHF was suspected by the infectious diseases physician who was initially consulted on September 3, 2009.

The National Center for Disease Control and Public Health of Georgia investigated the case by obtaining and testing clinical samples. Serum samples obtained on September 4, 2009, were analyzed by using a CCHF IgM ELISA Kit (Vector-Best, Novosibirsk, Russia) and found to be positive for antibodies against CCHF virus (optical density 0.760, cutoff value 0.457). Virus RNA was extracted by using a Mini RNA Extraction Kit (QIAGEN, Hilden, Germany). Samples were positive for CCHF virus by real-time PCR (Roche Diagnostics, Basel, Switzerland) with specific primers (Invitrogen, Carlsbad, CA, USA). The patient was then treated with oral ribavirin (600 mg 3×/d for 14 days), gradually recovered from the infection, and was discharged from the hospital on October 26.

The National Center for Disease Control and Public Health also conducted environmental sampling as part of their case investigation. Rodent brain and lung tissue homogenates were collected from 2 mice captured in the backyard of the patient. Samples were tested by using an antigen detection kit (#97, D-1154; Vector-Best) to confirm the diagnosis. Optical density values were 0.833 and 0.890, respectively (cutoff value 0.334).

This case has serious public health implications for Georgia. For example, laboratory capability to safely detect this virus should be evaluated. Also, healthcare personnel should receive additional education about this disease, particularly so that appropriate precautions can be implemented during initial evaluations. The case was typical of CCHF and showed the pattern of prehemorrhagic, hemorrhagic, and convalescent phases. Hematemesis, melena, and somnolence have been predictors of death in previous investigations (*2*). Frequency of patients with asymptomatic or mildly symptomatic disease should also be determined. Recognition and testing of mild-to-moderate cases may also increase in Georgia as a result of in-

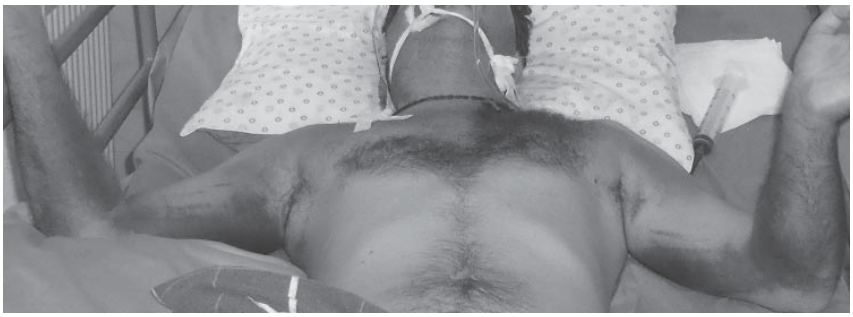

Figure. Intubated patient with Crimean-Congo hemorrhagic fever, Republic of Georgia, 2009, showing massive ecchymoses on the upper extremities that extend to the chest. A color version of this figure is available online (www.cdc.gov/EID/content/16/8/1326-F.htm).

creased awareness in the healthcare community.

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Mycobacterium avium subsp. *hominissuis* Infection in Horses

To the Editor: *Mycobacterium avium* subsp. *hominissuis* infection is often detected in pigs and humans (*1*–*3*). In most cases, the main sources of this agent are environmental (*4*,*5*). During the past few years, 2 hosts infected by this agent, dogs (*6*) and pet parrots (*7*), were identified as a possible source of infection for immunocompromised humans who may have close contact with animals. We report massive *M. avium* subsp. *hominissuis* infection in 2 sibling riding-type Fjord horses from an amateur-run horse-breeding farm.

The first horse, a 2-year-old colt, was admitted to a veterinary clinic in the Czech Republic in February 2009 with diarrhea and progressive weight loss of 3 weeks' duration. Multiple diagnostic procedures produced inconclusive results. Cyathostomosis was suspected, so moxidectin was administered twice, and prednisolone was given for 10 consecutive days. The clinical status of the horse initially improved but worsened after 3 weeks. Ultrasonographic examination of the peritoneal cavity showed a nodular mass and a nonperistaltic, thickened portion of the small intestine wall in the left ventrocranial region. Exploratory celiotomy showed enlargement of the mesenteric and colonic lymph nodes and multiple local thickenings of the small intestine wall, large colon, and cecum. The horse was euthanized. Specimens of enlarged lymph nodes and intestinal content were taken during necropsy for histopathologic and microbiologic examination. Microscopically, acid-fast rods (AFR) after Ziehl-Neelsen staining were observed, and quantitative real-time PCR (qPCR) showed 2.89×10^5 and 1.47×10^4 *M. avium* subsp. *hominissuis* cells per 1 g of intestinal content and mesenteric lymph node, respectively (*8*).

The second case, a 1-year-old full sister to the colt described above, was admitted in July 2009 after 1 month of lethargy, weight loss, diarrhea, and nasal discharge. Ultrasonographic examination of the abdominal cavity showed an increased amount of peritoneal fluid and nonperistaltic, corrugated, and thickened parts of the small intestine in the left caudal region. Local thickening of the jejunum and ileum were found during exploratory celiotomy; no lesions on the cecum or colon were observed macroscopically. Mesenteric lymph nodes were enlarged. Microscopically, AFR were observed, and qPCR showed 3.36×10^6 *M. avium* subsp. *hominissuis* cells per 1 g of mesenteric lymph node (*8*). Treatment with clarithromycin and rifampin was begun, but the condition of the filly improved only temporarily. She was euthanized after 4 months because of progressively worsening condition. Postmortem examination showed enlarged colonic lymph nodes with small nodular lesions, hyperemia of the colon mucosa, and corrugation and thickening of the colonic wall. For further examination, samples

of feces, colonic lymph nodes and wall, liver, mesenteric lymph nodes, kidney, spleen, and diaphragm were taken. Ziehl-Neelsen staining of tissue smears demonstrated AFR in different tissues. Culture examination following the described method (*2*) and qPCR confirmed *M. avium* subsp. *hominissuis* infection; quantities of this agent were 6.31×10^5 and 2.47×10^{11} in 1 g of feces and mesenteric lymph nodes, respectively (Table).

According to a review (*9*), infections caused by *M. avium* subsp. *hominissuis* have been described in only 6 horses until now. We presume that *M. avium* subsp. *hominissuis* infection in both these horses could have been caused by some immunodeficiency related to a genetic predisposition. The shedding of this agent in feces indicates that infected horses can also pose a health risk to humans, particularly immunocompromised persons. *M. avium* subsp. *hominissuis* infection is frequently observed in children, in whom it can cause peripheral lymphadenopathy (*10*). Currently, hippotherapy is a frequently used recreational activity in some countries for various patients, e.g., for children with cerebral palsy. Hippotherapy thus may be associated with a potential risk for humans in contact with clinically ill *M. avium* subsp. *hominissuis*–infected horses.

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Table. Detection of *Mycobacterium avium* subsp. *hominissuis* in tissues of a 1-year-old Fjord filly*

	Mycobacteria detection		qPCR	
Sample source	Microscopy	Culture	IS*1245*†	IS*901*
Feces	–	+	6.31×10^5	–
Lymph node of transversal colon	+	+	1.84×10^9	–
Lymph node of descending colon	+	+	5.89×10^9	–
Transversal colon wall	+	+	3.98×10^7	–
Descending colon wall	+	+	6.33×10^6	–
Liver	–	+	NT	NT
Mesenteric lymph node	+++	+	2.47×10^{11}	–
Kidney	–	+	NT	NT
Spleen	–	+	NT	NT
Diaphragm	–	–	8.22×10^4	–

*qPCR, quantitative real-time PCR; –, negative finding; +, few acid-fast rods; NT, not tested; +++, >100 AFR (per 50 microscopic fields).
†No. IS*1245* copies/g.

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Lethal Necrotizing Pneumonia Caused by an ST398 *Staphylococcus aureus* Strain

To the Editor: Several recent studies have shown massive colonization of livestock (especially pigs) and livestock workers by methicillin-resistant *Staphylococcus aureus* (MRSA) in western Europe, Canada, and the United States (*1*,*2*). Livestock MRSA isolates belong almost exclusively to a single sequence type, ST398. Evidence of zoonotic and interhuman transmission of methicillin-resistant and methicillin-susceptible variants of this hitherto unusual sequence type was recently reported (*1*,*3*). *S. aureus* ST398 infections in humans with or without a history of contact with livestock include bacteremia, endocarditis, ventilator-associated pneumonia, and wound infections, none of which involve the expression of specific toxins. Indeed, ST398 isolates are negative for all major virulence factors, with the exception of some rare isolates that harbor the genes that encode the Panton-Valentine leukocidin (PVL) (*1*), a toxin that is usually associated with community-acquired MRSA (*4*). We report a case of lethal necrotizing pneumonia caused by a PVL-positive methicillin-susceptible ST398 *S. aureus* isolate.

A previously healthy 14-year-old girl came to the emergency room with influenza-like illness, cough, fever, and a 2-day history of severe abdominal pain. She was given intravenous antibacterial chemotherapy with cefotaxime and amikacin. An exploratory laparotomy showed no signs of abdominal disease. Immediately after surgery, acute respiratory distress syndrome with hemodynamic instability developed in the patient; mechanical ventilation and inotropic support were required. A chest radiograph showed bilateral pulmonary infiltrates and pleural effusion. *S. aureus* was isolated by bronchoalveolar lavage fluid and blood culture, and staphylococcal necrotizing pneumonia was diagnosed. Clinical features, including the preceding influenza-like illness, were highly consistent with those previously reported (*5*). However, viral cultures and immunofluorescence assays were negative for all common respiratory viruses, and, although the patient had positive serologic test results for influenza B virus, antibody titers were too low to affirm influenza B infection. Severity factors were present (*5*), including leukopenia, airway bleeding, and multiorgan failure. She died 6 days after symptom onset, with refractory shock and respiratory failure caused by bilateral pneumothorax. The *S. aureus* strain, which was susceptible to all tested antimicrobial agents except macrolides, was *agr*1/ST398, *spa*-type t571 and nontypeable by *Sma*I pulsed-field gel electrophoresis, which showed its relatedness to livestock-associated strains. The origin of the infection could not be determined. The presence of the genes encoding PVL was confirmed by PCR.

Thus, the spread of *S. aureus* ST398 among livestock is a matter of increasing concern because strains of this sequence type were able to acquire PVL genes and cause necrotizing pneumonia in a young immunocompetent patient. Transmission control and surveillance efforts are urgently needed to prevent further spread of such strains.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Not-So-Novel Michigan Rabbit Calicivirus

To the Editor: A disease outbreak in a Michigan rabbitry led Bergin et al. (*1*) to identify a new rabbit calicivirus distinct from rabbit hemorrhagic disease virus, which they designated as Michigan rabbit calicivirus (MRCV). They found that in domestic rabbits from the United States, 2 different forms of rabbit calicivirus with differences in pathogenicity are circulating. Bergin et al. showed that, phylogenetically, MRCV was more closely related to the nonpathogenic rabbit calicivirus (RCV) than to pathogenic strains and used this observation as an argument for its classification as a novel calicivirus. However, they did not include the publicly available sequences of other nonpathogenic strains, such as Ashington (97% of the capsid viral protein [VP] 60) and the newly identified *Lagovirus* spp. RCV-A1 (complete genome) (*2*).

Using the same dataset as Bergin et al. and including these sequences, we performed genetic analyses focusing mainly on the capsid VP60. The lack of information for open reading frame 1 for the nonpathogenic strains led to this option. Independently of the sequences' length, RCV-A1 was more closely related to the *Lagovirus* spp. European brown hare syndrome virus, here used as an outgroup, and clearly apart from a highly supported primary group that was further subdivided into 2 also highly supported subgroups, 1 composed of pathogenic rabbit hemorrhagic disease virus strains and another encompassing the RCV-like group (RCV, Ashington and Lambay [*2*], and MRCV). Here, only the phylogenetic tree that corresponds to the more complete VP60 sequences is shown (Figure).

We conclude that MRCV is not a novel calicivirus but a new variant of the nonpathogenic RCV-like group. However, the low pathogenicity presented by MRCV and the presence of viral RNA in the liver rather than in the intestine are clearly new features among the nonpathogenic RCV-like group (*5*).

This work was supported by Foundation for Science and Technology Portugal grants SFRH/BD/31048/2006 and SFRH/BPD/27021/2006 to J.A. and P.J.E., respectively.

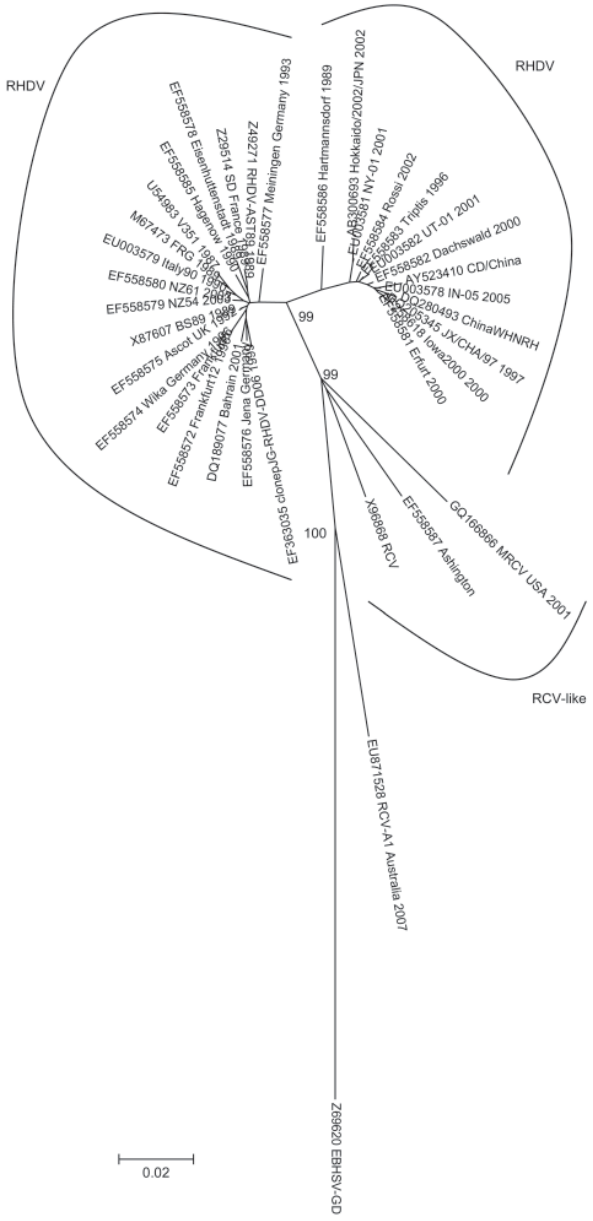

Figure. Evolutionary relationships of *Lagovirus* strains. The evolutionary history was inferred by using the neighbor-joining method (*3*) with the pairwise deletion option. The tree is drawn to scale. There were a total of 563 positions (97% of the capsid viral protein (VP [60 aa sequence]). Phylogenetic analyses were conducted in MEGA 4 (*4*). Reliability of the tree was assessed by bootstrap with 1,000 replicates and is indicated in the nodes (only relevant values are shown). Several genetic distance methods were used, and similar results were obtained, but only p-distance is shown. GenBank accession numbers of the sequences used are indicated. Scale bars indicates nucleotides substitutions per site. RHDV, rabbit hemorrhagic disease virus; RCV, rabbit calicivirus; EBHSV, European brown hare syndrome virus.

Joana Abrantes and Pedro J. Esteves

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In Response: We thank Abrantes and Esteves for their interest in our article (*1*). We demonstrated that a disease outbreak among rabbits in the United States was associated with a calicivirus distinct from members of the species *Rabbit hemorrhagic disease virus* (RHDV) (*1*). Rabbit hemorrhagic disease is classified as a disease of foreign animals in the United States and is caused by the only calicivirus in the genus *Lagovirus* previously associated with disease in rabbits. Our phylogenetic analysis established that Michigan rabbit calicivirus (MRCV) is distinct from RHDV and more closely related to a nonpathogenic rabbit calicivirus (RCV). We are pleased that Abrantes and Esteves agree with us on this point.

In regard to phylogeny, the additional analysis performed by Abrantes and Esteves is an extension to, not an omission of, the original disease-focused paper. It is difficult to understand their objection to the term novel, a point that seems semantic. This term has more than just a phylogenetic connotation, and our use of it is consistent with other reports in this journal (*3*,*4*).

In Abrantes and Esteves' analysis, although RCV, Ashington, MRCV, and RCV-A1 appear to share common ancestry, MRCV branches separately from Ashington and RCV. The limited sequence availability for RCV and Ashington hampers detailed analysis of the interrelatedness of these viruses.

In conclusion, we describe MRCV as a novel calicivirus on the basis of its identification as the first non-RHDV *Lagovirus* sp. detected in the United States, its unique pathogenic potential to rabbits among the currently described non-RHDV lagoviruses, and its genetic distinction from RHDV. The phylogenetic relationships of the non-RHDV caliciviruses will no doubt be further refined as more members with complete or near-complete sequences, like MRCV, become available. Perhaps this will shed further light on the apparent pathogenicity of MRCV under certain circumstances.

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DOI: 10.3201/1608.100711

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Note to Readers: The authority for formal recognition of any new viral species resides with the International Committee on Taxonomy of Viruses (ICTV; www.ictvonline.org). ICTV currently recognizes 2 species, *Rabbit hemorrhagic disease virus* and *European brown hare syndrome virus*, in the genus *Lagovirus*, family *Caliciviridae*. ICTV does not make policies for designation or recognition of strains or variants of viruses within a species. A proposal for formal recognition of any new virus species should be made through the appropriate ICTV study group.

Leptospira Serovar as Prognostic Factor

To the Editor: Herrmann-Storck et al. (*1*) investigated prognostic factors of leptospirosis and concluded that infection with *Leptospira interrogans* serovar Icterohemorrhagiae was linked to severe outcomes. We have concerns about this conclusion.

These researchers were comparing clinical severity of disease among patients for whom serovars of infecting isolates had been identified. However, in that study, blood culture was performed for only 88 (52%) of 168 case-patients, and serovars were identified for 40 (73%) of 55 *Leptospira* strains isolated. Expecting these 40 patients (24% of total) to represent all case-patients in the study is unjustified.

Also, the authors evaluated potential risk factors among these patients by applying a multivariable logistic regression model. This process is questionable. First, the sample size of 40 is not large enough to warrant multivariable analysis with 9 independent variables. Actually, only 8 case-patients had severe disease, although at least 10 outcomes are required for variables in a logistic regression model (*2*).

Second, the model selected is inappropriate. Variables such as thrombocytopenia, hyperneutrophilia, hyperamylasemia, and elevated asparate aminotransferase levels are laboratory findings of severe leptospirosis (and not risk factors of disease). These factors should not be included in a multiple logistic regression model as confounders. We believe it is premature to reach a conclusion about the association between *Leptospira* serovars and clinical severity from the data presented by Herrmann-Storck et al.

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In response: We thank Suzuki and Ariyoshi for their letter (*1*). Identification of serovar and species can only be accomplished by isolating *Leptospira* strains, and obtaining isolates and identifying the serovar and species are especially difficult for human cases of leptospirosis. The bacteria are present in blood for only 1 week after the onset of the disease; they also are fastidious and difficult to grow. Serologic testing gives only a possible serogroup, but it is the only diagnostic tool for confirming patients' infection after 1 week of disease. A strength of our study (*2*) is that it contains extensive epidemiologic, clinical, and biological data and provides a broad collection of identified strains.

Including laboratory findings such as thrombocytopenia, hyperneutrophilia, and hyperamylasemia in the model was appropriate for the following reasons. First, they were not used to establish the definition of severity. Second, the variables included in the model were defined, not according to the norms but at a given level far above the norms (thrombocytopenia <50 g/L, hyperneutrophilia >12 g/L, amylase >285 U/L), which have been recently suggested as possible predictors of severity in other reports.

We are aware that the statistical model has its limits in the context of this retrospective study. We must point out that the conclusion of the independent involvement of *Leptospira* serovar Icterhoaemorragiae in severity is made in the context of Guadeloupe with its particular ecology and insular features, and results were compared with those for given cocirculating strains that are sometimes different in other areas of the world. The real implication of our study is the opportunity it presents to explore some virulence factors in this particular serovar, to compare the results with those of other studies conducted in other areas with the same tools of identification, and to pave the way for a much larger prospective study in the region.

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DOI: 10.3201/eid1608.100763

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The Dictionary of Virology, 4th Edition

Brian W.J. Mahy

Academic Press, Burlington, Massachusetts, USA, 2009
ISBN: 978-0-12-373732-8
Pages: 520; Price: US $73.95

Rapidly expanding technologies in the field of virology, identification of novel viral agents, and the 2005 report (8th edition) of the International Congress of Taxonomy of Viruses (ICTV) addressing reclassification of several viruses generated the 20% new material in Mahy's 4th edition of The Dictionary of Virology. The previous edition of this book was published in 2001; the 2009 edition includes recent advancements, such as newly described viruses (e.g., severe acute respiratory syndrome (SARS) human coronavirus, human metapneumoviruses, bocaviruses, and Rabensburg virus), reclassification schemes of viruses (for instance, the unassigned *Anellovirus* genus), and descriptions of new technologies (e.g., microarray analyses and microRNAs) that have profoundly affected the field of virology. This comprehensive desk reference provides concise definitions of virologic terms; enables quick fact checking; and provides useful, often difficult to find, information—such as the origin of virus names, determination of ICTV-approved virus abbreviations, and locations and sources of viral isolations. An appendix of current ICTV-recognized virus families, subfamilies, genera, and type species is especially useful.

However, this reference is limited to viruses infecting vertebrate hosts; thus, it excludes viruses of plants, bacteria, fungi, invertebrates (except for arboviruses that have dual replication cycles within invertebrates and vertebrate hosts) or viruses (the newly described virophages of mimiviruses). The increasing quantity of information about viruses of vertebrates ranging from fish to primates presented the author with considerable space difficulties. He compensated for this situation, however, by citing literature sources at the end of entries for readers seeking more information. Additionally, considerable cross-referencing enhances the utility of the book. On the basis of inclusion of new information in the field and my personal experience with previous editions of The Dictionary of Virology, I highly recommend this volume to students, virologists, microbiologists, and public health professionals interested in viruses of vertebrate hosts.

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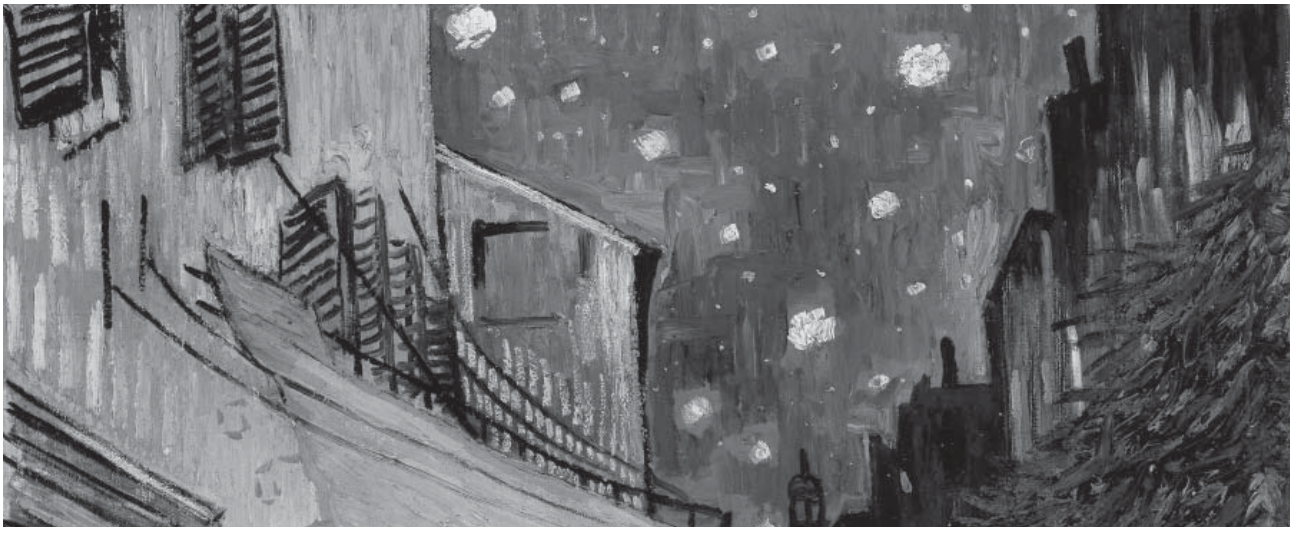

Vincent van Gogh (1853–1890) ***Terrace of a Café at Night (Place du Forum)*** **(c. 18 September 1888)** Oil on canvas (80.7 cm × 65.3 cm) Courtesy of Kröller-Müller Museum, Otterlo, the Netherlands

Not from the Stars Do I My Judgment Pluck[1]

Polyxeni Potter

"It amuses me enormously to paint the night right on the spot," wrote Vincent van Gogh to his brother Theo. "Normally, one draws and paints the painting during the daytime after the sketch. But I like to paint the thing immediately. It is true that in the darkness I can take a blue for a green, a blue lilac for a pink lilac, since it is hard to distinguish the quality of the tone. But it is the only way to get away from our conventional night with poor pale whitish light …." Despite this affection for the night, van Gogh described *Night Café,* one of his best known night paintings, as "one of the ugliest I have done." Though he loved the purity of the night outdoors, he loathed urban night life. "I have attempted to show that the café is a place where a man can ruin himself, become mad, commit a crime…." He moved away from Paris, where he lived with Theo, to Arles, "wishing to see a new light" and explore the calm.

In Paris he had come to know the impressionists and to experiment with broken brushstrokes and the style of the pointillists Georges Seurat and Paul Signac. He studied with Fernand Cormon and made friendships and contacts in the art world. His palette was transformed, from dark tones and stillness to yellows and blues and swirling lines. Yet, "When I left you at the station to go south," he told Theo, "I was very miserable, almost an invalid and almost a drunkard. Now at last something is beginning to show on the horizon: Hope." Moving to the countryside was an effort to get in touch with a more authentic way to live, to focus on ideas and nourish the spirituality he long sought, first as a student at the seminary and then in art.

The simplicity of rural life appealed to him on another level. "I will begin by telling you that this country seems to me as beautiful as Japan as far as the limpidity of the atmosphere and the gay color effects are concerned." Like many of his contemporaries, van Gogh was fascinated with art from the Orient. He collected and copied woodblock prints and welcomed Utagawa Hiroshige and Katsushika Hokusai into the western vernacular. "My whole work … builds so to speak on what the Japanese have done." Under their influence, he moved toward color and away from naturalism, volume and perspective, light and shadow. "I envy the Japanese artists for the incredible neat clarity which all their works have. It is never boring and you never get the impression that they work in a hurry. It is as simple as breathing; they draw a figure with a couple of strokes … as if it were as easy as buttoning one's waistcoat."

Van Gogh's meteoric rise to greatness in the so-brief span of his 37 years took place in various settings and was marked by emotional turmoil, from unrequited love and failure at evangelism to familial strife and poverty. Through it all, he assessed his own legacy as "of very secondary im-

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[1]William Shakespeare, Sonnet 14.

portance." Largely self-taught, he absorbed brief but potent influences. He took his first artistic steps in his native Holland, copying from art books, working as apprentice for an art dealer at age 16. He received formal instruction from leading Hague School artist Anton Mauve, then moved to London, where he taught school for a couple of years. He became interested in the Barbizon group, particularly Jean-François Millet, and started to paint peasants and rural life, a practice he would continue throughout his life. He traveled to Belgium to study at the Antwerp Academy, an unsuccessful venture, and soon after went to live with Theo in Paris. He took up painting in earnest in 1880 and continued until his death, producing in 10 years 900 paintings and more than 1,100 works on paper. Some of his masterpieces were created during the past 2 years of life when, overcome by mental illness, he committed himself to the asylum in Saint-Rémy. "I put my heart and my soul into my work and have lost my mind in the process."

The evening and night, recurring themes in van Gogh's work, interested him even before he began to paint. As a youth he was an avid reader, fluent in Dutch, German, English, and French. Many of the books he mentioned in his letters described the spiritual and poetic character of the night, the interval between sunset and dark, and the darkness between dusk and dawn. "It seems to me that the night is more alive and richly colored than the day." This time for reflection and introspection sparked his artistic imagination and produced, among other major works, *The Starry Night; Landscapes at Twilight; Peasant Life at Evening; Poetry of the Night;* and *Terrace of a Café at Night*, on this month's cover, a painting reminiscent of Hiroshige's *Scene of the Saruwaka-cho Theater Street by Night.*

"On the terrace there are small figures of people drinking," van Gogh wrote to his brother about this his first starry painting of an outdoor café. "An immense yellow lantern illuminates the terrace, the facade, the sidewalk, and even casts light on the paving stones of the road, which take a pinkish violet tone. The gables of the houses, like a fading road below a blue sky studded with stars, are dark blue or violet with a green tree." Excited about the results, he explained to Theo, "Here you have a night painting without black, with nothing but beautiful blue and violet and green and in this surrounding the illuminated area colors itself sulfur pale yellow and citron green."

In this and other night paintings, he struggled to achieve luminosity with contrasting or exaggerated colors and to demonstrate the superiority of natural light and the imagination over artificial light and reality. He struggled equally to express the mysterious influence of the night on the human heart as he understood it from his own tumultuous life. "I am a man of passion, capable and prone to undertake more or less foolish things which I happen to repent more or less." While he worked on his first painting of a starry night, he wrote, "It is good for me to work hard. But that does not keep me from having a terrible need of—shall I say the word—yes, of religion. Then I go out at night to paint the stars."

This need went back to van Gogh's days as evangelist in an impoverished mining town in Belgium. He was dismissed from that post for showing extreme charity and identifying too much with the flock. His religious zeal dampened, he vowed then to make art for the common people, to paint them and their concerns. And who among the common people has not gazed upward wishing to decipher the mysteries of the sky? "Looking at the stars always makes me dream," he wrote, "Why, I ask myself, shouldn't the shining dots of the sky be as accessible as the black dots on the map of France?" Like others throughout the ages, he sought solace in the stars' mysterious light and viewed them as symbols of hope. "Just as we take the train to get to Tarascon or Rouen, we take death to reach a star."

The stars, and their influence on human life— domain of the scientist, let alone the lover and the poet—have roots in antiquity and were examined long before van Gogh swirled them down to earth for all to see. In the 14th century, Italian physicians ascribed a mysterious illness often turned epidemic to the adverse influence of the stars and called it *influentia*. The term influenza was first used in English in 1743 during an outbreak of the disease in Europe. Despite our continued inability to prevent its global spread, we have learned since that viruses are the culprits and that influenza has less to do with ethereal substances emanating from the stars and more with tiny droplets shared generously between patrons under the café awning and in other gathering places. We are still just as intrigued with the stars and van Gogh's interpretations. And we have astronomy, as the Bard put it, "But not to tell of good or evil luck, / Of plagues, of dearths, or seasons' quality."

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Recurrent *Granulibacter bethesdensis* Infections and Chronic Granulomatous Disease

Legionnaires' Disease Outbreak and Asphalt Paving Machine

Influenza in Refugee Population on the Thailand–Myanmar Border

Comparison of Pandemic (H1N1) 2009 and Seasonal Influenza, Western Australia, 2009

Illicit Drug Use and Risk of USA300 MRSA Infections Complicated by Bacteremia

Worldwide Diversity of *Klebsiella pneumoniae* that Produce β-Lactamase blaKPC-2 Gene

Cotton Rats and House Sparrows as Hosts for Eastern Equine Encephalitis Virus

Cercarial Dermatitis Transmitted by Exotic Marine Snail

Pediatric Pneumococcal Serotypes in 4 European Countries

Determinants of Multidrug-Resistant Tuberculosis Clusters, California, 2004–2007

Long-Term Health Risks after Infective Gastroenteritis in Children and Young Adults

Trends in Peptic Ulcer Disease Hospitalizations, USA, 1998–2005

Extensively Drug-Resistant Tuberculosis, Pakistan

Increasing Incidence of Mucormycosis in University Hospital in Europe

Analysis of Avian Hepatitis E Virus from Chickens, China

Co-infections with *Plasmodium knowlesi* and Other Malaria Parasites, Myanmar

Rhinovirus Outbreaks in Long-term Care Facilities Ontario, Canada

Exposure of Dentists to *Mycobacterium tuberculosis* in a Dental Clinic, Ibadan, Nigeria

Typhoid Fever and Invasive Nontyphoid Salmonellosis, Malawi and South Africa

Complete list of articles in the September issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

August 28–September 1, 2010
Infectious Disease 2010 Board Review Course – 15th Annual Comprehensive Review for Board Preparation
Hyatt Regency Crystal City
Arlington, VA, USA
http://www.IDBoardReview.com

November 6–10, 2010
APHA 138th Annual Meeting and Expo
Denver, CO, USA
http://www.apha.org/meetings

November 11–13, 2010
European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE)
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Article Title

Clostridium difficile Bacteremia, Taiwan

CME Questions

1. You are seeing a 60-year-old woman who was admitted to the hospital for an infected foot ulcer. She has a history of poorly controlled diabetes, congestive heart failure, and stage III chronic kidney disease. Over the last 24 hours, she has developed fever and leukocytosis. Infection is suspected, but the source is not clear. The patient complains of fatigue and chills, but a review of systems is otherwise negative.

Based on data from the current study, which of the following statements regarding the possibility of *Clostridium difficile* bacteremia (CDB) in this patient is most accurate?

A. Most CDB is found in young, healthy people
B. CDB usually develops in association with hospitalizations or stays in chronic care facilities
C. Abdominal pain is the most common presenting symptom of CDB
D. CDB does not occur without preceding diarrhea

2. What was the most common source of bacteremia among patients in the current study?

A. Primary bacteremia
B. Intra-abdominal infection
C. Bone and joint infection
D. Soft tissue infection

3. The patient from question 1 is diagnosed with CDB. According to the current study, what is the best initial choice of antibiotic for her?

A. Metronidazole
B. Imipenem
C. Ertapenem
D. Clindamycin

4. The patient from question 1 is treated with an appropriate antibiotic. Which of the following statements regarding her prognosis is most accurate?

A. Mortality is 75% regardless of antibiotic therapy
B. Use of broad-spectrum antibiotics reduces the risk for mortality to less than 10%
C. Survivors of CDB usually remain in the hospital for 3–5 days
D. Treatment with appropriate antibiotics can reduce the risk for mortality to 12.5%

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Correlation of Pandemic (H1N1) 2009 Viral Load with Disease Severity and Prolonged Viral Shedding in Children

CME Questions

1. A 10-year-old boy is admitted to the emergency department (ED) with fever, cough, and lethargy of approximately 24 hours' duration. The ED staff recognizes that pandemic (H1N1) 2009 infection has been prevalent in the community, and the mother reports that the child's school recently sent a note home informing parents of several cases in the school. The patient's medical history is significant for asthma, but his mother reports that he has not had wheezing or coughing with this illness and she has not used his rescue medications.

Given this child's age and suspected diagnosis, the clinician should be particularly alert for which of the following potential complications?

A. Renal failure
B. Pneumonia
C. Septicemia
D. Bronchitis

2. Which of the following diagnostic tests for pandemic (H1N1) 2009 infection would be most useful to the ED in evaluating this patient?

A. DNA polymerase chain reaction (PCR)
B. Reverse transcription–PCR (RT-PCR)
C. Viral culture
D. Viral antibody levels

3. Diagnostic testing confirms pandemic (H1N1) 2009; comorbidities, such as pneumonia and sepsis, are ruled out. Oseltamivir therapy is begun. The patient is monitored in the ED over the next 24 hours, and is now afebrile, alert, and taking fluids. The mother reports that she has a 2-year-old child at home who appears to be well.

What education should be provided to this parent about the patient's contagiousness?

A. Viral shedding is reduced by use of oseltamivir and nondetectable within 24 hours
B. Viral shedding is higher in children under the age of 13 years, regardless of viral therapy
C. Comorbid respiratory illnesses, such as asthma, are associated with prolonged viral shedding
D. The patient's infection is not severe, and thus his period of viral shedding will be significantly shorter than average

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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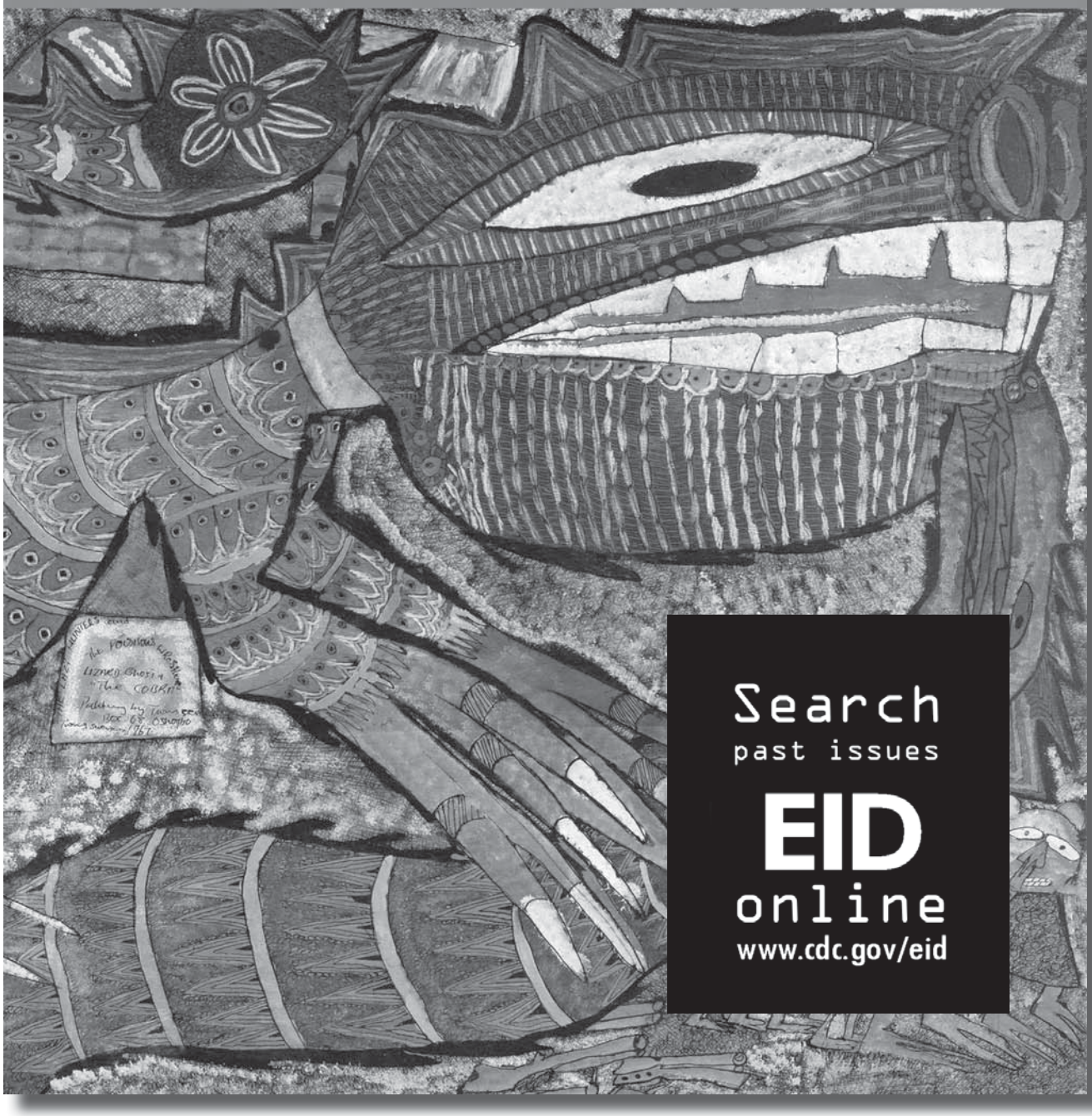
July 2010

Emergence in Africa



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Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

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Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

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Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

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

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

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