EMERGING INFECTIOUS DISEASES®

May 2012

Emerging Viruses

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EMERGING INFECTIOUS DISEASES®

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May 2012

On the Cover

Salvador Dalí (1904–1989)
Daddy Longlegs of the Evening—Hope! (1940)

Oil on canvas (25.4 cm x 50.8 cm)
Fundación Gala-Salvador Dalí, (Artists Rights Society), 2011
Collection of the Salvador Dalí Museum, Inc., St. Petersburg, FL, USA, 2011

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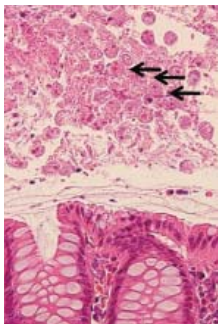
Research



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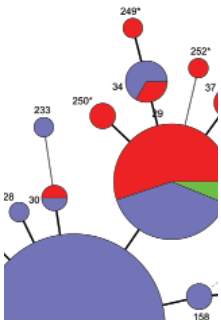

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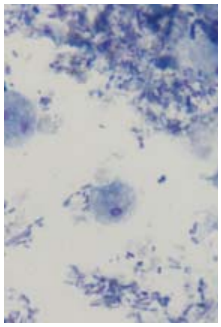

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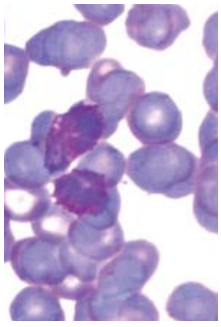

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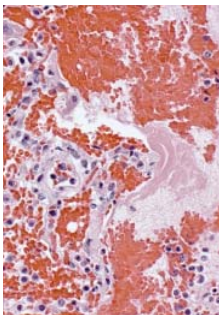

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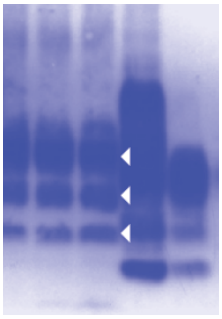

Corrections

About the Cover

Conference Summary

The EID journal is now using **Quick Response codes** (QR codes) to make its content more interactive, engaging, and accessible. QR codes can be interpreted by numerous smartphone apps. Free downloads of readers are available online. These codes will allow readers to access CME, articles, podcasts, and other relevant content, easily from smartphones.

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Risk Factors for Intestinal Invasive Amebiasis in Japan, 2003–2009

Naoyoshi Nagata, Takuro Shimbo, Junichi Akiyama, Ryo Nakashima, So Nishimura, Tomoyuki Yada, Koji Watanabe, Shinichi Oka, and Naomi Uemura

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Release date: April 16, 2012; Expiration date: April 16, 2013

Learning Objectives

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

- Describe yearly change in prevalence of amebic colitis, based on a Japanese study of persons who underwent endoscopy
- Describe independent risk factors for amebic colitis, based on a Japanese study of persons who underwent endoscopy
- Compare risk factors for amebic colitis between HIV-positive and -negative patients, based on a study of Japanese persons who underwent endoscopy.

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We determined yearly change in prevalence and risk factors for amebic colitis caused by intestinal invasive amebiasis among persons who underwent endoscopy and assessed differences between HIV-positive and HIV-negative persons in Japan. A total of 10,930 patients were selected for analysis, of whom 54 had amebic colitis. Prevalence was in 2009 (0.88%, 12/1360) compared with 2003 (0.16%, 3/1904). Male sex (odds ratio [OR] 8.39, 95% CI 1.99–35.40), age <50 years (OR 4.73, 95% CI 2.43–9.20), history of syphilis (OR 2.90, 95% CI 1.40–5.99), and HIV infection (OR 15.85, 95% CI 7.93–31.70) were independent risk factors. No differences in risk factors were identified between HIV-positive and HIV-negative patients. Contact with commercial sex workers was a new risk factor among HIV-negative patients. Homosexual intercourse, rather than immunosuppressed status, appears to be a risk factor among HIV-positive patients.

Amebiasis is caused by the protozoan *Entamoeba histolytica*. Each year, this disease develops worldwide in ≈40–50 million persons and causes 40,000 deaths (*1*,*2*). There are several amebic species of protozoans; *E. histolytica* is a pathogenic ameba that can cause invasive intestinal and extraintestinal disease (*1*–*3*). The most

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frequent manifestations of invasive amebiasis are colitis and liver abscess (*1*,*3*–*5*). Many persons with *E. histolytica* infection are asymptomatic, but invasive disease develops in 4%–10% of persons with symptomatic *E. histolytica* infections over a 1-year period (*1*,*6*–*8*).

Areas with high incidences of amebic infection include India, Africa, Mexico, and parts of Central and South America (*1*,*2*,*9*). In countries with low incidence, such as Taiwan, South Korea, and Australia, invasive amebiasis is uncommon, but reports have indicated that amebiasis is an emerging parasitic infection, particularly among men who have sex with men (MSM) (*10*–*13*). Although epidemics of amebiais have not occurred in Japan, reports from 2001 indicate that invasive amebiasis is common in middle-age men, MSM and HIV-infected patients (*8*,*14*,*15*). In Japan, the prevalence of amebiasis has been increasing according to data from the National Epidemiologic Surveillance of Infectious Diseases (*16*). However, the reasons amebiasis is increasing and the actual prevalence of amebic colitis in daily clinical practice have not been fully clarified. Moreover, some studies in Japan have examined risk factors, but most of these studies have reported case series or case reports without control patients (*14*,*15*,*17*,*18*).

Several studies have indicated that HIV infection is a risk for invasive amebiasis, but no consensus has been reached on this issue (*10*–*12*,*19*). Furthermore, some researchers have suggested that severe invasive amebiasis may develop in HIV-positive patients (*20*–*22*). Susceptibility and clinical factors differ between HIV-positive and -negative patients because of differences in immune status. However, the effect of HIV infection on these risk factors for invasive intestinal amebiasis remains unclear.

To address these issues, we clarified annual changes in prevalence and risk factors for amebic colitis among persons who had undergone endoscopy. These factors were then compared between HIV-positive and HIV-negative patients.

Methods

Study Design

We retrospectively reviewed endoscopy records for 14,923 consecutive patients who underwent colonoscopy at the National Center for Global Health and Medicine (NCGM) (Tokyo, Japan) during 2003–2009. Indications for endoscopy included screening for fecal occult blood test; colorectal cancer; anemia; examinations for symptoms such as constipation, loose stool, diarrhea, hematochezia, and abdominal pain; or therapies for colorectal adenoma, early colorectal cancer, and diverticular bleeding.

We excluded patients who had not been tested for HIV infection, syphilis, or hepatitis B virus (HBV) infection. Patients who underwent endoscopic observation only of the anorectal area and those <15 years of age were excluded. A total of 10,930 patients were selected for analysis.

NCGM has 900 beds and is the largest referral center for HIV/AIDS in Japan. Written informed consent for procedures was obtained from all patients before endoscopy and biopsy. The study protocol was approved by the ethics committee of NCGM.

Sexually Transmitted Diseases

We collected laboratory data for sexually transmitted diseases (STDs), such as HIV infection, syphilis, and HBV infection, before endoscopy. Histories of HBV infection and syphilis were defined as presence of antibody against as hepatitis B surface antigen and positive results in a *Treponema pallidum* hemagglutination test, respectively. In Japan, because only health care workers are vaccinated against hepatitis B, positive results for antibody against hepatitis B surface antigen were attributable to vaccination in few cases.

For HIV-positive patients, we determined CD4 cell counts within 1 week of endoscopy. We categorized CD4 cell counts into 4 groups: >300 cells/μL, 201–300 cells/μL, 101–200 cells/μL, and <100 cells/μL Routes of infection were determined by medical staff who questioned each patient at their first visit to the hospital. Routes were classified into 6 categories: homosexual, bisexual, heterosexual, drug use, untreated blood products, and unknown. We defined sexual preference into 2 categories: MSM and heterosexual. Patients who were not homosexual or bisexual were regarded as heterosexual.

Diagnosis of Amebic Colitis Caused by *E. histolytica* Infection

We performed a biopsy and aspirated intestinal fluid from lesions endoscopically when abnormal findings were seen by endoscopy. Amebic colitis was suspected on the basis of endoscopic findings, such as erythema, edematous mucosa, erosions, white exudates, and ulcers (Figure 1) (*22*,*23*). Negative results for intestinal fluid cultures for bacterial species or acid-fast bacillus were confirmed. Amebic colitis was defined as amebic trophozoites in biopsy specimens stained with both hematoxylin and eosin (Figure 2, panel A) and periodic acid–Schiff (Figure 2, panel B), negative intestinal fluid cultures for other species, negative histologic features for other colonic diseases, and a positive clinical response to metronidazole. Trophozoites showed characteristic hemophagocytosis, which is specific for *E. histolytica* infection (Figure 2, panel A).

Routes of Amebic Infection

When amebic colitis was diagnosed, the physician asked the patient directly for information about the route

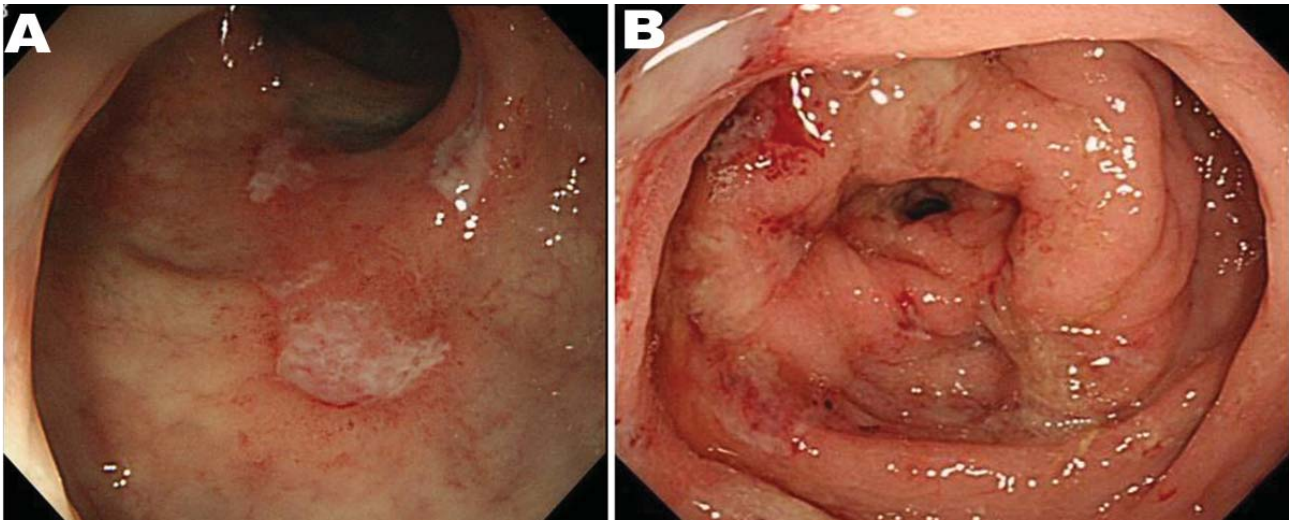

Figure 1. Endoscopic features of amebic colitis, Japan, 2003–2009. A) Colonoscopy showing ulcers in the rectum. B) Colonoscopy showing multiple erosions with exudates surrounded by edematous mucosa in the sigmoid colon.

of amebic infection. The physician confirmed whether the patient had traveled in tropical areas, resided in a facility for the intellectually disabled, was a male or female commercial sex worker (CSW), or had contact with a CSW or MSM. For travel exposure, history of overseas travel in the past year was elicited. Patients to whom none of the above applied were treated as unknown.

Statistical Analysis

We assessed changes in annual prevalence by using the χ^2 test for linear trends. We summarized descriptive data for patients with and without amebic colitis. To determine risk factors for amebic colitis, we estimated the odds ratio (OR) between amebic colitis and clinical factors including age, sex, sexual preference, and history of STDs. We divided patients into 2 age groups, $\geq$50 years and <50 years. We used a multiple logistic regression model with factors that showed $p<0.2$ by univariate analysis. A final model was then developed by backward selection of factors that showed $p<0.05$. The adequacy of this model was evaluated by using the Hosmer-Lemeshow goodness-of-fit test and a receiver operating characteristic area under the curve.

We also conducted subgroup analysis concerning HIV infection. We investigated interactions between the effect of HIV infection and risk factors for amebic colitis. In HIV-positive patients, the relationship between prevalence of amebic colitis and CD4 cell counts in 4 categories was evaluated by using the χ^2 test for linear trends. All statistical

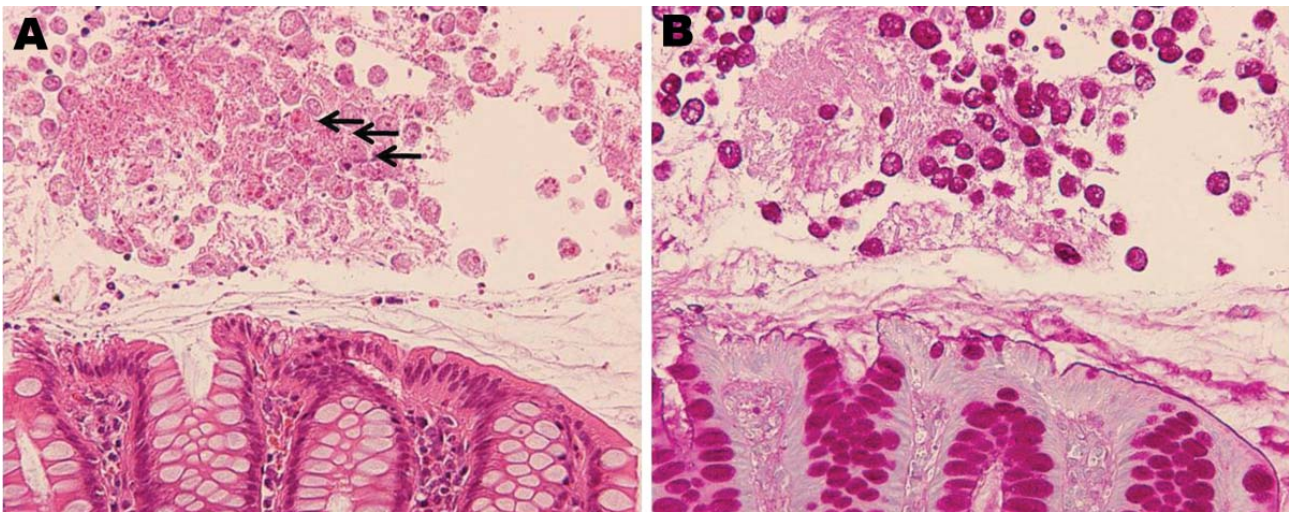

Figure 2. Histologic analysis of amebic colitis, Japan, 2003–2009. A) Trophozoites of *Entamoeba histolytica* ingesting erythrocytes (arrows) (hematoxylin and eosin stain). B) Numerous amebic trophozoites on the mucosal surface (periodic acid–Schiff stain). Original magnification ×200.

analyses were performed by using Stata version 10 software (StataCorp LP, College Station, TX, USA).

Results

Annual Prevalence of Amebic Colitis

Among 10,930 patients, 54 (0.5%) showed development of amebic colitis. Prevalence was 0.16% in 2003 but tended to increase over time (p<0.01 by trend test) (Figure 3). Prevalence was 5.6-fold higher in 2009 than in 2003.

Patient Characteristics

HIV-infected patients constituted 248 (2.3%) of 10,930 patients, and they had a median age of 43 years (interquartile range [IQR] 35–55 years) (Table 1). These HIV-infected patients were predominantly male (91.5%, 227/248). Median CD4 cell count was 230 cells/μL (IQR 89.5–401 cells/μL). Routes of HIV infection included homosexual (58.9%, 146/248), heterosexual (12.5%, 31/248), bisexual (10.5%, 26/248), unknown (12.1%, 30/248), untreated blood products (6.0%, 15/248), and drug use (0%).

Patients with a history of HBV infection constituted 184 (1.7%) of 10,390 patients, and they had a median age of 61 years (IQR 47.5–69 years). These patients were also predominantly male (69.0%, 127/184).

Patients with a history of syphilis constituted 266 (2.4%) of 10,390 patients, and they had a median age of 64 years (IQR 48–74 years). These patients were also predominantly male (76.3%, 203/266).

Risk Factors for Amebic Colitis

Risk factors for amebic colitis were age <50 years (OR 11.4, 95% CI 6.1–22.4), male sex (OR 18.5, 95% CI 4.9–156.7), HIV infection (OR 66.2, 95% CI 36.6–120.7), history of HBV infection (OR 9.0, 95% CI 3.4–20.4) and history of syphilis (OR 19.6, 95% CI 10.2–36.2) (Table 1). Multivariate analysis showed that age <50 years (OR 4.73, 95% CI 2.43–9.20, p<0.001), male sex (OR 8.39, 95% CI 1.99–35.40, p<0.01), HIV infection (OR 15.85, 95% CI 7.93–31.70, p<0.01), and history of syphilis (OR 2.90, 95% CI 1.40–5.99, p<0.01) were independent risk factors for amebic colitis. This logistic regression model was evaluated by using the Hosmer-Lemeshow test (p = 0.44) and receiver operating characteristic area under the curve (0.90).

Comparison of HIV-Positive and HIV-Negative Patients

Annual Prevalence of Amebic Colitis

Numbers of HIV-positive and HIV-negative patients have been increased annually during 2003–2009 in Japan (Figure 4). Among HIV-positive patients, the prevalence in 2009 increased by 2.1-fold over that in 2003 (Figure 4, panel A). Among HIV-negative patients, the prevalence in 2009 increased by 7.1-fold over that in 2003 (Figure 4, panel B).

Risk Factors for Amebic Colitis

Among HIV-positive patients, age <50 years, history of syphilis, and MSM status were risk factors for amebic colitis (Table 2). Immunosuppressed status, such as CD4 cell count <100 cells/μL, was not associated with amebic colitis among HIV-positive patients (Table 2). As CD4 cell counts decreased, the prevalence of amebic decreased (OR 0.3; p = 0.08 by trend test).

Among HIV-negative patients, age <50 years, male sex, history of HBV infection, and history of syphilis were risk factors for amebic colitis (Table 2). No interactions were apparent between HIV infection and risk factors, such as age, sex, history of syphilis, and history of HBV infection.

Route of Amebic Infection

Among HIV-positive patients, all 31 patients with amebic infection were male (Table 3). Of these patients, 28 were MSM and 2 were male CSWs. No patients reported contact with CSWs. The route of infection was unknown for 3 patients.

Among HIV-negative patients, 2 patients were female and 21 were male. Both female patients were CSWs. Of the 21 male patients, 8 had had sexual contact with a female CSW and 7 patients were MSM (2 bisexual and 5 homosexual). The route of infection was unknown for 6 patients.

Discussion

Endoscopic examination combined with biopsy sample collection is a valuable method for confirming suspected amebic colitis, which is often misdiagnosed as inflammatory bowel disease or other forms of infectious colitis caused by the similarity of associated gastrointestinal symptoms (e.g.,

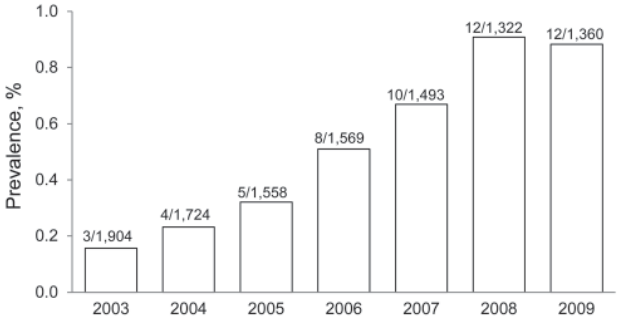

Figure 3. Annual prevalence of amebic colitis, Japan, 2003–2009. Values above bars are no. positive/no. tested.

Table 1. Characteristics and risk factors for 10,930 patients with amebic colitis, Japan, 2003–2009*

Characteristic	All, n = 10,930	Amebic colitis, n = 54	No amebic colitis, n = 10,876	Odds ratio (95% CI)
Median age (IQR)	64 (54–73)	41 (36–52)	65 (54–73)	NA
Age, y				
≥50	8,875 (81.2)	15 (27.7)	8,860 (81.5)	Referent
<50	2,055 (18.8)	39 (72.2)	2,016 (18.5)	11.4 (6.1–22.4)
Sex				
F	4,522 (41.4)	2 (3.7)	4,520 (39.1)	Referent
M	6,408 (58.6)	52 (96.3)	6,356 (58.4)	18.5 (4.9–156.7)
HIV infection				
Negative	10,682 (97.7)	23 (42.5)	10,659 (98.0)	Referent
Positive	248 (2.3)	31 (57.4)	217 (2.0)	66.2 (36.6–120.7)
HBV infection				
Negative	10,746 (98.3)	47 (87.0)	10,699 (84.0)	Referent
Positive	184 (1.7)	7 (13.0)	177 (1.6)	9.0 (3.4–20.4)
Syphilis				
Negative	10,664 (97.6)	37 (68.5)	10,627 (97.7)	Referent
Positive	266 (2.4)	17 (31.5)	249 (2.3)	19.6 (10.2–36.2)

*Values are no. (%) except as indicated. IQR, interquartile range; NA, not applicable; HBV, hepatitis B virus. p values for all comparisons were <0.05, by Mann-Whitney U test.

diarrhea, hematochezia, and abdominal pain) (*14*,*22*,*23*). However, only a few studies have included patients who had undergone endoscopy (*17*,*22*,*23*). In the present study, we performed a large number of endoscopic examinations. The prevalence of patients with amebic colitis was 0.5% (54/10,930) in this 7-year study. This prevalence was far lower than results from serum prevalence studies, which have shown prevalence in children of 8.4% in Mexico (*24*) and 4.2% in Bangladesh (*25*). However, the annual prevalence of the disease showed a tendency to increase to nearly 1% in recent years, and we assume the prevalence will continue to increase in the future.

In the past, amebic infection in Japan was reportedly caused by overseas travel to countries where epidemics occurred or where amebic infection was found in residents of facilities for the intellectually disabled (*16*,*26*). However, patients with these characteristics were not observed in this study. Multivariate analysis indicated that risk factors for amebic colitis in this study were male sex, age <50 years, and histories of syphilis and HIV infection.

The reason male sex was a risk factor might be related to specific sexual preference (*8*,*10*–*15*) because 52 male patients with amebic colitis often had contact with MSM (n = 35) or female CSWs (n = 8). In this study, MSM constituted 90% of men (OR 4.7 for patients with HIV infection), which is consistent with results of previous reports (*8*,*10*–*15*). However, HIV-negative male patients included heterosexual patients, and ≈35% of them had had contact with CSWs. We included CSWs as routes of infection for amebiasis because amebiasis among female CSWs has been reported in Japan (*27*). Therefore, new infection routes other than MSM, which has been considered a risk because of a diversity of sexual activities, should be considered.

Consistent with results of past reports (*8*,*14*,*15*), younger age was a risk factor. One possibility is that younger age represents a risk factor because younger persons are more sexually active, although this was not clarified in the present study.

Histories of syphilis or HIV infection have been noted as risk factors in previous case series (*7*,*15*,*28*). The present study included many patients with HIV infection or history of syphilis, which supports the hypothesis that these factors increase the risk for amebic colitis.

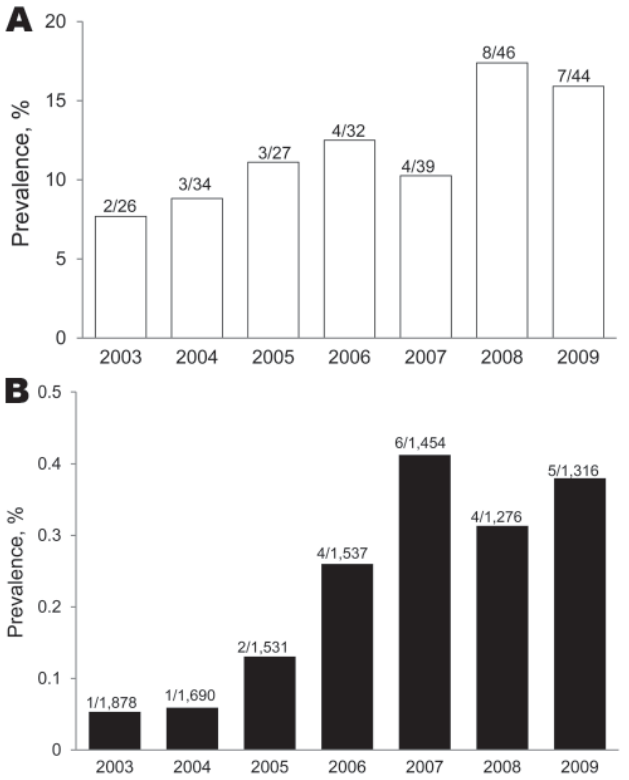

Figure 4. Annual prevalence of amebic colitis in persons with or without HIV infection, Japan, 2003–2009. A) HIV-positive patients. B) HIV-negative patients. Values above bars are no. positive/no. tested.

Table 2. Risk factors for amebic colitis among HIV-positive and -negative patients, Japan, 2003–2009*

Risk factor	HIV-positive patients: Amebic colitis, n = 31	HIV-positive patients: No amebic colitis, n = 217	HIV-positive patients: OR (95% CI)	HIV-positive patients: p value	HIV-negative patients: Amebic colitis, n = 23	HIV-negative patients: No amebic colitis, n = 10,659	HIV-negative patients: OR (95% CI)	HIV-negative patients: p value	p value for interaction
Age, y									
≥50	6	83	Referent		9	8,777	Referent		
<50	25	134	2.6 (1.0–8.0)	0.04	14	1,882	7.3 (2.9–19.0)	<0.01	0.11
Sex									
F	0	21	Referent		2	4499	Referent		
M	31	196	4.6† (0.8–∞)	0.11†	21	6,160	7.7 (1.9–67.5)	<0.01	0.56
HBV infection									
Negative	26	189	Referent		21	10,510	Referent		
Positive	5	28	1.3 (0.4–3.8)	0.62	2	149	6.7 (0.8–27.9)	<0.01	0.07
Syphilis									
Negative	16	163	Referent		21	10,464	Referent		
Positive	15	54	2.8 (1.2–6.5)	<0.01	2	195	5.1 (0.6–21.1)	0.01	0.48
Sexual preference									
Heterosexual	3	73	Referent		ND	ND	ND	ND	ND
MSM	28	144	4.7 (1.4–25.0)	<0.01	ND	ND	ND	ND	ND
CD4 cell count/μL									
>300	14	82	Referent	ND	ND	ND	ND	ND	ND
201–300	5	31	0.9 (0.3–2.8)	ND	ND	ND	ND	ND	ND
100–200	9	39	1.35 (0.5–3.4)	ND	ND	ND	ND	ND	ND
<100	3	65	0.3 (0.07–1.0)	0.15	ND	ND	ND	ND	ND

*OR, odds ratio; HBV, hepatitis B virus; ND, no applicable data; MSM, men who have sex with men.
†Analysis by using exact logistic regression model because number in cell was 0.

Among STDs, HIV infection showed the highest risk ratio, a ≈16-fold increase. HIV infection has been identified as a risk factor for invasive amebiasis in many studies (*10*–*12*,*21*), although many details of this risk remain unclear (*19*,*29*).

We presumed that compromised immune function increased the susceptibility of patients to invasive diseases. However, no relationship was seen between low CD4 cell counts and development of amebic colitis. Under existing conditions, the reason for HIV infection representing a risk factor for amebic colitis is considered the preference for oral–anal sex as a common risk factor for both infectious conditions.

We compared prevalence and risk factors between amebic colitis patients with and without HIV infection. An incidence of 0.1% (4/5,193) has been reported in studies of HIV-negative patients with positive results for occult blood in feces (*17*), and our results were similar. However, annual prevalence increased in 2009 (0.38%, 5/1,316) compared with 2003 (0.05%, 1/1,878), and the rate of increase was higher than that for HIV-positive patients. This result calls for careful attention in hospitals in which patients with HIV infection are not commonly encountered. In terms of risk factors, ORs for age, sex, and history of HBV infection or syphilis in our study did not vary according to HIV infection status.

Table 3. Route of amebic infection for 54 persons, Japan, 2003–2009*

Route	HIV positive, no. (%), n = 31	HIV negative, no. (%), n = 23
Travelers from tropical areas	0	0
Residents of facilities for intellectually disabled	0	0
MSM, male CSW	28, 2 (90.3)	7 (30.4)
Female CSW	0	2 (8.7)
Contact with female CSW	0	8 (34.8)
Unknown	3 (9.7)	6 (26.1)

*MSM, men who have sex with men; CSW, commercial sex worker.

Some limitations need to be considered in this study. First, Japan has not had epidemics of amebiasis, and data in this study were obtained from a metropolitan area. In addition, our hospital treats the largest number of patients with HIV infection in Japan. Second, selection bias was present because participants were patients who had undergone endoscopic examinations, which are highly likely to be performed for healthy patients. In addition, patients suspected before examination of having amebiasis might have been more likely to be actively included in the study. Third, the number of patients with amebic colitis was small; thus, the statistical power of the study might have been low. Fourth, a retrospective design was used for this investigation. With regard to HBV infection or history of syphilis, judgments had to be made for using results of serologic testing in some cases. In addition, determination of sexual preferences and overseas travel had to be based on the self-reports of patients.

In recent years, infectious diseases caused by *E. histolytica* and HIV have been increasing in Japan (*15*,*16*,*30*). HIV infection is a particularly serious problem because its incidence is consistently increasing in Japan while decreasing in western countries (*30*,*31*).

Numbers of patients with both infectious diseases studied are predicted to increase because little is known about measures to prevent infection in association with

a diversity of sexual activities. Amebic infection, in particular, is scarcely recognized as a sexually acquired infection, and improved education is needed to prevent these diseases. In Japan, measures to prevent the spread of HIV and amebic infections are urgently needed.

In conclusion, although this study was conducted at 1 center and involved retrospective analysis of a relatively small number of cases of amebic infection, the results suggest that the number of amebic colitis patients with or without HIV infection is tending to increase in Japan. Younger men with syphilis and HIV infections are at increased risk for amebic colitis. Route of infection differed slightly in that contact with CSWs was more frequent among HIV-negative patients than among HIV-positive patients. Among HIV-positive patients, homosexual intercourse, and not immunosuppressed status, seems to be a risk factor for amebic colitis.

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Dr. Nagata is a gastroenterologist at the NCGM in Tokyo, Japan. His research interests include gastrointestinal infections such as esophageal candidiasis, cytomegalovirus-related disease, mycobacterial infections, intestinal amebiasis, intestinal spirochetosis, chlamydial infection, and HIV-related gastrointestinal disease.

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Invasive *Haemophilus influenzae* Serotype e and f Disease, England and Wales

Shamez N. Ladhani, Sarah Collins, Anna Vickers, David J. Litt, Carina Crawford, Mary E. Ramsay, and Mary P.E. Slack

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Learning Objectives

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

- Analyze the epidemiology of Hie and Hif
- Distinguish the risk for mortality associated with invasive cases of Hie and Hif
- Evaluate invasive infection with Hie and Hif among children
- Evaluate invasive infection with Hie and Hif among older adults.

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Haemophilus influenzae infection causes serious invasive disease, but incidence of the most virulent serotype, Hib, has dropped since introduction of routine Hib vaccination. In England and Wales, the incidence of 2 other serotypes, Hie and Hif, is increasing; during 2001–2010, there was an 11.0% year-on-year increase in Hif and a 7.4% increase in Hie. In 2009–2010, Hif incidence was 0.090/100,000 persons and Hie incidence 0.030/100,000, with higher rates among infants and older adults. Hie had a more severe clinical course; although outcome at 6 months was comparable for the 2 serotypes, case-fatality rate within 7 days of diagnosis was higher for Hie, even after adjustment for age and comorbidities. Multilocus sequence typing revealed a single major circulating clone for both Hif (sequence type 124; 89/99 isolates, 90%) and Hie (sequence type 18; 21/33, 64%), but no association between type and clinical disease or outcome was found.

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Haemophilus influenzae is a gram-negative coccobacillus that inhabits the human upper respiratory tract and causes serious invasive infections. The organism can be nonencapsulated (ncHi) or classified into 1 of 6 serotypes (Hia–Hif) on the basis of capsular polysaccharide characteristics. Hib is the most virulent serotype; before routine vaccination was implemented, Hib was responsible for >80% of all invasive *H. influenzae* infections, mainly in children <5 years of age (*1*). Routine immunization with Hib conjugate vaccines, however, has greatly reduced the incidence of invasive Hib disease worldwide (*1*). As a consequence, invasive infections caused by other *H. influenzae* serotypes have become relatively more common.

The United Kingdom introduced the Hib conjugate vaccine in 1992 as a 3-dose infant schedule alongside a 12-month catch-up campaign for all children <5 years of age (*2*). This campaign resulted in a rapid reduction in invasive Hib disease across all age groups (*2*). After 1999, however, Hib disease increased, particularly among toddlers (*3*). Potential explanations for this increase include a greater than expected decline in Hib antibodies after primary immunization, waning of herd immunity after the initial catch-up campaign, and use of a less immunogenic Hib combination vaccine with diphtheria, tetanus, and acellular pertussis (DTaP-Hib) in 2000–2001 (*3*). This resurgence led to establishment of several control measures—re-introduction of a whole-cell pertussis–containing Hib vaccine (DTwP-Hib) in 2002, an Hib booster campaign for toddlers in 2003, and a routine 12-month Hib booster in 2006—that resulted in rapid control of Hib disease (*3*).

Because conjugate vaccines also reduce pharyngeal carriage (*4*), concerns have been raised that other *H. influenzae* strains could fill the ecologic niche and result in invasive disease (serotype replacement) (*5*), although no studies have demonstrated increased carriage rates of other *H. influenzae* serotypes after routine Hib vaccination (*6*). Some countries have reported small but significant increases in invasive ncHi disease after routine Hib vaccination (*7–10*); others have not (*11*,*12*). Increases in invasive Hif disease have also been reported, albeit with a small number of cases over years (*8*,*13–16*).

In England and Wales, the Health Protection Agency (HPA) has conducted enhanced national *H. influenzae* surveillance (encompassing a denominator population of ≈55 million persons) for >2 decades. The continuing decline in invasive Hib disease meant that in 2009, for the first time, other encapsulated *H. influenzae* serotypes overtook Hib as the most prevalent causes of invasive *H. influenzae* disease (*17*). During 2009–2010, therefore, the HPA investigated the clinical, epidemiologic, and microbiologic characteristics of invasive encapsulated *H. influenzae* disease.

Methods

The Health and Social Care Act 2001 authorizes the HPA to process confidential patient information for public health purposes (www.legislation.hmso.gov.uk/si/si2002/20021438.htm). HPA provides a national identification and serotyping service for invasive clinical *H. influenzae* isolates for all National Health Service hospital microbiology laboratories in England and Wales through its Haemophilus Reference Unit and conducts enhanced national surveillance through a combination of isolate submission, routine laboratory reporting, and clinical reporting schemes (*3*,*18*). Clinicians, microbiologists, and public health physicians are encouraged to report invasive *H. influenzae* cases to the HPA and submit isolates for species confirmation and serotyping (*19*). The HPA also receives electronic reports of clinically significant invasive pathogens from microbiologists in ≈400 National Health Service laboratories who are routinely contacted to submit isolates to the HPA if not already done, as well as notifications of all *H. influenzae*–related deaths from the Office for National Statistics (ONS) (www.statistics.gov.uk) (*18*).

We defined invasive *H. influenzae* disease as isolation of the organism from a normally sterile site. Localized infections such as pneumonia were included if accompanied by a sterile site isolate. Isolates were confirmed as *H. influenzae* by growth requirement for X and V factors (*20*) and *ompP2*-specific PCR positivity (*21*). Capsulation status was determined by PCR by using *bexA*-specific primers (*22*). Capsular type was confirmed by capsule-specific PCR by using primers for types Hia–Hif (*23*) and slide agglutination (*20*).

Laboratory-confirmed Hif and Hie infections diagnosed during 2009–2010 were followed up 3 months after diagnosis by sending a questionnaire to the patient's general practitioner (www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/HaemophilusInfluenzaeTypeB/EnhancedSurveillanceHaemophiliusInfluenzae) and requesting a copy of the hospital discharge summary. A reminder letter was sent to nonresponders after 8 weeks; missing or outstanding information was obtained 1 month later by telephone. Postmortem reports were obtained for all fatal cases that were referred to a coroner. In July 2011, the final outcomes of all patients were confirmed, and causes of death were obtained from the ONS death registrations dataset. Hie/Hif–attributable death was defined as isolation of a pure growth of Hie/Hif from a normally sterile site with clear clinical, radiologic, or histopathologic evidence of invasive bacterial infection and appropriate exclusion of other diagnoses.

Multilocus sequence typing (MLST) was performed according to methods previously described (*24*). Sequence types (STs) were assigned by using an online reference da-

tabase (http://haemophilus.mlst.net). Minimum-spanning trees were constructed by using Bionumerics version 6.1 software (Advanced Maths, Sint-Martins-Latem, Belgium) as described (*25*).

Data were analyzed by using Stata version 11.0 (StataCorp LP, College Station, TX, USA). Categorical variables were expressed as proportions and compared by using the χ^2 or Fisher exact test. Data that did not follow a normal distribution were presented as medians with interquartile ranges (IQR). Annual population estimates were obtained from ONS. The binomial method was used to calculate 95% CIs for incidence rates. To estimate any increase in Hie/Hif incidence over time, an overdispersed Poisson regression model was fitted for the annual number of Hie/Hif cases. Yearly changes in population and proportion of total *H. influenzae* isolates serotyped were included in the model as offsets.

Results

Epidemiology

During 2001–2010, incidence of invasive Hib disease rose and then declined, while Hif and Hie incidence remained stable (Figure 1). Hif incidence increased 11.0% (95% CI 8.3%–13.8%; p <0.0001) year-on-year, and Hif incidence increased 7.4% (95% CI 0.9%–14.2%; p = 0.024) over the 10 years. Only 6 Hia cases and 2 each of Hic and Hid were reported; none were reported during 2009–1010. In 2009, for the first time, Hif incidence exceeded that of Hib (Figure 1).

In 2009–2010, a total of 1,275 invasive *H. influenzae* infections were reported, including 715 (56.1%) ncHi, 69 (5.4%) Hib, 99 (7.8%) Hif, and 33 (2.6%) Hie. In 359 cases (28.2%), the serotype was not known because the isolate was not submitted to the reference laboratory or because the isolate died in transit. Annual incidence of invasive disease was 0.090 (95% CI 0.073–1.10) per 100,000 population for Hif and 0.030 (95% CI 0.021–0.042) per 100,000 for Hie; incidence was highest among infants and older adults (Figure 2).

Clinical Cases During 2009–2010

Clinical questionnaires were completed for all case-patients at a median of 6.8 (IQR 4.4–9.6) months after disease onset. Of the 99 Hif and 33 Hie infections, half (66/132, 50%) occurred in adults ≥65 years of age (Table). Most case-patients were white (121, 91.7%); 6 were Pakistani, 2 Black African, 2 Indian, and 1 Chinese. Underlying conditions (comorbidities) were present in 105/132 (79.6%) case-patients; these were more prevalent in adults (95/107 [88.8%]) than in children (10/25 [40.0%]; χ^2 = 29.6; p<0.001) and increased with age: 0/10 (0.0%) infants, 10/15 (66.7%) 1–14-year-olds, 7/10 (70.0%) 15–44-year-olds, 25/31 (79.6%) 45–64-year-olds, and 63/66 (95.5%) ≥65-year-olds (χ^2 for trend 41.5; p<0.0001). The prevalence of ≥2 comorbidities also increased with age, from 0 among infants to 40/66 (60.6%) among ≥65-year-olds. The overall case-fatality rate (CFR) was 47/132 (35.6%) and also increased with age (1/10 [10.0%], 1/15 [6.7%], 2/10 [20.0%], 8/23 [25.8%] and 35/66 [53.0%], respectively). CFR varied by clinical diagnosis and was highest for pneumonia (36/78, 46.2%) and septicemia (7/18, 38.9%) and lower for other clinical presentations (3/19, 15.9%) and meningitis (1/17, 5.9%)

Patients with Hif and Hie infections did not differ significantly by sex, age distribution, or presence or number of comorbidities, although malignancy/immunosuppression was overrepresented among Hif infections (Table). In particular, 27/99 (27.3%) patients with Hif infection had malignancy: 10 (37.0%) with solid organ tumors, 9 (33.3%) with myeloma, and 8 (29.6%) with other hematologic malignancies. By comparison, 1 Hie patient had a solid organ tumor (1/33, 3.0%; χ^2 = 8.7; p = 0.003). In 1 patient, Hif infection was the initial indication of myeloma. Initial signs and symptoms for Hif versus Hie were also similar, except for hepatobiliary infection, which was more prevalent among Hie case-patients (Table). Also, meningitis was ≈2× more common among patients with Hie, although this finding was not statistically significant. Among case-patients with meningitis, 5/7 (71.4%) Hie were previously healthy; 1/7 (14.3%) died, and half the survivors (3/6) had long-term sequelae. In contrast, only 3/10 (30.0%) Hif meningitis patients were previously healthy; all recovered without complications.

CFRs were similar for patients with Hif and Hie overall and for those with comorbidities but significantly lower for previously healthy patients with Hif versus Hie (0/18 [0.0%] vs. 3/9 [33.3%]; χ^2 = 6.75; p = 0.009). Among Hif

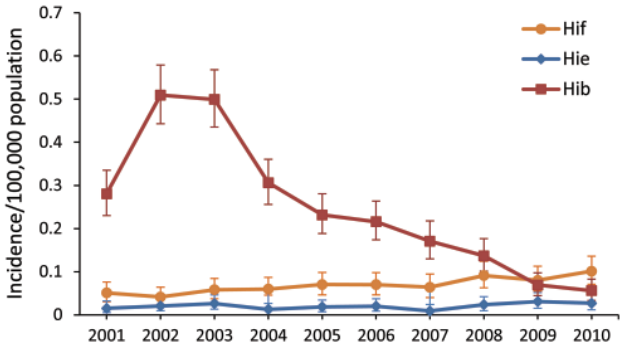

Figure 1. Epidemiology of invasive encapsulated *Haemophilus influenzae* disease, England and Wales, 2000–2009. Serotypes Hib, Hie, and Hif predominated, with Hib incidence dropping rapidly after re-introduction of a whole-cell pertussis–containing Hib vaccine in 2002, a Hib booster campaign for toddlers in 2003, and a routine 12-month Hib booster in 2006. During the period shown, only 6 cases of Hia and 2 each of Hic and Hid were reported. Error bars indicate 95% CIs.

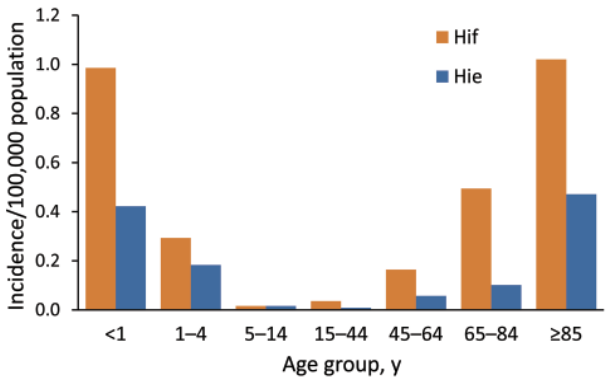

Figure 2. Incidence of invasive *Haemophilus influenzae* serotypes e and f infections, by age group, England and Wales, 2009–2010.

case-patients, CFR increased with the number of comorbidities: 0 for no comorbidities, 11/32 (34.4%) for 1 comorbidity, and 23/49 (46.9%) for ≥2 comorbidities ($\chi^2 = 11.7$; p = 0.002). This trend was not observed for Hie case-patients (3/9 [33.3%], 4/9 [55.5%], and 5/15 [33.3%], respectively). After adjustment for age and comorbidity, patients who had Hie had a 2.6× greater risk for death than did those who had Hif (hazard ratio 2.63, 95% CI 1.14–6.10; p = 0.024). Moreover, the interval between diagnosis and death was significantly shorter for Hie (Table), even after adjustment for age and comorbidities (Figure 3). Among case-patients with pneumonia, CFR within 7 days after infection was higher for those with Hie than Hif (Table); this difference remained significant even after adjusting for age and comorbidity (OR 5.1, 95% CI 1.4–18.6; p = 0.014).

Infections in Children

Children <15 years of age accounted for 25/132(18.9%) Hie/Hif infections. Ten cases (7 Hif, 3 Hie) occurred in the first year of life, with patient age evenly distributed from 1 to 10 months of age and no cases among neonates. None of the infants had been born prematurely or had comorbidities; 9/10 had meningitis, and 1 had Hif septic arthritis. All Hif case-patients recovered uneventfully, but all 3 Hie case-patients had meningitis and either died of purulent meningitis (1 patient) or had long-term sequelae (bilateral sensorineural deafness [1] or seizures [1]).

Among children 1–4 years of age, 8 had Hif and 5 had Hie infection. Of the 8 Hif case-patients, 6 had comorbidities: malignancy/immunosuppression (4 patients) or chronic lung disease of prematurity (2; pneumonia developed in both). There were 2 other cases of pneumonia, 1 of meningitis in a previously healthy child, 2 of septicemia, and 1 of cellulitis; none of the patients needed intensive care, and all survived. Two of the Hie case-patients had comorbidities, 1 with multiple complications of extreme prematurity who developed meningitis and 1 with complex congenital heart disease who developed endocarditis. Three previously healthy toddlers developed cellulitis (recovered without complications), meningitis (bilateral sensorineural deafness developed), and pneumonia (died).

Among older children 5–14 years of age, only 2 had Hie/Hif infections: 1 Hif septicemia case in a child with malignancy, and 1 Hie meningitis case after otitis media in a previously healthy child. Both patients recovered uneventfully.

Adults 15–64 Years of Age

Among 15–44-year-olds, 8 Hif and 2 Hie infections occurred. Comorbidities were present in 5/8 Hif case-patients. Pneumonia was the most common diagnosis (4 patients), followed by septicemia (3) and septic arthritis (1). Two Hif septicemia cases occurred in pregnant women at 8 and 25 weeks' gestation; 1 infection resulted in septic abortion. One man who had a comorbid malignancy died within 7 days after infection. Both Hie case-patients had comorbidities: 1 had chronic liver disease, had septicemia develop, and died >1 month later of the underlying disease; 1 had HIV, had pneumonia develop, and survived.

Among 45–64-year-olds, 23 had Hif and 8 had Hie infection. Most Hif cases had comorbidities (20/23, 87.0%) and an initial diagnosis of pneumonia (14/23, 60.9%); 3 cases each had an initial diagnosis of meningitis and septicemia. Of the 6/23 (26.1%) case-patients who died, 1 with chronic obstructive pulmonary disease died of pneumonia within 7 days, and 5 died of an underlying malignancy >3 months later. The 3 previously healthy Hif case-patients had pneumonia (2 patients) and septic arthritis (1) and recovered uneventfully. Hie case-patients had pneumonia (5), meningitis (1), cholangitis (1), and cholecystitis (1); 6/8 had comorbidities. Two Hie case-patients with comorbidities died, 1 within 7 days after multiorgan failure, and 1 after 3 months of underlying malignancy.

Adults ≥65 Years of Age

Half the infections (52 Hif, 14 Hie) occurred in this age group. Fifty (96.2%) Hif case-patients had comorbidities (11 with malignancy), and most had pneumonia (35 patients) or septicemia (9). CFR was highest in this age group (27/50, 54.0%), although only 6 (12.0%) case-patients died within 7 days and an additional 13 (26.0%) within 28 days. The 2 previously healthy Hif case-patients had initial diagnoses of pneumonia and supraglottitis but recovered uneventfully. Of the 14 Hie infections, 13 had comorbidities (only 2 had malignancy). Pneumonia (10 patient) was the most common clinical presentation; 1 case each of supraglottitis, urogenital septicemia, cholangitis, and cholecystitis occurred. Eight of 14 (57.1%) Hie case-patients died; 6 with pneumonia and 1 with biliary

Table. Characteristics of patients with invasive Hie and Hif infections, England and Wales, 2009–2010*

Characteristic	Hie, n = 33	Hif, n = 99	All, n = 132	p value
Median age, y (IQR)	62 (6–79)	65 (46–77)	65 (41–78)	0.54
Male sex	17 (51.5)	47 (47.5)	64 (48.5)	0.69
Underlying conditions				
0	9 (27.3)	18 (18.2)	10 (20.5)	0.26
1	9 (27.3)	32 (32.3)	41 (31.1)	0.59
≥2	15 (45.5)	49 (49.5)	64 (48.5)	0.69
Type of underlying condition†				
Heart disease	12 (36.4)	28 (28.3)	40 (30.3)	0.38
Lung disease	7 (21.2)	28 (28.3)	35 (26.5)	0.43
Gastrointestinal disease	4 (12.1)	7 (7.1)	11 (8.3)	0.36
Renal disease	6 (18.2)	12 (12.1)	18 (13.6)	0.38
Metabolic/endocrine	8 (24.2)	13 (13.1)	21 (15.9)	0.13
Malignancy/immunosuppression	4 (12.1)	31 (31.3)	35 (26.5)	0.031
Neurologic condition	3 (9.1)	6 (6.1)	9 (6.8)	0.55
Other risk factors‡	1 (3.0)	5 (5.1)	6 (5.3)	0.63
Type of *Haemophilus influenzae* illness				
Pneumonia	17 (51.5)	61 (61.6)	78 (59.1)	0.47
Meningitis	7 (21.2)	10 (10.1)	17 (12.9)	0.099
Septicemia	2 (6.1)	16 (16.2)	18 (13.6)	0.14
Hepatobiliary infection	3 (9.1)	1 (1.0)	4 (3.0)	0.019
Septic arthritis	0	4 (4.0)	4 (3.0)	0.24
Epiglottitis	1 (3.0)	3 (3.0)	4 (3.0)	1.00
Cellulitis	1 (3.0)	4 (4.0)	5 (3.8)	0.79
Urogenital infection	1 (3.0)	0	1 (0.8)	0.082
Endocarditis	1 (3.0)	0	1 (0.8)	0.082
CFR				
Overall	10/33 (41.7)	34/99 (42.0)	44/132 (41.9)	0.98
No underlying condition	3/9 (33.3)	0/18	3/27 (11.1)	0.009
1 underlying condition	5/24 (20.8)	9/81 (11.1)	14/105 (13.3)	0.22
≥2 underlying conditions	3/15 (20.0)	7/49 (14.3)	10/64 (15.6)	0.59
Attributable CFR	10/33 (30.3)	12/99 (12.1)	22/132 (16.7)	0.015
CFR within 7 d	8/33 (24.2)	8/99 (8.8)	16/132 (12.1)	0.014
CFR within 28 d	10/33 (30.3)	15/99 (15.2)	25/132 (18.9)	0.054
CFR for meningitis§	1/7 (14.3)	0/10	1/17 (5.9)	0.22
CFR for pneumonia§	7/17 (41.2)	7/61 (11.5)	14/78 (18.0)	0.005
CFR for septicemia§	0/2	1/16 (6.3)	1/18 (5.6)	0.72
CFR for other conditions§	0/7	0/12	0/19	

*Values are no. cases (%) or no. cases/no. in category (%), unless otherwise specified. Hie, *Haemophilus influenzae* serotype e; Hif, *H. influenzae* serotype f; IQR, interquartile range; CFR, case-fatality ratio.
†Some case-patients had ≥1 underlying condition.
‡For Hie, long-term complications of extreme prematurity (n = 1); for Hif, alcohol dependency (n = 3), long-term complications of extreme prematurity (n = 1), and schizophrenia (n = 1).
§Death within 7 d.

sepsis died within 7 days, and 1 died later of underlying malignancy.

Characterization of Hie and Hif Isolates

Characterization of clinical isolates by MLST showed 2 distinct phylogenetic clusters around a dominant ST within each serotype (Figure 4). Twenty-one (64%) Hie isolates were ST18, while other isolates diverged from this ST at 1 (single-locus variant) or 2 (double-locus variant) of 7 genomic MLST loci. Among Hif isolates, 89 (90%) were ST124; 4 STs were single- or double-locus variants of this ST, and the remaining 2 differed from ST124 at 3 (ST968) and 4 (ST16) loci. STs 966–970 and 973 are previously unreported types assigned as a result of this study. Except for ST16 (which shared its *atpG* allele with all Hie isolates) and ST66 (which shared its *fucK* allele with most Hif isolates), the 2 serotype clusters did not share any individual MLST alleles in common. Similarly, apart from occasional shared *fucK* alleles, neither cluster had alleles in common with contemporary Hib isolates (data not shown). A minimum-evolution tree constructed from concatenated DNA sequences from the 7 MLST loci suggested that the Hie and Hif isolates in this study were evolutionarily closely related to each other but distantly related to 64 Hib isolates from the same period (data not shown).

Discussion

In England and Wales, encapsulated *H. influenzae* other than Hib rarely cause invasive disease; among those, Hie and Hif are most common. These serotypes are similar to ncHi in that they cause invasive disease mainly in older adults, who often have comorbidities and present with pneumonia. However, although Hie is 3× less common than Hif, it appears to be more virulent, with more complications of meningitis and higher infection-attributable CFR, even after adjustment for age and comorbidities.

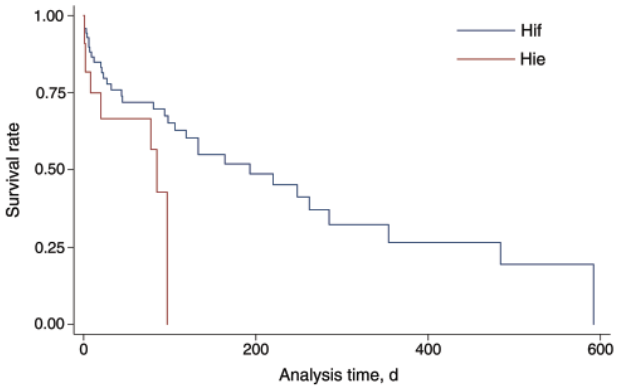

Figure 3. Kaplan-Meier curve for outcome among patients with *Haemophilus influenzae* serotype e and f infections, after adjustment for age and comorbidities, England and Wales, 2009–2010.

The HPA national reference service for *H. influenzae* species confirmation and serotyping provides a rich source of epidemiologic and molecular surveillance data, and the consistently high proportion of serotyped isolates with data enhancement from multiple sources further strengthens the quality and completeness of the surveillance program (*18*). This data source enables more accurate interpretation of trends in disease rates and pathogens over time, as demonstrated by the relatively stable trends in the rare *H. influenzae* serotypes over the past decade. Despite the small number of cases, our results show small but gradual annual increases in invasive Hie and Hif disease. These increases are most likely to be a consequence of 1) the expansion of at-risk populations in industrialized countries resulting from increased survival rates among those at the extremes of age, who often have long-term comorbidities; 2) better survival prognosis among patients with malignancy; and 3) increased use of immunosuppressive therapy for immunologic and rheumatologic conditions.

Other countries have also reported an increase in invasive Hif disease since routine Hib vaccination was introduced. In 1 surveillance study involving multiple states in the United States, Hif incidence increased from 0.14/100,000 population in 1989 to 1.9/100,000 in 1994 (*13*); in Utah, Hif incidence increased from 0.14/100,000 person-years during 1998–2008 to 0.48/100,000 during 2007–2008 (*8*). The US Active Bacterial Core surveillance also recently reported an increase in invasive Hif disease, from 0.06 cases/100,000 population in 1989 to 0.25/100,000 in 2008 (*16*). In Sweden, invasive Hif disease increased 2.3% annually during 1997–2009 (*10*); however, other studies, including a recent study in Europe involving national surveillance data from 14 countries over 11 years, did not show any changes in Hif incidence (*7*,*26*). In relation to Hie, perhaps because of the small number of invasive cases caused by this serotype, we identified only the recent US Active Bacterial Core surveillance study that reported a small increase in disease incidence over 2 decades (*16*).

In addition to national epidemiologic surveillance, we prospectively collected clinical information for all laboratory-confirmed infections. Most published reports of Hie and Hif infection are in the form of individual cases, with few studies reporting a sufficient number of cases focusing on clinical aspects of specific *H. influenzae* serotypes (*10*,*13*,*27*). Our results indicate that Hie and Hif share many features characteristic of invasive ncHi disease, such as patient age distribution, higher risk for infection among vulnerable populations, and higher CFR compared with Hib (*7*). Certain features that were previously attributed primarily to ncHi, such as causing hepatobiliary disease (*28*,*29*) and disease during pregnancy (*30*), were also observed among Hie and Hif case-patients, respectively, in our study. The proportion of case-patients with comorbidities in our cohort was similar to previous smaller studies (60%–80%) (*13*,*27*,*31*–*34*) and to that among ncHi case-patients (*13*,*26*,*33*,*35*–*37*). Several Hif case-patients had

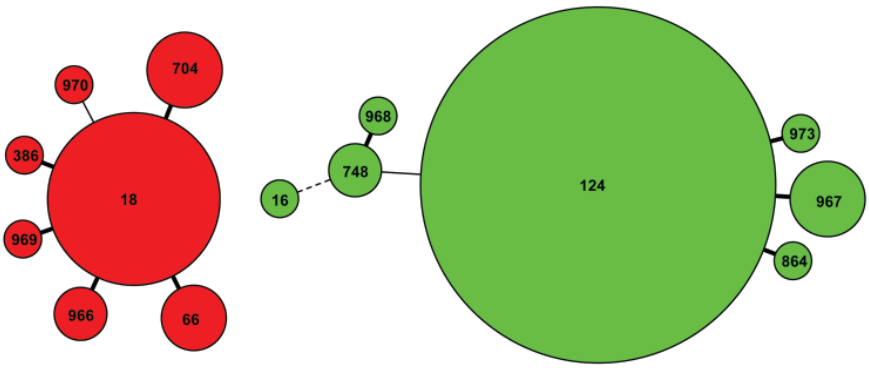

Figure 4. Minimum spanning tree showing the relationship between multilocus sequence types of 33 *Haemophilus influenzae* serotype e (Hie, red) and 99 Hif (green) strains isolated from patients in England and Wales, 2009–2010. The tree was derived from the 7 multilocus sequence type alleles of each isolate; the number within each circle represents a unique sequence type, and the size of the circle illustrates the proportion of strains with that sequence type (the smallest circle represents 1 isolate). Thick lines, thin lines, and dotted lines separating circles indicate single-, double-, and triple-locus variants, respectively.

disorders of B-cell immunity, such as chronic lymphocytic leukemia and multiple myeloma, which have also been reported among persons with invasive ncHi; this finding suggests a major role for humoral immunity (*10*). Declining B-cell function has also been speculated to explain the increasing incidence with age (*10*). The finding that none of the infants in this study had any reported comorbidities at infection, however, is intriguing. Most children and adults outside this age group had comorbidities, which suggests that some of these infants might have as-yet-undetected subtle immunologic abnormalities that made them vulnerable to opportunistic infections.

Although Hie and Hif should be compared with caution because of the relatively small number of cases, our results suggest that Hie causes more severe clinical disease. This observation is supported by recent population-based studies that have reported a worse outcome for patients with Hie than for those with Hif, including a CFR of 24% versus 10.0% (p = 0.003) in a study in Europe (*7*) and 32% versus 6% (p = 0.002) in a US study (*38*). One possible reason that Hie has a lower incidence but it is more fatal than Hif could be that this serotype is less pathogenic and infects persons who are older and/or in much poorer health and who, therefore, are more likely to die. This hypothesis, however, is not supported by our study because the median age at disease onset and the prevalence of any or multiple comorbidities were similar, and the 7-day CFR remained significant even after adjusting for these risk factors. However, the 6-month CFR for the 2 serotypes was similar, which suggests that these pathogens can infect highly vulnerable persons who, even if they survived the infection, subsequently succumbed to their underlying illness; this finding has been also observed for invasive ncHi disease (*10*,*35*). For example, a recent study in Sweden reported that the 28-day CFR for invasive ncHi disease was 8%, but 1-year CFR increased to 29% (*10*).

MLST analysis of the clinical isolates revealed dominant Hie (ST18) and Hif (ST124) types that have been reported among bacterial strains from Europe and North America (http://haemophilus.mlst.net) (*24*). In 2 recent independent studies in Canada, ST124 was also the dominant Hif type, and the Hie types detected differed from ST18 at only a single MLST locus (*9*,*39*). None of the Hie or Hif MLST types in our study have been reported outside of their respective serotypes. These observations, combined with phylogenetic analysis of MLST sequences across capsulated and noncapsulated *H. influenzae* isolates in our and other studies (*24*,*40*), suggest that any increase in Hie and Hif disease is not due to genetic capsule switching events between these serotypes and Hib.

In conclusion, invasive Hie and Hif infections are rare in England and Wales, but their incidence is increasing slowly. The clinical features are similar to ncHi, although Hie appears to be associated with more severe disease and worse infection-attributable outcome. Given that most infections occur among persons with comorbidities, it would be prudent to investigate all previously healthy persons in whom Hie/Hif infections develop for possible underlying immune deficiency, malignancy, or other undiagnosed conditions. Phylogenetic analysis of clinical isolates showed no evidence of serotype replacement by capsule switching between Hie or Hif and Hib and no association between sequence types and clinical disease or outcome.

Dr Ladhani is a pediatric infectious disease consultant with the HPA, London, UK. His research interests include vaccine-preventable diseases.

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Epidemic of Invasive Pneumococcal Disease, Western Canada, 2005–2009

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In Canada before 2005, large outbreaks of pneumococcal disease, including invasive pneumococcal disease caused by serotype 5, were rare. Since then, an epidemic of serotype 5 invasive pneumococcal disease was reported: 52 cases during 2005, 393 during 2006, 457 during 2007, 104 during 2008, and 42 during in 2009. Of these 1,048 cases, 1,043 (99.5%) occurred in the western provinces of Canada. Median patient age was 41 years, and most (659 [59.3%]) patients were male. Most frequently representing serotype 5 cases (compared with a subset of persons with non–serotype 5 cases) were persons who were of First Nations heritage or homeless. Restriction fragment-length polymorphism typing indicated that the epidemic was caused by a single clone, which multilocus sequence typing identified as sequence type 289. Large pneumococcal epidemics might go unrecognized without surveillance programs to document fluctuations in serotype prevalence.

Before the advent of antimicrobial drugs, outbreaks of invasive pneumococcal disease were numerous. Since then, however, outbreaks have been less frequently reported and have involved fewer persons, usually those confined to closed settings such as hospitals or military barracks (*1*,*2*). Even more rare have been large outbreaks or epidemics of invasive pneumococcal disease; if and when they do occur, they tend to be caused by a limited number of pneumococcal serotypes (*2*–*4*).

The serotype of a *Streptococcus pneumoniae* bacterium is designated according to the organism's polysaccharide capsule, its major virulence factor. Worldwide, 91 polysaccharide capsular serotypes have been identified (*5*,*6*). A small subset of serotypes is responsible for most large outbreaks; these serotypes typically include, but are not restricted to, serotypes 1, 4, 5, 9V, 12F, and 23F (*2*).

Before 2005, large outbreaks of pneumococcal disease, including invasive pneumococcal disease caused by serotype 5, were rare in Canada. In 2002, an outbreak caused by *S. pneumoniae* in northern Quebec, Canada, was reported, and blood culture identified 10 cases as being caused by a serotype 1 strain (*7*). We report a large epidemic of invasive pneumococcal disease caused by *S. pneumoniae* serotype 5 in Canada that occurred during 2005–2009. The study received approval from the institutional research review committees of the health regions and the University of Alberta ethics review board.

Materials and Methods

In Canada, invasive pneumococcal disease is nationally notifiable. For this study, cases of invasive pneumococcal disease were defined according to the national case definition: isolation of *S. pneumoniae* from a normally sterile site, such as blood, cerebrospinal fluid, pleural fluid, biopsy

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tissue, joint aspirate, pericardial fluid, or peritoneal fluid (*8*). In the provinces affected by the 2005–2009 epidemic, clinical diagnostic microbiology laboratories were required by provincial health authorities to submit isolates from patients with invasive pneumococcal infections to their respective provincial laboratories, which would then send them to the National Centre for Streptococcus, in Edmonton, Alberta, for capsular serotyping and antimicrobial drug resistance epidemiologic profiling. For this study, 1 isolate per case was counted. Multiple isolates of the same serotype collected from the same patient within a 30-day period were considered to account for 1 case. Regardless of serotype, isolates collected from the same patient >30 days after the first isolate were counted as separate cases.

Clinical Data Collection

To elucidate features of disease caused by *S. pneumoniae* serotype 5, we reviewed all cases of invasive pneumococcal disease in the northern Alberta area reported from 2005 through 2009. During the study period, Alberta was subdivided into 9 health regions. For cases originating in health regions 4 through 9 (located in northern Alberta), an extensive medical chart review was conducted. The total population for these health regions in 2008 was 1,888,881 (www.health.alberta.ca/documents/Population-Projections-2006.pdf). The clinical data collected were patient age, sex, aboriginal status (i.e., First Nations heritage), homelessness, substance abuse, type of invasive pneumococcal disease, outcome, and concurrent conditions (Table 1).

Table 1. Characteristics of 1,112 patients with pneumococcal disease, northern Alberta, Canada, 2005–2009*

Characteristic	Total	*Streptococcus pneumoniae* serotype		p value
		Not serotype 5, n = 827	Serotype 5, n = 285	
Demographic				
Age, mean ± SD, y	45.4 ± 22.5	47.1 ± 23.9	40.6 ± 16.7	<0.001
Age group, y				
<16	137 (12.3)	119 (14.4)	18 (6.3)	<0.001
16–65	771 (69.3)	522 (63.1)	249 (87.4)	
≥65	204 (18.4)	186 (22.5)	18 (6.3)	
Male sex	659 (59.3)	471 (57.0)	188 (66.2)	0.006
First Nations heritage	145 (13.0)	83 (10.0)	62 (21.8)	<0.001
Homeless.	85 (7.6)	39 (4.7)	46 (16.1)	<0.001
Substance abuse				
Tobacco	687 (61.8)	471 (57.0)	216 (75.8)	<0.001
Alcoholism	257 (23.1)	159 (19.2)	98 (34.4)	<0.001
Illicit drug	259 (23.3)	135 (16.3)	124 (43.5)	<0.001
Concurrent conditions				
Cancer	103 (9.3)	96 (11.6)	7 (2.5)	<0.001
≤5 y before IPD	103 (9.3)	96 (11.6)	7 (2.5)	<0.001
>5 y before IPD	41 (3.7)	33 (4.0)	8 (2.8)	0.361
Central nervous system disorder†	167 (15.0)	135 (16.3)	32 (11.2)	0.038
Cardiovascular disease‡	317 (28.5)	281 (34.0)	36 (12.6)	<0.001
Hematologic abnormality§	80 (7.2)	76 (9.2)	4 (1.4)	<0.001
Diabetes mellitus	134 (12.1)	119 (14.4)	15 (5.3)	<0.001
Cirrhosis	53 (4.8)	48 (5.8)	5 (1.8)	0.006
Chronic renal failure¶	48 (4.3)	46 (5.6)	2 (0.7)	<0.001
HIV/AIDS	46 (4.1)	29 (3.5)	17 (6.0)	0.072
Rheumatoid arthritis	21 (1.9)	19 (2.3)	2 (0.7)	0.088
Systemic lupus erythematosus	9 (0.8)	8 (1.0)	1 (0.4)	0.461
Mental problem#	180 (16.2)	141 (17.0)	39 (13.7)	0.183
Musculoskeletal impairment**	201 (18.1)	172 (20.8)	29 (10.2)	<0.001
Chronic obstructive pulmonary disease	161 (14.5)	131 (15.8)	30 (10.5)	0.028
Hepatitis C	157 (14.1)	91 (11.0)	66 (23.2)	<0.001
Type of pneumococcal disease				
Bacteremia	1054 (94.8)	782 (94.6)	272 (95.4)	0.564
Pneumonia	887 (79.8)	617 (74.6)	270 (94.7)	<0.001
Meningitis	68 (6.1)	66 (8.0)	2 (0.7)	<0.001
Outcome				
Death	126 (11.3)	117 (14.1)	9 (3.2)	<0.001
Hospitalization				
No. hospitalized	826	581	245	
Length of stay, d, mean ± SD	17.9 ± 39.7	20.1 ± 46.4	12.9 ± 13.1	0.001

*All values are no. (%) unless indicated otherwise. IPD, invasive pneumococcal disease.
†Chronic central nervous system leak, epilepsy or other seizure disorder, Parkinsonism or other neurodegenerative disorder, Alzheimer or other dementia, or stroke or other neurologic disease.
‡Congestive heart failure, coronary artery disease, myocardial infarction, arrhythmia, congenital defect, atrial fibrillation, hypertension, or other.
§Sickle cell anemia, other anemia, bleeding disorder/coagulopathy, or other.
¶Nephritic syndrome or other.
#Depression or other.
**Osteoarthritis, osteoporosis, or other.

Identification and Serotyping

As part of its serotyping program, the National Centre for Streptococcus pneumococcal surveillance confirmed isolates as *S. pneumoniae* according to morphologic appearance and optochin susceptibility (*9*). All pneumococcal isolates that exhibited a positive quellung reaction when commercial type-specific antiserum (Statens Serum Institute, Copenhagen, Denmark) was used were assigned a serotype (*10*). Strains that were susceptible to optochin but for which no serotype was assigned were further tested by using the AccuProbe *Streptococcus pneumoniae* Culture Identification Test (Gen-Probe, San Diego, CA, USA) to confirm species identification.

Antimicrobial Drug Susceptibility Testing

Drug susceptibility was determined by using the reference broth microdilution method described by the Clinical and Laboratory Standards Institute (*11*). The following antimicrobial drugs were tested: penicillin, cefotaxime, ceftriaxone, chloramphenicol, erythromycin, clindamycin, tetracycline, trimethoprim/sulfamethoxazole, levofloxacin, and vancomycin. All antimicrobial agents were purchased from Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada. Interpretation of MICs was based on Clinical and Laboratory Standards Institute performance standards that were current at the time of testing (M100-S15 through M100-S17) (*12*,*13*).

Pulsed-field Gel Electrophoresis and Multilocus Sequence Typing

S. pneumoniae chromosomal DNA was prepared as described (*14*). Chromosomal DNA was restricted with 20 U of *Sma*I (New England Biolabs, Beverly, MA, USA), and pulsed-field gel electrophoresis (PFGE) was performed by using a CHEF DR-III apparatus (Bio-Rad Laboratories [Canada] Limited, Mississauga, ON, Canada) for 23 h. The parameters used were as follows: initial pulse 3.5 s, final pulse 23.5 s, voltage 6 V/cm, and temperature 13°C. *Salmonella* Braenderup U9812 was used as a molecular size marker. The macrorestriction pattern was analyzed by using Bionumerics version 5 (Saint-Martens-Latem, Belgium).

Multilocus sequence typing was performed as described (*15*). The multilocus sequence type (MLST) was searched against the online pneumococcal database (http://spneumoniae.mlst.net).

Statistical Analyses

Data were analyzed by using SAS software version 9.1 (SAS Institute, Inc., Cary, NC, USA). Possible factors associated with serotype 5 among patients with pneumococcal disease were assessed. We examined the association of each demographic, substance abuse, and concurrent condition variable with outcome variable serotype 5 (yes or other serotype). For continuous variables, we used the *t* test or Mann Whitney U test as appropriate. For categorical variables, we used the χ^2 or Fisher exact test. For variables that were significant ($p<0.20$) on univariable analyses, we used the following multivariable logistic regression model:

$$\text{LOGIT P (Serotype 5)} = B_0 + B_1X_1 + B_2X_2 + \ldots + B_pX_p \ldots\ldots$$

In model 1, the significance of variables was assessed by using the Wald statistic. The variables that were significant at $p<0.05$ were retained in the model. All others were removed from the model unless they were possible confounders. In the final model, we tested βs, the effect of each variable on log odds of serotype 5 after adjustment for other associated variables. To calculate rates, we used the populations that were current for each province in January 1, 2009 (*16*).

Results

The Epidemic

From January 1, 2000, through December 31, 2004, the National Centre for Streptococcus serotyped 5,509 *S. pneumoniae* isolates from patients with invasive pneumococcal disease (1 isolate counted per case) from across Canada and identified 7 as serotype 5: one each in 2001, 2002, and 2003 and 4 in 2004. Since then, the Centre identified 52 isolates from patients with invasive pneumococcal disease as serotype 5 in 2005, 393 in 2006, 457 in 2007, 104 in 2008, and 42 in 2009, for a total of 1,048 cases (Figure 1). The number of cases caused by serotype 5 peaked in December 2006 and then slowly declined in 2007, 2008, and 2009 (Figure 1). Patients with invasive pneumococcal disease caused by *S. pneumoniae* serotype 5 were from British Columbia (343 [32.7%], 7.8 cases/100,000 population) Alberta (523 [49.9%], 14.4/100,000), Saskatchewan (85 [8.1%], 8.3/100,000), and Manitoba (92 [8.8%], 7.6/100,000) (Figure 1). During this 5-year period, only 5 isolates of serotype 5 were detected from elsewhere in Canada: 1 from Ontario in March 2007; 1 from Quebec in June 2009; and 3 from Northwest Territories in April, July, and December 2007.

In western Canada during 2000–2009, the numbers of serotype 5 and other serotype isolates identified increased (Figure 2). The increased number of isolates submitted for typing after the onset of the epidemic indicates greater interest on the part of public health officials in western Canada in identifying circulating serotypes from patients with invasive pneumococcal disease in their provinces.

The epidemic primarily affected young adults (median age 41 years) (Figure 3). Only a small subset of cases

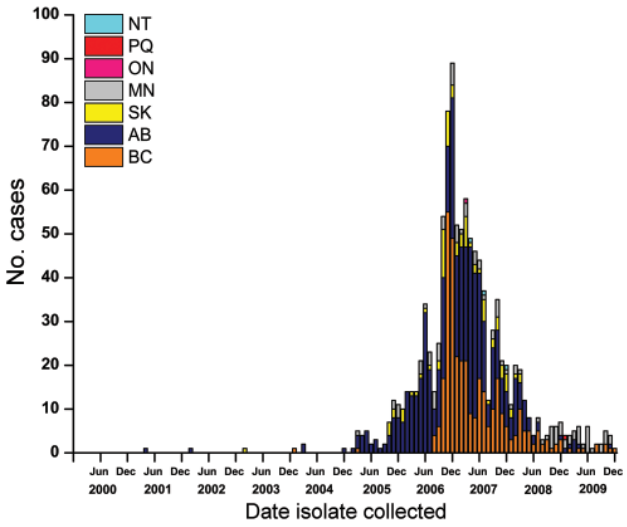

Figure 1. *Streptococcus pneumoniae* serotype 5 isolated in western Canada, 2000–2009, by province and month. NT, Nunavut; PQ, Quebec; ON, Ontario; MN, Manitoba; SK, Saskatchewan, AB, Alberta; BC, British Columbia.

occurred among patients <5 years of age and even fewer in those >65 years of age. Most patients were male (637 male, 395 female, and 16 unknown).

Specimen Source

The sources of specimens for the serotype 5 isolates from across Canada were as follows: 988 isolates from blood, 33 from lung/pleural fluid, 9 from cerebrospinal fluid, 7 from synovial fluid, 7 from chest/hip/leg fluid, 3 from pericardial fluid, and 1 from peritoneal fluid. For the univariable and multivariable analyses, isolates from patients with serotype 5 and nonserotype 5 invasive *S. pneumoniae* were collected from northern Alberta only (1,112 cases).

Patient Characteristics

According to univariable analysis, serotype 5 was more prevalent than other serotypes among patients who were male (66.2% vs. 57.0%), of First Nations heritage (21.8% vs. 10.0%), or homeless (16.1% vs. 4.7%) (Table 1). Among the substance-abuse categories, associations with tobacco use, alcoholism, and illicit drug use were considered significant ($p<0.001$ for each; Table 1). With respect to concurrent conditions, cases of invasive pneumococcal disease caused by serotype 5 were significantly associated with cancer within 5 years before onset of invasive pneumococcal disease, cardiovascular disease, hematologic abnormalities, diabetes mellitus, cirrhosis, chronic renal failure, musculoskeletal impairment, and hepatitis C (Table 1). For patients with bacteremia and pneumonia, invasive pneumococcal disease caused by *S. pneumoniae* serotype 5 occurred significantly more often with pneumonia than did that caused by other serotypes (94.7% vs. 74.6%; Table 1). In addition, meningitis was more common for patients in the non–serotype 5 group than in the serotype 5 group (8.0% vs. 0.7%, respectively; $p<0.001$; Table 1). Death was less associated with infection caused by serotype 5 than by other serotypes (3.2% vs. 14.1%, respectively; Table 1).

The multivariable logistic regression model used to examine the associations of different factors with *S. pneumoniae* serotype 5 that were identified by univariable analyses found that First Nations heritage and homelessness were significantly associated with serotype 5 (adjusted odds ratio [aOR] 2.34; 95% CI 1.53–3.57 and aOR 1.83, 95% CI, 1.07–3.12, respectively) (Table 2). Tobacco use (aOR 1.90, 95% CI 1.29–2.81) and illicit drug use (aOR 1.89, 95% CI, 1.31–2.73) were also significantly associated, whereas alcoholism was not (Table 2). Among concurrent conditions, the following were significantly associated: cancer within 5 years before invasive pneumococcal disease (aOR 0.32, 95% CI 0.14–0.71), cardiovascular disease (aOR 0.51, 95% CI 0.32–0.82), hematologic abnormalities (aOR 0.19, 95% CI 0.06–0.55), and cirrhosis (aOR 0.18; 95% CI 0.06–0.50) (Table 2). Associations with musculoskeletal disease and hepatitis C infection, although significant according to univariable analysis, were not significant according to multivariable analysis.

S. pneumoniae Serotype 5 Characteristics

Antimicrobial drug susceptibility testing of 1,009 isolates indicated that all *S. pneumoniae* serotype 5 isolates tested were susceptible to cefotaxime, ceftriaxone,

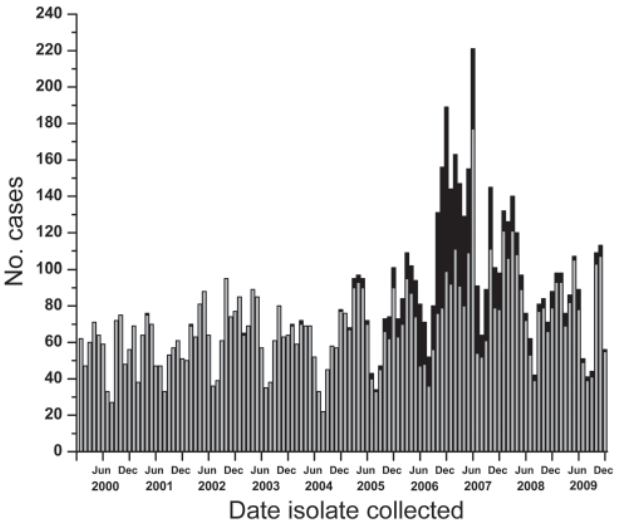

Figure 2. Total pneumococci serotyped in British Columbia, Alberta, Saskatchewan, and Manitoba, Canada, by month collected, 2000–2009. Gray bars represent all *Streptococcus pneumoniae* serotypes except serotype 5; black bars represent serotype 5 isolates only.

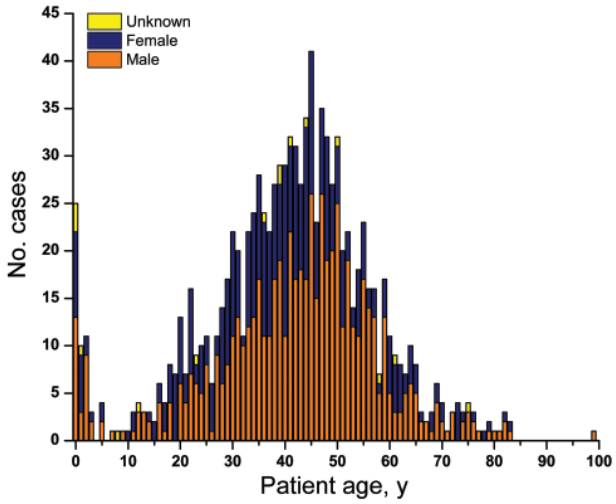

Figure 3. Age and sex of patients with invasive pneumococcal pneumonia caused by *Streptococcus pneumoniae* serotype 5, western Canada, 2000–2009. Median age 41 years.

tetracycline, levofloxacin, and vancomycin. A small percentage (5 [0.5%]) of the 1,009 were intermediately resistant to penicillin (MIC ≈0.125 mg/L), 2 (0.2%) were resistant to chloramphenicol, 4 (0.4%) were resistant to clindamycin, 2 (0.2%) were intermediately resistant to erythromycin, and 4 (0.4%) were fully resistant to erythromycin. For trimethoprim/sulfamethoxazole, 976 (96.7%) isolates showed intermediate resistance (MICs 1.0–2.0 mg/L) and 18 (1.8%) showed resistance (4.0 to >16.0 mg/L). The remaining 39 isolates were not available for testing.

During the epidemic, a subset of *S. pneumoniae* serotype 5 isolates (91 isolates), encompassing each year of the epidemic from the 4 affected western provinces were randomly selected and subjected to PFGE for restriction fragment-length polymorphism (RFLP) analysis (12 isolates in 2005, 26 in 2006, 13 in 2007, 20 in 2008, and 20 in 2009). All isolates typed by PFGE had either an identical RFLP pattern or differed by 1 band (Figure 4). Extending the RFLP analysis back to the 7 serotype 5 isolates from Canada from 2000 through 2004 showed that the first similar fingerprint detected was from a person who lived in a small town in rural southeastern Alberta in March 2004.

To determine whether this clone had been found in the United States, we compared it with 6 serotype 5 isolates from the US Centers for Disease Control and Prevention. Three isolates were from a small cluster of cases in San Francisco, California, in 2002 and 3 were from sporadic cases in the United States in 2006 (B. Beall, pers. comm.). Of these 6 isolates, the RFLP pattern for 5 isolates was identical to that of the epidemic clone and 1 isolate had a single band difference, suggesting that the serotype 5 clone in western Canada had been circulating in the United States in 2002 and 2006. This clone might have been imported into Canada from the United States; however, it might also have been imported from elsewhere in the world because sequence type (ST) 289 is the major circulating serotype 5 clone.

MLST analysis showed the allelic profile of the *S. pneumoniae* serotype 5 clone to be ST289 (*aroE16*, *gdh12*, *gki9*, *recP1*, *spi41*, *xpt33*, *ddl33*). ST289 has been listed in the MLST database (http://spneumoniae.mlst.net). The ST289 clone was originally reported from Colombia and is contained in the Pneumococcal Molecular Epidemiology Network list of worldwide antimicrobial drug–resistant clones (designation Colombia[5]-19) (www.sph.emory.edu/PMEN) (*17*).

Discussion

Large epidemics of pneumococcal disease might go unrecognized unless surveillance programs are in place to document fluctuations in serotype prevalence, as reported here. The year-to-year variability of invasive pneumococcal disease caused by *S. pneumoniae* serotype 5 seen in some countries might actually reflect serotype 5 outbreaks similar to what we have described (*18*). For example, in 2000 in Mali, Africa, 50% of the isolates recovered from children with invasive pneumococcal disease were serotype 5, yet 2 years later; this percentage had dropped to a small portion of the total cases (*19*,*20*). This serotype 5 variability has also been reported in Chile and Israel (*21*,*22*). In Israel during 1989–1998, serotype 5 was the second most common serotype (serotype 1 was the most common) that caused invasive pneumococcal disease (12%–13% of cases among children <15 years of age) (*21*).

Although in other countries the number of *S. pneumoniae* serotype 5 cases might vary from year to year, in Canada no variability for serotype 5 was evident until the 2005–2009 epidemic. Few serotype 5 isolates had been documented since 1991, when the National Centre for Streptococcus first began performing pneumococcal serotyping to support national surveillance in Canada, until 2005. This serotype 5 strain has been demonstrated elsewhere in the world, not just Canada. Data from the MLST database and published reports indicate that the Colombia[5] ST289 clone has been reported in countries in Europe, Latin America, and Africa and in the United States (http://spneumoniae.mlst.net) (*23*–*26*). In addition, the rate of resistance to trimethoprim/sulfamethoxazole by the Colombia[5] ST289 clone has been reported as 80.8% (*27*) and 58.2% (*17*) of the Colombia[5] ST289 strains from Latin American countries.

The *S. pneumoniae* serotype 5 epidemic mostly affected middle-aged men (median 41 years of age). Other

Table 2. Patient characteristics associated with *Streptococcus pneumoniae* serotype 5 invasive pneumococcal disease, northern Alberta, Canada, 2005–2009*

Characteristic	Adjusted odds ratio (95% CI)	p value
Demographic		
Age group, y		
<16	0	
16–65	2.10 (1.14–3.89)	0.018
≥65	1.09 (0.48–2.47)	0.846
Male sex	1.13 (0.82–1.56)	0.443
First Nations heritage	2.34 (1.53–3.57)	<0.001
Homeless	1.83 (1.07–3.12)	0.026
Substance abuse		
Tobacco	1.90 (1.29–2.81)	0.001
Alcoholism	1.19 (0.82–1.75)	0.363
Illicit drug	1.89 (1.31–2.73)	0.001
Concurrent condition		
Cancer within 5 y before IPD	0.32 (0.14–0.71)	0.005
Central nervous system disorder†	0.83 (0.52–1.33)	0.445
Cardiovascular disease‡	0.51 (0.32–0.82)	0.006
Hematologic abnormality§	0.19 (0.06–0.55)	0.002
Diabetes mellitus	0.60 (0.32–1.12)	0.109
Cirrhosis	0.18 (0.06–0.50)	0.001
Chronic renal failure¶	0.34 (0.08–1.55)	0.155
Rheumatoid arthritis	0.40 (0.09–1.84)	0.239
Mental problem#	0.64 (0.41–0.99)	0.045
Musculoskeletal impairment**	0.72 (0.44–1.18)	0.191
Chronic obstructive pulmonary disease	1.19 (0.71–2.00)	0.516

*IPD, invasive pneumococcal disease. Adjusted odds ratios were obtained by multivariable logistic regression analysis.
†Chronic central nervous system leak, epilepsy or other seizure disorder, Parkinsonism or other neurodegenerative disorder, Alzheimer or other dementia, or stroke or other neurologic disease.
‡Congestive heart failure, coronary artery disease, myocardial infarction, arrhythmia, congenital defect, atrial fibrillation, hypertension, or other.
§Sickle cell anemia, other anemia, bleeding disorder/coagulopathy, or other.
¶Nephritic syndrome or other.
#Depression or other.
**Osteoarthritis, osteoporosis, or other.

risk factors were homelessness and First Nations heritage, although these factors accounted for a small percentage of the population. Because invasive pneumococcal disease reportedly affects homeless populations, the finding that homelessness was a major demographic factor associated with this epidemic is not surprising (*28*–*30*). A recent study from Toronto, Ontario, Canada, found that incidence of invasive pneumococcal disease was greater in the homeless population than in the general population (*30*) and that the variables associated with the serotype 5 epidemic (tobacco use, alcohol abuse, illicit drug use) were associated with invasive pneumococcal disease. Serotype 5 pneumococci were not identified in this study.

In December 2006, investigators found *S. pneumoniae* serotype 5 affecting persons of First Nations heritage living near the city of Calgary, Alberta, and persons living in inner-city Calgary; Edmonton; and Vancouver, British Columbia (*31*–*33*). These reports indicated that the variables associated with invasive pneumococcal disease caused by this serotype were homelessness, use of illicit drugs, First Nations heritage, alcoholism, and hepatitis B or C, thereby corroborating our findings for those cases in northern Alberta (*31*,*32*). Recovery of this serotype in locations other than inner cities in western Canada (including northern Saskatchewan) suggests its spread beyond the larger metropolitan areas of western Canada (*34*).

A public health response to the epidemic occurred throughout western Canada. Regional health authorities conducted vaccination programs focused primarily on homeless populations in large metropolitan areas. They used the 23-valent pneumococcal polysaccharide vaccine, which contains serotype 5. As a result of these large-scale pneumococcal vaccination campaigns, the National Advisory Committee on Immunization issued an advisory statement recommending use of the 23-valent pneumococcal vaccine for homeless persons and injection drug users (*35*). Examples of public health measures used to address the outbreak in 2 health regions are contained in reports from British Columbia focusing on *S. pneumoniae* serotype 5 outbreaks in the Vancouver downtown eastside and in the city of Kelowna (*35*,*36*). In Vancouver, investigators found that the serotype 5 strain accounted for 78% of cases of invasive pneumococcal disease. The major risk factors reported were use of crack cocaine and residence in Vancouver's downtown eastside, an impoverished part of that city where most of the illicit-drug users and homeless persons live (*36*). As a result, Vancouver Coastal Health authorities targeted rooming houses, shelters, food banks, and other community locations (*32*). In Kelowna, public health nurses and health care providers focused a pneumococcal vaccination program on persons who were homeless and/or addicted to illicit drugs or alcohol; at the

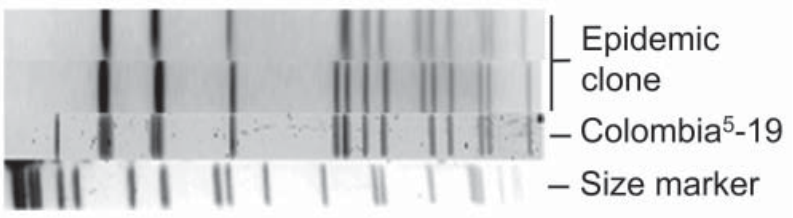

Figure 4. Restriction fragment length polymorphism pattern of *Streptococcus pneumoniae* serotype 5 from epidemic in western Canada, 2000–2009 (epidemic clone), determined by pulsed-field gel electrophoresis. The Colombia^5-19 strain is from the Pneumococcal Molecular Epidemiology Network (www.sph.emory.edu/PMEN) (*17*).

time of their report, they had vaccinated ≈1,000 at-risk persons (*37*).

A strength of our study is the ability of the centralized laboratory to capture and document shifts in the epidemiology of pneumococci in Canada. Regionalization of serotyping of pneumococci has the potential to miss changes in serotypes that can occur rapidly.

A weakness of our study is the lack of clinical data for all cases of invasive pneumococcal disease caused by *S. pneumoniae* serotype 5 that occurred during this epidemic. Logistically, gathering all of these data was not possible; however, the clinical data from northern Alberta do indicate some of the clinical variables involved and the concurrent conditions associated with serotype 5 cases. Another limitation might be that the variables for persons with invasive pneumococcal disease caused by *S. pneumoniae* serotype 5 (patient demographics, substance-abuse associations, concurrent conditions, type of pneumococcal disease, and outcomes) were compared with those for persons with other pneumococcal disease rather than with a healthy (nondiseased) control group. However, we thought it useful to try and determine among those with invasive pneumococcal disease whether differences existed among disease caused by serotype 5 and other serotypes.

We do not know why the epidemic was focused in western Canada and why large numbers of cases did not spread to eastern Canada or the United States. Clearly, we do not understand all the dynamics associated with large invasive pneumococcal disease epidemics.

In conclusion, we document a rare large-scale outbreak of invasive pneumococcal disease in western Canada caused by a single clone of *S. pneumoniae*. The clone possessed a serotype 5 polysaccharide capsule and ST289, indicating that the clone is derived from the international Pneumococcal Molecular Epidemiology Network clone Colombia^5-19 originally described in Colombia (*18*). RFLP comparing a collection of *S. pneumoniae* serotype 5 isolates from the United States with the epidemic clone from western Canada showed that all isolates were identical, suggesting that this strain has been circulating within the United States. However, without direct evidence, we do not know from what part of the world this clone was originally imported into Canada.

Acknowledgments

We thank Bernard Beall for providing *S. pneumoniae* serotype 5 strains from the United States; James D. Kellner for contributing to the study design, case report form, and database for the clinical data collection portion of this study; and Wyeth-Pfizer for continued support for data analysis for invasive pneumococcal disease in Alberta. We acknowledge the use of the pneumococcal MLST database, located at Imperial College London and funded by the Wellcome Trust. We also acknowledge the tremendous effort and skill of the acute care diagnostic laboratories throughout Canada used to identify patients with *S. pneumoniae* infection and with suspected cases of invasive pneumococcal disease and forwarding them to their public health laboratories for serotyping.

Dr Tyrrell is a clinical microbiologist in the Provincial Laboratory for Public Health–Alberta and a professor in the Department of Laboratory Medicine and Pathology, University of Alberta. His research interests include bacterial epidemiology and bacterial pathogenesis with a major focus on streptococci.

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Antimicrobial Drug Resistance in *Escherichia coli* from Humans and Food Animals, United States, 1950–2002

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We conducted a retrospective study of *Escherichia coli* isolates recovered from human and food animal samples during 1950–2002 to assess historical changes in antimicrobial drug resistance. A total of 1,729 *E. coli* isolates (983 from humans, 323 from cattle, 138 from chickens, and 285 from pigs) were tested for susceptibility to 15 antimicrobial drugs. A significant upward trend in resistance was observed for ampicillin (p<0.001), sulfonamide (p<0.001), and tetracycline (p<0.001). Animal strains showed increased resistance to 11/15 antimicrobial agents, including ampicillin (p<0.001), sulfonamide (p<0.01), and gentamicin (p<0.001). Multidrug resistance (≈3 antimicrobial drug classes) in *E. coli* increased from 7.2% during the 1950s to 63.6% during the 2000s. The most frequent co-resistant phenotype observed was to tetracycline and streptomycin (29.7%), followed by tetracycline and sulfonamide (29.0%). These data describe the evolution of resistance after introduction of new antimicrobial agents into clinical medicine and help explain the range of resistance in modern *E. coli* isolates.

Antimicrobial drugs have played an indispensable role in decreasing illness and death associated with infectious diseases in animals and humans. However, selective pressure exerted by antimicrobial drug use also has been the major driving force behind the emergence and spread of drug-resistance traits among pathogenic and commensal bacteria (*1*). In addition, resistance has developed after advent of every major class of antimicrobial drugs, varying in time from as short as 1 year (penicillin) to >10 years (vancomycin) (*2*,*3*).

Escherichia coli is usually a commensal bacterium of humans and animals. Pathogenic variants cause intestinal and extraintestinal infections, including gastroenteritis, urinary tract infection, meningitis, peritonitis, and septicemia (*4*,*5*). Therapeutic options vary depending on the type of infection. For example, for urinary tract infections, trimethoprim/sulfamethoxazole and fluoroquinolones are treatments of choice (*6*), whereas for Shiga toxin–producing *E. coli* infections, antimicrobial drug therapy is not recommended (*7*). *E. coli* is sometimes used as a sentinel for monitoring antimicrobial drug resistance in fecal bacteria because it is found more frequently in a wide range of hosts, acquires resistance easily (*8*), and is a reliable indicator of resistance in salmonellae (*9*).

Surveillance data show that resistance in *E. coli* is consistently highest for antimicrobial agents that have been in use the longest time in human and veterinary medicine (*10*). The past 2 decades have witnessed major increases in emergence and spread of multidrug-resistant bacteria and increasing resistance to newer compounds, such as fluoroquinolones and certain cephalosporins (*3*). For example, a study of the susceptibility of *E. coli* isolates recovered from hospitals during a 12-year period (1971–1982) showed no major change in resistance to any of the antimicrobial drugs tested (*11*). In contrast, a retrospective analysis of *E. coli* from urine specimens collected from patients during 1997–2007 showed an increasing resistance trend for ciprofloxacin, trimethoprim/sulfamethoxazole, and amoxicillin/clavulanic acid (*12*). Similarly a 30-year (1979–2009) follow-up study on *E. coli* in Sweden showed

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an increasing resistance trend for ampicillin, sulfonamide, trimethoprim, and gentamicin (*13*). Although studies of farms have shown an association of multidrug-resistant *E. coli* with chronic antimicrobial drug exposure (*14*,*15*), there are few data on temporal trends of antimicrobial drug resistance in food animal *E. coli* isolates, particularly those recovered before 1980. Recent data are available in several countries that established resistance monitoring programs during the mid-1990s.

In the United States, the National Antimicrobial Resistance Monitoring System (NARMS) was established in 1996 to prospectively monitor changes in antimicrobial drug susceptibilities of zoonotic foodborne bacteria, including *E. coli* from retail meats (chicken breast, pork chops, ground beef, ground turkey), and chickens at slaughter. During 2000–2008, NARMS laboratories tested 13,521 *E. coli* isolates from chickens to determine the MIC to antimicrobial drugs essential in human and veterinary medicine. The resistance trend in chickens observed during this period varied on the basis of the antimicrobial agents. For example, resistance during 2000–2008 decreased slightly for kanamycin (16.1% to 10.2%), streptomycin (77.5% to 54.6%), trimethoprim/sulfamethoxazole (17.2% to 9.1%), and tetracycline (68.4% to 47.4%). Cefoxitin resistance increased from 7.4% in 2000 to 15% in 2006, and ceftriaxone resistance increased from 6.3% to 13.5%. Ciprofloxacin resistance remained low (<1%) during this period.

To better understand the historical emergence of resistance since the advent of the antimicrobial drug age, which led to baseline data in the first year of NARMS testing, we assayed *E. coli* collections from human and animal sources obtained during 1950–2002 for antimicrobial drug susceptibility. This information, when coupled with secular surveillance data, will provide a broader picture of evolution of resistance and lay the groundwork for understanding genetic mechanisms of resistance development and dissemination.

Materials and Methods

Bacterial Strains

A total of 1,729 *E. coli* isolates from human and animal samples obtained from different US states were used in this study. Isolates were obtained by the American Type Culture Collection (ATCC) (Manassas, VA, USA) from the *E. coli* Reference Center (ECRC) at Pennsylvania State University (University Park, PA, USA) and the Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA) under contract with the US Food and Drug Administration Center for Veterinary Medicine (Rockville, MD, USA). These isolates were recovered from human and animal specimens (e.g., feces, blood, kidney, lymph nodes, urine, cerebrospinal fluid, peritoneal fluid, pleural fluid) submitted to ECRC and CDC from state public health and veterinary diagnostic laboratories. For human isolates obtained from CDC, most acquired during 1948 through the late 1980s were maintained on trypticase soy agar stabs sealed with paraffin and stored at room temperature. Starting in the late 1980s, strains were frozen in trypticase soy broth containing 30% glycerol at −70°C. Similarly, isolates were stored, according to the ECRC standard protocol at −70°C to −80°C in trypticase soy broth containing 30% glycerol until further processing.

Of 1,729 *E. coli* isolates, 983 (56.8%) were recovered from humans during 1950–2001, and the remaining 746 (43.2%) were recovered from animals during 1962–2002. Three hundred twenty-three (43.2%) *E. coli* isolates of animal origin were from cattle, 138 (18.5%) from chickens, and 285 (38.2%) from pigs. Fifty percent were from 10 states: Pennsylvania (183, 11.2%), California (100, 5.8%), Ohio (90, 5.2%), Maryland (83, 4.8%), Minnesota (82, 4.7%), Illinois (72, 4.2%), Texas (72, 4.2%), New York (70, 4.1%), North Carolina (61, 3.5%), and Virginia (58, 3.4%). Distribution of isolates by source and year are shown in Table 1.

Antimicrobial Drug Susceptibility Testing

Each isolate was streaked on trypticase soy agar supplemented with 5% defibrinated sheep blood (Becton Dickinson, Sparks, MD, USA) before antimicrobial drug susceptibility testing. MICs were determined by using the Sensititer automated antimicrobial susceptibility system (Trek Diagnostic Systems, Cleveland, OH, USA) according to the manufacturer's instructions. Results were interpreted according to National Committee for Clinical and Laboratory Standards criteria (*16*) where available (Table 2). Antimicrobial drugs tested were ampicillin, amoxicillin/clavulanic acid, cefoxitin, ceftiofur, cephalothin, ceftriaxone, ciprofloxacin, nalidixic acid, streptomycin, gentamicin, kanamycin, chloramphenicol, tetracycline, sulfonamide, and trimethoprim/sulfamethoxazole. *E. coli* ATCC 25922 and ATCC 35218, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms in MIC determinations. Multidrug resistance was defined as resistance to ≥3 classes of antimicrobial drugs.

Statistical Analysis

The Mann-Kendall test, a nonparametric statistical test, was performed to detect a monotone increasing or decreasing resistance trend over time. Magnitude of annual change was estimated by using a slope parameter, Q, and the Sen nonparametric method (*20*). Calculations were performed by using the Excel (Microsoft, Redmond,

Table 1. Period of isolation and source of *Escherichia coli* isolates tested, United States, 1950–2002

Period	Source, no. isolates			
	Human	Cattle	Chickens	Pigs
1950–1959	180	0	0	0
1960–1969	112	17	0	15
1970–1979	292	32	11	25
1980–1989	211	86	65	96
1990–1999	182	108	44	81
2000–2002	6	81	18	68

WA, USA) template Mann-Kendall test for trend and Sen slope estimates (*21*). For time series <10 annual percentage resistance values, significance of the trend was determined from the exact distribution of the S test statistic, and a normal approximation (z statistic) was used when there were ≥10 values. Significance was assessed at 4 levels (α = 0.001, 0.01, 0.05, and 0.1); p values <0.05 were considered significant. Comparisons of drug resistance profiles between different sources (human, cattle, chicken, and pigs) were conducted by using the χ^2 test; p values <0.05 was considered significant.

Results

Antimicrobial Drug Susceptibility

Overall, 934 (54.0%) of 1,729 *E. coli* were resistant to ≥1 antimicrobial drug. As expected, the most common resistance phenotypes were to older drugs such as tetracycline (40.9%) (introduced in 1948), sulfonamide (36.2%) (introduced in 1936), streptomycin (34.2%) (introduced in 1943), and ampicillin (24.1%) (introduced in 1961). A much smaller number of isolates were resistant to antimicrobial drugs introduced for clinical use since 1980, such as amoxicillin/clavulanic acid (5.6%) (introduced in 1984), ceftriaxone (2.4%) (introduced in 1984), ceftiofur (2.3%) (introduced in 1988), and ciprofloxacin (0.4%) (introduced in 1987) (Table 2).

When analyzed by source, *E. coli* isolates of animal origin were more resistant than those of human origin. Among 983 human isolates, resistance was observed most often to sulfonamide (19.9%), followed by tetracycline (18%) and ampicillin (16.5%). No human *E. coli* isolates showed resistance to ciprofloxacin, and 1 isolate (0.1%) from 1997 showed resistance to ceftiofur, ceftriaxone, and gentamicin. Of 746 isolates recovered from animal sources, 531 (71.1%) were resistant to tetracycline, 441 (59%) to streptomycin, 431 (57.7%) to sulfonamide, 277 (37.1%) to kanamycin, and 255 (34.1%) to ampicillin. Among animal *E. coli* isolates, the rate of resistance was significantly higher in cattle isolates than in pig isolates for chloramphenicol (p = 0.039), amoxicillin/clavulanic acid (p = 0.03), sulfonamide (p = 0.038), and trimethoprim/sulfamethoxazole (p = 0.022). There was a significant difference in resistance rate between cattle and chicken isolates to ceftiofur (p = 0.008), ceftriaxone (p = 0.008), chloramphenicol (p = 0.011), and kanamycin (p = 0.037) (Table 2).

Antimicrobial drug resistance was observed for drugs tested at different frequencies (Table 2). Seven hundred ninety-six (46%) *E. coli* isolates analyzed were susceptible to all 15 drugs tested. Among these pan-susceptible isolates, 637 (80%) were from humans, 69 (8.7%)

Table 2. Antimicrobial drug resistance phenotypes of *Escherichia coli* isolates from different sources, United States, 1950–2002*

Drug class	Drug	Resistance breakpoint, μg/mL	% Resistance					Timeline for clinical use of drugs (reference)
			Overall, n = 1,729	Human, n = 983	Cattle, n = 323	Chickens, n = 138	Pigs, n = 285	
Penicillins	AMP	≥32	24.1	16.5	35	34.1	33.3	1961 (*17*)
β-lactam/β-lactamase inhibitor combinations	AUG	≥32	5.6	2.4	12.7	7.3	7.4	1984 (*17*)
Cephems	CEP	≥32	12.9	8.8	20.1	12.3	19.3	1964 (*18*)
	FOX	≥32	4.4	1.5	9.3	5.1	8.4	1977 (*19*)
	TIO	≥8	2.3	0.1	7.4	1.5	4.2	1988 (FDA Green Book)†
	AXO	≥4	2.4	0.1	7.7	1.5	4.6	1984 (FDA Orange Book)‡
Phenicols	CHL	≥32	8.1	3.7	18	8.7	11.9	1947 (*17*)
Aminoglycosides	GEN	≥16	6.7	0.1	16.1	16.7	14	1963 (*17*)
	KAN	≥64	19.3	5.7	39.9	29.7	37.5	1957 (*17*)
	STR§	≥64	34.2	15.3	61.3	58	57.2	1943 (*17*)
Quinolones	CIP	≥4	0.4	0	1.9	0.7	0	1987 (*17*)
	NAL	≥32	1.7	1	3.7	1.5	1.8	1962 (*19*)
Tetracyclines	TET	≥16	40.9	18	71.2	68.8	72.3	1948 (*17*)
Folate pathway inhibitors	SUL	≥512	36.2	19.9	61	60.9	52.6	1936 (*19*)
	TMP/SMX	≥4	7	2.2	16.1	13.8	9.8	1968 (*19*)

*AMP, ampicillin; AUG, amoxicillin/clavulanic acid; CEP, cephalothin; FOX, cefoxitin; TIO, ceftiofur; FDA, Food and Drug Administration; AXO, ceftriaxone; CHL, chloramphenicol; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; CIP, ciprofloxacin; NAL, nalidixic acid; TET, tetracycline; SUL, sulfonamide; TMP/SMX, trimethoprim/sulfamethoxazole.
†www.fda.gov/ AnimalVeterinary/Products/ApprovedAnimalDrugProducts
‡www.fda.gov/drugs/developmentapprovalprocess/ucm079068.htm
§No Clinical and Laboratory Standards Institute breakpoint. The National Antimicrobials Resistance Monitoring System breakpoint was used.

Table 3. Multiple antimicrobial drug resistance among *Escherichia coli* isolates analyzed by source, United States, 1950–2002

No. drug classes to which isolates are resistant	% Resistant				
		Animal			
	Human, n = 983	Cattle, n = 323	Chickens, n =138	Pigs, n = 285	Total, n = 1,729
0*	64.8	21.4	21.0	21.1	46
1	12.4	9	7.2	9.1	10.8
2	7.5	10.5	16.7	16.1	10.2
3	6.5	22.6	26.8	23.9	14
4	4.5	15.5	14.5	13.3	8.8
5	3.8	7.7	7.2	8.4	5.5
6	0.4	6.2	4.3	2.8	2.2
7	0.1	5.3	2.2	5.3	2.1
8	0	1.9	0	0	0.3

*Susceptible to all drug classes.

from cattle, 60 (7.5%) from pigs, and 29 (3.6%) from chickens. Approximately 65% of human isolates were pan susceptible, compared with ≈20% of cattle, chicken, and pig isolates (Table 3). The proportion of pan-susceptible *E. coli* isolates decreased from 73.9% in 1950–1959 to 18.5% in 2000–2002. Conversely, multidrug resistance increased from 7.2% in 1950–1959 to 63.6% in 2000–2002 (Figure 1). Five hundred seventy (32.9%) *E. coli* isolates showed multidrug-resistant phenotypes, and 176 (10.2%) showed resistance to ≥5 drug classes. A larger proportion of multidrug-resistant isolates was recovered from animals than humans (Figure 2). One hundred ninety-one (59.1%) isolates from cattle, 153 (53.7%) from pigs, and 76 (55.1%) from chickens were resistant to ≥3 drug classes (Table 3), compared with 15.3% from humans. Two strains showed resistance to all 15 drugs tested; both strains were recovered from cattle in 2001.

Concurrent resistance to tetracycline and streptomycin was the most common co-resistance phenotype (29.7%), followed by resistance to tetracycline and sulfonamide (29.0%); tetracycline, sulfonamide, and streptomycin (23.9%); tetracycline and ampicillin (18.8%); and tetracycline, ampicillin, streptomycin, and sulfonamide (12.9%). A total of 130 (92.9%) of 140 chloramphenicol-resistant *E. coli* isolates were also resistant to tetracycline. Resistance to ceftriaxone, ceftiofur, and ciprofloxacin was rare and found only in isolates resistant to ≈7 drugs. More than 80% of these isolates were resistant to ampicillin, amoxicillin/clavulanic acid, cephalothin, cefoxitin, streptomycin, tetracycline, and sulfonamide.

Antimicrobial Drug Resistance Trends

The major goal of this study was to document antimicrobial drug resistance among historical bacteria from humans and animals to associate emergence of resistance with approval of new antimicrobial classes. Animal *E. coli* isolates showed an increasing resistance trend to 11 antimicrobial agents (ampicillin, sulfonamide, tetracycline, cephalothin, trimethoprim/sulfamethoxazole, streptomycin, chloramphenicol, cefoxitin, gentamicin, amoxicillin/clavulanic acid, and kanamycin), and human *E. coli* isolates showed an increasing trend in resistance only to ampicillin, sulfonamide, and tetracycline (Figure 3, Table 4).

Human *E. coli* isolates showed an increased resistance trend for ampicillin (0.59%/year, 95% CI 0.38%–0.81%; p<0.001), sulfonamide (0.49%/year, 95% CI 0.23%–0.73%; p<0.001), and tetracycline (0.45%/year, 95% CI 0.22%–0.70%; p<0.001), and this trend ranged during the study period from 0% to 66.7% for ampicillin, 0% to 50% for sulfonamide, and 0% to 58% for tetracycline (Table 4). The resistance rate to ampicillin in animal *E. coli* isolates

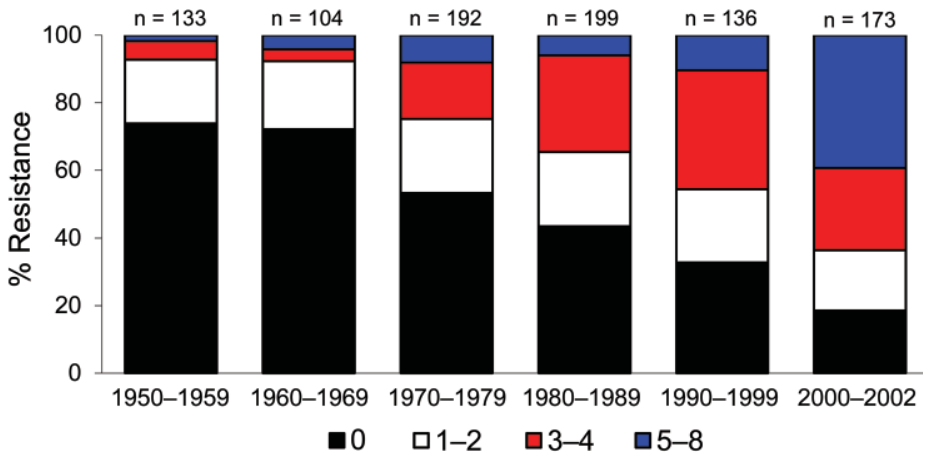

Figure 1. Change in antimicrobial drug resistance patterns among *Escherichia coli* isolates, United States, 1950–2002.

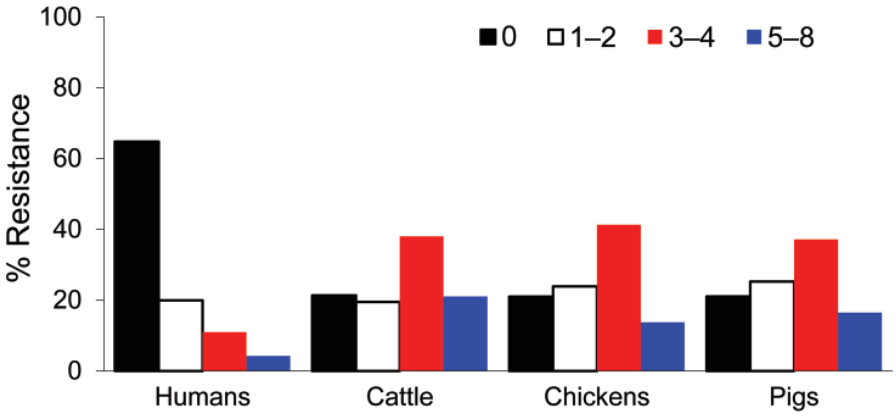

Figure 2. Distribution of multidrug resistance patterns among *Escherichia coli* isolates recovered from different sources, United States, 1950–2002.

was similar to that in human isolates and ranged from 0% to 69.4%. In contrast, resistance rates for sulfonamide and tetracycline among animal *E. coli* isolates ranged from 0% to 73.7% and 0% to 85.5%, respectively, and were higher than those for human isolates. There was a linear increasing trend in resistance to ampicillin (0.97%/year, 95% CI 0.60%–1.33%; p<0.001), sulfonamide (1.11%/year, 95% CI 0.34%–1.85%; p<0.01), and tetracycline (0.7%/year, 95% CI 0.21%–1.21%; p<0.01) (Table 4).

Cephalothin resistance significantly increased over time (0.43%/year, 95% CI 0.08%–0.77%; p<0.05) among animal *E. coli* isolates. In contrast, a decreasing resistance trend to cephalothin (–0.2%/year, 95% CI –0.34% to 0%; p<0.05) was observed among human isolates (Table 4). Although animal *E. coli* isolates showed an increasing rate of streptomycin resistance over time (0.9%/year, 95% CI 0.14%–1.41%; p<0.05), there was no significant increase among human *E. coli* isolates during the study period (0.15%/year, 95% CI –0.10% to 0.39%; p>0.1) (Table 4). In our study isolates, gentamicin resistance was observed in the 1980s among animal *E. coli* isolates but not until the late 1990s in human isolates. In animal *E. coli* isolates, the prevalence of gentamicin resistance increased from 0% during the 1970s to 28.1% during 2000–2002, and an increasing trend for resistance to gentamicin (1.28%/year, 95% CI 0.90%–1.69%; p<0.001) was first observed in the 1980s and reached a prevalence of 40% in 2002. Chloramphenicol resistance varied widely between human and animal isolates (yearly range 0%–46.8% among animal isolates and 0%–20% among human isolates). A linear increase in chloramphenicol resistance was observed among animal isolates (p<0.01), which increased 0.30%/year. Ceftiofur-resistant and ceftriaxone-resistant strains were not detected until 1990–1999 among human and animal *E. coli* isolates. None of the human *E. coli* isolates showed resistance to ciprofloxacin.

There was no monotonic resistance trend for trimethoprim/sulfamethoxazole, chloramphenicol, cefoxitin, ceftiofur, ceftriaxone, gentamicin, amoxicillin/clavulanic acid, nalidixic acid, and kanamycin among human *E. coli* isolates. Similarly, animal *E. coli* isolates did not show a monotonic resistance trend for ceftiofur, ceftriaxone, ciprofloxacin, and nalidixic acid.

Discussion

To help characterize evolution of drug resistance in *E. coli* since antimicrobial drugs were first widely used, we tested existing strain collections of *E. coli* for their susceptibility to a common panel of 15 antimicrobial agents. We tested 1,729 *E. coli* isolates from human and animal sources for susceptibility trends during the past 6 decades.

Resistance to sulfonamide was one of the most common resistance profiles identified among our study isolates and showed a monotone increasing resistance trend over time. Sulfonamide resistance has been observed in human *E. coli* isolates since 1950 and in animal isolates since 1964. Sulfonamides were introduced in the 1930s and have been in continuous use for >70 years. These drugs were administered alone from the 1930s through the 1960s in humans and were almost exclusively combined with diaminopyrimidines (e.g., trimethoprim) since the 1970s. In animal production systems, SUL is one of the most commonly used drugs as a single agent or in combination with diaminopyrimidines (e.g., ormetoprim) (*14*). A high prevalence of clinical resistance to sulfonamides was reported in enteric bacteria isolated from healthy food animals and humans (*10*,*22*,*23*) and is often associated with acquisition of the resistance genes *sul1* and *sul2* (*23*).

Sulfonamide resistance genes are commonly associated with mobile genetic elements, and these elements play a major role in dissemination of multiple antimicrobial drug resistance genes in *E. coli* isolates (*24*–*26*). In addition, despite a major reduction in the rate of sulfonamide use in the United Kingdom in 1995, resistance to sulfonamides persisted at high rates among clinical *E. coli* isolates

(*22*,*25*). Similarly, a 30-year (1979–2009) follow-up study on antimicrobial drug resistance at the Karolinska Hospital in Stockholm, Sweden, reported an increase in sulfonamide resistance despite decreased use (*13*).

Linkage of sulfonamide resistance genes, particularly as a constituent of class I integrons, to determinants conferring resistance to antimicrobial drugs that are still commonly used might help explain persistence of sulfonamide resistance (*22*). In our study, 80% (502/627) and 74% (462/627) of sulfonamide-resistant *E. coli* isolates were also resistant to tetracycline and streptomycin, respectively. Wu et al. (*27*) demonstrated that streptomycin and ampicillin are the 2 most frequently co-transferred resistance phenotypes among sulfonamide-resistant *E. coli* isolates recovered from pigs, pig carcasses, and humans. In addition to co-selection by drugs still commonly used, Enne et al. (*28*) and Bean et al. (*25*) suggested that lack of selective disadvantage of *sul2* (the most prevalent determinant of sulfonamide resistance) carriage and the genetic mobility of *sul2* might account for persistence in the absence of clinical selection pressure.

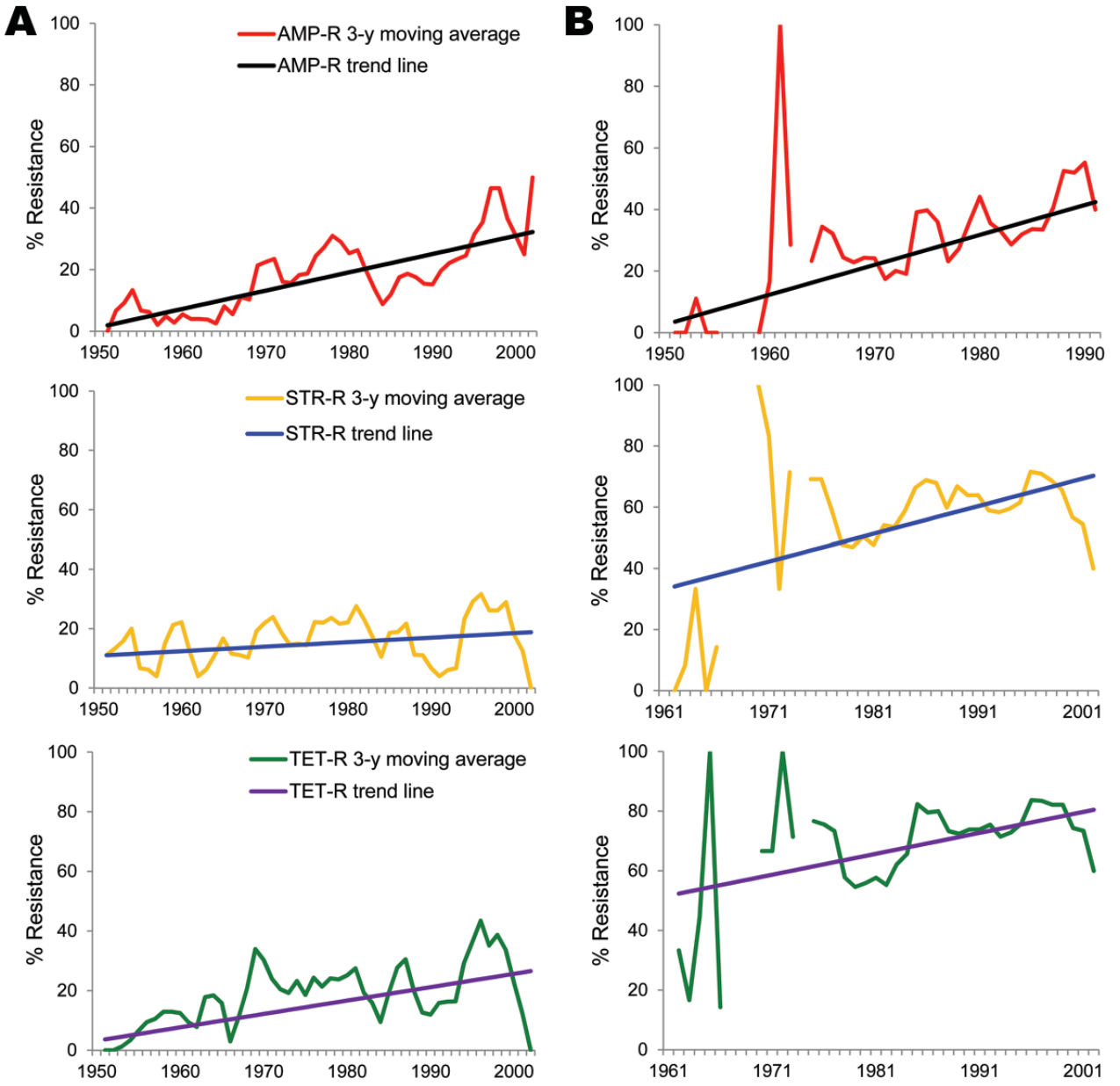

Figure 3. Trend analysis of selected antimicrobial agents among *Escherichia coli* isolates from humans (A) and animals (B), United States, 1950–2002. AMP-R, ampicillin resistance; STR-R, streptomycin resistance; TET-R, tetracycline resistance.

Table 4. Mann-Kendall and Sen estimates for trend statistics of antimicrobial drug resistance among *Escherichia coli* isolates of human and animal origin, United States, 1950–2002

Drug	Source	Years of time series	No.*	Mann-Kendall trend test Z value	Sen's slope estimate	
					p value	Q† (95% CI)
Ampicillin	Human	1950–2001	52	4.91	<0.001	0.59 (0.38–0.81)
	Animal	1962–2002	37	3.95	<0.001	0.97 (0.60–1.33)
Cephalothrin	Human	1950–2001	52	–2.38	<0.05	–0.20 (–0.34 to 0)
	Animal	1962–2002	37	2.52	<0.05	0.43 (0.08–0.77)
Sulfonamide	Human	1950–2001	52	3.41	<0.001	0.49 (0.23–0.73)
	Animal	1962–2002	37	3.24	<0.01	1.11 (0.34–1.85)
Streptomycin	Human	1950–2001	52	1.16	>0.1	0.15 (–0.10 to 0.39)
	Animal	1962–2002	37	2.24	<0.05	0.90 (0.14–1.41)
Tetracycline	Human	1950–2001	52	3.84	<0.001	0.45 (0.22–0.70)
	Animal	1962–2002	37	2.78	<0.01	0.70 (0.21–1.21)
Trimethoprim/sulfamethoxazole	Animal	1982–2002	21	2.64	<0.01	1.17 (0.32–2.15)
Chloramphenicol	Animal	1962–2002	37	2.75	<0.01	0.30 (0–0.59)
Cefoxitin	Animal	1982–2002	21	3.72	<0.001	0.88 (0.28–1.38)
Gentamicin	Animal	1978–2002	25	4.53	<0.001	1.28 (0.90–1.69)
Amoxicillin/clavulanic acid	Animal	1962–2002	37	3.09	<0.01	0.14 (0–0.38)
Kanamycin	Animal	1962–2002	7	4.38	<0.001	1.11 (0.59–1.50)

*Number of annual values for calculation of trend analysis.
†Magnitude of annual percentage increase or decrease.

Tetracycline resistance was the most common type of resistance observed and the most prevalent resistance phenotype in animal isolates (71.1%). This finding is not surprising because tetracycline has been widely used in therapy and to promote feed efficiency in animal production systems since its approval in 1948 (*2*,*14*). Persistence of tetracycline resistance was reported in animal coliforms a decade after it was no longer used in feed or for treatment (*29*). We commonly found co-resistance for tetracycline with streptomycin, sulfonamide, ampicillin, and chloramphenicol, as in other studies (*23*,*30*,*31*).

A small percentage of *E. coli* showed resistance to chloramphenicol, a drug approved in 1947 for human clinical use. Chloramphenicol is not approved for use in food animals in the United States. Persistence of chloramphenicol resistance in *E. coli* has been observed by other authors (*10*,*32*). Florfenicol, a closely related drug, was approved for treatment of respiratory diseases in cattle in the United States in 1996. Florfenicol resistance mediated by the *flo* gene confers nonenzymatic cross-resistance to chloramphenicol (*33*,*34*) and might select for nascent resistance in recent strains. Of known chloramphenicol-resistance genes, only a small number mediate resistance to florfenicol (*34*). For example, chloramphenicol-resistant strains in which resistance is exclusively based on activity of chloramphenicol acetyltransferases do not show resistance to florfenicol (*35*). Of 104 chloramphenicol-resistant animal *E. coli* isolates, 35.6% were isolated before approval of florfenicol. More than 90% of chloramphenicol-resistant *E. coli* isolates were concurrently resistant to tetracycline. In addition, our data showed not only persistence of chloramphenicol but an increasing tetracycline and SUL resistance trend over time among animal *E. coli* isolates. These observations could be explained by co-selection of mobile resistance elements or by unknown substrate(s) for the chloramphenicol-resistance determinants that serve as a selection pressure (*23*,*36*).

Gentamicin was approved for use in 1963 (*2*). Although gentamicin resistance was rare in human *E. coli* isolates, we found resistance rates ≤40% among animal *E. coli* in 2002. Since 1980, resistance to gentamicin has increased among animal *E. coli* isolates. The overall rate of gentamicin resistance was slightly higher in chicken (16.6%) and cattle (16%) isolates than in pig (14%) isolates. Gentamicin is widely used in the poultry industry. Aminoglycosides approved for use in food animals in the United States include dihydostreptomycin, efrotomycin, hygromycin B, neomycin, spectinomycin, streptomycin, and apramycin (*37*). A correlation between use of apramycin at the farm level and apramycin/gentamycin–resistant *E. coli* in diseased pigs and healthy finishers was reported (*15*). Yates et al. (*38*) reported apramycin-resistant *E. coli* isolates that were resistant to gentamicin and tobramycin, which are drugs used in human medicine. In our study, 93% of gentamicin-resistant *E. coli* isolates were multidrug resistant (≥3 classes of drugs). Eighty-one percent (94/116) were resistant to ≥5 antimicrobial drugs, including 95.7% (111/116) to streptomycin, 93.1% (108/116) to sulfonamides, and 91.4% (106/116) to tetracycline.

Our data showed lack of a monotonic trend for extended-spectrum cephalosporins resistance. Ceftiofur, a third-generation cephalosporin, was first approved in 1988 for veterinary use in food animals to treat a variety of gram-negative bacterial infections, including acute bovine respiratory diseases (*39*). In our culture collection, ceftiofur resistance was not detected before 1993 in animal isolates and before 1997 in human isolates. In NARMS *E. coli* collections, ceftiofur resistance was detected in the first years of testing among chicken carcasses (6.3% in 2000) and retail chicken breast samples (7.1% in 2002) (*10*). Studies

have shown ceftiofur use in animals can select for extended-spectrum cephalosporin resistance, including ceftriaxone resistance in bacteria isolated from animals and humans (*40*).

In the present study, 1 human *E. coli* isolate recovered in 1997 showed resistance to ceftiofur and ceftriaxone. This isolate was also resistant to 9 other antimicrobial drugs. Studies on *E. coli* isolates with decreased susceptibilities to ceftiofur and ceftriaxone showed carriage of a bla_{CMY} allele that conferred resistance to cephalothin, ampicillin, and amoxicillin/clavulanic acid, as in salmonellae (*24,40*). Additional data that include more years are needed to determine the resistance trend over time because third-generation cephalosporins were introduced in the 1980s.

A recent NARMS report showed that resistance to ceftriaxone ranged from 6.3% to 13.5% among *E. coli* isolates from chickens during 2000–2008; resistance to ceftiofur ranged from 4.4% to 10.5% during the same period (*10*). Currently, the molecular mechanisms of antimicrobial drug resistance development and evolution of these resistance genes over time are being investigated.

Our study has limitations because of its retrospective nature and reliance on preexisting culture collections for analysis. These limitations resulted in an uneven distribution of isolates per year and decade, incomplete or absent patient/host information regarding prior treatment history, and potential for bias in selecting isolates that were ultimately tested in this study. ECRC and CDC accept clinical samples for diagnostic purposes. Thus, isolate sets cannot be considered truly random. Also, patient information was limited; we had no data for prior antimicrobial drug exposure, travel, and other epidemiologic information. Therefore, analyses of resistance as a function of time were confounded. We selected the nonparametric tests of Mann-Kendall and Sen for trend analysis because they are suitable for non-normally distributed data and data with small number of observations.

Despite these limitations, this analysis provides foundational information for resistance development over time, laying the groundwork for understanding evolution of multidrug resistance at the genetic level. In addition, these data show that multidrug resistance is not a congenital feature of *E. coli*, and that drug use and resistance are closely related temporally. Work is ongoing to analyze this isolate set for alleles underlying resistance and compare them with recent isolates. This work will provide more definitive data on how resistance gene clusters have evolved and the context in which genes are maintained in the absence of known selection pressures.

Study isolates were collected by ATCC through contract agreement (223-02-7010) with Center for Veterinary Medicine, Office of Research, US Food and Drug Administration.

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Novel Strain of Andes Virus Associated with Fatal Human Infection, Central Bolivia

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To better describe the genetic diversity of hantaviruses associated with human illness in South America, we screened blood samples from febrile patients in Chapare Province in central Bolivia during 2008–2009 for recent hantavirus infection. Hantavirus RNA was detected in 3 patients, including 1 who died. Partial RNA sequences of small and medium segments from the 3 patients were most closely related to Andes virus lineages but distinct (<90% nt identity) from reported strains. A survey for IgG against hantaviruses among residents of Chapare Province indicated that 12.2% of the population had past exposure to ≥1 hantaviruses; the highest prevalence was among agricultural workers. Because of the high level of human exposure to hantavirus strains and the severity of resulting disease, additional studies are warranted to determine the reservoirs, ecologic range, and public health effect of this novel strain of hantavirus.

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) are trisegmented negative-strand RNA viruses in which the small (S), medium (M), and large (L) genomic segments encode for the nucleocapsid protein (N), 2 envelope glycoproteins (Gn and Gc), and the viral polymerase, respectively. Hantaviruses are maintained in rodent reservoirs, and human exposure typically results from inhalation of aerosols from infectious urine or feces, although human-to-human transmission of Andes virus (ANDV) has also been described (*1*). Human hantavirus infection in South America is often associated with rapid onset of severe disease manifestations, such as respiratory failure and cardiac dysfunction referred to as hantavirus pulmonary syndrome (HPS) and case-fatality rates ≥50% (*2*,*3*). Despite the public health effects, in most cases of human infection, the precise etiologic agent is not identified. Thus, the extent of genetic diversity and geographic distribution of distinct hantavirus strains is not well understood.

Since the first identification of HPS in 1993, many new hantaviruses have been described throughout North, Central, and South America. Studies of rodent reservoirs in South America have identified an increasingly complex picture of hantavirus diversity and ecology (*2*,*4*). Unique strains of hantavirus have been identified in rodents in Venezuela (*5*,*6*), Peru (*7*), Brazil (*8*–*10*), Argentina (*11*–*13*), Paraguay (*14*,*15*), and Chile (*11*,*16*), many of which have also been associated with human illness. In Bolivia, the first hantavirus identified was Río Mamoré virus (RIOMV), which was isolated from a pygmy rice rat (*Oligoryzomys microtis*) (*17*) but has not been associated with human disease. In 1997, a Laguna Negra virus (LNV) variant was identified in an HPS patient in Chile who had traveled extensively in Bolivia (*18*,*19*). An ecologic assessment of reservoir hosts identified the large vesper mouse (*Calomys callosus*) as reservoir host of LNV in Bolivia (*20*). The association of ANDV (Nort lineage) and Bermejo virus (BMJV) with 2 HPS cases in southern Bolivia in 2000 documented the first human infection by BMJV (*21*).

To further describe the diversity of hantavirus strains associated with human disease in Bolivia, we screened febrile patients reporting to 2 health centers in Chapare Province for serologic and molecular evidence of hantavirus infection. We describe the clinical signs and symptoms and

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genetic characterization (partial S and M segment) of a novel strain of hantavirus in 3 patients, including 1 who died. In addition, we report results of a survey to determine the prevalence of previous hantavirus exposure in the region.

Materials and Methods

Study Site and Human Participant Issues

Patients were recruited when they reported acute febrile illness (≤7 days) at the Hospital San Francisco de Asis or Centro de Salud Eterezama (16°55′S, 65°22′W; 265 m above sea level), located in the Chapare Province of the Department of Cochabamba in central Bolivia (*22*) (Figure 1). Chapare is a rural province with tropical rainforests surrounding the Chapare River, the main waterway of the region. The health centers are located in the towns of Villa Tunari and Eterezama, which had 2,632 and 2,001 inhabitants, respectively, at the time of the 2001 census (*23*).

Study protocols were approved by Servicio Departamental de Salud Santa Cruz, and Colegio Medico de Santa Cruz. Study protocols (NMRCD.2000.0008 and NMRCD.2005.0002) were also approved by the US Naval Medical Research Unit Institutional Review Board in compliance with all US Federal regulations governing the protection of human subjects. Written consent was obtained from patients ≥18 years of age. For patients <18 years of age, written consent was obtained from a parent or legal guardian. Written assent was also obtained from patients 8–17 years of age.

A survey for prior exposure to arenaviruses and hantaviruses was conducted in Chapare Province during April 25–May 2, 2005, after a reported outbreak of febrile illness and hemorrhagic fever in the region (*24*). Adults (≥18 years of age) were invited to participate in the study. Blood samples (10 mL) were collected by venipuncture for screening of antibodies against hantaviruses, and demographic data were collected for risk factor analysis in assorted villages in Chapare Province (Figure 1). Data included age, occupation, self-reported exposure to rodents, house construction materials, and recent health history.

Serologic Analyses

Serum samples from febrile patients were screened for IgM against ANDV or LNV antigens by ELISA. In brief, 96-well plates were coated with anti-human IgM, human serum samples (1:100 dilution) were added, and plates were incubated for 1 h at 37°C. Wells were subsequently incubated with ANDV or LNV antigen for 1 h at 37°C. Viral antigens were recognized by the addition of hyperimmune mouse ascitic fluid for 1 h at 37°C and incubation with horseradish peroxidase–conjugated anti-mouse IgG for 1 h. Finally, substrate (2,2′-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid]; Kirkegaard and Perry, Inc., Gaithersburg, MD, USA) was added, and optical density at a wavelength of 405 nm was measured by using a spectrophotometer. Patient serum specimens were also screened for IgM against a panel of arboviral pathogens, including dengue viruses, yellow fever virus, and Venezuelan equine encephalitis virus. Virus culture and identification was attempted in African green monkey Vero cell cultures by indirect immunofluorescence assay and Sin Nombre virus (SNV) polyclonal antibodies, as described for arboviruses (*22*).

Figure 1. Location of Villa Tunari, Department of Cochabamba, Bolivia, the area where patients with hantavirus infection were recruited. The constitutional (Sucre) and administrative (La Paz) capitals of Bolivia are shown for reference.

For the seroprevalence study, serum samples from healthy participants were screened by indirect ELISA for IgG against SNV antigen (Centers for Disease Control and Prevention, Atlanta, GA, USA). Serum samples were diluted 1:100 and incubated in SNV recombinant antigen–coated wells and then with horseradish peroxidase–conjugated mouse anti-human IgG (1:8,000 dilution). Finally, substrate (2,2′-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid]) was added, and absorbance was measured at 405 nm with a Dynex ELISA MRX Revelation absorbance reader (Dynex Technologies, Chantilly, VA, USA). Samples with optical densities greater than the mean of 5 negative controls plus 5 SD at a 1:100 dilution were considered positive.

Molecular Analyses

After serologic screening, RNA was extracted from whole blood and serum samples of patients positive for hantavirus IgM by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). A 1-step reverse transcription PCR (RT-PCR) was performed by using the Access RT-PCR system (Promega, Madison, WI, USA). Nested PCRs were performed by using the FastStart PCR Master (Roche, Indianapolis, IN, USA). Initial screening was performed by using primers specific for the S segment as described (*20*). Additional primers were designed on the basis of preliminary sequences to generate additional S segment coding region sequence (forward: HANSF3 5′-TGGATGTTAATTCCATCGA-3′ and reverse: HANSR4 5′-GATAATGTTTCGTGCTTTCA-3′; forward: HANF0001 TAGTAGTAGACTCCTTGAGAAGCTACT and reverse: HANTASR2 TAGTATGCTCCTTGAR AAGC). A 1,287-bp region of the S segment was generated, which included positions 43–1329 of the full-length S segment of ANDV strain Chile R123 (*25*).

For the M segment, RT-PCR and nested PCR were performed by using specific primers (*18*), which generated a 1,330-bp sequence of the M segment that included positions 1678–3007 of the full-length M segment of ANDV strain Chile R123. RT-PCR amplicons were purified by agarose gel electrophoresis and sequenced directly by using the Big Dye Terminator v3.1 Cycle Sequencing Kit on a 3100 Avant-Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic Analysis

S segment and M segment sequences (submitted to GenBank under accession nos. JF750417–JF750422) were compared with sequences from other members of the genus *Hantavirus*, including Puumula virus strain Umea (Genbank accession nos. S segment: AY526219, M segment: AY526218), RIOMV strain HTN-007 (S: FJ532244, M: FJ608550), SNV strain NMH10 (S: L25784, M: L24783), El Moro Canyon virus strain RM97 (S: U11427, M: U26828), Choclo virus (S: DQ285046, M: DQ285047), Caño Delgadito virus (S: DQ285566; M: DQ284451), Pergamino virus (PRGV; S: AF482717, M: AF028028), ANDV strain AH-1 (S: AF324902, M: AF324901), ANDV strain CHI7913 (S: AY228237, M: AY228238), ANDV strain Chile-9717869 (S: AF291702, M: AF291703), Maciel virus strain 13796 (MACV; S: AF482716, M: AF028027), Catacamas virus (CATV; S: DQ256126, M: DQ177347), Paranoa virus (S: EF576661), Oran virus (S: AF482715, M: AF028024), LNV (S: AF005727, M; AF005728), BMJV (S: AF482713, M: AF028025), Lechiguanas virus strain 22819 (S: AF482714, M: AF028022), ANDV strain Hu39694 (S: AF482711, M: AF028023), Playa de Oro virus (S: EF534079, M: EF534082), Neembucu virus (S: DQ345763), Alta Paraguay virus (S: DQ345762), Itapua virus (S: DQ345765), Araraquara virus (ARAV; S: AF307325, M: AF307327), Araucaria virus strain HPR/03–99 (S: AY740630), Jabora virus strain Akp8048 (S: JN232080), Juquitiba virus strain Olfo_777 (S: GU213198), and Castelo dos Sonhos virus (CASV; S: AF307324, M: AF307326).

Sequences were aligned by using ClustalW (www.clustal.org) with manual adjustments, and pairwise genetic distances were calculated by using MEGA4.0 (*26*). For phylogenetic analysis, maximum-likelihood (ML) and Bayesian approaches were used. ML phylogenetic trees were estimated by using PhyML (*27*,*28*) and 100 bootstrap replications to place confidence intervals at nodes. Phylogenetic reconstructions were also conducted in MrBayes version 3.1 (*29*,*30*) under the general time reversible + Γ + proportion invariant model of evolution, with 1 million Markov Chain Monte Carlo generations, and sampling every 100 generations with a burn-in of 25,000. Puumula virus S and M segments were included as outgroups in the phylogenetic reconstructions.

Results

Patient Identification

During January 2008–June 2009, serum samples from 372 febrile patients reporting to clinics in Chapare Province, Bolivia (Figure 1) were tested for serologic evidence of recent infection by ≥1 hantaviruses. Of these 372 patients, 199 (53.5%) were male patients with a median age of 31 years (range 7–95 years). IgM against ANDV (n = 8) or LNV (n = 1) antigen was identified in acute-phase or convalescent-phase samples from 9 (2.4%) patients. No evidence of recent arbovirus infection was detected in these samples. Of the 9 patients with IgM against hantaviruses, 7 (77.8%) were male patients with a median age of 32 years (range 15–49 years). Three of the 9 patients were positive for hantavirus RNA.

Patient 1 (FVB0554) was an 18-year-old man (student) from the town of Pedro Domingo Murillo, Bolivia, who came to Hospital San Francisco de Asis in January 2008 He reported 7 days of fevers, chills, and malaise. Other symptoms included oliguria, arthralgias, myalgias, bone pain, headache, and retroocular pain. Gastrointestinal (abdominal pain, diarrhea, nausea, emesis, and icterus) and respiratory (cough, dyspnea, and cyanosis) manifestations were also prominent. The patient died the next day. IgM against LNV antigen (reciprocal titer 1,600) was detected in a serum sample collected before his death.

Patient 2 (FVB0640) was a 27-year-old man (agricultural worker) from Samuzabety, Bolivia, who came to Hospital San Francisco de Asis in March 2008. The patient had a temperature of 39.9°C and reported 8 days of

fever, chills, and malaise. Other symptoms included cough, arthralgias, myalgias, bone pain, headache, and retroocular pain. On examination, multiple cutaneous manifestations were noted, including petechiae, purpura, a maculopapular rash, and a diffuse erythematous rash. The patient was hospitalized for 4 days and recovered. IgM against ANDV was detected in an acute-phase serum sample (reciprocal titer 6,400); no convalescent-phase sample was obtained.

Patient 3 (FVB0799) was a 49-year-old man (farmer) from Flor de San Pedro, Bolivia, who came to Hospital San Francisco de Asis in June 2009. He reported 4 days of fever, chills, and malaise. Other symptoms included arthralgias, myalgias, bone pain, abdominal pain, headache, cough and dyspnea. The patient survived. IgM against ANDV was detected in an acute-phase serum sample (reciprocal titer 6,400); no convalescent-phase sample was available for additional analysis.

Molecular Analyses

Viral sequences generated from samples from the 3 patients were highly conserved over the gene regions analyzed; >99.6% pairwise nucleotide identity in the S segment (3–5-nt differences) and >99.2% pairwise nucleotide identity in the M segment (1–10-nt differences). Nucleotide sequences were compared with those of hantavirus strains available in GenBank (Table 1). In pairwise comparisons of S segment gene sequences, we observed the highest identity with CASV (*31*), which showed 89.3% identity at the nucleotide level and 98.6% identity at the amino acid level, although only limited sequence (643 nt) was available for comparison. In comparison with other Western Hemisphere hantaviruses for which more extensive sequences were available (1,287 nt) 75.8%–84.1% nucleotide sequence identity and 85.3%–97.7% amino acid identity were observed, and the highest similarity was with members of the species *Andes virus* (Table 1).

In pairwise comparisons of M segment gene sequences, the highest nucleotide identity (83.3%) was observed in comparison with CASV. Similar amino acid identities were observed with CASV (95.1%), Oran virus (95.3%), Lechiguanas virus (95.0%), and ANDV Hu39694 (95.3%) (Table 1). Viral sequences amplified from patient samples were more distantly related to LNV, Caño Delgadito virus, and Maporal virus; all showed <80% pairwise identity at the nucleotide level and <90% pairwise identity at the amino acid level (Table 1).

Table 1. Percent pairwise nucleotide and amino acid identity between select Western Hemisphere hantaviruses and virus sequences amplified from patients from central Bolivia*

		S segment (1,287 bp)		M segment (1,330 bp)	
Virus strain	Country	Nucleotide	Amino acid	Nucleotide	Amino acid
PRGV	Argentina	81.4	94.6	80.8	93.0
ANDV AH1	Argentina	83.5	96.0	81.7	93.9
ANDV Hu39694	Argentina	82.0	97.4	81.7	95.3
MACV	Argentina	81.7	94.2	80.2	91.4
BMJV	Argentina	83.7	97.7	80.2	93.9
LECV	Argentina	84.1	97.4	81.1	95.0
ORNV	Argentina	83.5	97.4	80.3	95.3
CASV†‡	Brazil	89.3	98.6	83.3	95.1
PARV	Brazil	82.9	95.3	NA	NA
ARAV§	Brazil	84.0	94.9	79.5	93.2
JABV	Brazil	77.3	88.6	NA	NA
ARCV	Brazil	82.2	95.8	NA	NA
ANDV 9717869	Chile	83.5	96.0	80.7	93.7
ANDV CHI7913	Chile	82.7	95.6	81.1	92.8
CATV	Honduras	76.9	88.1	76.0	86.2
PDOV	Mexico	77.4	87.4	75.8	85.4
CHOV	Panama	78.9	89.3	77.8	88.0
NEMV	Paraguay	84.9	97.0	NA	NA
ALPV	Paraguay	80.3	89.3	NA	NA
ITAPV	Paraguay	81.7	95.8	NA	NA
JUQV‡	Paraguay	82.5	95.5	NA	NA
LNV	Paraguay	79.4	90.2	79.2	90.5
RIOMV	Peru	80.1	90.0	80.6	91.4
SNV NMH10	USA	76.5	87.2	76.0	86.2
ELMCV RM97	USA	76.8	83.9	73.8	82.8
MAPV	Venezuela	79.6	91.1	77.8	89.8
CADV	Venezuela	75.8	85.3	74.3	83.1

*S, small; M, medium; PRGV, Pergamino virus; ANDV, Andes virus; MACV, Maciel virus; BMJV, Bermejo virus; LECV, Lechiguanas virus; ORNV, Oran virus; CASV, Castelo dos Sonhos virus; PARV, Paranoa virus; ; NA, sufficient sequence not available for comparison; ARAV, Araraquara virus; JABV, Jabora virus; ARCV, Araucaria virus; CATV, Catacamas virus; PDOV, El Moro Canyon virus; CHOV, Choclo virus; NEMV, Neembucu virus; ALPV, Alta Paraguay virus; ITAPV, Itaporanga virus; JUQV, Juquitiba virus; LNV, Laguna Negra virus; RIOMV, Río Mamoré virus; SNV, Sin Nombre virus; ELMCV, El Moro Canyon virus; MAPV, Maporal virus; CADV, Caño Delgadito virus.
†S segment sequence comparison was limited to the homologous 999 bp (JUQV) or 643 bp (CASV) available from GenBank.
‡M segment sequence comparison was limited to the homologous 1,246 bp available from GenBank.

To further explore genetic relationships between the novel viral sequences and previously described hantaviruses, we conducted ML and Bayesian analyses on the basis of S segment and M segment nucleotide sequences. Similar results were obtained for ML and Bayesian approaches (Figure 2). Viral sequences derived from patient samples grouped with other strains of ANDV (www.ncbi.nlm.nih.gov/ICTVdb/index.htm); formed a clade with ARAV, MACV, PRGV, and other ANDV strains; and formed a subclade with CASV (Figure 2). Similar tree topologies for other strains of ANDV were obtained on the basis of analysis of S segment and M segment sequences. Genetic differences between CASV and the novel sequences were well supported by posterior probabilities (Figure 2) and ML bootstrap values.

Prevalence of IgG against Hantaviruses among Humans in the Chapare Region

To determine the extent of human exposure to hantaviruses in the region, we screened serum samples from residents of villages in Chapare Province for IgG against SNV antigen. A total of 500 participants >18 years of age residing in villages in the region were enrolled during April 25–May 2, 2005 (Table 2). Participants had a median age of 31 years (range 18–99 years); 54.9% were women (Table 2).

Sixty-one (12.2%; 95% CI 9.3%–15.1%) had IgG against SNV antigen (Table 2), and the highest prevalences were in the towns of Samuzabety (18.6%) and San Gabriel (17.2%). No differences were observed between sexes or among different age groups (Table 2). The highest prevalence of IgG against SNV was among agricultural workers (15.0%) and housewives (13.5%) (Table 2). No differences in seropositivity were observed for participants with differing house construction materials or quality.

Discussion

We demonstrated the association of a novel genotype of ANDV with fatal human infection in central Bolivia and extended the known genetic diversity of hantaviruses circulating in South America. One fatal case occurred among the 3 patients described, which was consistent with high mortality rates observed with infections with ANDV lineages in neighboring Brazil and Argentina (*3*). The International Committee on Taxonomy of Viruses has provided guidelines for the demarcation of hantaviruses (www.ictvdb.org/Ictv/index.htm), which include a ≥7% difference in amino acid identity in comparison with previously identified complete S segment and M segment gene sequences, a ≥4-fold difference in results of 2-way cross-neutralization tests, and occupation of a unique ecologic niche, such as a different primary rodent reservoir. As with other hantavirus strains, attempts to isolate virus in

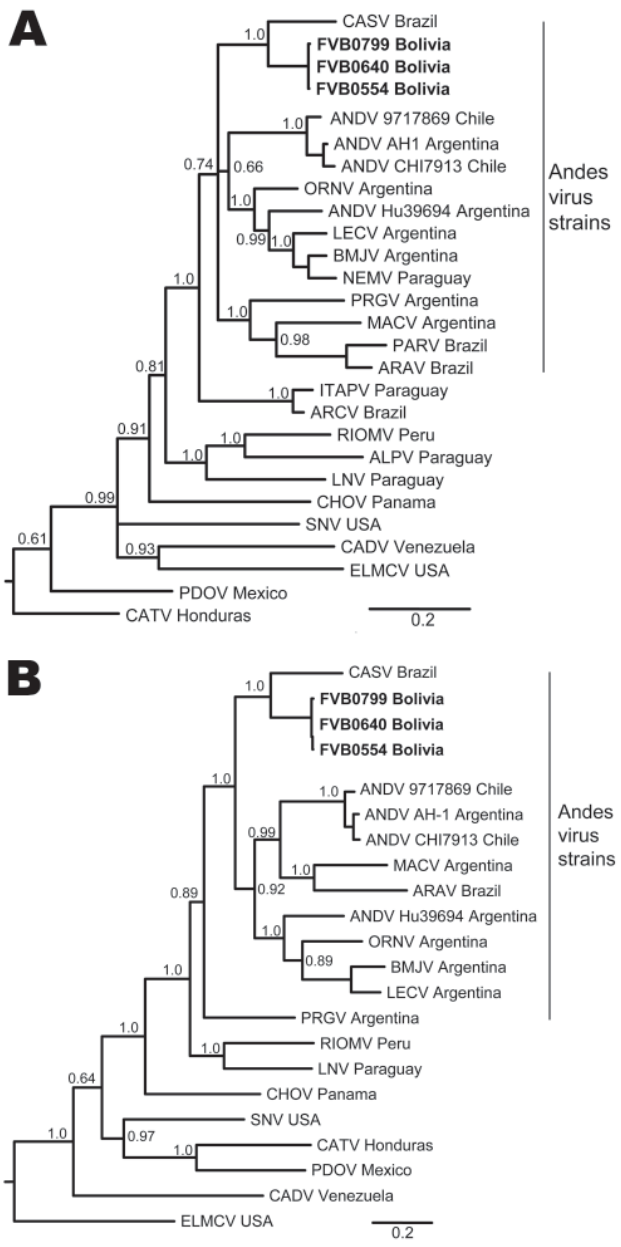

Figure 2. Phylogenetic analysis of hantaviruses from the Western Hemisphere on the basis of partial A) small and B) medium segments. Novel strains described in this study are indicated in **boldface**. Depicted phylogenetic reconstructions are based on Bayesian inference conducted in MrBayes (*29,30*). Posterior probabilities are indicated at relevant nodes. Scale bars indicate nucleotide sequence divergence. CASV, Castelo dos Sonhos virus; ANDV, Andes virus; ORNV, Oran virus; BMJV, Bermejo virus; LECV, Lechiguanas virus; BMJC, Bermejo virus; NEMV, Neembucu virus; PRGV, Pergamino virus; MACV, Maciel virus; PARV, Paranoa virus; ARAV, Araraquara virus; ITAPV, Itaporanga virus; ARCV, Araucaria virus; RIOMV, Río Mamoré virus; ALPV, Alta Paraguay virus; LNV, Laguna Negra virus; CHOV, Choclo virus; SNV, Sin Nombre virus; CADV, Caño Delgadito virus; ELMCV, El Moro Canyon virus; PDOV, El Moro Canyon virus; CATV, Catacamas virus.

Table 2. Characteristics of patients tested for IgG against Sin Nombre virus, central Bolivia*

Characteristic	No. positive/no. tested (%)
Sex	
M	28/224 (12.5)
F	32/273 (11.7)
Age, y	
18–30	28/244 (11.5)
31–50	28/207 (13.5)
≥50	4/43 (9.3)
Occupation	
Agricultural worker	25/167 (15.0)
Housewife	26/193 (13.5)
Student/teacher	3/57 (5.3)
Health professional	0/20 (0)
Other/unknown	7/62 (11.3)
Village	
Eterazama	13/116 (11.2)
Isinuta	6/71 (8.5)
Primero de Mayo	1/ 20 (5.0)
Samuzabety	13/70 (18.6)
San Gabriel	5/ 29 (17.2)
San Julian	2/24 (8.3)
Urkupina	2/22 (9.1)
Other	19/148 (12.8)
Total	61/500 (12.2)

*Complete demographic data were not available for all participants.

tissue culture were unsuccessful; thus, cross-neutralization studies could not be conducted. Genetic criteria, amino acid and nucleotide comparisons, and phylogenetic analysis clearly support inclusion of this strain in the species *Andes virus*.

No guidelines have been proposed for demarcation of viruses below the species level, and there does not appear to be consensus on the designation of novel genotypes. We observed the highest identity with CASV, which has been associated with human illness near the border of the Mato Grosso and Pará States of Brazil (*31*,*32*), ≈1,500 km from the Chapare study sites. We observed ≈11% and 17% divergence at the nucleotide level for the S segment and M segment, respectively, in comparison with CASV. However, the true difference between the strains might be underestimated because higher nucleotide and amino acid conservation was observed among ANDV strains overall in the limited S region available for comparison (14%), relative to other genome regions (16%). No other hantavirus was found to be <15% divergent at the nucleotide level in the S segment or <18% divergent in the M segment. If one considers that the strains identified in our study segregate with other strains of ANDV but are genetically distinct, we provisionally propose to name this novel genotype Tunari virus (TUNV), after the town of Villa Tunari, where all 3 patients sought medical attention.

On the basis of data in this report, we found that human hantavirus exposure is common in the Chapare Province. Including the 3 TUNV cases, in 2008 and 2009, >2% of febrile cases analyzed had evidence of recent hantavirus infection, which is consistent with the 4% of febrile cases reported for the region in 2005 and 2006 (*33*). In addition, the 2005 serosurvey of healthy persons indicated that a high percentage (≈12%) of adults had evidence of exposure to ≥1 hantaviruses at some time in their lives.

The extent of exposure we found is similar to that reported in neighboring Brazil, which was 13.3% in Maranhao State in northeastern Brazil (*34*) and 14.6% in southern Brazil (*35*), and in northern Argentina, which was 6.5%–17.1%, depending on the population studied (*13*,*36*). Occupational exposure appears to be a risk factor because the highest antibody prevalence and 2 of 3 TUNV cases were identified among agricultural workers. We did not observe an age-dependent increase in antibody prevalence among adults sampled, a finding also reported in southern Brazil (*35*). There are several possible explanations for this observation, including relatively recent emergence of hantaviruses in the region, high mortality rates among infected persons, and preponderance of risk for exposure during early adulthood.

Broad antigenic cross-reactivity that prevents discrimination among diverse hantavirus groups is 1 of the major limitations of ELISA-based serologic studies, whether used in screening febrile patients for IgM against hantaviruses or healthy persons for IgG against hantaviruses. Co-circulation of heterologous hantaviruses has been described in Bolivia in rodent reservoirs and in ill persons. RIOMV has been identified in the pigmy rice rat (*Oligoryzomys microtis*) in Bolivia (*17*). In 2000, HPS cases associated with BMJV and ANDV strain Nort were identified along the southern border of Bolivia with Argentina (*21*). LNV had been amplified from an HPS patient in Chile with recent travel history to Bolivia (*19*). In addition to these cases are many additional suspected cases of HPS in Bolivia for which no specific virus was identified. Of the 246 reported cases from 2007 through 2010, a total of 74 occurred in the Department of Cochabamba (*37*). Future studies with more specific serologic assays are necessary to determine the true extent of TUNV circulation in this population.

In this study, we made no effort to incriminate the reservoir host for TUNV. The only hantavirus reservoir identified in South America is rodents of the subfamily *Sigmodontinae*. *Oligoryzomys* spp. rodents appear to be the principal reservoirs for most ANDV variants, including CASV (*32*,*38*). In addition to *Oligoryzomys* spp. rodents, ANDV variants have been identified in *Akodon* spp. (PRGV), *Necromys* spp. (MACV and ARAV), and *Bolomys* spp. (MACV) rodents. Potential reservoir species are abundant in Bolivia, including *Oligoryzomys* spp., *Akodon* spp., and *Calomys* spp. (LNV) rodents. Increased rodent population density has been associated with the emergence of hantavirus infection in humans (*4*). Therefore identifying the TUNV reservoir host and understanding its

ecology could lead to interventions for reducing human exposure.

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Transmission Dynamics, Border Entry Screening, and School Holidays during the 2009 Influenza A (H1N1) Pandemic, China

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Pandemic influenza A (H1N1) 2009 virus spread rapidly around the world in 2009. We used multiple data sources from surveillance systems and specific investigations to characterize the transmission patterns of this virus in China during May–November 2009 and analyze the effectiveness of border entry screening and holiday-related school closures on transmission. In China, age distribution and transmission dynamic characteristics were similar to those in Northern Hemisphere temperate countries. The epidemic was focused in children, with an effective reproduction number of ≈1.2–1.3. The 8 days of national holidays in October reduced the effective reproduction number by 37% (95% credible interval 28%–45%) and increased underreporting by ≈20%–30%. Border entry screening detected at most 37% of international travel–related cases, with most (89%) persons identified as having fever at time of entry. These findings suggest that border entry screening was unlikely to have delayed spread in China by >4 days.

Pandemic influenza A (H1N1) 2009, hereafter referred to as A(H1N1)pdm09, spread rapidly, resulting in millions of cases and ≈18,000 deaths in ≈200 countries (*1*). On August 10, 2010, the World Health Organization (WHO) declared that the world had entered the postpandemic period (*2*). Much has been published about the epidemiology of the pandemic in Western countries (*3*–*9*), but far less has been published about the experience of a large and diverse country, such as the People's Republic of China. In addition, although many countries adopted so-called early containment strategies, data on their effectiveness are rare (*7*,*10*,*11*).

In response to the evolving global spread of A(H1N1) pdm09 virus infection, China established national surveillance on April 30, 2009. Initially, the country implemented an aggressive containment strategy based on the national pandemic preparedness plan, including isolation of all suspected case-patients in designated hospitals, contact tracing, medical observation of persons exposed to patients with confirmed cases, and border entry screening (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-0356-Techapp.pdf). On May 11, the first case of A(H1N1)pdm09 in mainland China was identified in a traveler returning from the United States (*12*). We report the transmission patterns of A(H1N1)pdm09 in China from that time through November 2009 and analyze the effectiveness of border entry screening and holiday-related school closures on transmission using multiple data sources from surveillance systems and specific investigations.

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Methods

Sentinel Surveillance for Influenza-like Illness

National sentinel hospital-based surveillance for influenza-like illness (ILI) was launched in China in 2005. This type of surveillance is primarily dedicated to virologic surveillance with a goal of providing information for annual WHO influenza vaccine selection (online Technical Appendix). Each week, 193 sentinel hospitals in 30 provinces report the total number of outpatient visits and the number of those patients with ILI by age group to a centralized online system maintained by the Chinese Center for Disease Control and Prevention (China CDC). In addition, respiratory specimens are collected each day from the first or second ILI case-patient who visits each hospital's outpatient clinic. This collection results in virologic samples from 10–15 respiratory tract specimens per hospital each week. Specimens are sent to 1 of the 62 province- or prefecture-level disease control centers for testing. Laboratory results are reported weekly online to China CDC. These data are collected systematically throughout the year and are an unbiased sample of the timing of influenza activity.

Individual Case-based Surveillance

During the early containment phase of the 2009 pandemic (until mid-July 2009), an individual case–based surveillance system was implemented. A(H1N1)pdm09 virus infection was added to China's list of notifiable communicable diseases on April 30, 2009. Persons with suspected A(H1N1)pdm09 infection were identified through active surveillance with border entry screening and medical monitoring of close contacts exposed to confirmed case-patients or through passive reporting by clinicians when those patients sought health care. Any person entering China was required to undergo screening at the border (any point of entry into China from another country or from a neighboring region, such as Hong Kong Special Administrative Region), regardless of border type or travel mode. All patients with suspected A(H1N1) pdm09 virus infection, regardless of its clinical severity, were admitted to designated hospitals for containment (*13*,*14*). Upper respiratory specimens were collected and sent to the national sentinel ILI surveillance network of 62 laboratories for A(H1N1)pdm09 testing by real-time reverse transcription PCR (rRT-PCR) (online Technical Appendix). All suspected and laboratory-confirmed cases were reported online within 24 hours to China CDC by public health officers in county-, prefecture-, and province-level disease control centers and clinicians nationwide. Data posted on a standardized reporting card included sex, age, place, overseas travel history, and date of symptom onset.

Outbreak Surveillance

In accordance with recommendations from the Ministry of Health of China, local disease control centers were asked to investigate all institutional or community outbreaks (e.g., associated with particular schools or shared public transport vehicles) by using the case definition for acute respiratory illness (ARI). Data on all suspected cases, probable cases, and confirmed cases were reported online to China CDC.

Investigation of Cases Linked to International Travel

In addition, through July 31, a joint team from local disease control centers and China CDC investigated confirmed international travel–related cases (online Technical Appendix) to collect detailed epidemiologic information. A standardized questionnaire was used to collect data about international travel histories, date of symptom onset, and reported symptoms on arrival in China. Data on contacts were also obtained. In accordance with Ministry of Health recommendations, all close contacts of confirmed case-patients were quarantined at home or in designated hotels and monitored daily for fever and respiratory symptoms for 7 days after their last exposure to a confirmed case-patient.

We also learned whether the case was detected at the border. Data were not available on how these case-patients entered mainland China (e.g., by air, sea, or land).

Case Definitions

A case-patient with ARI had fever (temperature ≥37.3°C), and/or recent onset of ≥1 of the following: rhinorrhea, nasal congestion, sore throat, or cough. A case-patient with ILI had a body temperature ≥38°C with either cough or sore throat in the absence of an alternative diagnosis. A person with a suspected case of A(H1N1) pdm09 virus infection had ARI and 1 of the following: illness onset within 7 days after travel to an area with ≥1 confirmed A(H1N1)pdm09 cases or within 7 days after close contact with a confirmed case-patient. A person with a confirmed case had ARI and laboratory evidence of A(H1N1)pdm09 virus infection diagnosed by rRT-PCR of respiratory specimens. A person with a probable case had ARI that was epidemiologically linked to a patient with a confirmed case. On the basis of information about overseas travel and any identified links to other known case-patients, all reported confirmed cases were classified as international travel–related cases, individual domestic cases, and institutional or community outbreaks.

Change in Surveillance Strategy

By mid-August 2009, as A(H1N1)pdm09 activity expanded, the national surveillance strategy changed from individual case-based surveillance to identification of

hospitalized patients who required medical treatment for complications, identification of outbreaks, and ongoing routine sentinel ILI surveillance. Only patients who required hospital care were admitted; patients with milder infection were cared for at home.

Statistical Analysis

The serial interval of an infectious disease is defined as the time between onset of symptoms in an index patient and onset of symptoms in an infected contact. We analyzed data on transmission among the first 47 identified clusters we investigated, each with a single index case, to estimate the serial intervals associated with 60 infected contacts.

We estimated the incubation period distribution using data from the 22 persons with identified single-day exposures and the 35 persons with identified multiple-day exposure intervals (online Technical Appendix Table 2), excluding 3 persons with exposures implying incubation periods of >20 days (online Technical Appendix). We report the posterior median and 95% credible interval (CrI) of the mean and SD of the incubation period.

Doubling times in case numbers were estimated from the epidemic curve of weekly ILI incidence attributable to A(H1N1)pdm09 virus infection, obtained by multiplying raw ILI data by the weekly proportion of ILI case-patients who tested positive for A(H1N1)pdm09 virus. Those estimates, along with the evidence-based assumption that the generation time of influenza A(H1N1)pdm09 had a mean of 2.6 days and an SD of 1.3 days (*3*,*5*,*6*,*15*,*16*) (consistent with data analyzed on the serial interval), were used to estimate the effective reproduction number of A(H1N1)pdm09 virus infections in China. A simple epidemic model was fitted to the A(H1N1)pdm09 virus–attributable ILI case curve on the calendar weeks before and after the National Day Holiday (October 1–8) to estimate the effect of holidays on effective reproduction numbers and reporting rates. The model is based on the observation that numbers of cases increase at a rate that is a function of the reproduction number and the generation time of the disease (*17*). From the rate of growth of case numbers observed in the epidemic and the generation time of influenza A(H1N1)pdm09, the model can be used to derive the reproduction

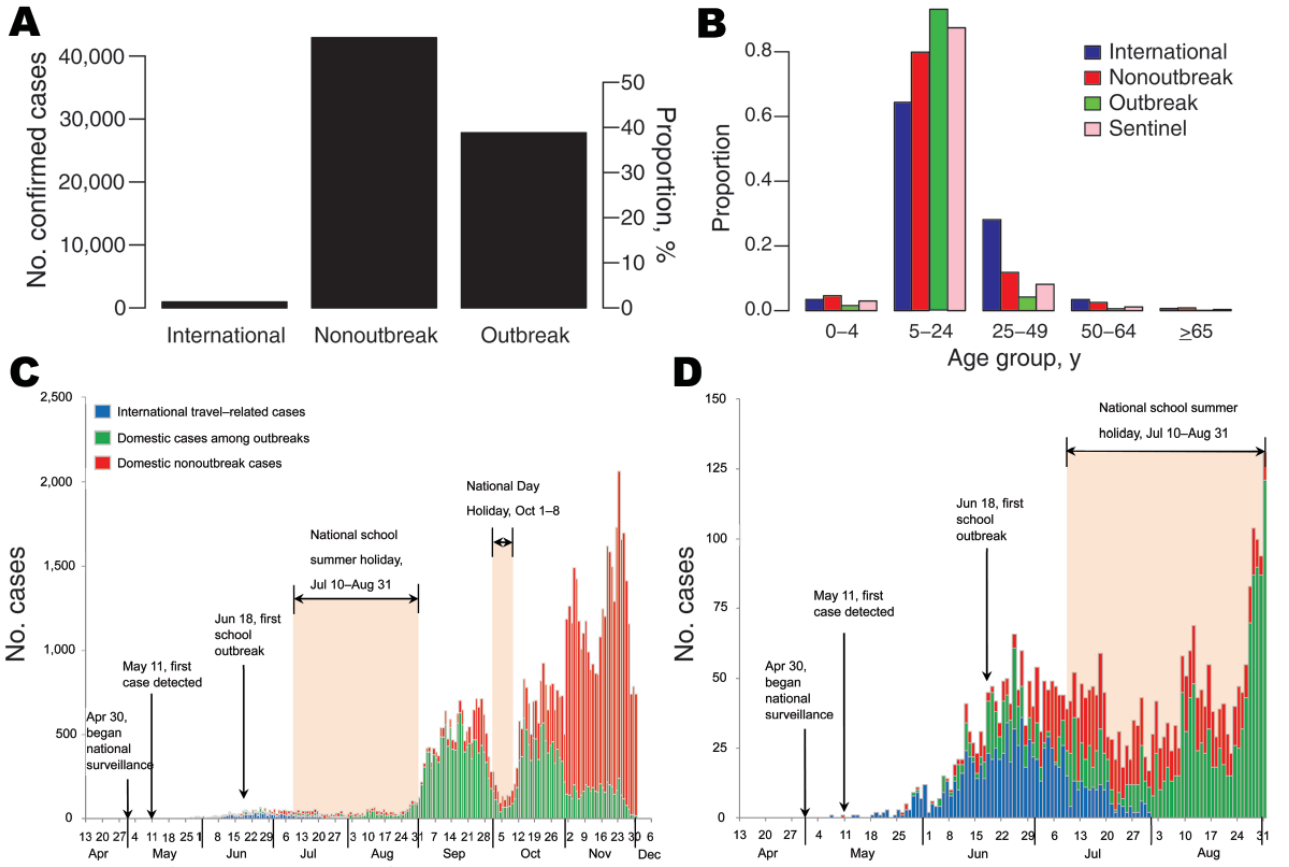

Figure 1. Confirmed cases of influenza A(H1N1)pdm09 virus infection, People's Republic of China, 2009. A) Number and proportion of confirmed A(H1N1)pdm09 cases by type (international travel–related cases, nonoutbreak cases, outbreak cases). B) Age distribution of patients with confirmed cases of A(H1N1)pdm09 infection gathered from different data sets. C, D) Number of confirmed A(H1N1)pdm09 cases by date of illness onset during May–August 2009 (C) and May–November 2009 (D) from case-based surveillance and outbreak investigations.

number of the disease for different intervals. In the past, this approach has been used to estimate the reproduction number of 1918 pandemic influenza in US cities (*18*).

International travel–related case-patients who had symptoms on arrival were classified as either "having fever" or "without fever but having respiratory symptoms" (online Technical Appendix). Frequency tables (with χ^2 tests) were constructed to examine the univariate associations between the probability of detection at the border and patients' characteristics. Univariable and multivariable logistic regression models were used to examine potential predictors of the probability of detection at the border (fever on arrival, time between onset and arrival, age group, and province) individually and simultaneously (i.e., using univariable and multivariable regression models, respectively) and to quantify their effects.

Results

Confirmed Cases

During May 7–November 30, 2009, a total of 71,665 persons with confirmed A(H1N1)pdm09 virus infection were reported to China CDC. Of those, 932 (1%) were related to international travel; 27,806 (39%) cases were detected during domestic outbreak investigations; and 42,917 (60%) were domestic nonoutbreak cases (Figure 1, panel A). The first case-patient was a traveler who returned from the United States with illness onset on May 7; the first domestic case-patient had symptom onset on May 10 (Figure 1, panels C, D). The origin of reported cases slowly shifted: most cases were international travel related until early June; in June, roughly half were international travel related and the other half were domestic; in July, most cases were domestic (Figure 1, panel C; online Technical Appendix Figure 2). The last known international travel–related case was reported on July 31, after which intensive border entry screening was gradually reduced. Irrespective of the type of case, persons 5–24 years of age were most affected, with the proportion of cases ranging from 64% in international travelers to 94% in outbreak cases (Figure 1, panel B). The proportion of persons 25–49 years was <12% in all categories, except international travel–related cases, for which it was 28% (likely because persons 25–49 years were overrepresented among international travelers).

The infection spread rapidly throughout China; 11 provinces (containing many of the most globally connected cities) reported confirmed cases in May, and all but 5 western provinces reported cases in July (Figure 2). By September, all provinces reported confirmed cases. Geographic variation occurred in the incidence of confirmed cases per 1 million persons throughout the epidemic, but how much this variation was caused by surveillance system variation (e.g., differences in access, use of health care, in laboratory capacity) is difficult to determine.

Sentinel ILI Surveillance

The percentage of visits for ILI from sentinel surveillance increased slowly from May 2009 through the end of August 2009, although the percentage was lower than that observed during the same months in 2007 and 2008 (Figure 3, panel A). In September 2009, ILI activity increased substantially and was higher than in the 2 previous seasons. ILI activity decreased sharply during the National Day Holiday, then rebounded at the end of the holiday period. Similar fluctuations were observed for other influenza viruses (Figure 3, panel B). The number and proportion of influenza-positive cases from sentinel ILI surveillance increased stably from May 2009 onward; A(H1N1)pdm09 became the predominant strain at the end of September and subsequently declined after early December.

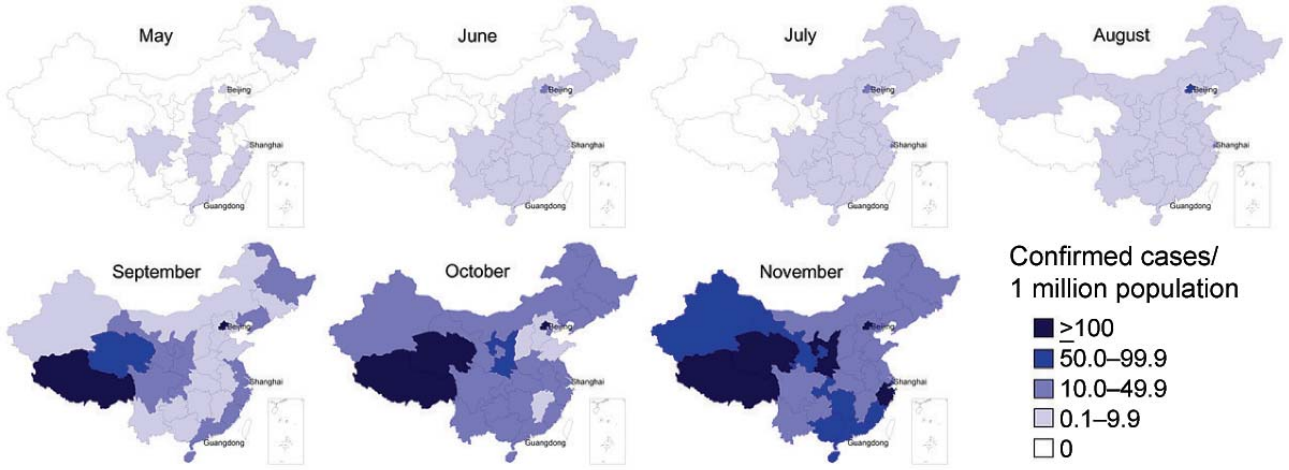

Figure 2. Incidence of confirmed cases of influenza A(H1N1)pdm09 virus infection per 1,000,000 inhabitants, by month and province, People's Republic of China, May–November 2009.

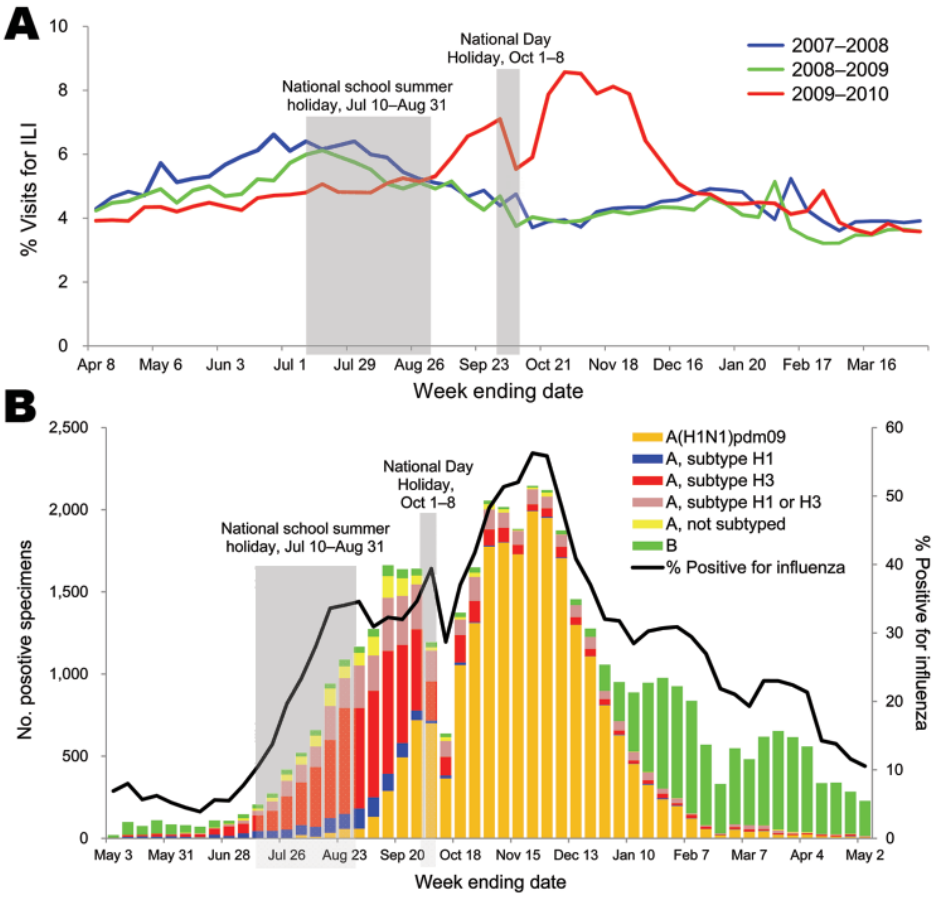

Figure 3. Sentinel surveillance for influenza-like illness (ILI) and virologic surveillance, People's Republic of China, 2007–2010, A) Weekly percentage of visits for ILI, sentinel ILI surveillance, People's Republic of China, 2007–08 through 2009–10. B) Number and percentage of specimens positive for influenza, by week of specimen collection during sentinel ILI surveillance in China, May–November 2009.

Serial Interval and Incubation Period

In the household setting, the average serial interval was 2.6 days (95% CI 2.2–3.0 days; Figure 4, panel A). Similar results were obtained in the analysis restricted to data from the 38 clusters in which the single index case-patient transmitted infection to a single contact. The incubation period had a mean of 2.2 days (95% CrI 1.9–2.5 days) and an SD of 1.0 days (95% CrI 0.8–1.2 days) (Figure 4, panel B).

Transmissibility and Effect of Holidays on Spread

We estimated that the effective reproduction number changed from 1.25 (95% CrI 1.22–1.28) before the National Day Holiday (August 31–September 30) to <1 during that holiday (0.79; 95% CrI 0.69–0.90) and back to 1.23 (95% CrI 1.15–1.32) after that holiday (October 7–October 25) (Figure 5, panel A; Table 1; online Technical Appendix Table 1). The National Day Holiday was therefore found to reduce the effective reproduction number by 37% (95% CrI 28%–45%). Our model also predicted that underreporting had increased by 19% (95% CrI 6%–31%) and 32% (95% CrI 11%–48%), respectively during the first and second calendar weeks of the National Day Holiday. However, the 8-week summer school holiday appeared to have had a limited effect on transmission as measured by A(H1N1)pdm09 virus–attributable ILI incidence, in contrast to what was observed in other countries, such as the United Kingdom (*19*). The doubling time during the summer school holiday (8.7 days during July 13–August 30) was similar to that observed in the month after schools reopened in September (7.1 days) (Figure 5, panel B). Using the rate of growth observed during July–August (Figure 5, panel B), we extrapolated the A(H1N1)pdm09 virus–attributable ILI case curve back in time and inferred that the first sentinel-detected ILI case caused by A(H1N1)pdm09 virus occurred in China in week 19 (May 11–17), near the date when the first imported case was detected (May 11).

Effectiveness of Border Entry Screening

International travel–related cases were detected either at the border or later by contact tracing and passive case finding within the country. Overall, 37% of international travel–related cases ever detected were detected at the border. The timing of onset of symptoms affected the probability of detection by symptom screening at the border. Half (468/932) of international travel–related case-patients ever detected had onset of symptoms ≥1 days after arrival (Figure 5, panel C).

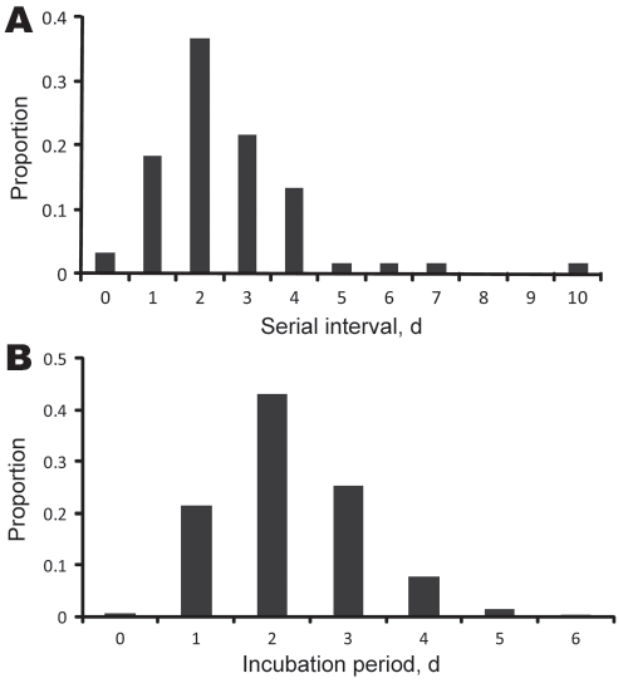

Figure 4. Natural history of influenza A(H1N1)pdm09 virus infection, People's Republic of China, 2009. A) Distribution of serial intervals among clusters of cases, each with a single index case. B) Incubation period distribution estimated from the 22 persons with identified single-day exposures and the 35 persons with identified multiple-day exposure intervals.

Among international travel–related case-patients who had symptoms on arrival, those with fever were significantly more likely to be detected at the border. The percentage of patients detected at the border was as follows: 76% for those with fever, 63% for those without fever but with respiratory symptoms (χ^2 4.41; df 1; p = 0.036; n = 464). Overall, 74% of persons ever detected with symptom onset on or before the day of arrival were identified at the border. Multiple logistic regression modeling showed a significant interaction (p = 0.023) between whether a case-patient had a fever on arrival and the time between symptom onset and arrival (stratified by those with onsets 0 or 1 day before arrival and those with onsets >1 day before arrival; Tables 2, 3). Thus, if a case-patient had a fever on arrival, then the time since onset was irrelevant. Similarly, the odds ratios (ORs) were similar for those with fever on arrival and onset 0 or 1 day before arrival (OR 1.80) relative to those with no fever and onset 0 or 1 day before arrival) and those with fever on arrival and onset >1 day before arrival (OR 1.91 relative to those with no fever and onset 0 or 1 day before arrival) (Table 3). However, among persons who did not have a fever on arrival, those who had been ill longer before arrival (>1 day) were more likely to be detected at the border (the percentage of detection = 83%, Table 1; OR 2.36, Table 3). After adjusting for these effects, neither age group nor province affected the probability of a case being detected at the border.

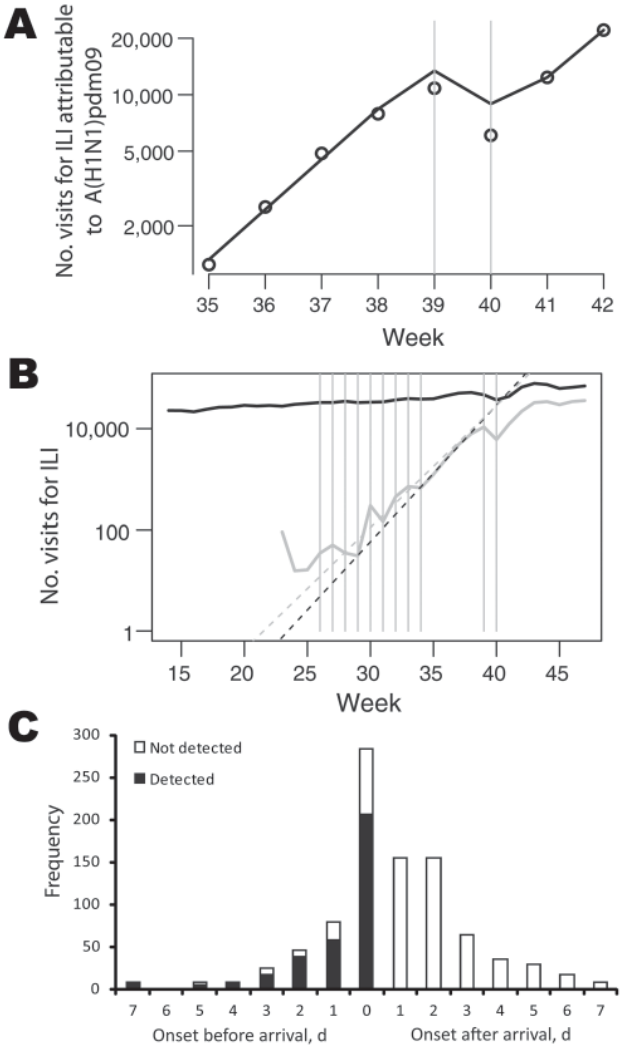

Figure 5. Effects of school holidays and border entry screening on influenza A(H1N1)pdm09 virus infections, People's Republic of China, 2009. A) Observed (black points) and predicted (solid line) number of visits for influenza-like illness (ILI) attributable to A(H1N1)pdm09 from week 35 (ending September 6) through week 42 (ending October 25). National Day Holiday occurred from Thursday, October 1 (week 39), through Thursday, October 8 (week 40). A simple epidemic model was fitted to data for calendar weeks before and after the National Day Holiday (gray bars) so that potential changes in reporting rates during holidays could be estimated. B) Number of visits for ILI. The black solid line shows raw numbers of visits for ILI; the gray solid line shows numbers corrected by the weekly proportion of ILI cases that are positive for A(H1N1)pdm09 virus. Gray dashed line shows growth rate in July-August. Black dashed line shows growth rate during the first 3 weeks of September. Gray bars indicate holiday periods. C) The distribution of intervals between symptom onset and arrival in China among confirmed international travel–related case-patients (N = 932).

Table 1. Reproduction numbers obtained by fitting a simple epidemic model to numbers of influenza-like illness cases attributable to pandemic influenza A (H1N1) 2009 virus, People's Republic of China, September–October 2009*

Interval	Mean (95% credible interval)
School term 1 (Sep 6–Oct 1)	1.25 (1.22–1.28)
Holidays (Oct 1–8)†	0.79 (0.69–0.90)
School term *2* (Oct 9–25)	1.23 (1.15–1.33)

*Fitted on data for the calendar weeks before and after the National Day Holiday.
†Period of National Day Holiday. Details can be found in online Technical Appendix Table 1 (wwwnc.cdc.gov/EID/pdfs/11-0356-Techapp.pdf).

Discussion

We described transmission patterns of A(H1N1) pdm09 virus infection in China during 2009 by using multiple epidemiologic data collected from surveillance and investigations. The age distribution and transmission dynamic parameters, including incubation period and serial interval, are consistent with those observed in other countries (*3*–*6*).

We can put an upper boundary only on the effectiveness of the border screening adopted early in the pandemic because data are available only on cases detected (either at the border or later through case-tracing), rather than missed cases. Given that travelers with mild illness or subclinical infection might not seek health care, a substantial proportion of international travel–related cases were likely never detected and therefore did not appear in our dataset. Hence, the proportion of imported cases that were detected at the border was, at most, 37%. Assuming the doubling time of the global epidemic in May was similar to that seen in China during July–August (8.7 days), and if the border screening reduced transmission from case-patients with imported cases by 37% (i.e., isolation of detected cases was 100% effective), the epidemic in China would have been delayed by 4 days (the additional time taken for cumulative imported cases to reach the level they would have reached in the absence of border controls). Thus, border controls likely delayed the epidemic by only a few days, even assuming few imported cases were missed altogether. This conclusion is supported by the observation that the trajectory of the epidemic in China appears relatively similar to that seen in the United Kingdom, another country to which infection had spread early in May but that did not employ border screening. Clearly, symptom-based border screening cannot detect infections among persons who are asymptomatic on arrival.

Our analysis suggests that the October national holiday might have reduced transmission by as much as 37% and reporting by ≈20%–30%. The National Day Holiday in China is similar in scope to the Christmas holiday in Western countries, with all kindergartens, schools, and universities and many businesses being closed. Most citizens leave their routine work, but festivals, mass gatherings, and travel occur during this period. However, the Summer School Holiday appears to have reduced transmission by a minimal amount (no more than 3% reduction in the effective reproduction number), in contrast to the large drop seen in other countries such as the United Kingdom. Why this discrepancy exists are unclear but might relate to the much more frequent use of collective childcare and summer camps and schools by Chinese parents during summer holidays than is typical in many European countries. In addition, seasonal factors that can limit transmission in temperate countries in summer might have had more limitedly affected the southern subtropical provinces of China.

The effective reproduction number for A(H1N1) pdm09 in China ranged from 1.2 to 1.3, which is consistent with that observed in other countries, although in the lower range. In comparison, the effective reproduction number was ≈1.4 in the United Kingdom in June–July 2009 (*19*). Because the proportion of the population <15 years of age is similar in both countries, demographic differences would not appear to explain these differences. However, spatial heterogeneity in the efficiency of spread and desynchrony between the epidemics in different regions of China might lead to the underestimation of transmissibility on a national scale. This remains a topic for future analysis. We relied on A(H1N1)pdm09 virus–attributable ILI incidence to estimate the epidemic growth rate because the proportion of ILI case-patients who tested positive for influenza increased substantially during the pandemic (Figure 3, panel B). As a consequence, the growth rate of the ILI incidence curve underestimates the epidemic growth rate (Figure 5, panel B). A similar approach was used by Baguelin et al. (*19*).

Our study has several limitations, which are inevitable, given that many of the data were collected as part of public health control rather than specifically to inform epidemiologic characterization of the pandemic. Case-based surveillance established by many countries in the early phase of the pandemic was critical to monitor early emergence and extent of geographic spread. However, in retrospect, those systems were not able to monitor the growth in case numbers over time because the ability to identify cases and conduct outbreak investigations could

Table 2. Percentage of case-patients detected by symptom status and interval between symptom onset and arrival among international travel–related case-patients who were symptomatic on arrival, People's Republic of China, 2009

Onset	Fever on arrival, %	No fever on arrival, %	Total, %
0 or 1 d before arrival	75, n = 329	49, n = 35	73, n = 364
>1 d before arrival	76, n = 76	83, n = 24	78, n = 100
Total	76, n = 405	63, n = 59	74, n = 464

Table 3. Potential predictors of the probability of detection at the border of international travel–related pandemic influenza A (H1N1) 2009 infection, People's Republic of China, 2009*

Model	OR (95% CrI)	p value
Univariable		
Fever on arrival	1.84 (1.04–3.27)	0.042
Onset of symptoms >1 d before arrival	1.05 (0.91–1.22)	0.288
Age group, y†		0.126
0–4	1	
5–24	1.67 (0.55–5.03)	
25–49	1.12 (0.36–3.50)	
50–64	0.58 (0.13–2.69)	
Province‡		0.400
44	1	
31	1.05 (0.49–2.23)	
35	0.66 (0.30–1.45)	
Multivariable§		
Fever on arrival	1.80 (1.26–2.57)	0.001
Onset of symptoms >1 d before arrival	2.36 (1.18–4.73)	0.016
Interaction between fever on arrival and onset of symptoms >1 d before arrival	0.45 (0.22–0.89)	0.023

*OR, odds ratio; CrI, credible interval.
†n = 463. Only 1 such person was >64 y of age, and this person was excluded from this analysis.
‡n = 302. Only 3 provinces had >40 persons so only they are included here.
§Likelihood ratio–based p value for the global hypothesis that all ORs equal 1 (3 df 0.008). The deviance (that is minus twice the log likelihood) for the multivariable model presented was 520.6, whereas the deviance for the model with only an intercept (i.e., no effects of fever or time from onset to arrival) was 532.5.

quickly be limited by saturated resources, for example, laboratory diagnostic capacity. Furthermore, the change from reporting individual cases regardless of clinical severity to reporting hospitalized cases likely affected the reporting rate of confirmed A(H1N1)pdm09 cases during mid-July and mid-August. In contrast, sentinel surveillance was not influenced by the change in case-based surveillance during the pandemic. However, for a country as large and diverse as China, some geographic variability is almost unavoidable in the quality of the surveillance system and capacity of health care system. This variability could make comparison of incidence levels by geographic zone somewhat difficult.

Improving and monitoring the homogeneity of the Chinese surveillance and health care system are challenging, yet vital, tasks to improve the monitoring of future pandemics. The effects of other interventions also need to be assessed, for example, strict case isolation, contact tracing, and medical observation, which might have helped delay the spread at early containment stage of the pandemic.

Thus, the overall picture of the epidemiology and transmission dynamics of A(H1N1)pdm09 that emerges from the surveillance data is comparable to that in many European countries and the United States. Border entry screening during the influenza pandemic delayed spread in China by a few days, at most, but the autumn school holidays reduced the effective reproduction number by ≈40%.

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H. Yu, S. Cauchemez, C.A. Donnelly, N.M. Ferguson, and Z. Feng conceived, designed and supervised the study, finalized the analysis, and interpreted the findings. L. Zhou, L. Feng, N. Xiang, J. Zheng, M. Ye, Y. Huai, Q. Liao, Z. Peng, Y. Feng, H. Jiang, and W. Yang assisted in data collection and analysis. H. Yu, S. Cauchemez, C.A Donnelly, and N.M. Ferguson drafted the manuscript. All other co-authors participated in data collection and management.

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Temporal Trends in *Bordetella pertussis* Populations, Denmark, 1949–2010

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We used multilocus variable-number tandem repeat analysis and multiple antigen sequence typing to characterize isolates of *Bordetella pertussis* strains circulating in Denmark during periods with and without pertussis vaccination coverage. Our results show substantial shifts in the *B. pertussis* population over time and a reduction in genetic diversity. These changes might have resulted from the introduction of pertussis vaccines in Denmark and other parts of Europe. The predominant strains currently circulating in Denmark resemble those in other European countries.

Whooping cough is a vaccine-preventable disease caused by the bacterium *Bordetella pertussis*; however, the protection conferred by vaccination does not last throughout life. In Denmark, as in other industrialized countries with high vaccination coverage, the disease is still endemic despite ≈50 years of vaccination. In 1961, a whole-cell *B. pertussis* (wP) vaccine was introduced into the childhood vaccination schedule in Denmark. In January 1997, it was replaced by a monocomponent acellular *B. pertussis* (aP) vaccine (DiTeKiPol or DiTeKiPol/Act-Hib; Statens Serum Institut [SSI], Copenhagen, Denmark) containing hydrogen peroxide–inactivated *B. pertussis* toxoid as the sole pertussis antigen. This vaccine was originally described and patented by the National Institutes of Health (Bethesda, MD, USA) (*1*,*2*). In 2003, a booster dose for preschool-age children (5 years of age) was introduced (diTekiBooster or DiTeKiPol Booster; SSI). Coverage among infants in Denmark for the third dose of pertussis vaccine has traditionally been high (≈87%–91%) (*3*). Acellular pertussis vaccines can contain up to 5 different antigens from *B. pertussis*, and Denmark is the only country using a monocomponent vaccine for both primary and booster vaccination (*4*).

The incidences of whooping cough and related deaths in Denmark have decreased dramatically since the introduction of pertussis vaccines. During a whooping cough epidemic in the early 1950s, before vaccine was introduced, the incidence of infection was ≈11,000 per 100,000 infants (*5*). In 2010, however, the incidence of infection among infants 0–1 year old had dropped to 110 per 100,000 infants and the incidence among the whole population had dropped to 7 per 100,000 persons (*6*). In addition, the last 2 pertussis–related deaths among infants in Denmark were notified in 2010 and 2005 (*7*). In general, the population-wide incidence of whooping cough in Denmark has been low since implementation of the pertussis vaccination program. However, occasional epidemic peaks have occurred, and the latest 2 were in 2002 (incidence, 36 cases/100,000 persons) and 2004 (incidence, 24 cases/100,000 persons) (*8*).

To determine the predominant strains of *B. pertussis* circulating in Denmark, we characterized clinical isolates obtained during 3 periods. Period 1, comprised 1949–1961, the year vaccine was introduced; period 2 comprised 1962–1996, during which wP vaccine was used; and period 3 comprised the years after 1996, during which aP vaccine has been used. We characterized the isolates by using multilocus variable-number tandem repeat analysis (MLVA) and multiple antigen sequence typing (MAST) to partially sequence the genes encoding pertactin (*prn*), *B. pertussis* toxin S1 subunit (*ptx*A, also designated *ptxS1*), *B.*

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pertussis toxin promoter (*ptxP*), and tracheal colonization factor A (*tcfA*). Because there is no consensus about which genes should be included in MAST of *B. pertussis,* we designated the results obtained in Denmark as MASTdk.

Materials and Methods

Strain Collection

B. pertussis isolates were available from the strain collection at SSI. Strains in the collection for 1950–1974 were lyophilized; for 1974–1994, isolates were either lyophilized or stored in liquid nitrogen, and after 1995, they were mostly stored at −80°C. The isolates were cultured on *B. pertussis* charcoal agar plates containing cephalexin and 10% horse blood (SSI). Plates were incubated at 36°C for up to 5 days.

Selection Criteria

Strains were selected to cover whooping cough patients from all over Denmark during 1 prevaccine and 2 postvaccine periods. A total of 24 isolates were selected from period 1 (1961 and earlier); 51 were selected from period 2 (1962–1996); and 185 were selected from period 3 (1997 forward). Strains were selected regardless of the patient's sex, age, clinical complications, and vaccine history; limited or no information was known about the patients.

DNA and Primers

Approximately 1 μL (1 inoculation loop) from a pure 4-day culture of *B. pertussis* on agar was suspended in 200 μL of sample preparation reagent (PrepMan; Applied Biosystems, Foster City, CA, USA), boiled for 10 min, and centrifuged for 10 min at maximum speed. For PCR, 2 μL of a 1:400 dilution of lysate was used. Primers used in the study are shown in Table 1.

MLVA

Six loci, originally identified by Schouls et al. (*9*), were included in the typing of isolates: variable-number tandem repeats (VNTRs) 1, 3a, 3b, 4, 5, and 6. VNTR3b is a duplication of VNTR3a and is present only in a subset of isolates.VNTR2 was excluded from the analysis because it did not exhibit substantial variation between isolates (*9*). Amplification was performed in a total volume of 20 μL; 2 μL of a 1:400 dilution of lysate was added to a mix of 1 μL of each primer (10 pmol/μL), 10 μL of HotStarTaq Master Mix Kit (QIAGEN, Hilden, Germany), and 4 μL of 5M betaine (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) for VNTR1, 3, 4, and 5 or 6 μL of 5 M betaine for VNTR 6.

The PCR protocol we used was a modified version of protocols published by Schouls et al. (*9*) and Kurniawan et al. (*14*). PCRs were set up as monoplex reactions and amplified in similar PCR programs, except that the annealing temperature was 68°C for primers 1, 5, and 6 and 60°C for primers 3–4. Amplification was initiated by denaturation at 96°C for 15 min and followed by 40 cycles at 95°C for 20 s, 68°C or 60°C for 30 s, 72°C for 90 s, and a final extension step at 72°C for 20 min. Final PCR products of VNTR 1, 5, and 6 and VNTR 3 and 4 were diluted 1:100 and 1:200, respectively, before the fragments were separated on an ABI 3130 DNA analyzer (Applied Biosystems).

Table 1. Primers used for MLVA and MASTdk typing in a study of temporal trends in *Bordetella pertussis* populations, Denmark, 1949–2009*

Primer name	Target	Sequence, 5′ → 3′	Reference
BP-VNTR1-DF	VNTR1	FAM-CCTGGCGGCGGGAGACGTGGTGGTG	(*9*)
BP-VNTR1-DR	VNTR1	AAAATTGCGGCATGTGGGCTGACTCTGA	(*9*)
BP-VNTR3-BF	VNTR3	FAM -GCCTCGGCGAAATTGCTGAAC	(*9*)
BP-VNTR3-BR	VNTR3	GCGGGCGAGGAAACGCCCGAGACC	(*9*)
BP-VNTR4-CF	VNTR4	FAM-CGTGCCCTGCGCCTGGACCTG	(*9*)
BP-VNTR4-BR	VNTR4	GCCGCTGCTCGACGCCAGGGACAA	(*9*)
BP-VNTR5-BF	VNTR5	FAM-GAAGCCGGCCCACCCGAGCTCCAGGCTCTT	(*9*)
BP-VNTR5-BR	VNTR5	TGCCGGGTTTCGGCATCTCGATGGGATACG	(*9*)
BP-VNTR6-EF	VNTR6	FAM-CCAACGGCGGTCTGCTGGGTGGTC	(*9*)
BP-VNTR6-FR	VNTR6	AGGGCGCTGGTCACGCCACCGAGGAT	(*9*)
BP-PtxP-F	Pertussis toxin promoter	AATCGTCCTGCTCAACCGCC	(*10*)
BP-PtxP-R	Pertussis toxin promoter	GGTATACGGTGGCGGGAGGA	(*10*)
BP-Ptx S1-F2	Pertussis toxin Subunit 1	CCCCCTGCCATGGTGTGATC	(*11*,*12*)
BP-Ptx S1-R2	Pertussis toxin Subunit 1	AGAGCGTCTTGCGGTCGATC	(*11*,*12*)
BP-prn-AF	Pertactin region 1	GCCAATGTCACGGTCCAA	(*11*–*13*)
BP-Prn-AR	Pertactin region 1	GCAAGGTGATCGACAGGG	(*11*–*13*)
BP-Prn-BF	Pertactin region 2†	AGCTGGGCGGTTCAAGGT	(*11*–*13*)
BP-Prn-BR/	Pertactin region 2†	CCGGATTCAGGCGCAACTC	(*11*–*13*)
BP-tcfAF	Tracheal colonization factor	TTCTTGCGCGTCGTGTCTTC	(*9*)
BP-tcfAR3	Tracheal colonization factor	GCGGTTGCGGACCTTCAT	(*9*)

*MLVA, multilocus variable-number tandem repeat analysis; MASTdk, multiple antigen sequence typing results obtained in Denmark; VNTR, variable-number tandem repeat.
†Pertactin region 2 was sequenced to confirm *prn* allele type (1 or 7).

Data Analysis

We converted the DNA analyzer–determined size of each VNTR into the number of repeat units by using a custom-made conversion table. Data were imported to BioNumerics version 6.1 software (Applied Maths, Sint-Martens-Latem, Belgium) and analyzed. Each isolate was defined by an MLVA profile containing a string of numbers representing the number of repeats at each allele in the following order: VNTR1, 3a, 3b, 4, 5, and 6. Each unique MLVA profile was assigned an MLVA type (MT) and named according to the Dutch scheme (*15*).

To verify the correct conversion from sequence sizes to number of repeats, we sequenced 1–3 representatives of each VNTR size. Sequencing results showed that there is a difference between the size determined by fragment analysis and the size obtained by sequencing in our setup of the ABI 3130 DNA analyzer. VNTR1 (15-bp unit) had a difference of 26 bp, VNTR3 (5-bp unit) 10 bp, VNTR4 (12 bp-unit) 3–4 bp, VNTR5 (6 bp-unit) 9–11 bp, and VNTR6 (9-bp unit) 9–12 bp (data not shown). In all cases, the sequencing size was larger than the DNA analyzer–determined size, and the difference for each VNTR was stable for all investigated sizes. Schouls et al. suggested that the inaccurate sizing obtained by the DNA analyzer resulted from the secondary structure of the PCR product (*9*).

MAST (MASTdk)

Four genetic loci known to be polymorphic for *B. pertussis* were selected for the MASTdk analysis: *ptxP* (*9–11*), *ptxA* (*11,12*), and 2 genes encoding surface proteins, tracheal colonization factor A (*tcfA*) (*9,11*) and pertactin (*prn*) (*11–13*). Sequence typing was performed as described for the individual genes (Table 1).

Bioinformatics

MLVA profiles were clustered in the BioNumerics version 6.1 software by using a categorical coefficient and visualized by using the minimum spanning tree method. Temporal tendencies of MLVA and MAST were determined by using the statistics tool in the BioNumerics program. The genetic diversity (Simpson's index of diversity) was calculated by using Comparing Partitions, an online tool for quantitative assessment of partition congruence (www.comparingpartitions.info).

Results and Discussion

MLVA

We used MLVA to type 260 clinical *B. pertussis* isolates collected in Denmark during 1949–2010. The isolates were resolved into 40 MTs, 27 of which have been published (*15*). These new types derived from the entire study period. Two MTs, MT27 and MT29, were predominant among all isolates investigated, representing 47% and 19% of the isolates, respectively. We found 7 MTs in ≥4 isolates, and 31 were in 1–3 isolates only and were compiled into a single group, denoted minor types, that represented ≈20% of all types (Table 2). The allelic profiles of all MTs found in this study are summarized in the online Appendix Table (wwwnc.cdc.gov/EID/article/18/5/11-0812-TA1.htm).

The clonal relationship between different MTs was investigated and visualized by constructing a minimum spanning tree based on the categorical clustering of MLVA profiles (Figure 1). The tree showed that the majority of isolates belonged to 1 of the dominant MTs, MT27 or MT29, or were single-locus variants to 1 of these. MT27 and MT29 differed from each other by a single locus. A smaller proportion of isolates were more divergent, showing multiple locus differences between

Table 2. MLVA typing results for isolates analyzed in a study of temporal trends in *Bordetella pertussis* populations, Denmark, 1949–2009*

	No. isolates detected (% frequency)			
MT	Total period, n = 260 isolates, 40 MTs	Period 1, n = 24 isolates, 8 MTs	Period 2, n = 51 isolates, 17 MTs	Period 3, n = 185 isolates, 23 MTs
Major MTs				
27	122 (47)	ND	3 (6)	119 (65)
29	49 (20)	3 (12)	27 (53)	19 (10)
36	8 (3)	ND	ND	8 (4)
253†	7 (3)	7 (29)	ND	ND
34	6 (2)	ND	2 (4)	4 (2)
141	5 (2)	4 (17)	1 (2)	ND
18	4 (1)	ND	ND	4 (2)
256†	4 (1)	4 (17)	ND	ND
16	4 (1)	ND	1 (2)	3 (2)
Minor MTs‡	51 (20)	6 (25)	17 (33)	28 (15)

*Genetic diversity was calculated as Simpson index of diversity for each period. The genetic diversity index was calculated for MLVA types by using the online tool Comparing Partitions (www.comparingpartitions.info). Simpson index of diversity (95% CI) was 0.74 (0.69–0.79) for the total period, 0.84 (0.75–0.92) for period 1, 0.71 (0.57–0.85) for period 2, and 0.57 (0.48–0.65) for period 3. MLVA, multilocus variable-number tandem repeat analysis; MT, MLVA type; ND, none detected.
†MTs found in 1–3 isolates.
‡New MTs detected in this study.

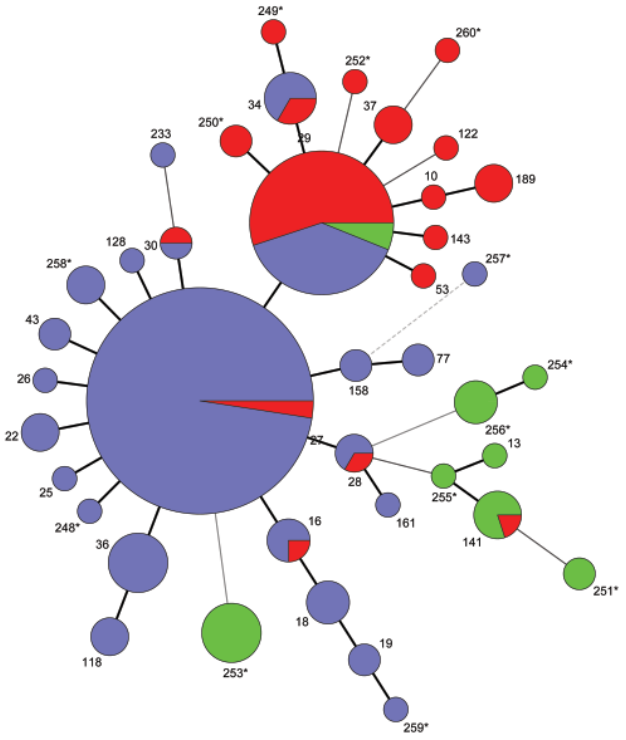

Figure 1. Multilocus variable-number tandem repeat analysis types (MT) of 260 *Bordetella pertussis* isolates collected in Denmark during 1949–2010. A minimum spanning tree based on the categorical clustering of MTs. Each MT is represented by a circle, and the Dutch type name (*15*) is indicated. MTs connected by heavy short lines, thinner lines, dashed lines, and spotted lines designate 1, 2, 3, and 4 loci differences, respectively. *New MTs detected in this study. Circle size indicates the number of isolates with the particular MT. Colors indicate the time period of isolation: green, period 1 (1961 and earlier); red, period 2 (1962–1996); purple, period 3 (1997 forward).

the MTs. The more divergent MTs primarily derived from period 1, and most isolates from period 2 were MT29 or single-locus variants of this type; period 3 was dominated by MT27 or single-locus variants of this type (Figure 2; Table 2). MT29 was first detected in Denmark in 1951, and during period 2, the proportion of MT29 increased to constitute 53% of isolates during that period. During period 3, MT27 gradually replaced MT29 to become the predominant type, constituting 65% of the isolates in period 3.

We determined the genetic diversity (Simpson's index of diversity) of MLVA for periods 1, 2, and 3 to be 0.84, 0.72, and 0.57, respectively (Table 2). These numbers reflect a tendency of decreasing genetic diversity from period 1 to 3, i.e., a change from a more even distribution of isolates among the detected MTs toward single dominant types (Figure 2). Throughout the 3 study periods, we detected a proportion of minor and new MTs, which indicated the continuous appearance of new genetic *B. pertussis* types, regardless of the use or type of vaccine. Our results are in line with observations from the Netherlands (*9*), the United Kingdom (*16*), and Australia (*14*).

MAST

We analyzed the following genes of a selection of *B. pertussis* isolates by using previously published sequencing methods: *prn* (153 isolates), *ptxA* (151 isolates), *ptxP* (148 isolates), and *tcfA* (153 isolates). These genes were selected because they are the most polymorphic genes found in *B. pertussis*, and we were particularly interested in variation in *PtxA* and the *Ptx* promoter because Denmark has used a monocomponent vaccine containing only pertussis toxoid since 1997. Before this study, 13 *prn*, 8 *ptxA* (*17*), 4 *tcfA* (*11*), and 14 *ptxP* (*10*,*18*) alleles had been identified. In our study, we detected 4 *prn* alleles (*prn1*, *prn2*, *prn3,* and *prn7*), 3 *ptxA* alleles (*ptxA1*, *ptxA2*, and *ptxA4*), 5 *ptxP* alleles (*ptxP1*, *ptxP2*, *ptxP3*, *ptxP15*, and *ptxP17*), and 2 *tcfA* alleles (*tcfA*2 and *tcfA*3).

We analyzed the frequencies of the individual alleles and their temporal trends (Table 3; Figure 3). In the case of *prn* (Figure 3, panel A), there was a replacement of

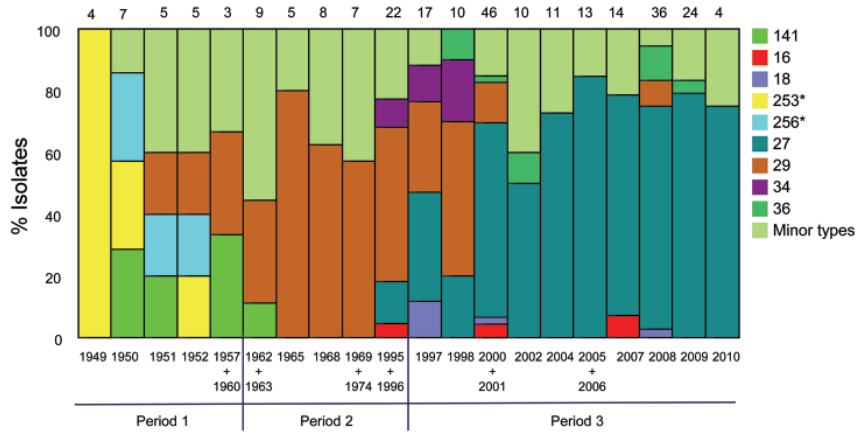

Figure 2. Temporal trends in the frequency of multilocus variable-number tandem repeat analysis types (MTs) of *Bordetella pertussis* isolates collected in Denmark, 1949–2010. MTs represented in 1–3 isolates are compiled into 1 group denoted minor types. Years containing 1 or 2 isolates are combined with another year in the same time period, as indicated. The number of isolates analyzed each year/years is given above each column.

Table 3. Results of partial sequencing of isolates analyzed in a study of temporal trends in *Bordetella pertussis* populations, Denmark, 1949–2009*

Partially sequenced gene, alleles detected†	No. isolates (% frequency)			
	Total period	Period 1	Period 2	Period 3
prn	153 (100)	24 (100)	50 (100)	79 (100)
prn1	41 (27)	15 (63)	22 (44)	4 (5)
prn2	86 (56)	ND	18 (36)	68 (86)
prn3	17 (11)	ND	10 (20)	7 (9)
prn7	9 (6)	9 (38)	ND	ND
ptxA	151 (100)	23 (100)	47 (100)	80 (100)
ptxA1	125 (83)	3 (13)	42 (89)	80 (100)
ptxA2	14 (9)	8 (35)	5 (11)	ND
ptxA4	12 (8)	12 (52)	ND	ND
ptxP	148 (100)	24 (100)	44 (100)	80 (100)
ptxP1	82 (55)	12 (50)	42 (96)	28 (35)
ptxP2	12 (8)	12 (50)	ND	ND
ptxP3	52 (35)	ND	2 (4)	50 (63)
ptxP15	1 (1)	ND	ND	1 (1)
ptxP17	1 (1)	ND	ND	1 (1)
tcfA	153 (100)	24 (100)	51 (100)	78 (100)
tcfA2	141 (92)	24 (100)	45 (88)	72 (92)
tcfA3	12 (8)	0	6 (12)	6 (8)

*ND, none detected.
†Genes encoding pertactin (*prn*), *B. pertussis* toxin S1 subunit (*ptx*A), *B. pertussis* toxin promoter (*ptxP*), and tracheal colonization factor A (*tcfA*).

predominant alleles from period 1 to periods 2 and 3. The *prn1* allele dominated in period 1 and 2 but was rarely observed in period 3. The *prn2* allele appeared in period 2 and dominated in period 3 (86%). The *prn3* allele was present at a low level in period 2 and 3, and the *prn7* allele was present in period 1 but was not found later.

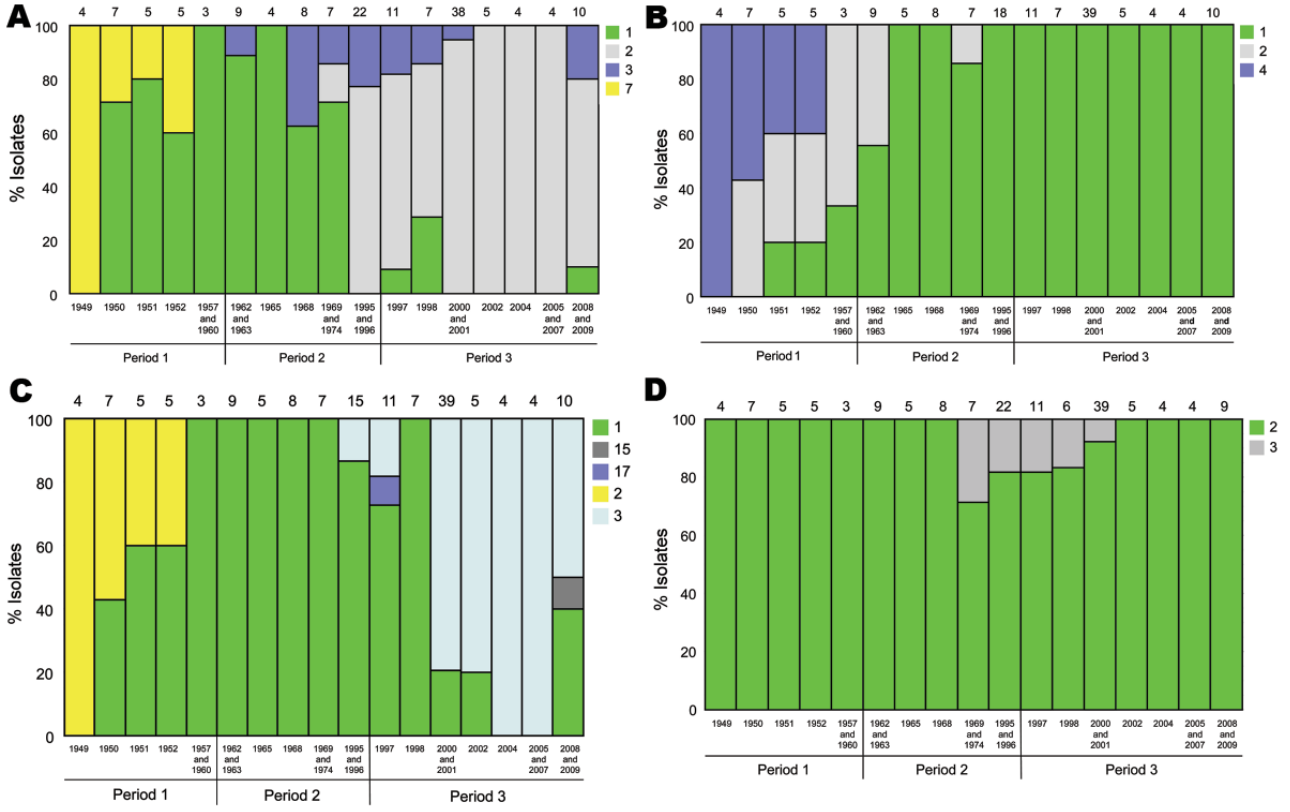

Figure 3. Temporal trends in the frequencies of the alleles of pertactin (*prn*) (A), the pertussis toxin subunit S1 (*ptxA*) (B), the pertussis toxin promoter (*ptxP*) (C), and the tracheal colonization factor A (*tcfA*) (D) of *Bordetella pertussis* isolates collected in Denmark, 1949–2010. The number of isolates analyzed each year/years is given above each column. Years containing 1 or 2 isolates are combined with another year in the same time period, as indicated

We observed a similar replacement of dominant alleles for *ptxA*. Three alleles were detected in period 1: *ptxA1* (13%), *pxtA2* (35%), and *ptxA4* (52%). In period 2, *ptxA4* was not detected, *ptxA2* decreased to 11%, and *ptxA1* increased to 89%. In period 3, the *ptxA1* allele was the only allele detected. For *tcfA*, the *tcfA2* allele dominated throughout all 3 periods (Figure 3, panel D). The *tcfA3* allele was detected in few isolates during 1969–2001. In the case of *ptxP*, the *ptxP1* allele gradually replaced *ptxP2* during period 1, and *ptxP2* was not found in isolates collected after 1952. The *ptxP1* allele dominated in period 2 (96%); however, in 1995 the *ptxP3* allele was detected and increased to constitute 63% of all isolates in period 3. Sporadic isolates of *ptxP15* and *ptxP17* were detected in period 3.

Similar observations of the temporal changes of *prn* and *ptxA* alleles have been reported from other European countries, such as United Kingdom (*16*), Finland (*19*,*20*), France (*20*), and the Netherlands (*11*,*12*). The current population of *B. pertussis* in Europe is now dominated by the *prn2* allele and almost exclusively the *ptxA1* allele (*16*,*20*,*21*).

The recent emergence and current dominance of the *ptxP3* allele has also been reported for other European countries (*9*,*10*,*22*,*23*). In the Netherlands, *ptxP3* strains produced more *B. pertussis* toxin than did *ptxP1* strains; in addition, the emergence of *ptxP3* was associated with increased whooping cough notifications and a shift in disease prevalence toward older age groups (*10*). In Denmark, however, the emergence of *ptxP3* has not been associated with increased whooping cough notification, although a shift in age distribution has been observed (*6*). The emergence of *ptxP3* strains is unrelated to the introduction of acellular vaccines because in Denmark and the Netherlands, these strains circulated when wP vaccines were used. In the Netherlands, *ptxP3* strains reached frequencies of ≥90% when the wP vaccine was used for the primary series (*10*). The changes in the *B. pertussis* population in Denmark appear to be independent of epidemics because isolates from the 2002 and 2004 epidemics follow the ongoing trends in the period.

MASTdk

We identified 14 combinations of the sequence types of *prn*, *ptxA*, *ptxP*, and *tcfA* (MASTdk); some MASTdk types appeared to be dominant and were detected in multiple isolates, whereas others were seen in few or a single isolate (Table 4). Similar to our MLVA findings, we found that the genetic diversity determined by using Simpson's index of diversity based on the MASTdk results decreased from 0.78 in period 1 to 0.77 in period 3 to 0.56 in period 3 (Figure 4; Table 4).

The trends for *B. pertussis* MTs and sequence types (or MAST) are similar in Denmark and the Netherlands (*9*,*10*,*23*). Different vaccines have been used in these countries, which may suggest that the most important factor driving these changes is not the type of vaccine used, but the removal, by vaccination, of immunologically naive infants as a major source of *B. pertussis* transmission. This might, as suggested (*10*), have selected for strains that are more efficiently transmitted by adolescents and adults, in whom immunity has waned.

Conclusions

By using MLVA and sequence typing of *B. pertussis* isolates collected in Denmark during 1949–2010, we showed that the population of this pathogen has changed over time. In general, the *B. pertussis* population in Denmark has changed from having a higher genetic diversity, as measured by MLVA and sequence typing, toward the

Table 4. MASTs detected in isolates analyzed in a study of temporal trends in *Bordetella pertussis* populations, Denmark, 1949–2009*

	Allele types in MASTdk				No. isolates (% frequency)†			
MASTdk	*prn*	*ptxA*	*ptxP*	*tcfA*	Total period, n = 142	Period 1, n = 24	Period 2, n = 41	Period 3, n = 77
MAST1.dk	2	1	3	2	50 (35)	ND	2 (5)	48 (63)
MAST2.dk	2	1	1	2	26 (18)	ND	9 (22)	17 (22)
MAST3.dk	1	1	1	2	22 (15)	3 (13)	17 (42)	2 (3)
MAST4.dk	1	2	1	2	13 (9)	8 (33)	5 (12)	ND
MAST5.dk	7	4	2	2	8 (6)	8 (33)	ND	ND
MAST6.dk	3	1	1	3	8 (6)	ND	3 (7)	5 (7)
MAST7.dk	3	1	1	2	5 (3)	ND	4 (10)	1 (1)
MAST8.dk	1	4	2	2	3 (2)	3 (13)	ND	ND
MAST9.dk	2	1	1	3	2 (1)	ND	1 (2)	1 (1)
MAST10.dk	2	1	15	2	1 (1)	ND	ND	1 (1)
MAST11.dk	7	2	2	2	1 (1)	1 (4)	ND	ND
MAST12.dk	3	1	3	2	1 (1)	ND	ND	1 (1)
MAST13.dk	1	1	17	2	1 (1)	ND	ND	1 (1)
MAST14.dk	1	4	1	2	1 (1)	1 (4)	ND	ND

*Sequence types are given in the order *prn*-*ptxA*-*ptxP*-*tcfA*. MASTs, multilocus sequence types; MASTdk, MAST types detected in Denmark during 1949–2010. ND, none detected.

†Genetic diversity index calculated on multilocus variable-number tandem repeat analysis types. Calculations were done by using the online tool Comparing Partitions (www.comparingpartitions.info). Simpson's index of diversity (95% CI) was 0.80 (0.76–0.84) for the total period, 0.77 (0.68–0.86) for period 1, 0.76 (0.67–0.85) for period 2, and 0.56 (0.45–0.67) for period 3.

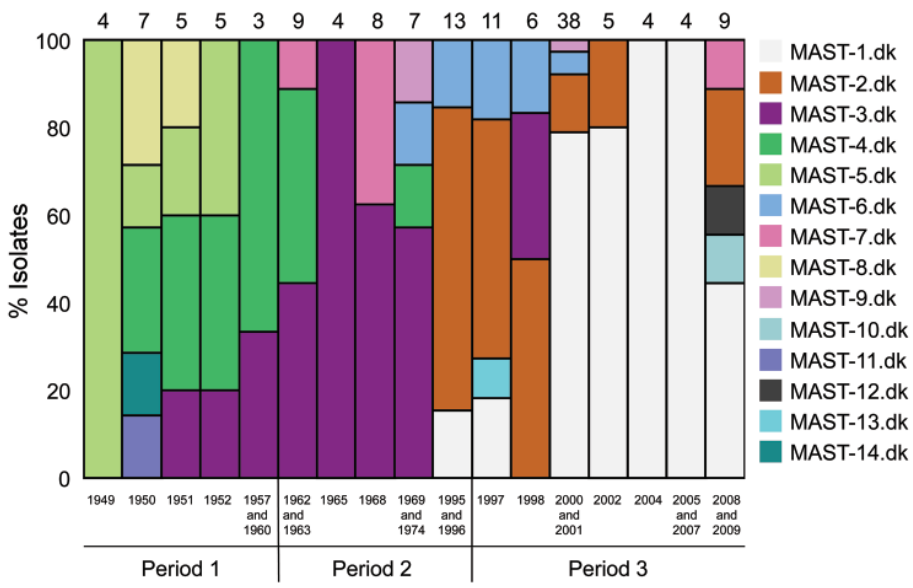

Figure 4. Temporal trends in the frequency of multilocus sequence type types (MASTdk) of *Bordetella pertussis* isolates collected in Denmark, 1949–2010. Years with 1 or 2 isolates are combined with another year in the same time period, as indicated. The number of isolates analyzed each year/years is given above each column.

dominance of single types. The predominant MLVA- and MASTdk-identified strains currently circulating in Denmark resemble the types observed in other European countries.

The genetic diversity of the *B. pertussis* isolates in Denmark was highest during period 1 (1949–1961), i.e., before introduction of pertussis vaccine. Period 2, when wP vaccine was used, was dominated by few or single types, as is the case in period 3, when aP vaccine has been used. The observed genetic changes of *B. pertussis* could therefore be related to the introduction of vaccines. However, there is no evidence that the wP or aP vaccines used in Denmark have selected for other dominant MT or sequence types than those observed in other European countries. Also, since the aP vaccine used in Denmark contains only *B. pertussis* toxoid, changes in the *prn* alleles in period 3 must have occurred independently of that vaccine. Travel within Europe has increased substantially since the prevaccine era; thus, *B. pertussis* imports from neighboring countries might explain the shift in the *B. pertussis* populations in Denmark. This explanation is supported by the appearance of similar *B. pertussis* types around Europe and by the occurrence of such types in Denmark before the introduction of change in pertussis vaccines in Denmark. The true explanation for the changes in genetic diversity among *B. pertussis* isolates in Denmark is probably a combination of those 2 theories.

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A Spatial Analysis of Individual- and Neighborhood-Level Determinants of Malaria Incidence in Adults, Ontario, Canada

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Malaria, once endemic in Canada, is now restricted to imported cases. Imported malaria in Canada has not been examined recently in the context of increased international mobility, which may influence incidence of imported and autochthonous cases. Surveillance of imported cases can highlight high-risk populations and help target prevention and control measures. To identify geographic and individual determinants of malaria incidence in Ontario, Canada, we conducted a descriptive spatial analysis. We then compared characteristics of case-patients and controls. Case-patients were significantly more likely to be male and live in low-income neighborhoods that had a higher proportion of residents who had emigrated from malaria-endemic regions. This method's usefulness in clarifying the local patterns of imported malaria in Ontario shows its potential to help identify areas and populations at highest risk for imported and emerging infectious disease.

Malaria is a parasitic, vector-borne disease that causes ≈1 million deaths each year and substantial global public health costs (*1*,*2*). The disease was previously endemic in North America, with transmission in most of the United States and parts of southern Canada (*3*). The malaria parasite, *Plasmodium* spp., was introduced into North America during the 16th–17th centuries through the arrival of European colonists and African slaves (*3*). Malaria was eliminated in North America by the 1950s through several different interventions, including vector control by changes in vector habitat, introduction of new antimalarial medications (such as quinine) for improved treatment, and decreased contact between humans and mosquitoes, in part because of changes in housing conditions (*3*).

Cases of locally acquired, mosquito-transmitted (autochthonous) malaria still occur in the United States, particularly in the Northeast. Most recently, reported autochthonous cases in the United States have occurred in suburban or urban areas (*3*). Although no confirmed cases of autochthonous malaria have been recorded in Canada in recent years, ≈400 cases of imported malaria are identified in Canada each year, a significantly higher prevalence than in the United States (*4*,*5*). Increased international travel and immigration have the potential to change the probability of autochthonous transmission in Canada and the United States, where competent vectors and suitable regional climates already exist (*3*,*6*).

Malaria incidence is affected by socioeconomic inequality and human movement (*7*). Malaria transmission in Canada was associated historically with socioeconomic inequality and migration; in particular, malaria transmission surged among migrant workers on the Rideau Canal in Ontario during 1826–1832 (*8*). Regional and international travel and migration patterns have also been implicated in malaria incidence across the globe, including reports of so-called airport malaria, refugee outbreaks, and cases in migrant populations (*9*–*12*). Rates of transmission in highly traveled areas have been found to affect rates of imported malaria cases (*13*). Length and type of travel and travel behavior are associated with risk for malaria transmission (*14*–*16*). At particularly high risk of acquiring malaria and importing it to their country of residence are travelers who visit friends and relatives

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(VFRs) in countries where malaria is endemic (*16*–*21*). Research has recently shown that VFRs are also at risk for multidrug-resistant malaria because of the inappropriate use of antimalarial drugs available over the counter in malaria-endemic countries (*22*).

Emerging and reemerging disease risk interacts with socioeconomic vulnerability to determine the populations at highest risk for infection and those most likely to import pathogens into the country. In the context of changing patterns in international travel, immigration, and global disease spread, understanding the socioeconomic determinants of existing disease risks is prudent.

In this study, we first conducted a descriptive spatial analysis to identify the geographic and individual determinants of malaria incidence in Ontario, Canada. We then tested the hypothesis that malaria case-patients do not differ significantly from controls in terms of their individual and geographic characteristics.

Methods

Study Design

The study location was Ontario, Canada. Data on positive and negative malaria tests conducted in Ontario were used to assign persons to 1 of 2 groups: case-patients (persons who tested positive for *Plasmodium* spp. by standard microscopy using Giemsa stain) and controls (persons who tested negative for *Plasmodium* spp.). First, descriptive statistics, mapping, and space–time cluster analysis were used to examine the spatial patterns of malaria incidence in Ontario and associated individual and geographic risk factors. Second, logistic regression was used to test the hypothesis that malaria case-patients and controls did not differ significantly in their individual and geographic determinants.

Location Context

Toronto, the largest city in the province and country, experiences a high volume of international travel, with 3.5 million passengers arriving at Toronto's Pearson International Airport in 2007 (*23*). Toronto is a major immigration destination, globally and within Canada (*24*). One fifth of Canadians were born outside of Canada, and in the census metropolitan area of Toronto, the proportion is more than twice the national rate, with 45.7% of the population born outside Canada (*25*). Locations where immigrants choose to settle within the greater Toronto area (GTA) show the strongest growth of cities outside the city of Toronto (*25*).

Data Sources

Data on malaria cases were obtained from the Malaria Reference Laboratory, Public Health Laboratories (PHL), Ontario Agency for Health Protection and Promotion (Toronto). These data included results of malaria tests conducted in the 12-month period from May 2008 through April 2009, and they identified patients as either positive (case-patients) or negative (controls) by reference thick and thin blood films. PHL receives blood specimens from other laboratories throughout the province and from hospitals and clinics. The data include the results of any test conducted at PHL for malaria diagnosis, confirmation, or malaria parasite speciation. PHL conducts ≈75% of the malaria testing for Ontario. An ethics certificate was obtained from the McGill University Research Ethics Board, and the de-identified data were stored in a confidential and secure manner.

The dataset from the Ontario Agency for Health Protection and Promotion contained 990 specimens. A specimen represented a blood sample that was tested for malaria. Specimens from persons for whom home postal code information was not available and those for whom home addresses were outside of Ontario were excluded, leaving 770 specimens. Data were converted from specimens to observations (persons) to avoid duplication in the dataset because some persons had results for multiple tests. Conversion from specimen to person was based on home postal code, birth date, and sex. This conversion resulted in 562 observations (persons). Malaria can be more severe in children, and symptoms in children are similar to those of other diseases (*26*,*27*); these facts may prompt a higher index of suspicion and different patterns of diagnostic testing for children and adults. The dataset included many negative test results for children, and combined with a low number of child case-patients, stratified analysis of malaria in children was not feasible; persons <18 years of age were thus excluded.

The final dataset included 354 observations of individual adults who tested positive or negative for *Plasmodium* spp. These persons were categorized as case-patients (n = 94) and controls (n = 260). A travel history was reported for 151 of these persons. Data on immigration status were not available. Such information would have been a valuable component of the analysis.

Census data describing population size, residents' immigration status, and median household income were compiled for all Ontario census tracts. Census tracts were used as a proxy for neighborhoods (*28*), and population density was calculated for each area. Proportion of residents who are immigrants from malaria-endemic countries for each neighborhood was calculated by dividing the number of respondents reporting immigration from an area where malaria is endemic (Southeast Asia, South Asia, East Africa, Central Africa, West Africa) by the number of census respondents. Geographic boundaries of both census tracts (population size ≈2,000–8,000 persons) and dissemination

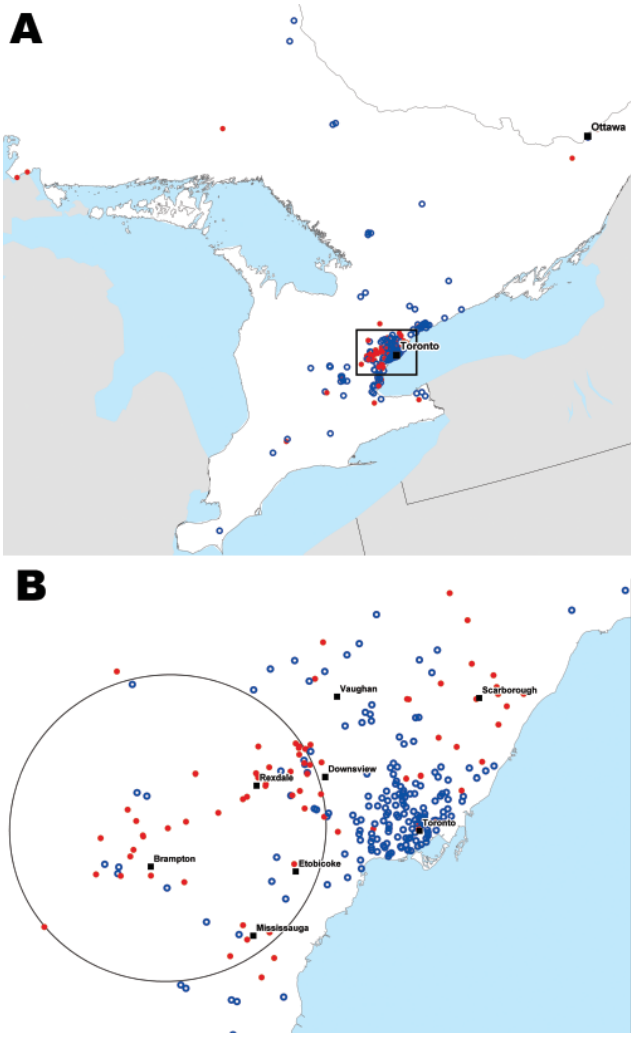

Figure 1. Locations of persons from whom 990 blood samples were taken and tested for malaria by the Ontario Agency for Health Protection and Promotion, Ontario, Canada, 2008–2009. Red dots, malaria case-patients (positive test results); blue circles, controls (negative test results). A) All observations; B) the most significant space–time cluster for malaria cases (circle), greater Toronto area, during May 15–November 6, 2008 (relative risk 3.54; p<0.01).

areas (population size ≈400–700 persons) were used for mapping, area calculation, and spatial reference.

Descriptive Analysis

All cartography was carried out by using ArcGIS version 9.3 software (ESRI, Redlands, CA, USA). Several key variables were used in the descriptive mapping of malaria case-patients: case-control status, parasite species, and age and sex of participant. For case–control data, observations were placed at the geographic center of the postal code recorded as the home address. Six-digit postal codes were used for all observations retained in the dataset, and those without appropriate postal codes were removed. Placing the observation at the geographic center of the postal code ensured accurate representation of location while maintaining participant privacy. Week codes (a numerical designation by the US Centers for Disease Control and Prevention for each week of the year, 1–52) were assigned to observations in the dataset to facilitate temporal graphing. Timeline graphs were created for evaluating the seasonal and temporal distribution of malaria observations. Incidents were stratified by parasite species.

Travel history was categorized by using the regional groupings provided in the 2006 Canadian census. Univariate tests and graphing were performed for the variables of travel, neighborhood immigration characteristics, case or control status, and parasite species. For travel and immigration analysis, observations that noted a parasite species other than *P. falciparum* and *P. vivax* (n = 9) were excluded.

Cluster Analysis

Because of a high concentration of cases in the GTA, a subset of the malaria case dataset was created, with only observations in the GTA. For purposes of analysis, Canadian regional municipalities were used to delimit the dataset, and the GTA subset thus included Toronto, Halton, Peel, York, and Durham municipalities. Cluster analysis was performed on the GTA subset to assess the significance of any potential spatial clustering. A space–time scan statistic (*29*) was performed on case–control status and parasite species by using SaTScan version 8.1.1 software (M. Kulldorff, Boston, MA, USA). The scan statistic compares observed case counts within a range of alternate scan windows, in both space and time, to expected case counts derived from Monte Carlo random simulation. The scan window with the maximum likelihood ratio statistic is identified and compared with the null hypothesis of no significant clustering. Analyses assumed a binomial distribution in which the 2 possible outcomes were to be either a case-patient or a control. The maximum cluster size was 50%, so that no more than 50% of the observations could be classified as being part of the detected cluster.

Logistic Regression

The key variables in the logistic regression model were population density, median household income, proportion of residents who are immigrants from malaria-endemic countries, and sex. Predictor variables were chosen on the basis of individual and ecologic risk factors for travel-related disease and infectious disease. The outcome variable was case–control status. All statistical analyses were assessed for significance at the 95% CI. Univariate tests were conducted to assess the relationship between the individual predictor variables and case–control status. One observation was

removed because no census data were available for the postal code geographic center. All variables were checked for co-linearity and normality, and the model was checked for notable interaction, outliers, confounding, predictive ability and accuracy, leverage, and goodness-of-fit. The best-fit model was chosen on the basis of the inclusion of the key variables related to imported malaria cases, given data availability and the results of the tests for goodness-of-fit and other postestimation procedures. The best-fit model was interpreted by using quartile values of each of the predictor variables, comparing the first and fourth quartiles. All statistical analyses were performed by using Stata version 11 software (StataCorp, College Station, TX, USA).

Results

The general distributions of sex, age, and relative parasite proportions did not differ greatly between case-patients (n = 94) and controls (n = 260) for all of Ontario and the GTA subset (Table 1). The spatial distribution of the case-patients and controls was consistent with Ontario's settlement patterns, with most of the observations located in southern Ontario and the GTA (Figure 1). The downtown core of Toronto had a large concentration of controls, whereas more case-patients lived in suburban areas.

Significant clustering of case-patients occurred in suburban Toronto during the summer months (Figure 1). A significant space–time cluster was identified during May 15, 2008–November 6, 2008, in the region northwest of Toronto near Brampton ($p<0.01$; relative risk 3.54). This area is near the main international airport in the GTA. In this area during the summer months, tested persons were 3.5× more likely to receive a diagnosis of malaria than were persons outside of this area and period. No significant clustering of individual parasite species (*P. falciparum* or *P. vivax*) was identified.

Cases were not significantly more likely to occur in the summer months (June–September) than in the nonsummer months for the GTA and for all of Ontario (χ^2; $p = 0.14$ and $p = 0.11$, respectively). This result is consistent with that of the space–time cluster analysis, which found that the significant space–time cluster extended through summer into autumn. Visual observation of monthly incidence, however, did not indicate any strong or clear trends, possibly because of the influence of low observation numbers, and incidence and seasonality in malaria-endemic countries from which the disease is imported (Figure 2).

Population density was highly variable in the study area, with more densely populated areas located near the downtown core of the GTA. Median household income, in contrast, exhibited negligible clustering except for low-income suburbs east and west of the downtown core. The proportion of residents who were immigrants from malaria-endemic regions was highest in the suburban areas to the east and west of Toronto (Figure 3). More than 94% of case-patients for whom travel history was recorded indicated recent travel to Africa or Asia (Figure 4), with travel to Africa reported most frequently. These results are consistent with known spatial ranges of malaria-endemic areas (*30*).

Case-patients were significantly more likely than controls to be male and live in low-income neighborhoods

Table 1. Summary characteristics of imported malaria case-patients and controls, Ontario, Canada, 2008–2009

Characteristic	Case-patients	Controls	Total
Ontario	94 (27)	260 (73)	354 (100)
Sex, no. (%)			
M	65 (33)	131 (67)	196 (100)
F	23 (15)	129 (85)	152 (100)
Not available	6 (100)	0	6 (100)
Mean age, y	42.5	41.3	41.7
Species, no. (%)			
Plasmodium falciparum	54		
P. vivax	31		
P. ovale	4		
Plasmodium spp. (unidentified)	3		
Plasmodium spp. (mixed)	1		
Babesia spp.	1		
Greater Toronto area subset	84 (29)	209 (71)	293 (100)
Sex, no. (%)			
M	58 (37)	98 (63)	156 (100)
F	20 (15)	111 (85)	131 (100)
Not available	6 (100)	0	6 (100)
Mean age, y	42.5	40.6	41.2
Species, no. (%)			
P. falciparum	48		
P. vivax	30		
P. ovale	3		
Plasmodium spp. (unidentified)	2		
Plasmodium spp. (mixed)	1		
Babesia spp.	0		

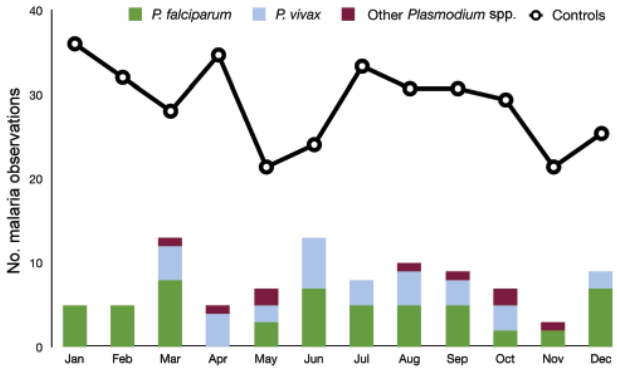

Figure 2. Case-patients, by *Plasmodium* species with which infected, and controls who tested negative for *Plasmodium* spp., by month, Ontario, Canada, 2008–2009.

with a higher proportion of residents who immigrated from malaria-endemic regions (Table 2). The proportion of residents who were immigrants from malaria-endemic countries was, for example, >2× as high in neighborhoods with case-patients than in neighborhoods where controls were identified. The average income of case-patient neighborhoods was ≈$28,000, compared with ≈$31,000 in control neighborhoods (Student *t* test, p = 0.04). Case-patients with a travel history (n = 47) were more likely to report having traveled to Africa and to be living in a neighborhood with a higher proportion of residents who are immigrants from malaria-endemic countries than controls with a travel history (Table 3).

The regression results are consistent with univariate analyses: case-patients were more likely to be male (odds ratio [OR] 2.24, 95% CI 1.24–4.05) and live in neighborhoods with high levels of immigration from malaria-endemic countries (OR 1.09, 95% CI 1.06–1.12) (Table 4). Population density was a weak predictor, but was retained in the model to account for potential confounding with proportion of residents who are immigrants from malaria-endemic regions. This variable had a wide range (0%–58%) and showed the strongest effect on case status: for every 1% increase in the proportion of residents who are immigrants from malaria-endemic regions of Africa, the odds of being a case-patient increased by 7%. The odds of being a case-patient were >17× higher in neighborhoods in the top quartile of proportion of residents who are immigrants from malaria-endemic areas than in the neighborhoods in the lowest quartile. There were slight negative effects from median household income and population density. For every 1 unit increase in either, the odds of being a case-patient decreased by 1%.

The pseudo R^2 of the model was 0.19, with a predictive accuracy of 70% correctly classified by using a cutoff of 0.25 (67% sensitivity, 71% specificity). The model fit the data well (Hosmer-Lemeshow test, p = 0.76), and leverage values did not indicate any outliers that required removal. Several high positive residuals were found, because of the low sensitivity of the model that used a default cutoff (0.5). This low sensitivity reflects the limited number of variables available for analysis. The model was developed, however, for explanatory rather than predictive purposes; low sensitivity reflects the small number of covariates and use of neighborhood-level variables.

The distribution of parasite species infection in case-patients reflects the patterning of global malaria; *P. vivax* cases are associated with travel to or immigration from Asia, and *P. falciparum* cases are associated with travel to Africa (Table 3). Globally, a significant spatial patterning of parasite species exists, with *P. falciparum* found predominantly in Africa and *P. vivax* found predominantly in Asia (*30*). Case-patients infected with *P. falciparum*, for example, were significantly more likely to report recent travel to Africa than case-patients infected with *P. vivax* (Fisher exact test, p<0.01). Conversely, case-patients infected with *P. vivax* were more likely to report travel to malaria-endemic regions of Asia (Fisher exact test, p<0.01). Case-patients with *P. vivax* lived in neighborhoods with a significantly higher proportion of residents who are immigrants from malaria-endemic regions (Mann-Whitney, p<0.01). In contrast, however, *P. falciparum* case-patients did not necessarily live in neighborhoods with a high proportion of residents who are immigrants from malaria-endemic regions of Africa (Mann-Whitney, p = 0.33). This finding may be due to the much lower overall level of immigration from Africa.

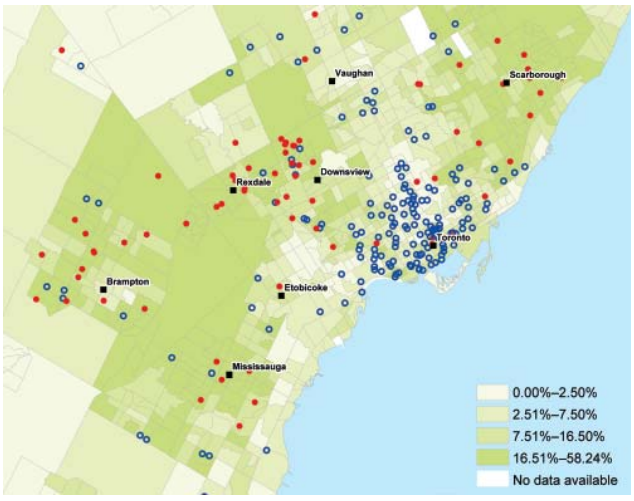

Figure 3. Percentage of residents in a neighborhood reporting immigration from malaria-endemic areas, greater Toronto area, Ontario, Canada, 2008–2009. Red dots, malaria case-patients (positive test results); blue circles, controls (negative test results).

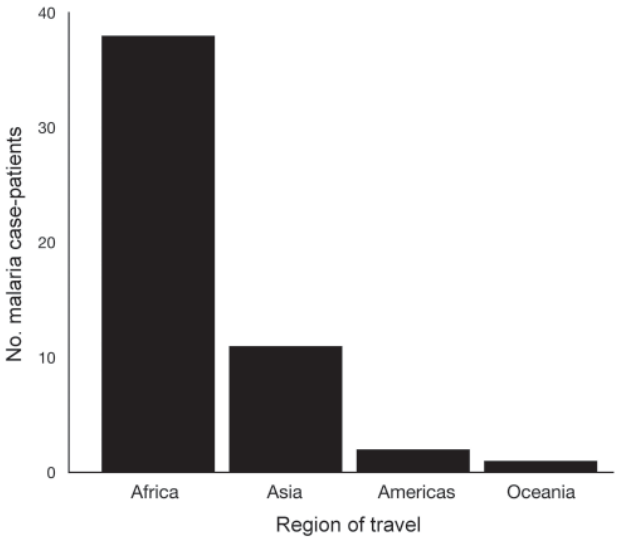

Figure 4. Number of malaria case-patients by region of travel, Ontario, Canada, 2008–2009.

Discussion

Malaria case-patients in Ontario were found predominantly in the GTA, with more case-patients in suburban areas outside the city center. Malaria case-patients were more likely to live in neighborhoods with a high proportion of residents who emigrated from malaria-endemic areas. Case-patients were more likely than controls to report travel to areas with endemic malaria, and there was concordance between the parasite species and the region of travel. The association between parasite species and geography held when neighborhood immigration was examined as well—cases of *P. vivax* malaria corresponded with immigration from malaria-endemic areas of Asia, and cases of *P. falciparum* malaria were found in areas with immigration from malaria-endemic areas of Africa.

Case-patients were more likely to be male than female. This finding is consistent with the literature on sex and travel-associated diseases, which finds that men are more likely than women to have vector-borne diseases, including malaria (*31*). Whether this finding is due to a biologic predisposition or to behavioral differences associated with increased transmission risk is not known (*31*). Whether potential differences in patterns of health care use between men and women could have affected the results in this study also is not known. Further research into how men and women seek pretravel and posttravel medical care in Ontario would improve the understanding of this result.

When assessing imported malaria cases, underreporting is a substantial issue. In Ontario, underreporting of malaria is likely to range from 10% to 40% (*5*,*32*). In Ontario, 226 malaria cases were documented by laboratories in 1998 (*32*). However, in that same year, only 160 cases in Ontario were reported to Health Canada (*32*). No recent published or quantified estimates of actual incidence are available, and the true level of current underreporting of malaria in Ontario and Canada more generally remains unclear. The data used in the study are strengthened by the inclusion of negative and positive malaria test results. The use of controls enables the factors that correlate with malaria cases to be better understood, unbiased by underlying patterns of malaria testing.

The odds of becoming infected and importing malaria to Canada were >17× higher for residents of neighborhoods with high immigration from malaria-endemic areas than those with low immigration from malaria-endemic countries. These results, combined with the geographic findings of the cluster analysis, are supported by census data on immigration. Brampton (the location of the significant space–time cluster of malaria cases), Markham, and Ajax all showed a significant increase in the proportion of residents born outside Canada in the 2006 census (*25*). As immigration to the neighborhoods in the suburbs of the GTA grows, more imported malaria cases could result. These results have implications for potential preventative measures that could be taken before persons travel abroad. The use of cluster detection methods has been proposed in other areas to target prevention methods (*33*,*34*). Targeted prevention could focus on hospitals and clinics found in

Table 2. Univariate analysis of malaria case-patients and controls, Ontario, Canada, 2008–2009

Variable	Case-patients	Controls	p value
Total, no. (%)	94 (100)	259 (100)	
Individual level			
Sex, no. (%)			
M	65 (69)	130 (50)	<0.01*
F	23 (25)	129 (50)	
Not reported	6 (6)	0	
Age, mean (95% CI)	42.5 (39.4–45.7)	41.4 (39.3–43.5)	0.57†
Neighborhood-level, mean (95% CI)			
Population density, persons/km	4,633 (3,926–5,340)	6,498 (5,634–7,363)	0.12‡
Median income (Canadian dollars)	28,140 (26,232–30,047)	30,802 (29,416–32,187)	0.04†
Residents who are immigrants from malaria-endemic areas, %	18.1 (15.5–20.7)	8.8 (7.6–10.0)	<0.01‡

*χ^2 test.
†Student *t* test.
‡Mann-Whitney U test.

Table 3. Univariate analysis of travel and immigration and case–control and parasite species variables, Ontario, Canada, 2008–2009

Variable	Patients			Parasite species		
	Case-patients	Controls	p value	*Plasmodium falciparum*	*P. vivax*	p value
Neighborhood-level, mean %						
Residents who are immigrants from malaria-endemic Africa	3.1	1.5	<0.01*	3.8	2.0	0.33*
Residents who are immigrants from malaria-endemic Asia	15.5	7.2	<0.01*	12.4	20.8	<0.01*
Individual level, no.						
Travel to malaria-endemic Africa	36	27	<0.01†	35	1	<0.01‡
Travel to malaria-endemic Asia	11	25	0.61†	0	11	<0.01‡

*Mann-Whitney test.
†χ^2 test.
‡Fisher exact test.

the cluster area and in regions with a high proportion of residents who are immigrants from malaria-endemic areas, with the goal of implementing targeted travel medicine screenings and education regarding malaria prophylaxis.

Predeparture travel medicine services are not covered under Ontario's provincial insurance plan (*35*). However, the use of travel clinics is not associated with income in Canada (*36*). As a high-risk group, VFRs are characterized by differing preventive care choices and lower use of pretravel medical services (*37*). In Canada, 1 study found that VFRs consulted family practitioners more often than travel clinics and that the family practitioners were more likely to prescribe inappropriate chemoprophylaxis (*38*). Another reason for the increased risk for imported malaria among VFRs could be differing perceptions of the risk for malaria, leading to different behavior in malaria-endemic areas (*16*,*21*,*39*). The recent growth of immigrants in Brampton and related Toronto suburbs may merit targeted programming by physicians and public health agencies, including translation of public health prevention materials (*25*).

Imported malaria in Canada is of public health significance given current, and likely substantially underestimated, cases of imported disease in Canada. Our characterization of imported malaria cases in Ontario highlights increased risk for infection in immigrant and low-income neighborhoods, with associations between immigration from countries where malaria transmission and particular pathogen types are endemic. These results suggest a pattern of inequality in tropical health outcomes in Ontario with implications for prevention and control of current incidence.

Imported malaria cases represent the potential for geographic emergence of a range of travel-associated diseases. The current landscape of emerging infectious diseases demands that we improve our understanding of the mechanisms by which diseases are imported and the characteristics of neighborhoods and populations at highest risk. The results of this study point to potential individual- and neighborhood-level risk factors that might be relevant to emerging infectious diseases more generally. As new infectious diseases emerge, identifying the processes of emergence and the areas and persons at greatest risk is critical. An analysis of the local patterns of imported malaria in Ontario can help the public health community better understand the ways in which global travel and immigration can affect the spatial distribution of other travel-related diseases.

Ms Eckhardt completed this study at McGill University in Montreal. She is currently affiliated with the Keenan Research Centre, Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto. Her research interests include the spatial epidemiology of infectious diseases, social determinants of infectious diseases, and international infectious disease epidemics.

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Table 4. Results of regression analyses of individual- and neighborhood-level variables of malaria incidence, Ontario, Canada, 2008–2009*

Variable	Odds ratio (95% CI)		
	Null model	Fully adjusted	1st vs. 4th quartile
Residents who are immigrants from malaria-endemic areas, %	1.07 (1.05–1.09)	1.09 (1.06–1.12)	17.66 (7.17–43.48)
Median income, CAD$	0.99 (0.99–0.99)	0.99 (0.99–0.99)	3.29 (1.34–8.09)†
Population density	0.99 (0.99–0.99)	0.99 (0.99–0.99)	6.39 (2.48–16.49)†
Male sex	2.80 (1.64–4.78)	2.24 (1.24–4.05)	NA

*Dependent variable: case-patients (positive malaria test); goodness-of-fit, Hosmer-Lemeshow test, p = 0.76. CAD$, Canadian dollars; NA, not applicable.
†Reverse coded to show 4th vs. 1st quartile.

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Bartonella spp. Bacteremia and Rheumatic Symptoms in Patients from Lyme Disease–endemic Region

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Bartonella spp. infection has been reported in association with an expanding spectrum of symptoms and lesions. Among 296 patients examined by a rheumatologist, prevalence of antibodies against *Bartonella henselae, B. koehlerae,* or *B. vinsonii* subsp. *berkhoffii* (185 [62%]) and *Bartonella* spp. bacteremia (122 [41.1%]) was high. Conditions diagnosed before referral included Lyme disease (46.6%), arthralgia/arthritis (20.6%), chronic fatigue (19.6%), and fibromyalgia (6.1%). *B. henselae* bacteremia was significantly associated with prior referral to a neurologist, most often for blurred vision, subcortical neurologic deficits, or numbness in the extremities, whereas *B. koehlerae* bacteremia was associated with examination by an infectious disease physician. This cross-sectional study cannot establish a causal link between *Bartonella* spp. infection and the high frequency of neurologic symptoms, myalgia, joint pain, or progressive arthropathy in this population; however, the contribution of *Bartonella* spp. infection, if any, to these symptoms should be systematically investigated.

The genus *Bartonella* comprises at least 26 species or subspecies of vector-transmitted bacteria, each of which has evolved to cause chronic bacteremia in ≥1 mammalian reservoir hosts (*1*–*4*). Among these, bartonellae of 14 species or subspecies have been implicated in zoonotic diseases (*5*,*6*), including cat-scratch disease, which is caused by *B. henselae* transmission during a cat bite or scratch and characterized by acute onset of self-limiting fever and regional lymphadenopathy (*7*–*9*). Recent observations, however, are causing a paradigm shift from the assumption that infection with a *Bartonella* sp. consistently induces an acute, self-limiting illness to the realization that subsets of infected, immunocompetent patients can become chronically bacteremic (*10*–*15*).

After *B. henselae* was confirmed as the primary cause of cat-scratch disease in the early 1990s, several reports described an association between the newly identified bacterium and rheumatic disease manifestations, variously described as rheumatoid, reactive, or chronic progressive polyarthritis (*16*–*20*). One study, however, failed to isolate *B. henselae* from synovial fluid of 20 patients with chronic arthritis (*21*). Because epidemiologic evidence supports an association between rheumatic symptoms and cat-scratch disease and because arthritis is a primary disease manifestation of *Borellia burgdorferi* infection (Lyme disease), we explored whether antibodies against and bacteremia with *Bartonella* spp. can be detected in patients examined for arthropathy or chronic myalgia. Our primary objective was to determine the serologic and molecular prevalence of *Bartonella* spp. bacteremia in patients referred to a clinical rheumatologist. We also compared self-reported symptoms, health history, and demographic factors with *Bartonella* spp. bacteremia as determined by an enrichment blood culture platform combined with PCR amplification and DNA sequencing, when possible, to determine the *Bartonella* species and strain. This study was conducted in conjunction with North Carolina State University Institutional Review Board approval (IRB# 164–08–05).

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Materials and Methods

Study Population

For this cross-sectional study, we enrolled only patients examined by a rheumatologist in the Maryland–Washington, DC, USA, area from August 25, 2008, through April 1, 2009. Because *Bartonella* spp. are known to primarily infect cells within the vascular system, including erythrocytes, endothelial cells, and potentially circulating and tissue macrophages (*1*,*5*,*6*), selection was biased by patients who had historical, physical examination, or laboratory evidence of small vessel disease, including a subset of patients with a prior diagnosis of Lyme disease or chronic post–Lyme syndrome. We also included patients with chronic joint pain, prior documentation of synovial vascular inflammation, or a diagnosis of rheumatoid arthritis.

A standardized 5-page questionnaire was mailed to each participant for self-report. The questionnaire collected information about demographics, animal/arthropod exposure, history of visiting a medical specialist, outdoor activity, self-reported clinical symptoms, and concurrent conditions. Questionnaires were returned to the Intracelluar Pathogens Research Laboratory at North Carolina State University, College of Veterinary Medicine, Raleigh, North Carolina, USA, where results were entered into an electronic database.

Sample Collection

From each patient, the attending rheumatologist aseptically obtained anticoagulated blood samples (in EDTA tubes) and serum samples and shipped them overnight to the laboratory. Patient variations included timing of sample collection relative to onset of illness, duration of illness, current illness severity, and prior or recent use of antimicrobial drugs. The samples were then processed in a limited-access laboratory.

Sample Processing

Immunofluorescence Antibody Assay

To determine the antibody titer to each *Bartonella* species or subspecies, we used *B. henselae*, *B. koehlerae*, and *B. vinsonii* subsp. *berkhoffii* (genotypes I, II, and III) antigens in a traditional immunofluorescence antibody (IFA) assay with fluorescein conjugated goat anti-human IgG (Pierce Antibody; Thermo Fisher Scientific, Rockford, IL, USA) (*10*,*12*,*22*). To obtain intracellular whole bacterial antigens for IFA testing, we passed isolates of *B. henselae* (strain Houston-1, ATCC #49882); *B. koehlerae* (NCSU FO-1–09); and *B. vinsonii* subsp. *berkhoffii* genotypes I (NCSU isolate 93-CO-1, ATCC #51672), II (NCSU isolate 95-CO-2), and III (NCSU isolate 06-CO1) from agar-grown cultures into *Bartonella*-permissive tissue culture cell lines: AAE12 (an embryonic *Amblyomma americanum* tick cell line) for *B. henselae*, DH82 (a canine monocytoid cell line) for *B. koehlerae*, and Vero (a mammalian fibroblast cell line) for the *B. vinsonii* genotypes. Heavily infected cell cultures were spotted onto 30-well Teflon coated slides (Cel-Line; Thermo Fisher Scientific), air dried, acetone fixed, frozen, and stored. Serum samples were diluted in a phosphate-buffered saline solution containing normal goat serum, Tween-20, and powdered nonfat dry milk to block nonspecific antigen binding sites and then incubated on antigen slides. All available patient serum was screened at dilutions from 1:16 to 1:64. Samples reactive at a 1:64 dilution were further tested with 2-fold dilutions to 1:8192. As in previous studies, we defined a seroreactive antibody response against a specific *Bartonella* sp. antigen as a threshold titer of 64 (*10–15*,*23*,*24*).

Bartonella α Proteobacteria Growth Medium Enrichment Culture

Each sample was tested by PCR amplification of *Bartonella* spp. DNA before and after enrichment of blood and serum in *Bartonella* α Proteobacteria growth medium (BAPGM) (*10–14*,*23–26*). The BAPGM platform incorporates 4 PCR steps, representing independent components of the testing process for each sample, as follows: step 1) PCR amplifications of *Bartonella* spp. after DNA extraction from whole blood and serum; steps 2 and 3) PCR after whole blood culture in BAPGM for 7 and 14 days; and step 4) PCR of DNA extracted from subculture isolates (if obtained after subinoculation from the BAPGM flask at 7 and 14 days onto plates containing trypticase soy agar with 10% sheep whole blood, which are incubated for 4 weeks). To avoid DNA carryover, we performed PCR sample preparation, DNA extraction, and PCR amplification and analysis in 3 separate rooms with a unidirectional work flow. All samples were processed in a biosafety cabinet with HEPA (high-efficiency particulate air) filtration in a limited-access laboratory.

Methods used to amplify *Bartonella* DNA from blood, serum, and BAPGM liquid culture and subculture samples included conventional PCR with *Bartonella* genus primers targeting the 16S-23S intergenic spacer region (ITS) and a second PCR with *B. koehlerae* ITS species-specific primers, as described (*13*,*25–29*). Amplification of the *B. koehlerae* ITS region was performed by using oligonucleotides Bkoehl-1s: 5′-CTT CTA AAA TAT CGC TTC TAA AAA TTG GCA TGC-3′ and Bkoehl1125as: 5′-GCC TTT TTT GGT GAC AAG CAC TTT TCT TAA G-3′ as forward and reverse primers, respectively. Amplification was performed in a 25-μL final volume reaction containing 12.5 μL of Tak-Ex Premix (Fisher Scientific), 0.1 μL of 100 μM of each forward and reverse primer (IDT; DNA Technology,

Coralville, IA, USA), 7.3 μL of molecular grade water, and 5 μL of DNA from each sample tested.

Conventional PCR was performed in an Eppendorf Mastercycler EPgradient (Hauppauge, NY, USA) under the following conditions: 1 cycle at 95°C for 2 s, followed by 55 cycles with DNA denaturing at 94°C for 15 s, annealing at 64°C for 15 s, and extension at 72°C for 18 s. The PCR was completed by a final cycle at 72°C for 30 s. As previously described for the *Bartonella* ITS genus and *B. koehlerae*–specific PCRs, all products were analyzed by using 2% agarose gel electrophoresis and ethidium bromide under UV light, after which amplicon products were submitted to a commercial laboratory (Eton Bioscience Inc., Research Triangle Park, NC, USA) for DNA sequencing to identify the species and ITS strain type (*13*,*15*,*28*,*30*).

To check for potential contamination during processing, we simultaneously processed a noninoculated BAPGM culture flask in the biosafety hood in an identical manner for each batch of patient blood and serum samples tested. For PCR, negative controls were prepared by using 5 μL of DNA from the blood of a healthy dog. All controls remained negative throughout the course of the study.

Statistical Analysis

Descriptive statistics were obtained for all demographic variables, self-reported clinical symptoms and concurrent conditions, previous specialist consultation, and self-reported exposures. The χ^2 test was used to assess associations between self-reported clinical symptoms and previous specialist consultation separately with PCR results for *B. henselae*; *B. koehlerae*; and *B. vinsonii* subsp. *berkhoffii* genotypes I, II, and III. The Fisher exact test was used when expected cell value was <5. For the initial analysis, a liberal α value ($\alpha \leq 0.10$) was selected. The effect of each significant variable on the outcome variables was adjusted in separate multivariate logistic regression models controlling for age, sex, and health status. The models were repeated for different possible outcomes: PCR results for *B. henselae* or PCR results for *B. koehlerae*. Variables maintaining $p \leq 0.05$ were considered significant. For some comparisons of potential interest, we were unable to estimate associations with the outcome(s) of interest because of low numbers (e.g., *B. vinsonii* subsp. *berkhoffii* genotypes I, II and III). Statistical analyses were performed by using SAS/STAT for Windows version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Patient Characteristics

The age range of the 296 patients was 3–90 years; median ages were 46 years for women and 36 years for men (Table 1). Women made up ≈70% of the study population. Most (68.2%) patients reported that they felt ill, whether chronically or infrequently, and 27.7% considered themselves to be generally healthy. The most common animal exposure reported was dog (n = 252; 85.1%), followed by cat (n = 202; 68.2%) and horse (n = 86; 29.0%). Most patients reported having been bitten or scratched by an animal (n = 202; 68.2%) or exposed to ticks (n = 229; 77.4%) and biting flies (n = 160; 54.0%). Hiking was the predominant outdoor activity reported (52.0%). Most (273 [92.2%]) patients reported having had a condition diagnosed before visiting the rheumatologist. Previously diagnosed conditions included Lyme disease (46.6%), arthralgia/arthritis or osteoarthritis/rheumatoid arthritis (20.6%), chronic fatigue (19.6%), and fibromyalgia (6.1%) (Figure 1).

Serologic and BAPGM Findings

Of the 296 patients, 185 (62.5%) were seroreactive to ≥1 *Bartonella* sp. antigens and 122 (41.1%) were infected with *B. henselae*, *B. koehlerae*, *B. vinsonii* subsp. *berkhoffii*, or *Bartonella* spp. Of the 122 patients with *Bartonella* spp. infection, PCR results were positive but DNA sequencing was unsuccessful or did not enable species identification for 29 (23.7%). After subculture, 6 isolates were obtained from 5 samples: 3 *B. henselae* isolates, 2 *B. koehlerae* isolates, and 1 *Bartonella* sp. isolate that was not fully characterized. Of the *Bartonella*-infected patients, 120 (98.4%) had a positive PCR result after DNA extraction from blood, serum, or enrichment culture (Figure 2), and 2 (1.6%) had a positive PCR result only after subculture isolation.

For *B. henselae,* 67 (22.6%) patients were seroreactive and 40 (13.5%) had positive PCR results. Of these 40 patients, only 7 (17.5%) were concurrently *B. henselae* seroreactive, whereas 33 (82.5%) patients who had a positive PCR result were not seroreactive to *B. henselae* antigens. There was no association between *B. henselae* antibodies and bacteremia (p = 0.37).

For *B. koehlerae*, 89 (30.1%) patients were seroreactive and 54 (18.2%) had positive PCR results. Of these 54 patients, 24 (44.4%) were seroreactive to *B. koehlerae* by IFA assay, whereas 29 (53.6%) were not seroreactive to *B. koehlerae* antigens. One patient with a positive *B. koehlerae* PCR result did not have a concurrent IFA test result (serum not submitted). There was an association between *B. koehlerae* seroreactivity and bacteremia (p = 0.008); seroreactive patients were more likely to be infected (odds ratio [OR] 2.25 [1.22–4.15]).

For *B. vinsonii* subsp. *berkhoffii*, 148 (50.0%) patients were seroreactive by IFA testing to at least 1 of 3 genotypes, and 10 (3.4%) had a positive PCR. Of these 10 patients, 3 were infected with genotype I, 6 were infected with genotype II, and for 1 patient the genotype could

not be defined on the basis of readable DNA sequence. Seroreactivity to genotypes I, II, and III was found for 77 (26.0%), 102 (34.5%), and 82 (27.7%) patients, respectively. There was no association between *B. vinsonii* subsp. *berkhoffii* seroreactivity and bacteremia. Combined PCR and IFA assay results are summarized in Table 2. Of the patients with a positive PCR, 65% reported a prior diagnosis of Lyme disease (n = 138), bartonellosis (n = 29), or babesiosis (n = 14). Among the 138 patients with a prior diagnosis of Lyme disease, the prevalence of *Bartonella*

Table 1. Characteristics and *Bartonella* spp. PCR results for 296 patients examined by a rheumatologist, Maryland–Washington, DC, USA, August 25, 2008–April 1, 2009*

Characteristic	Overall study population, no. (%)	Positive *Bartonella* sp. result by PCR, no. (%)				
		Overall positive	*B. henselae*	*B. koehlerae*	*B. vinsonii* subsp. *berkhoffii*	*Bartonella* spp.†
Total	296 (100)	122 (62.5)	40 (13.5)	54 (18.2)	10 (3.4)	29 (9.8)
Sex						
F	205 (69.3)	86 (29.0)	24 (11.7)	38 (18.5)	7 (3.4)	21 (10.3)
M	91 (30.7)	36 (12.2)	16 (17.6)	16 (17.5)	3 (3.3)	8 (8.8)
State of residence						
Maryland	148 (50.0)	58 (39.2)	20 (13.5)	27 (18.2)	5 (3.4)	13 (8.8)
Virginia	76 (25.7)	37 (48.7)	13 (17.1)	19 (25.0)	0	7 (9.2)
Pennsylvania	26 (8.8)	9 (34.6)	2 (7.7)	3 (11.5)	2 (7.7)	3 (11.5)
District of Columbia	16 (5.4)	5 (31.3)	1 (6.3)	1 (6.25)	1 (6.3)	2 (12.5)
Other	30 (10.1)	13 (43.3)	4 (13.3)	4 (13.3)	2 (6.7)	4 (13.3)
Immunofluorescence antibody results						
All *Bartonella* spp.	185 (62.5)	77 (41.6)	25 (13.5)	33 (17.8)	4 (2.1)	20 (10.8)
B. henselae	67 (22.6)	24 (35.8)	7 (10.3)	8 (11.7)	2 (2.9)	8 (11.7)
B. koehlerae	89 (30.1)	38 (42.7)	10 (11.2)	24 (26.9)	3 (3.4)	5 (5.6)
B. vinsonii subsp. *berkhoffii*	148 (50.0)	59 (39.8)	21 (14.1)	21 (14.1)	3 (2.0)	18 (12.1)
Self-report health assessment						
Healthy	82 (27.7)	32 (39.0)	12 (14.6)	13 (15.8)	3 (3.6)	7 (8.5)
Infrequently Ill	53 (17.9)	26 (49.1)	7 (13.2)	14 (26.4)	3 (5.6)	5 (9.4)
Chronically Ill	149 (50.3)	54 (36.2)	17 (11.4)	31 (14.1)	4 (2.7)	15 (10.1)
No response	12 (4.0)	10 (83.3)	4 (33.3)	6 (50.0)	0	2 (16.7)
Animal contact						
Yes	283 (95.6)	116 (40.9)	38 (13.4)	51 (18.0)	9 (3.2)	27 (9.5)
No	13 (4.4)	6 (46.2)	2 (15.4)	3 (23.1)	1 (7.7)	2 (15.4)
Type						
Dog	252 (85.1)	104 (41.3)	33 (13.1)	45 (17.9)	7 (2.8)	27 (10.7)
Cat	202 (68.2)	77 (38.1)	24 (11.8)	34 (16.8)	7 (3.5)	19 (9.4)
Horse	86 (29.0)	41 (47.7)	12 (13.9)	14 (16.3)	2 (2.3)	13 (15.1)
Bird	59 (19.3)	26 (44.0)	8 (13.5)	8 (13.5)	2 (3.4)	9 (15.2)
Cattle	32 (10.8)	11 (34.4)	3 (9.3)	4 (12.5)	0	4 (12.5)
Poultry	30 (10.1)	13 (43.3)	6 (20.0)	3 (10.0)	0	4 (30.7)
Swine	25 (8.5)	10 (25.0)	5 (20.0)	2 (8.0)	0	3 (12.0)
Sheep	25 (8.5)	12 (48.0)	6 (24.0)	2 (8.0)	0	4 (16.0)
Other	12 (4.0)	12 (58.3)	4 (33.3)	1 (8.3)	0	2 (16.7)
Animal bites/scratches						
Cat	154 (52.0)	64 (41.6)	21 (13.6)	27 (17.5)	6 (3.9)	14 (9.1)
Dog	118 (39.8)	52 (44.1)	18 (15.3)	22 (18.6)	2 (1.7)	13 (11.0)
Bird	12 (4.0)	10 (83.3)	3 (25.0)	4 (33.3)	2 (16.7)	3 (25.0)
Horse	14 (4.7)	9 (64.2)	2 (14.3)	3 (21.4)	1 (8.3)	3 (21.4)
Insect exposure						
Mosquitoes	256 (86.5)	106 (41.4)	37 (14.4)	46 (17.9)	8 (3.1)	24 (9.4)
Ticks	229 (77.4)	96 (41.9)	29 (12.6)	43 (18.7)	10 (4.3)	23 (10.0)
Fleas	148 (50.0)	66 (44.5)	23 (15.5)	26 (17.5)	7 (4.7)	16 (10.8)
Biting Flies	160 (54.0)	68 (42.5)	25 (15.6)	27 (16.9)	5 (3.1)	16 (10.0)
Lice	38 (12.8)	17 (44.7)	7 (18.4)	3 (7.8)	0	7 (18.4)
Spiders	5 (1.7)	4 (80.0)	1 (20.0)	2 (40.0)	0	1 (20.0)
Sarcoptes mite	3 (1.0)	1 (33.3)	0	0	0	1 (33.3)
Outdoor exposure						
Hiking	154 (52.0)	66 (42.9)	21 (13.6)	28 (18.2)	5 (3.3)	16 (10.4)
Wildlife rescue/rehabilitation	22 (7.4)	7 (31.8)	2 (9.1)	2 (9.1)	0	3 (14.3)
Hunting	21 (7.1)	9 (42.9)	1 (4.7)	4 (19.0)	0	4 (19.1)
Other	36 (12.2)	16 (44.4)	6 (16.7)	8 (22.2)	2 (5.6)	1 (2.8)

*Positive sample and exposure categories are not mutually exclusive (i.e., some persons had positive test results by both IFA and PCR, or could have been exposed to both cats and dogs). Median patient ages, for women and men, respectively, were as follows: overall study population, 46.0 and 36.0 y; those with positive results for overall *Bartonella*, 47.0 and 38.0 y, *B. henselae*, 44.0 and 41.0 y, *B. koehlerae*, 49.0 and 40.5 y, *B. vinsonii* subsp. *berkhoffii*, 43.0 and 64.0 y, and *Bartonella* spp., 48.0 and 24.0 y.
†Positive PCR results after using *Bartonella* genus primers but unable to obtain a clean sequence to determine species.

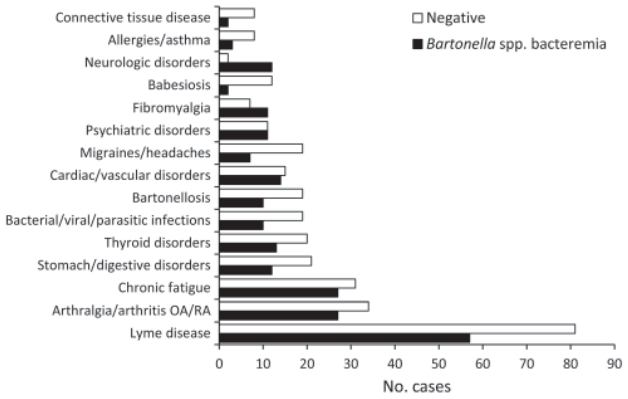

Figure 1. *Bartonella* spp. PCR results for the 15 most frequently reported previous diagnoses. OA, osteoarthritis; RA, rhuematoid arthiritis.

spp. antibodies and bacteremia were 93 (67.4%) and 57 (41.3%), respectively.

Factors Associated with *Bartonella* spp.

PCRs indicated the following: *B. henselae* positivity was associated ($p<0.05$) with blurred vision and numbness (Table 3), patients who had visited a neurologist were more likely than those who had not to be *B. henselae* positive, older median age was significantly associated with *B. koehlerae* positivity, and patients who reported paralysis were more likely to be positive for *B. vinsonii* subsp. *berkhoffii*. No associations were found for self-reported exposures (e.g., insect or animal exposure) and positive PCR for *B. henselae*, *B. koehlerae*, or *B. vinsonii* subsp. *berkhoffii*. No associations were found for *B. henselae*, *B koehlerae*, or *B vinsonii* subsp. *berkhoffii* positivity and seroreactivity.

Logistic Regression Analysis

To identify factors associated with PCR positivity for *B. henselae* or *B. koehlerae*, we adjusted the models for 3 biological confounders: age, sex, and health status (Table 4). We identified the following factors as associated with *B. henselae*–positive PCR result: blurred vision (adjusted OR [aOR] 2.37, 95% CI 1.13–4.98), numbness (aOR 2.74, 95% CI 1.26–5.96), and previous consultation with a neurologist (aOR 2.76, 95% CI 1.33–5.73). No self-reported symptoms were significantly associated with PCR positivity for *B. koehlerae*. However, patients who had visited an infectious disease physician were more likely to have a *B. koehlerae*–positive PCR result (aOR 1.98, 95% CI 1.05–3.75).

Discussion

We identified unexpectedly high serologic and molecular prevalence for *B. henselae*, *B. koehlerae*, and *B. vinsonii* subsp. *berkhoffii* in patients who had been examined by a rheumatologist, of whom more than half reported a prior diagnosis of Lyme disease, bartonellosis, or babesiosis. However, the diagnostic criterion upon which these infections were based was not available for review because all prior diagnoses were self-reported. Overall, 185 (62.5%) of 296 patients had antibodies to *B. henselae*, *B. koehlerae,* or *B. vinsonii* subsp. *berkhoffii*, and 122 (41.1%) were positive for *Bartonella* spp. according to PCR. In most instances, DNA sequencing of the amplified product facilitated identification of the infecting species. The prevalence of antibodies against *Bartonella* spp. (93 [67.4%]) and bacteremia [57 [1.3%]) among 138 patients with a prior diagnosis of Lyme disease did not differ from that of the overall study population. Because our analysis was restricted to patients selected by a rheumatologist practicing in a Lyme disease–endemic region, extrapolations to other regions or other rheumatology practices might not be applicable. Also, because the survey was self-administered, objective confirmation of symptoms, conditions, and diagnoses was not always possible; therefore, responses might have been subject to respondent bias. Similarly, because responses associated with symptoms, conditions, and exposures might have occurred over a protracted time, survey responses might also be subject to recall bias.

Despite these study limitations, *B. henselae* infections seemed to be more common in patients who reported blurred vision, numbness in the extremities, and

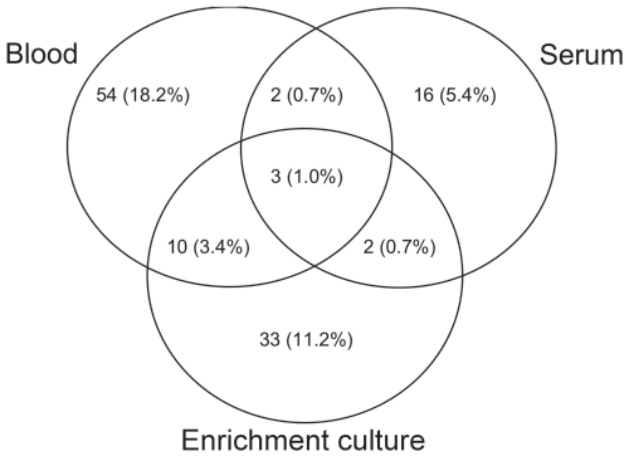

Figure 2. *Bartonella* PCR amplification results from blood, serum, and enrichment blood culture with the *Bartonella* α Proteobacteria growth medium. Of 296 patients, 120 had positive PCR results in 1 component. Two patients, who had positive PCR results only after enrichment culture incubation and subculture onto agar, are not included. Each circle represents *Bartonella* PCR amplification results from blood, serum, or after enrichment blood culture. Each number represents the total (%) positive for each of the 4 possibilities within each of the 3 circles. For example, only 3 (1%) patients had positive results from blood, serum, and enrichment blood culture.

Table 2. Test results for *Bartonella* spp. in 296 patients examined by a rheumatologist, Maryland–Washington, DC, USA, August 25, 2008–April 1, 2009*

Bartonella sp.	IFA–/PCR–	IFA+/PCR–	IFA+/PCR+	IFA–/PCR+
B. henselae	196	60	7	33
B. koehlerae	177	65	24	29
B. vinsonii subsp. *berkhoffii*	141	145	3	7
Genotype I	217	75	2	1
Genotype II	189	101	1	5
Genotype III	213	82	0	0

*IFA, indirect immunofluorescent antibody results obtained by using *B. henselae*, *B. koehlerae,* and *B. vinsonii* subsp. *berkhoffii* antigens; PCR, results obtained after PCR amplification by using *Bartonella* intergenic spacer primers, followed by attempted DNA sequencing of each amplicon.

previous consultation with a neurologist before referral to the rheumatologist. In a case series of 14 patients, the following were reported by 50% of patients infected with a *Bartonella* species, specifically *B. henselae*, *B. vinsonii* subsp. *berkhoffii*, or both: memory loss, numbness or a loss of sensation, balance problems, and headaches (*10*). Another 6 *B. henselae*–bacteremic patients reported seizures, ataxia, memory loss, and/or tremors; 1 of these patients was co-infected with *B. vinsonii* subsp. *berkhoffii*, and another was positive for *B. henselae* by PCR after enrichment of cerebrospinal fluid in BAPGM (*23*). An enrichment culture approach also identified an association between intravascular infection with *B. vinsonii* subsp. *berkhoffii* genotype II and *B. henselae* and neurologic symptoms in a veterinarian and his daughter (*12*). Symptoms in the father included progressive weight loss,

Table 3. Factors associated with *Bartonella spp.* positivity by PCR, among 296 patients examined by a rheumatologist, Maryland–Washington, DC, USA, August 25, 2008–April 1, 2009*

	B. henselae, no. (%)			*B. koehlerae,* no. (%)			*B. vinsonii subsp. berkhoffii,* no. (%)		
Variable	Positive, n = 40	Negative, n = 256	p value†	Positive, n = 54	Negative, n = 242	p value†	Positive, n = 10	Negative, n = 286	p value†
Sex									
F	24 (60.0)	181 (70.7)	0.17	38 (70.4)	167 (69.0)	0.84	7 (70.0)	198 (69.2)	0.99
M	16 (40.0)	75 (29.3)		16 (29.6)	75 (30.1)		3 (30.0)	88 (30.7)	
Self-reported health status									
Healthy	12 (33.3)	70 (29.2)	0.77	13 (27.1)	69 (29.2)	0.11	3 (30.0)	79 (29.8)	0.59
Infrequently Ill	7 (19.4)	46 (18.5)		14 (29.2)	39 (16.5)		3 (30.0)	50 (18.3)	
Chronically Ill	17 (47.2)	132 (53.2)		21 (43.7)	128 (54.3)		4 (40.0)	145 (52.9)	
Signs or symptoms									
Fatigue	38 (95.0)	226 (88.3)	0.27	48 (88.9)	216 (89.3)	0.93	9 (90.0)	255 (89.2)	0.93
Headache	25 (62.5)	155 (60.5)	0.81	32 (59.2)	148 (61.2)	0.79	8 (80.0)	172 (60.2)	0.32
Difficulty remembering	32 (80.0)	174 (84.5)	0.12	38 (70.4)	168 (69.4)	0.89	4 (40.0)	202 (70.6)	0.07
Confusion	25 (62.5)	132 (51.5)	0.20	29 (53.7)	128 (52.9)	0.91	4 (40.0)	153 (59.6)	0.52
Disorientation	18 (45.0)	82 (32.0)	0.10	14 (25.9)	86 (35.5)	0.17	2 (20.0)	98 (34.3)	0.50
Irritability	30 (75.0)	153 (59.7)	0.06	32 (59.3)	151 (62.4)	0.66	5 (50.0)	178 (62.2)	0.51
Blurred vision	23 (57.5)	100 (39.1)	0.03	23 (42.6)	100 (41.3)	0.86	3 (30.0)	120 (41.9)	0.45
Eye pain	16 (40.0)	78 (30.5)	0.23	18 (33.3)	76 (31.4)	0.78	3 (30.0)	91 (31.8)	0.99
Sleeplessness	32 (80.0)	188 (73.4)	0.37	36 (66.7)	184 (76.0)	0.15	8 (80.0)	212 (74.1)	0.67
Insomnia	22 (55.0)	153 (59.7)	0.56	33 (61.1)	142 (58.7)	0.74	5 (50.0)	170 (59.4)	0.55
Balance problems	24 (60.0)	123 (48.0)	0.16	26 (48.2)	121 (50.0)	0.80	4 (40.0)	143 (50.0)	0.75
Tremors/shaking	17 (42.5)	92 (35.9)	0.42	17 (31.5)	92 (38.0)	0.36	5 (50.0)	104 (36.4)	0.51
Muscle weakness	28 (70.0)	161 (62.9)	0.38	36 (66.7)	153 (63.2)	0.63	8 (80.0)	181 (63.3)	0.34
Paralysis	3 (7.5)	13 (5.1)	0.52	3 (5.6)	13 (5.4)	0.95	2 (20.0)	14 (4.9)	0.04
Muscle pain	31 (77.5)	176 (68.7)	0.26	36 (66.7)	171 (70.6)	0.56	6 (60.0)	201 (70.3)	0.49
Numbness	28 (70.0)	128 (50.0)	0.01	25 (46.3)	131 (54.1)	0.29	7 (70.0)	149 (52.1)	0.34
Joint pain	31 (77.5)	199 (77.3)	0.97	41 (75.9)	189 (78.1)	0.73	10 (100)	220 (76.9)	0.12
Chronic fatigue	27 (67.5)	180 (70.3)	0.71	37 (68.5)	170 (70.3)	0.80	7 (70.0)	200 (69.9)	0.99
Bowel/bladder dysfunction	17 (42.5)	95 (37.1)	0.51	19 (35.2)	93 (38.4)	0.66	5 (50.0)	107 (37.4)	0.41
Shortness of breath	19 (47.5)	98 (38.3)	0.26	21 (38.9)	96 (39.7)	0.91	3 (30.0)	114 (39.8)	0.74
Poor appetite	8 (20.0)	75 (29.3)	0.22	12 (22.2)	71 (29.3)	0.29	1 (10.0)	82 (28.6)	0.29
Weight loss	7 (17.5)	52 (20.3)	0.67	6 (11.1)	53 (21.9)	0.07	1 (10.0)	58 (20.3)	0.69
Depression	20 (50.0)	126 (49.2)	0.92	28 (51.9)	118 (48.7)	0.68	4 (40.0)	142 (49.6)	0.75
Syncope	4 (10.0)	41 (16.0)	0.32	8 (14.8)	37 (5.3)	0.93	2 (20.0)	43 (15.0)	0.66
Consultation with neurologist	23 (57.5)	87 (33.9)	<0.01	22 (40.7)	88 (36.4)	0.56	3 (30.0)	107 (37.4)	0.63
Consultation with infectious disease physician	16 (40.0)	104 (40.6)	0.94	29 (53.7)	91 (37.6)	0.03	4 (40.0)	116 (40.7)	0.97

*Median ages, compared by using Wilcoxon rank-sum test, for positive and negative results, respectively, were *B. henselae*, 42.5, 44.0, p = 0.43; *B. koehlarae*, 48.0, 43.0, p = 0.03; and *B. vinsonii* subsp. *berkhoffii*, 46.5, 44.0, p = 0.64.
†Results of χ^2 analysis (Fisher exact test used when expected cell value <5).

Table 4. Factors associated with positive PCR result for *Bartonella henselae* and *B. koehlerae among* 296 patients examined by a rheumatologist, Maryland–Washington, DC, USA, August 25, 2008–April 1, 2009*

Variable	Positive vs. negative result, adjusted odds ratio (95% CI)	
	B. henselae	*B. koehlerae*
Blurred vision	2.37 (1.13–4.98), p = 0.03	NS
Numbness	2.74 (1.26–5.96), p = 0.01	NS
Consultation with infectious disease physician	NS	1.98 (1.05–3.75), p = 0.04
Consultation with neurologist	2.76 (1.33–5.73), p<0.01	NS

*Results of logistic regression analysis. Variables adjusted for age, sex, and duration of illness. NS, not significant.

muscle weakness, and lack of coordination; symptoms in the daughter were headaches, muscle pain, and insomnia. For each patient, after repeated courses of antimicrobial drugs, blood cultures became negative, antibody titers decreased to nondetectable levels, and all neurologic symptoms resolved.

Although no symptoms were statistically associated with *B. koehlerae* infection, patients infected with *B. koehlerae* were more likely to have previously consulted an infectious disease physician. Of the 54 *B. koehlerae* patients with a positive PCR result, 54% reported a prior diagnosis of Lyme disease (n = 25), bartonellosis (n = 3), or babesiosis (n = 1). Fatigue, insomnia, memory loss, and joint and muscle pain were frequent complaints among those with a positive PCR result for *B. koehlerae*, but these symptoms did not differ in frequency from those in patients with negative PCR. Similar symptoms were previously reported in a small case series involving *B. koehlerae*–bacteremic patients (*13*). Peripheral visual deficits, sensory loss, and hallucinations resolved in a young woman after antimicrobial drug treatment for *B. koehlerae* infection (*30*). Because of the small number of patients with positive PCR results for *B. vinsonii* subsp. *berkhoffii*, we restricted the multivariate analysis to those with positive results for *B. henselae* and *B. koehlerae*. Because limited sample size affected our ability to conduct multivariate analysis to control for potential confounders for *B. vinsonii* subsp. *berkhoffii* positivity, the χ^2 associations with *B. vinsonii* subsp. *berkhoffii* positivity should be interpreted with caution.

Although the pathogenic relevance of the high *Bartonella* spp. seroprevalence and bacteremia in this patient population are unclear, these results justify additional prospective studies involving more narrowly defined patient and control populations. Of the 92 patients infected with *B. koehlerae, B. henselae,* or *B. vinsonii* subsp. *berkhoffi*, 69 (75%) had at least 1 discordant IFA assay result for *Bartonella* spp. antigen seroreactivity and only 34 (30.6%) had a concordant species-specific PCR and IFA result. Also, consistent with previous study findings (*15*), the PCRs depicted in Figure 2 illustrate an increased likelihood of positivity if blood, serum, and enrichment blood cultures are independently tested. According to these and previous results (*7*,*18*,*31*,*32*), a subset of *Bartonella* spp.–bacteremic patients could be anergic and might not produce a detectable IFA response, or alternatively, the substantial antigenic variation among various *Bartonella* strains might result in false-negative IFA assay results for some patients. In a study on *Bartonella* serology conducted by the Centers for Disease Control and Prevention, IFA cross-reactivity among *Bartonella* species occurred in 94% of patients with suspected cat-scratch disease (*33*). Despite the lack of concordance between serologic results and BAPGM enrichment PCR results, most (185 [62.5%]) patients in our study were seroreactive to *Bartonella* spp., suggesting prior exposure to ≥1 *Bartonella* spp. Because serologic cross-reactivity to *Chlamydia* spp. and *Coxiella burnettii* antigens has been reported, exposure to these or other organisms might have contributed to the high seroprevalence. In a previous study involving 32 healthy volunteers and patients at high risk for *Bartonella* spp. bacteremia, seroprevalence rates for *B. henselae*, *B. koehlerae* and *B. vinsonii* subsp. *berkhoffii* genotypes I and II were 3.1%, 0%, 0,%, and 50%, respectively, for the healthy population compared with 15.6%, 9.2%, 19.8%, and 28.1%, respectively, for the high-risk population (*15*). Although in that study and the study reported here, the same test antigens and identical IFA assays were used and the same research technologist interpreted the results, the overall seroprevalence in the study reported here was higher than that among high-risk patients with extensive arthropod or animal contact (49.5%) and differed substantially from serologic results from healthy volunteers (*15*). However, in the study reported here, a large portion of the population (34.5%) was also seroreactive to *B. vinsonii berkhoffii* genotype II. Immunophenotypic properties giving rise to seroreactivity to this particular antigen among healthy control and patient populations have not been clarified but could be related to polyclonal B-cell activation, commonly found in patients with rheumatologic or chronic inflammatory diseases.

It is becoming increasingly clear that no single diagnostic strategy will confirm infection with a *Bartonella* sp. in immunocompetent patients. Before the current study, we primarily used BAPGM enrichment blood cultures and PCR to test symptomatic veterinarians, veterinary technicians, and wildlife biologists, who seem to be at occupational risk for *Bartonella* sp. bacteremia because of animal contact and frequent arthropod exposure (*10*–

15,*23*). Cats are the primary reservoir hosts for *B. henselae* and *B. koehlerae,* whereas canids, including dogs, coyotes and foxes, are the primary reservoir hosts for *B. vinsonii* subsp. *berkhoffii (4*,*6*,*29*,*34)*. Although infrequent when compared with cat transmission of *B. henselae* resulting in classical cat-scratch disease, dogs have been implicated in the transmission of *B. vinsonii* subsp. *berkhoffii* and *B. henselae* to humans (*35*,*36*). The predominant symptoms reported among occupationally at-risk patient populations have included severe fatigue, neurologic and neurocognitive abnormalities, arthralgia, and myalgia (*10–13*,*23*). In the study reported here, dog (85%) and cat (68%) contact were reported by most respondents; however, no associations were found between infection with a *Bartonella* sp. and contact with a specific animal. Similarly, exposure to mosquitoes, ticks, fleas, and biting flies were all reported by >50% of the study population. The results of this study support documentation of *Bartonella* spp. bacteremia in patients seen by a rheumatologist in a Lyme disease–endemic area and provides the basis for future studies to ascertain the prevalence of *Bartonella* spp. in patients with rheumatic and neurologic symptoms.

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Dr Maggi is a research assistant professor in the Department of Clinical Sciences at North Carolina State University College of Veterinary Medicine. His research has focused on the development of novel or improved molecular diagnostic and culture methods for detection of *Bartonella* spp. infections in animals and humans.

E.B.B., in conjunction with Sushama Sontakke and North Carolina State University, holds US Patent No. 7,115,385, Media and Methods for Cultivation of Microorganisms, which was issued October 3, 2006. E.B.B. is chief scientific officer for Galaxy Diagnostics, a newly formed company that provides diagnostic testing for the detection of *Bartonella* species infection in animals and human patients. R.G. Maggi has lead research efforts to optimize the BAPGM platform and is the scientific technical advisor for Galaxy Diagnostics. R. Mozayeni was the attending physician for the patients described in this study and has recently joined Galaxy Diagnostics as the chief medical officer. All other authors have no potential conflicts.

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Characterization of Virulent West Nile Virus Kunjin Strain, Australia, 2011

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To determine the cause of an unprecedented outbreak of encephalitis among horses in New South Wales, Australia, in 2011, we performed genomic sequencing of viruses isolated from affected horses and mosquitoes. Results showed that most of the cases were caused by a variant West Nile virus (WNV) strain, $WNV_{NSW2011}$, that is most closely related to WNV Kunjin (WNV_{KUN}), the indigenous WNV strain in Australia. Studies in mouse models for WNV pathogenesis showed that $WNV_{NSW2011}$ is substantially more neuroinvasive than the prototype WNV_{KUN} strain. In $WNV_{NSW2011}$, this apparent increase in virulence over that of the prototype strain correlated with at least 2 known markers of WNV virulence that are not found in WNV_{KUN}. Additional studies are needed to determine the relationship of the $WNV_{NSW2011}$ strain to currently and previously circulating WNV_{KUN} strains and to confirm the cause of the increased virulence of this emerging WNV strain.

In Australia, Murray Valley encephalitis virus (MVEV) and West Nile virus (WNV) Kunjin (KUN) strain are the main etiologic agents of arboviral encephalitis in humans, which usually occurs as isolated sporadic cases or occasional small outbreaks, mainly in northwestern Australia and rarely in southern regions (Figure 1, panel A) (*1*). MVEV is the more virulent pathogen and the only proven cause of fatal arboviral encephalitis in humans in Australia (*2*). WNV_{KUN} infections are infrequent and less severe (*3*). Horses are also susceptible to these viruses and have been involved in WNV outbreaks elsewhere, most notably in the United States in an outbreak that began in 1999. In Australia, infection with WNV_{KUN} has been detected intermittently in horses in the Southeast, but reports of encephalitis caused by this virus are rare (*3*). In New South Wales (NSW), Australia, the seroprevalence of WNV_{KUN} in horses is <5% (P.D. Kirkland and A. Read, unpub. data); infection is confined to inland areas where flooding supports large mosquito populations and water birds are a reservoir and amplifying host. Even in years when WNV_{KUN} has caused disease in humans, disease has rarely been observed or confirmed in horses (*3*,*4*).

In 2011, an outbreak of encephalitis occurred among horses in NSW. To analyze this strain of WNV_{KUN}, we conducted genomic sequencing, antigenic profiling, in vitro growth kinetics, and mouse virulence studies on virus isolates from diseased animals and mosquitoes.

Materials and Methods

Disease Outbreak

In late February 2011, neurologic disease was reported in several horses in northwestern and southwestern NSW. The number of cases and geographic distribution gradually increased. By mid-June 2011, specimens from ≈300 horses were submitted to the virology laboratory at Elizabeth Macarthur Agriculture Institute (Menangle, NSW, Australia). Many more horses probably were affected.

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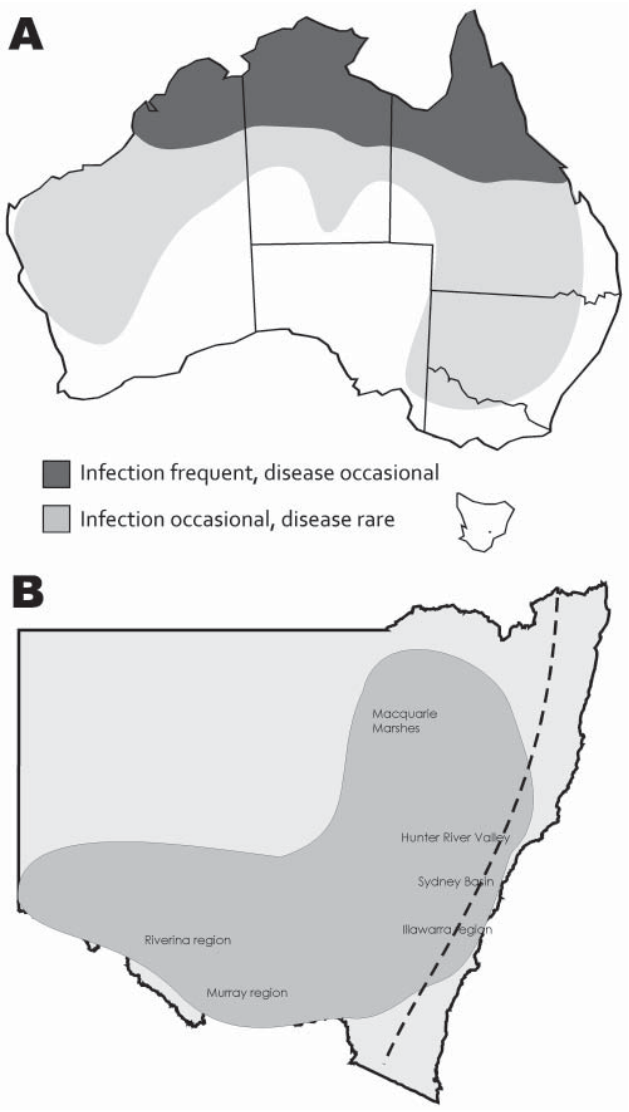

Figure 1. Known distribution of West Nile virus infection and disease caused by Kunjin strain (A) and distribution of encephalitis cases among equids (B), New South Wales, Australia, 2011. Dashed line indicates the Great Dividing Range.

Diseased horses were located throughout most of NSW, west of the Great Dividing Range, but also extending through the Hunter River Valley region, Sydney Basin, and Illawarra coastal region immediately south of Sydney (Figure 1, panel B). Cases also occurred in other southern Australia states. Clinical signs were generally consistent with those described in horses infected with WNV in the United States (*5*). A detailed report of the clinical signs, virology, and pathology of equine cases will be published elsewhere.

Specimen Collection

Whole brains were removed from 12 horses at postmortem examination. Half of each brain was fixed in 10% neutral buffered formalin; the other half was held fresh at 4°C. Upon receipt, we collected small pieces of fresh and formalin-fixed tissue from several locations in the cerebrum and cerebellum and along the brain stem and cervical spinal cord. If virus isolation could not be performed on fresh samples within 24 h after receipt, we held the samples at –80°C until tested. Before testing, we prepared 10% tissue homogenates in RPMI medium (Life Technologies, Carlsbad, CA, USA) containing antimicrobial drugs.

Mosquitoes were collected throughout NSW, as part of the NSW Arbovirus Surveillance and Mosquito Monitoring Program, by using dry ice–baited light traps. The mosquitoes were submitted live to the Medical Entomology Laboratory at Westmead Hospital (Westmead, NSW, Australia) for species identification, arbovirus isolation, and virus identification (*6*).

Cells and Viruses

We propagated Vero 76 cells in Dulbecco modified minimum essential medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS). C6/36 *Aedes albopictus* mosquito cells were maintained in RPMI medium supplemented with 10% FBS, and BHK21 cells were maintained in DMEM containing 5% FBS. All cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). We used prototype WNV_{KUN} MRM61C (*7*) and WNV New York 99 (WNV_{NY99}) 4132 strains (*8*) for comparison with $WNV_{NSW2011}$. Stocks of WNV_{KUN} MRM61C and WNV_{NY99} 4132 that most closely resembled the low-passage level $WNV_{NSW2011}$ were prepared by electroporation of BHK21 cells with WNV_{KUN} or WNV_{NY99} RNA (prepared from corresponding infectious cDNA clones) and passaging them 1× in C6/36 cells. Viral supernatants were harvested 5 days later. Viral titers for each viral stock were determined by plaque assay on Vero 76 cells.

Virus Neutralization Tests

We conducted microneutralization tests (*9*) in Vero cells by using 25–100 infectious units (measured as 50% tissue culture infective doses) of $WNV_{NSW2011}$, WNV_{KUN}, and WNV_{NY99}; we used 2-fold dilutions of serum from an initial dilution of 1:20. Results were scored as 80% reduction in virus growth or 100% inhibition of virus growth. Reduction in virus growth was determined by assessing the extent of cytopathic effect in each well. Inhibition of virus growth was determined by the absence of viral antigen in the cells of each well when tested with a WNV-reactive monoclonal antibody (mAb) in ELISA.

Nucleic Acid Purification

We used the MagMax-96 Viral RNA Isolation Kit (Ambion, Austin, TX, USA) on a magnetic particle handling system (Kingfisher 96; Thermo Electron Corporation, Vantaa, Finland) to extract total nucleic acid from clarified 10% brain homogenate (50 μL) or tissue culture fluid. Purified nucleic acids were eluted in 50 μL of kit elution buffer and used immediately for PCR amplification or stored frozen at ≈–20°C

Real-Time Reverse Transcription PCR

We used a published WNV real-time reverse transcription PCR (rRT-PCR) (*10* [assay 2]) with the following variations: Black Hole Quencher (Biosearch Technologies, Novato, CA, USA) was used instead of TAMRA on the probe, the internal control system was not used, and 5 μL of RNA was used as template. The AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) was used for the rRT-PCR on a 7500HT Fast Real-Time PCR System (Applied Biosystems). We ran rRT-PCR reactions in standard mode, according to conditions recommended by the mastermix manufacturer.

Virus Isolation

Supernatant from the 10% brain homogenate was placed on monolayers of *A. albopictus* C6/36 mosquito cells in cell culture tubes. The cultures, which were maintained in RPMI medium containing antimicrobial drugs at standard concentrations and supplemented with 2% FBS, were incubated at 28°C for 5–7 days. Culture supernatants were then passaged up to 3× on BHK21 cells maintained in Basal Medium Eagle (MP Biomedicals, Sydney, Australia) containing antimicrobial drugs and 2% FBS. We regularly examined cultures by light microscopy for cytopathic effects. We used rRT-PCR to confirm the identity of virus isolates in culture supernatants or to confirm that there was no virus replication in the absence of cytopathology. Viral RNA recovered from culture fluid at the first or second passage in BHK21 cells was sequenced as described below. A virus isolate obtained from the first brain examined was designated $WNV_{NSW2011}$. Virus isolation was conducted on homogenates of mosquitoes by similar methods but with passage onto BHK and PSEK cells after initially being placed on C6/36 cells. We identified virus isolates by using immunoassays with generic and specific mAbs.

$WNV_{NSW2011}$ virus harvested from the first passage in C6/36 cells was used to examine plaque morphology and virulence in mice. The virus was passaged 1× in Vero76 cells for 4 d and 1× in C6/36 cells for 5 d. Virus supernatant was centrifuged at 500 × *g* at 4°C for 5 min before being stored at −80°C

Reactivity with mAbs

Reactivity of the new isolate with a panel of mAbs was compared with that of WNV_{KUN} and WNV_{NY99} by using a fixed-cell ELISA (*11*). The mAbs and their characteristics follow: mAb 10C6, anti–nonstructural protein (NS) 1 (reactive with MVEV); mAb 10A1, anti-envelope glycoprotein (anti-E; specific for WNV_{KUN}); mAb 2B2, anti-E (reactive with WNV); mAb 3.1112G, anti-NS1 (reactive with WNV); mAb 5H1, anti-NS5 (strong reaction with WNV_{KUN} strains, weak for WNV_{NY99}, nonreactive with WNV strains from other lineages); mAb 17D7, anti-E (specific for strains of WNV with glycosylated E); mAb 3.101C, anti-E (specific for strains of WNV with unglycosylated E); and the pan–flavivirus-reactive mAbs (i.e., 4G4, anti-NS1; and 4G2, anti-E) (*12–15*; J. Hobson-Peters et al., unpub. data).

Nucleic Acid Sequencing

For sequencing of the whole genome, we used total nucleic acid purified from virus-infected cell culture supernatant as template in 5 RT-PCRs with primers designed to cover the coding regions of any WNV genome (Table 1). RT and amplification were performed by using the SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA) with primers at 20 μM. RT was performed at 50°C for 30 min, followed by denaturation at 94°C for 2 min. PCR amplification involved 40 cycles (95°C for 30 s, 55°C for 1 min, 68°C for 4.5 min) followed

Table 1. Primers used for viral RNA amplification and genomic sequencing of WNV isolates from horses and mosquitoes, Australia, 2011*

RT-PCR region	Forward primer, 5′ → 3′ (relative genome position†)	Reverse primer, 5′ → 3′ (relative genome position†)
Amplification and sequencing of whole genome		
5′ NTR capsid	TAGTTCGCCTGTGTGAGCTG (5′ NTR-2)	TTGAAAATTCCACAGGAATGG (capsid-1772)
Capsid-NS2A	GTGATAGCATTGGGCTCWCA (capsid-1720)	ATCTTGAAGGYYGCCATGAG (NS2A-1760)
NS2A-NS3	CACTGATGTGTTACGCTATGTCA (NS2A-3678)	CAAAGTCCCAATCATCGTTCT (NS3-5807)
NS3-NS5	CGGTTTGGTTTGTGCCTAGT (NS3-5687)	CCAACTTCACGCAGGATGTA (NS5-9235)
NS5–3′ NTR	GACCACTGGCTTGGAAGAAA (NS5-9169)	CTGGTTGTGCAGAGCAGAAG (3′ NTR-10955)
Partial sequencing of key regions of genome		
NS3	GTGCTGGTAAAACAAGGAGG (NS3-5201)	TGTATCCTCTAGCCGCGATG (NS3-5493)
NS5	TCGGCCCAGATGATGTG (NS5-9575)	CGGCATGGAACCACCAGTGTTC (NS5-9860)

*Primers were designed from available sequences in GenBank to cover the coding regions of any WNV genome. WNV, West Nile virus; RT, reverse transcription; NTR, nontranslated region; NS, nonstructural protein.
†WNV_{NY99} GenBank accession no. NC_009942.1.

by a final extension at 68°C for 10 min. Reaction products were visualized after electrophoresis on a ethidium bromide–stained 1% agarose gel. Reaction products were purified directly (MinElute PCR Purification Kit; QIAGEN, Valencia, CA, USA) or excised from the gel and cleaned (Gel MinElute PCR Purification Kit; QIAGEN). Purified nucleic acid was sequenced at the Australian Genome Research Facility (Sydney) by using the primers used to generate the PCR product. Each RT-PCR was run 3× and sequenced in both forward and reverse orientation. Sequence data were assembled by using Sequencher software (Gene Codes Corp., Ann Arbor, MI, USA). For subsequent isolates from horses and mosquitos, we used primers designed from the sequence of the $WNV_{NSW2011}$ genome (Table 1) to amplify and sequence the NS3 and NS5 regions, in which changes had been identified. The same RT-PCR and sequencing methods were used, except that the annealing temperature was 50°C and extension time was 1 min. The nucleic acid sequences were translated and then aligned with $WNV_{NSW2011}$ and WNV_{KUN} (GenBank accession nos. JN887352 and D00246.1, respectively) by using ClustalW (www.clustal.org).

Bioinformatics Analysis

Complete coding regions of selected WNV isolates, representing all lineages and clades and including all complete KUN sequences, were aligned with the $WNV_{NSW2011}$ sequence as described (*16*). This alignment was transferred to BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html) for manual editing before construction of phylogenetic trees. Maximum-likelihood trees were constructed by using PhyML (*17*). Trees were rooted by using the Japanese encephalitis virus Nakayama sequence (GenBank accession no. EF571853), which was removed from the final tree for clarity.

Endoglycosidase Digestion

To examine glycosylation of the E protein, viral proteins from cultures of infected C6/36 cells were digested as described (*18*). Proteins were separated and analyzed by Western blot. Samples were loaded with reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis buffer (NuPAGE LDS Sample Buffer; Invitrogen) on a 4%–12% NuPAGE Gel (Invitrogen). Electrophoresed proteins were electroblotted onto nitrocellulose paper (Hybond C; GE Healthcare, Little Chalfont, UK) and immunostained with anti-E mAb (*11*).

Plaque Morphology

We allowed the virus to adsorb to monolayers of Vero 76 cells in 6-well plates for 2 h at 37°C. The cells were overlaid with DMEM containing 0.75% low melting point agarose and 2% FBS. Four days after infection, the cells were fixed with 4% formaldehyde solution and stained with 0.2% crystal violet.

Virulence in Mice

Groups of 10 weanling (18–19 days old) or young adult (4 weeks old) Swiss outbred CD1 mice were injected intraperitoneally with 10-fold dilutions of virus. The mice were monitored for 21 days after injection and euthanized when signs of encephalitis were evident. All animal procedures had received prior approval from The University of Queensland Animal Ethics Committee.

Results

Virus Isolation and Initial Characterization

Viral RNA was detected by WNV-specific rRT-PCR in fresh brain tissue from 6 of 12 horses showing signs of encephalitis. Viruses were isolated from 4 of these samples; each showed distinct cytopathology in BHK21 and Vero cells. rRT-PCR of the culture fluids and immunoperoxidase staining of the cells with pan–flavivirus-reactive and WNV-specific mAbs confirmed the isolation of a West Nile–like virus. The first isolate was named NSW2011 and designated $WNV_{NSW2011}$. Eight isolates of $WNV_{NSW2011}$ were isolated from *Culex annulirostris* Skuse mosquitoes during the 2011 vector season. Of the 8 isolates, 5 were from mosquitoes collected in the Riverina region of southwestern NSW (Hanwood, 4 isolates; Barren Box, 1 isolate); 2 were from the Murray region in southern NSW (Mathoura, 1 isolate; Moama, 1 isolate); and 1 was collected at Lower Portland in the outer western Sydney region of NSW. No other isolates of WNV were obtained.

Antigenic Analysis of $WNV_{NSW2011}$

To antigenically type $WNV_{NSW2011}$ in a fixed-cell ELISA (*11*), we used a panel of mAbs previously shown to differentiate between strains of WNV_{KUN} and other WNVs (*11*,*13*–*15*,*19*,*20*). The recognition patterns showed that the $WNV_{NSW2011}$ isolate most closely resembled Australian WNV_{KUN} strains; the WNV_{KUN}-specific mAb 10A1 reacted strongly with prototype WNV_{KUN} and $WNV_{NSW2011}$ but not with WNV_{NY99} (Table 2). However, the anti-NS5 mAb 5H1, which is also specific for WNV_{KUN} isolates from Australia (*15*), failed to react with $WNV_{NSW2011}$ and WNV_{NY99}, but it bound strongly to WNV_{KUN}. The reaction patterns of mAbs 17D7 and 3.101C, which react specifically with glycosylated and unglycosylated WNV E antigens, respectively (*11*,*14*; J. Hobson-Peters et al., unpub. data), indicated that, unlike the E protein of WNV_{KUN}, the E protein of $WNV_{NSW2011}$ is glycosylated.

To assess the level of antigenic crossreactivity between $WNV_{NSW2011}$, WNV_{KUN}, and WNV_{NY99}, we assessed neutralization titers for homologous and heterologous

Table 2. Binding pattern of monoclonal antibodies to the viral antigens of 3 WNV strains in fixed-cell ELISA, Australia, 2011*

	Monoclonal antibody, by specificity								
	Pan-flavivirus		WNV group		WNV_{KUN}		Unglycosylated WNV E protein	Glycosylated WNV E protein	MVEV
Virus	4G4, anti-NS1	4G2, anti-E	2B2, anti-E	3.91D, anti-E	10A1, anti-E	5H1, anti-NS5	3.101C	17D7	10C6, anti-NS1
$WNV_{NSW2011}$	+	+	+	+	+	–	–	+	–
WNV_{KUN}†	+	+	+	+	+	+	+	–	–
WNV_{NY99}‡	+	+	+	+	–	–	–	+	–

*WNV, West Nile virus; KUN, Kunjin; E, envelope; MVEV, Murray Valley encephalitis virus; NS, nonstructural protein; NS, nonstructural protein; NSW, New South Wales; + positive; –, negative; NY, New York.
†Prototype WNV_{KUN} strain MRM-61C.
‡North American WNV strain.

viruses in immune serum samples from the following sources: horses infected during the 2011 outbreak, horses infected with WNV_{KUN} in the Northern Territory of Australia several years earlier, and horses infected with WNV in the United States. Convalescent-phase serum samples from $WNV_{NSW2011}$-immune horses had neutralizing titers similar to those of the homologous virus ($WNV_{NSW2011}$) and of WNV_{KUN} and WNV_{NY99} (Table 3). Serum samples from WNV_{KUN}-immune horses from Northern Territory and from WNV-immune animals from the United States showed a similar pattern of cross-neutralization. However, serum samples from horses from Northern Territory and the United States showed slightly less neutralizing efficiency of $WNV_{NSW2011}$; this was likely due to a higher dose (≈4-fold) of virus in the assay (Table 3). Overall, these results are consistent with those in our previous reports showing a high level of cross-neutralization between WNV_{KUN} and WNV_{NY99} strains (*21*,*22*).

Nucleotide and Amino Acid Sequence Analysis of $WNV_{NSW2011}$

A comparison of the nucleotide sequence of the complete coding region of the first isolate of $WNV_{NSW2011}$ with sequences available in GenBank confirmed that $WNV_{NSW2011}$ was genetically most closely related to Australian WNV_{KUN} isolates (Figure 2). A detailed

Table 3. Neutralizing titers of serum samples from WNV–infected horses against 3 WNV strains, Australia, 2011*

	% Inhibition of CPE/growth†					
	$WNV_{NSW2011}$, 100 infectious units		WNV_{KUN}, 26 infectious units		WNV_{NY99}, 32 infectious units	
Horse serum samples	80‡	100§	80	100	80	100
Control¶						
1	<20	<20	<20	<20	<20	<20
2	<20	<20	<20	<20	<20	<20
3	<20	<20	<20	<20	<20	<20
4	<20	<20	<20	<20	<20	<20
5	<20	<20	<20	<20	<20	<20
NSW#						
04	640	320	1,280	1,280	640	320
06	320	160	640	640	1,280	160
08	320	320	1,280	1,280	640	640
28	320	320	640	640	320	320
36	320	160	640	640	320	640
NT**						
111473	**80**	**20**	**640**	**320**	**160**	**160**
104714	**320**	**80**	**640**	**640**	**640**	**640**
110910	80	40	160	160	160	160
98727	40	40	160	160	80	80
WNV††						
1	**160**	**40**	**640**	**40**	**320**	**320**
2	**320**	**160**	**1,280**	**640**	**160**	**160**
3	**80**	**160**	**320**	**320**	**640**	**640**
4	**40**	**20**	**160**	**160**	**320**	**320**
5	**320**	**80**	**1,280**	**320**	**640**	**640**
mAb 3.91D‡‡	>2,560	>2,560	>2,560	>2,560	>2,560	>2,560

*Determined, as described (*14*), by microneutralization assay in Vero cells. WNV, West Nile virus; CPE, cytopathic effect; NSW, New South Wales; KUN, Kunjin; NY, New York; NT, Northern Territory; mAb, monoclonal antibody.
†**Boldface** indicates serum samples with >4-fold difference in titer between virus strains.
‡Determined by using a microscope to assess the level of CPE in each well compared with that in control wells.
§Determined by the absence of viral antigen in the cell monolayer of each well when tested with a WNV-reactive mAb in ELISA.
¶Samples from uninfected horses.
#Samples from horses infected with WNV during the 2011 outbreak in New South Wales, Australia.
**Samples from horses infected with WNV_{KUN} in Northern Territory, Australia.
††Samples from horses infected with WNV in the United States.
‡‡This mAb has potent WNV-neutralizing activity (*11*).

comparison of deduced amino acid sequences for the entire coding region of $WNV_{NSW2011}$, WNV_{KUN}, and WNV_{NY99} further confirmed a closer relationship between $WNV_{NSW2011}$ and WNV_{KUN} than between $WNV_{NSW2011}$ and WNV_{NY99}. There was a 42-aa difference (18 nonconserved changes) between $WNV_{NSW2011}$ and WNV_{KUN} and an 89-aa difference (38 nonconserved changes) between $WNV_{NSW2011}$ and WNV_{NY99} (online Appendix Table, wwwnc.cdc.gov/EID/article/18/5/11-1720-TA1.htm). At least 2 of the known WNV virulence markers present in WNV_{NY99} but not in WNV_{KUN} were found in $WNV_{NSW2011}$ (online Appendix Table). The glycosylation tripeptide (N-Y-S) at residues $E_{154–156}$, which allows N-linked glycosylation at a conserved site on the E protein, domain I, and is associated with virulence in most WNV strains (*24*), was present in $WNV_{NSW2011}$; its presence is consistent with the mAb recognition profile. A phenylalanine residue at aa 653 in NS5, which also is associated with enhanced virulence of WNV strains (*24*), was present in $WNV_{NSW2011}$. Because the WNV_{KUN} strain–specific mAb 5H1 did not react with $WNV_{NSW2011}$, we also examined the predicted amino acid sequence of the NS5 protein that corresponded to the linear epitope previously mapped for 5H1 to residues 41–53 in the methyltransferase domain (*15*). $WNV_{NSW2011}$ and WNV_{NY99} contain a substitution (I—V) at residue 49 that is not contained in WNV_{KUN}, confirming the critical role of this residue for 5H1 binding. Together these data suggest that $WNV_{NSW2011}$ represents a virulent WNV strain that has emerged in Australia. However, the amino acid substitution in NS3, which is believed to be associated with increased virulence of WNV_{NY99} in birds in North America (*25*), was not present in isolate $WNV_{NSW2011}$. Sequencing of the key regions of the other WNV isolates obtained during this outbreak (3 from horses and 8 from mosquitoes) showed that each was indistinguishable from $WNV_{NSW2011}$.

In vitro Growth Properties and E Protein Glycosylation Status of $WNV_{NSW2011}$

The average plaque size of $WNV_{NSW2011}$ (Figure 3, panel A) was 4.2 mm ± 0.5 mm, much closer to WNV_{NY99} (4.7 mm ± 0.8 mm) than to the prototype WNV_{KUN} (2.8 mm ± 0.4 mm). To confirm the presence of an N-linked glycan on the E protein of $WNV_{NSW2011}$, we used Western blot to analyze endoglycosidase-digested viral protein. Analysis showed that the E protein of $WNV_{NSW2011}$ and WNV_{NY99} migrated slightly faster than the undigested control protein. This result is consistent with N-linked glycosylation (Figure 3, panel B). However, consistent with the lack of a potential glycosylation site on the E protein of most WNV_{KUN} isolates, we found no evidence of N-linked glycosylation for WNV_{KUN} (*11,20,26*).

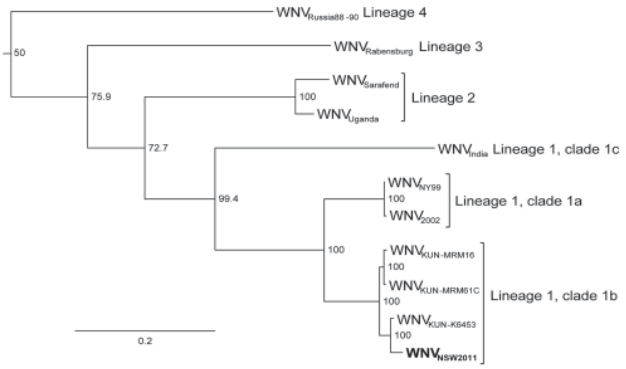

Figure 2. Maximum-likelihood tree based on nucleotide sequences of the complete open reading frame of genomes of West Nile virus (WNV) NSW2011 (**boldface**) and representative strains of WNV from the different lineages and clades. All published complete Kunjin (KUN) virus sequences are included. Bootstrap values are shown on the nodes and are expressed as a percentage of 1,000 replicates. Sequences downloaded from GenBank were $WNV_{Russia88–90}$, AY277251; $WNV_{Rabensburg}$, AY765264; $WNV_{Sarafend}$, AY688948; WNV_{Uganda}, AY532665; WNV_{India}, DQ256376; WNV_{NY99}, AF196835; WNV_{2002}, GU827998; $WNV_{KUNV-MRM16}$, GQ851602; $WNV_{KUNV-MRM61C}$, AY274504; and $WNV_{KUNV-K6453}$, GQ851603. NY, New York; NSW, New South Wales. Horizontal branch lengths indicate genetic distance proportional to the scale bar.

Neuroinvasive Properties of $WNV_{NSW2011}$ in mice

Injection of 18- to 19-day-old (weanling) mice with 10-fold dilutions of virus showed that substantially lower doses of $WNV_{NSW2011}$ (50% lethal dose [LD_{50}] 0.5 PFU), compared with WNV_{KUN} (LD_{50} 13.4 PFU), induced neurologic signs (Table 4); and the time to disease onset was substantially shorter (Table 4; Figure 3, panel C). In contrast, the LD_{50} for WNV_{NY99} (0.1 PFU) was lower than that for $WNV_{NSW2011}$, and neurologic signs developed more rapidly (Table 4; Figure 3, panel C). Only WNV_{NY99} and $WNV_{NSW2011}$ caused a substantial number of deaths among 4-week-old (young adult) mice. Compared with $WNV_{NSW2011}$, WNV_{NY99} exhibited a lower LD_{50} (240 PFU vs. 0.7 PFU, respectively) and a shorter time to death (10.7 days vs. 6.6 days, respectively, at 1,000 PFU) (Table 5; Figure 3, panel C).

Discussion

It is estimated that at least 1,000 horses were affected during an unprecedented outbreak of encephalitis in southeastern Australia during 2011. The case-fatality rate was 10%–15%, and diseased animals had clinical signs consistent with those observed during a WNV outbreak in the United States. Not only was the Australian outbreak unique and unprecedented in size and disease severity, but its epidemiologic features also differed from those

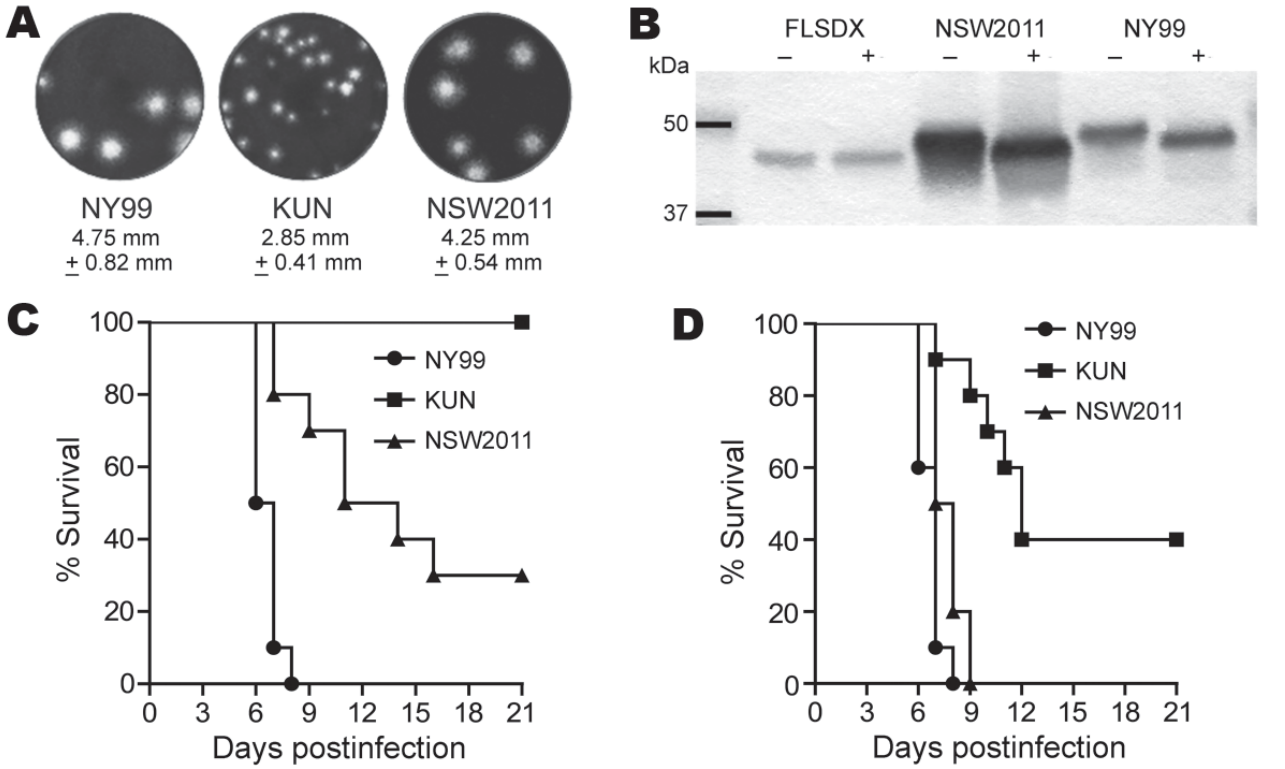

Figure 3. Studies of West Nile virus (WNV) properties in cell cultures and mice. A) Plaque morphology of WNV_{NY99}, prototype WNV_{KUN}, and $WNV_{NSW2011}$ in Vero cells. Cells in 6-well plates were infected with specified virus and overlaid with 0.75% low melting point agarose in Dulbecco modified minimum essential medium (Life Technologies, Carlsbad, CA, USA) containing 2% fetal bovine serum. Four days after infection, the cells were fixed with 4% formaldehyde and stained with 0.2% crystal violet. B) Assessment of envelope (E) protein glycosylation of $WNV_{NSW2011}$, WNV_{KUN} and WNV_{NY99} by endoglycosidase digestion (PNGase F; Roche Diagnostics, Basel, Switzerland). Viral proteins in culture supernatant were digested by PNGase F (+) or undigested (–) and then resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The migration rate of the E protein in each sample was determined by Western blot with E glycoprotein–specific monoclonal antibodies. C) Young adult (4 weeks old) or D) weanling (18–19 days old) Swiss outbred mice survival after intraperitoneal injection with 1,000 PFU (adult) or 10 PFU (weanling) of WNV_{NY99}, WNV_{KUN}, or $WNV_{NSW2011}$. The mice were monitored for 21 days after injection for signs of encephalitis and then euthanized. The differences in virulence in weanling and adult mice between different pairs of viruses were all highly significant, as calculated by log rank Mantel-Cox algorithm with exact p values: for adult mice, WNV_{NY99} vs. WNV_{KUN} $p<0.0001$, WNV_{NY99} vs. $WNV_{NSW2011}$ $p = 0.0001$, and WNV_{KUN} vs. $WNV_{NSW2011}$ $p = 0.0012$; and for weanling mice, WNV_{NY99} vs. WNV_{KUN} $p<0.0001$, WNV_{NY99} vs. $WNV_{NSW2011}$ $p =0.0004$, and WNV_{KUN} vs. $WNV_{NSW2011}$ $p = 0.0006$. NY, New York; KUN, Kunjin; NSW, New South Wales.

observed previously in Australia. In particular, WNV_{KUN} now has been detected on the eastern seaboard of NSW, close to major urban areas, including the largest 3 cities (Sydney, Newcastle, and Wollongong). This detection occurred despite relatively small mosquito populations in many of these areas, suggesting that the virus is more virulent and probably transmitted more efficiently than other strains between mosquito vectors and mammalian hosts. Characterization of virus isolated from the brain of an animal that died showed a variant strain of WNV most closely related to WNV_{KUN}. Typing of $WNV_{NSW2011}$ by reactivity with a panel of mAbs indicated the virus was antigenically more similar to the native Australian WNV_{KUN} strains than to exotic WNV strains. However, for $WNV_{NSW2011}$, the reaction profile of mAbs 17D7 and 3.101C differed from that of the prototype WNV_{KUN}. Similar to WNV_{NY99} and other virulent strains of WNV, $WNV_{NSW2011}$ E protein was glycosylated at residue 154. This finding was further confirmed by gene sequencing and endo-glycosidase F digestion analysis. Glycosylation of WNV E protein at this site is thought to enhance virus dissemination in the infected host by increasing the efficiency of assembly and release of virus particles from infected cells (*27*). Previous studies showed that a phenylalanine residue at aa 653 in NS5, observed in $WNV_{NSW2011}$ and WNV_{NY99} but not in WNV_{KUN}, is associated with increased resistance to interferon, which may also enhance virulence in the host (*24*). Virulence studies in weanling and young adult mice clearly demonstrate that $WNV_{NSW2011}$ is substantially more neuroinvasive than the

Table 4. Virulence of 3 WNVs in 18- to19-day-old mice after intraperitoneal injection, Australia, 2011*

Virus and dose, PFU	No. mice/no. died	Average survival time, d	LD_{50}
WNV_{NY99}			
100	10/10	6.1	
10	10/10	6.7	
1	10/10	6.9	0.1 PFU
0.1	5/10	7.8	
WNV_{KUN}			
1,000	9/10	8.4	
100	4/10	8	13.4 PFU
10	6/10	10.2	
1	3/10	12	
$WNV_{NSW2011}$			
1,000	10/10	7.1	
100	10/10	7.4	
10	10/10	7.7	0.5 PFU
1	7/10	8.3	
0.1	1/10	10	

*WNV, West Nile virus; LD_{50}, dose at which 50% of the mice died; NY, New York; KUN, Kunjin; NSW, New South Wales.

prototype strain of WNV_{KUN}, which might explain the severity of the 2011 outbreak. However, the association between the identified and perhaps other amino acid changes and increased virulence of $WNV_{NSW2011}$ in horses and mice will require further confirmation by using site-directed mutagenesis of an infectious cDNA clone.

Another unusual aspect of the 2011 outbreak was the absence of encephalitis caused by WNV_{KUN} in humans. In contrast, several confirmed cases of Murray Valley encephalitis in humans were recorded in southeastern Australia during this time. This absence of disease in humans suggests that ecologic and/or epidemiologic features of the virus transmission cycle, such as small mosquito populations and timely alerts, probably resulted in less exposure of the human population to $WNV_{NSW2011}$.

The US outbreak of WNV was associated with high mortality among several bird species, particularly American crows (*Corvus brachyrhynchos*). In contrast, increased mortality among birds of any species was not reported during the 2011 outbreak in southeastern Australia. The lack of disease in birds in Australia supports the hypothesis that the amino acid substitution observed in WNV_{NY99} (Ala—Pro at aa 249 in NS3) (*25*) is associated with increased virulence in birds because this change was not present in the $WNV_{NSW2011}$ isolate. However, this observation should be viewed with some caution because of the species differences between birds in Australia and the United States and because disease was limited or absent when a species of Australian crow (Little Raven [*Corvus mellori*]) was experimentally infected with WNV_{NY99} (*28*).

Table 5. Virulence of 3 WNVs in 4-week-old mice after intraperitoneal injection, Australia, 2011*

Virus and dose, PFU	No. mice/no. died	Average survival time, d	LD_{50}
WNV_{NY99}			
100	10/10	8.3	
10	10/10	8.2	0.7 PFU
1	5/10	8.6	
0.1	2/10	10	
WNV_{KUN}			
1,000	0/10	21	>1,000 PFU
$WNV_{NSW2011}$			
1,000	7/10	10.7	
100	2/10	11	240 PFU
10	3/10	10.3	

*WNV, West Nile virus; LD_{50}, dose at which 50% of the mice died; NY, New York; KUN, Kunjin; NSW, New South Wales.

Taken together, our results show that the $WNV_{NSW2011}$ isolate is closely related to Australian WNV_{KUN} strains. However, in contrast to the prototype WNV_{KUN} strain (MRM-61C), the new virus has several amino acid substitutions that are likely to be the reason for enhanced virulence in horses. More extensive epidemiologic studies in the field and experimental studies in the laboratory are required to determine the relation of $WNV_{NSW2011}$ to other currently and previously circulating WNV_{KUN} strains and to confirm which viral proteins and amino acid residues are associated with increased virulence of $WNV_{NSW2011}$ in horses.

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Dr Frost is a scientist engaged in research to characterize new and emerging viral pathogens among animals. She also has a special interest in the application of molecular methods for the diagnosis of viral diseases.

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No Association between 2008–09 Influenza Vaccine and Influenza A(H1N1)pdm09 Virus Infection, Manitoba, Canada, 2009

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We conducted a population-based study in Manitoba, Canada, to investigate whether use of inactivated trivalent influenza vaccine (TIV) during the 2008–09 influenza season was associated with subsequent infection with influenza A(H1N1)pdm09 virus during the first wave of the 2009 pandemic. Data were obtained from a provincewide population-based immunization registry and laboratory-based influenza surveillance system. The test-negative case–control study included 831 case-patients with confirmed influenza A(H1N1)pdm09 virus infection and 2,479 controls, participants with test results negative for influenza A and B viruses. For the association of TIV receipt with influenza A(H1N1)pdm09 virus infection, the fully adjusted odds ratio was 1.0 (95% CI 0.7–1.4). Among case-patients, receipt of 2008–09 TIV was associated with a statistically nonsignificant 49% reduction in risk for hospitalization. In agreement with study findings outside Canada, our study in Manitoba indicates that the 2008–09 TIV neither increased nor decreased the risk for infection with influenza A(H1N1)pdm09 virus.

The nature of the relationship between receipt of the 2008–09 seasonal inactivated trivalent influenza vaccine (TIV) and the risk for infection with the pandemic (H1N1) 2009 virus strain, hereafter referred to as A(H1N1)pdm09, remains unclear. A case–control study in Canada that used data from a network of sentinel physicians monitoring influenza vaccine effectiveness in the provinces of Alberta, British Colombia, Ontario, and Quebec found an increased risk for influenza A(H1N1)pdm09 virus infection among persons who received the 2008–09 TIV (odds ratio [OR] 1.7, 95% CI 1.0–2.7); an increased risk for severe illness was not detected (*1*). In addition, 3 other studies conducted by the same team found a 1.4- to 2.5-fold increased risk for infection (laboratory confirmed) with influenza A(H1N1)pdm09 virus among persons who received the 2008–09 TIV (*1*). The 3 studies were 1) a test-negative case–control study in Ontario; 2) a household transmission cohort study in Quebec; and 3) a conventional case–control study using population controls in Quebec.

The results of these studies in Canada were not confirmed by studies conducted elsewhere. In fact, several studies using different designs found that TIV partially prevented or had no effect on infections with the pandemic strain (*2–16*). It has been suggested that the finding in Canada of an increased risk for influenza A(H1N1)pdm09 virus infection among persons who received the 2008–09 TIV might be unique to Canada; the increased risk might be related to the use of the domestically manufactured vaccine (*1*) or to the timing of the pandemic in relation to the most recent influenza season and the types of circulating influenza strains during that season (*17*,*18*). At the time of the pandemic, the Canadian province of Manitoba was not part of the Canadian vaccine effectiveness monitoring network. However, the availability of a provincewide,

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population-based immunization registry and laboratory-based influenza surveillance system provided a unique opportunity to investigate these issues in Manitoba.

In the first wave of the pandemic (May–August 2009), Manitoba was more severely affected than any other Canadian province, accounting for 50% of hospital intensive care unit admissions attributable to the virus in Canada (*19*,*20*). TIVs used in Manitoba during the 2008–09 influenza season were identical to those used elsewhere in Canada; they included 15 μg hemagglutinin each of A/Brisbane/59/2007 (H1N1)–like virus, A/Brisbane/10/2007 (H3N2)–like virus, and B/Florida/4/2006-like virus. These were the 3 strains recommended that year by the World Health Organization for influenza vaccines in the Northern and Southern Hemispheres (*21*). In Manitoba, as in other provinces, ≈75% of the administered seasonal influenza vaccine doses were manufactured domestically (Fluviral; GlaxoSmithKline, Mississauga, Ontario, Canada); imported vaccines, predominantly Vaxigrip (Sanofi Pasteur Ltd, Toronto, Ontario, Canada), comprised the remaining 25%. The live attenuated influenza vaccine was not available in Canada during the 2008–09 season.

To investigate whether use of TIV in Manitoba was associated with influenza A(H1N1)pdm09 virus infection during the first wave of the pandemic, we conducted a population-based case–control study using data from Cadham Provincial Laboratory (CPL) and the Manitoba Immunization Monitoring System. The test-negative case–control design used in this study is similar to the design of the Ontario study (*1*).

Methods

Data Sources

This study was conducted using de-identified records obtained by linking the CPL database, the Manitoba Immunization Monitoring System, and other Manitoba Health (MH) administrative databases after securing the approval of the Research Ethics Board of the University of Manitoba and the Health Information Privacy Committee of MH. MH provides publicly funded health insurance coverage to 99% of the 1.2 million residents of Manitoba. The coverage includes laboratory testing and hospital and outpatient physician services, including immunization and laboratory services. Eligibility for coverage is not based on age or income. For administrative purposes, MH maintains several centralized electronic databases that can be linked by using a unique health services number.

Study Population

All Manitoba residents ≥6 months of age who had respiratory specimens submitted to CPL, the province's only public health laboratory, for influenza testing during April 27–August 21, 2009, were included in this analysis. During the pandemic, guidelines for testing patients seeking care for influenza-like illness were issued by MH; anecdotal evidence indicated that, to a great extent, physicians followed the guidelines. Patients were tested in hospital and ambulatory care settings; the specimens obtained were predominantly nasopharyngeal and nasal swab samples. For the duration of the first pandemic wave, all influenza testing in Manitoba was completed at CPL by using a real-time duplex reverse transcription PCR (RT-PCR) developed by the National Microbiology Laboratory (*22*). We obtained information about influenza testing from CPL's electronic database.

Study Design

Consistent with the design of the Ontario test-negative case–control study (*1*), we identified 3 nonoverlapping study groups: 1) the hospitalized cases group comprised persons in the study population (as defined above) who had real-time duplex RT-PCR test results positive for influenza A(H1N1)pdm09 virus and who had been admitted to a hospital in Manitoba around the time of testing (within ± 1 week of collection of their first influenza A(H1N1)pdm09 virus–positive specimen); 2) the community cases group comprised A(H1N1)pdm09-positive persons who had not been hospitalized during April–August 31, 2009; 3) the community controls group comprised persons who were not hospitalized during this period and who tested negative for influenza A and B. Identification of these 3 groups enabled us to assess whether use of TIV was associated with the detection of influenza A(H1N1)pdm09 virus infection (by contrasting the odds of TIV use among community case-patients and community controls). Identification of the 3 groups also enabled us to assess whether use of TIV was associated with increased risk for hospitalization as an indication of the severity of disease (by contrasting the odds of TIV use among hospitalized and community case-patients). Information about hospitalization status was obtained from the Hospital Separation database.

Determination of Vaccination Status

For all study participants, information about receipt of TIV and polyvalent pneumococcal polysaccharide (PPV23) vaccine during or before the 2009–10 influenza season was obtained from the Manitoba Immunization Monitoring System, the population-based provincewide registry that has recorded virtually all vaccinations administered to Manitobans since 1988. In addition to details about patients, the database stores information about the date of vaccination and the type and dose, but not the brand name, of the vaccine administered. The recorded vaccination information is considered highly complete and accurate (*23*).

Information about Potential Confounders

Study participants were assigned to a neighborhood of residence based on their postal code as recorded in the MH Population Registry. Information about socioeconomic status was obtained by using the postal code of residence and a previously validated area-based Socioeconomic Factor Index (SEFI) (*24*).

Information about coexisting diseases and propensity to seek health care (measured as the number of hospital and family physician visits in the previous 12 months) was obtained from the Hospital Separation and Physician Claims databases. Since 1971, these databases have recorded information about most hospital admissions and outpatient physician visits, respectively. Previously validated algorithms were used to identify various chronic diseases and other indications for vaccination (*25*) (Table 1). Immunosuppression was defined as having a diagnosis of cancer, AIDS, or another immunodeficiency disorder or as receiving prescriptions for immunosuppressive drugs. Information about the use of immunosuppressant and antimicrobial drugs and neuraminidase inhibitors was obtained from the Drug Program Information Network, the comprehensive database of all out-of-hospital prescriptions dispensed in Manitoba. Pregnancy status was determined from the databases mentioned above by using disease and tariff codes for different conditions and procedures indicative of ongoing pregnancy or the completion of pregnancy (*26*) (Table 1).

Statistical Analysis

We used unconditional logistic regression models (fitted to community case-patients and community controls) to estimate odds ratios (ORs) for the association between the receipt of the 2008–09 TIV and subsequent infection with laboratory-confirmed influenza A(H1N1)pdm09 virus while adjusting for confounding. Results are presented for unadjusted models (model A) and for models that were adjusted a priori for age, sex, place of residence, SEFI, and week of specimen collection (to account for changes in infection incidence and laboratory testing practices) (model B). Other potential confounders (Table 1) were included in the fully adjusted models if their inclusion resulted in a >2% change in crude ORs. Using this criterion, we also adjusted the final models (model C) for pregnancy, antiviral drug use, presence of a chronic or immunocompromising medical condition, and number of hospital admissions and family physician visits in the previous 12 months. Model C also included mutual adjustment for the 2007–08 TIV and the PPV23.

These analyses were repeated after stratification by potential confounders and effect modifiers, such as age group, place of residence, epidemic phase (before and after the peak), and presence of chronic conditions. We also assessed for possible effect modification between the 2008–09 TIV and the 2007–08 TIV and the PPV23. The statistical significance of adding the interaction terms was assessed by using a likelihood ratio test. Similar analyses, contrasting the odds of TIV use among hospitalized case-patients with those among community case-patients, were performed to assess whether use of the 2008–09 TIV was associated with increased risk for hospitalization.

Results

During the study period, 4,275 persons were tested for influenza. Of them, 879 (20.6%) were positive for influenza A(H1N1)pdm09 virus, 3,391 (79.3%) were negative for all influenza viruses, and 5 who were positive for influenza A but negative for the pandemic virus were excluded from study. We also excluded 35 persons (8 case-patients, 27 controls) who did not usually reside in Manitoba and 185 infants (26 case-patients, 159 controls) who were <6 months of age. A total of 726 hospitalized test-negative controls and 14 case-patients who were hospitalized during the study period but not around the time of testing were also excluded. Thus, there was a total of 3,310 study participants: 205 hospitalized case-patients, 626 community case-patients, and 2,479 community test-negative controls.

Consistent with previous reports from Manitoba (*19*,*27*), we found that the influenza A(H1N1)pdm09 virus–positive case-patients during the first pandemic wave were younger and more socioeconomically disadvantaged than controls (Table 2). Probably because they were younger, community case-patients had fewer prior hospitalizations and physician visits and were less likely than controls to have had a diagnosed chronic or immunocompromising medical condition. Consistent with the literature (*20*,*27*), we also found that younger children, pregnant women, residents of northern Manitoba, socioeconomically disadvantaged persons, and persons with chronic diseases were more likely to be hospitalized for infection with the pandemic virus.

About 17% of the community case-patients and 23% of the community controls received TIV during the 2008–09 influenza season (Table 3). The crude OR for the association of TIV receipt with subsequent infection with influenza A(H1N1)pdm09 virus was 0.7 (95% CI 0.6–0.9), corresponding to a vaccine effectiveness estimate of 30%. Adjusting for age, sex, region of residence, SEFI, and week of specimen collection (model B) resulted in an OR of 1.1 (95% CI 0.8–1.4). Additional adjustment for all other measured confounders (model C) did not appreciably change the OR estimates (OR 1.0, 95% CI 0.7–1.4).

In analyses limited by small numbers, study participants who received the seasonal 2007–08 and the 2008–09 TIV had a 40% increased risk for influenza

A(H1N1)pdm09 virus infection compared with those who received neither vaccine. In general, there was a trend of increasing risk for influenza A(H1N1)pdm09 virus detection with the receipt of more TIVs over the preceding 5 years (Table 3). However, annual receipt of TIV over the preceding 5 years was inversely associated with the risk for pandemic virus detection. In addition, having ever received PPV23 was not associated with increased risk for influenza A(H1N1)pdm09 detection (OR 0.9, 95% CI 0.6–1.5).

There was no evidence that the association between receipt of the 2008–09 TIV and influenza A(H1N1)pdm09

Table 1. Definitions of variables used in analyses to determine possible association between receipt of 2008–09 TIV and subsequent infection with influenza A(H1N1)pdm09 virus, Manitoba, Canada, 2009*†

Variable	Definition
Drugs‡	
For HIV	Protease inhibitors (J05AE*), nucleoside and nucleotide reverse transcriptase inhibitors (J05AF*), nonnucleoside reverse transcriptase inhibitors (J05AG*), antivirals for treatment of HIV infections, combinations (J05AR*)
For influenza	Neuraminidase inhibitors (J05AH*) or cyclic amines (J05AC*)
For diabetes	Drugs used in diabetes (A10*), insulins and analogs (A10A*), blood glucose lowering drugs, excluding insulins (A10B*)
Immunosuppressants	Antineoplastic agents (L01*), immunosuppressants (L04A*)
Systemic antimicrobials	Antibacterials for systemic use (J01*), antimycotics for systemic use (J02*), antimycobacterials (J04*)
Systemic steroids	Corticosteroids for systemic use, plain (H02A*), glucocorticoids (H02AB*), corticosteroids for systemic use, combinations (H02B*)
Pregnancy§	
Ongoing pregnancy	>1 admission code (O10–16, O20–29, O30–48, O94–99, Z32–36) or ≥2physician claims (640–649, V22) or ≥1 tariff code for prenatal services; must be within ±30 d of the index date (*26*)
Completion of pregnancy	>1 admission code (O8, O65–75, O80–84, O85–92, Z37–39) or ≥2 physician claims (650–659, 670–676) or ≥1 tariff code for delivery, abortion or postnatal services; must be within 270 d following the index date (*26*)
Medical condition§¶	
Alcoholism	>1 admission (E24.4, E51.2, E52, F10, G31.2, G62.1, G72.1, I42.6, K29.2, K70, K86.0, O35.4, P04.3, Q86.0, R78.0, T50.6, T51.0, T51.1, T51.9, X45, X65, Y15, Y57.3, Y90, Y91, Z50.2, Z71.4, Z72.1, Z81.1) or ≥2 physician claims (291, 303)
Anemia	>1 admission (D50–64) or ≥2 physician claims (280–285)
Asthma	>1 admission (J45, J46) or ≥2 physician claims (493)
Cancer, excluding NMS	≥1 admission (C00–43, C45–97) or ≥1 physician claim (140–172, 174–209, 235–239)
Cardiovascular disease	>1 admission (I00–99, O11) or ≥2 physician claims (390–519)
Chronic renal failure	>1 admission (I12.0, I13.1, N18, N19, N25.0, Z49, Z99.2) or ≥2 physician claims (403–404, 586–587)
Chronic respiratory condition	>1 admission (J40–99, O24) or ≥2 physician claims (490–496, 500–508)
COPD	>1 admission (J40–44, O24) or ≥2 physician claims (630–633, 634–639, 490–492, 496)
Diabetes	>1 admission (E10–14, G590, G632, H280, H360, M142, M146, N083, O24) or ≥2 physician claims (250) or ≥2 prescriptions for drugs used in treatment of diabetes
HIV/AIDS	≥1 admission (B20–24, R75, Z21) or ≥2 physician claims (042 V08) or ≥1 prescriptions for drugs used in treatment of HIV
Hypertension	>1 admission (I10–15, I67.4, O11) ≥2 physician claims (401–405)
Immunodeficiency	≥1 admission (D80–D84, D89) or ≥2 physician claims (288, 279)
Immunosuppressed	Having an organ transplant or a diagnosis of HIV/AIDS, other immunodeficiency disorders or cancer (other than nonmelanoma skin cancer), or receiving prescriptions for immunosuppressants or systemic steroids
Ischemic heart disease	>1 admission (I20–25) or ≥1 physician claims (410–414)
Organ transplant	≥1 admission (T86, Y83.0, Z94) or ≥2 physician claims (V42)
Stroke	≥1 admission (I61, I63, I64, I67.9, I69) or ≥2 physician claims (431, 434, 436–438)
Substance abuse	>1 admission (F11–16, F18–19) or ≥2 physician claims (292, 304,305)
Other	
Area of residence	Based on postal code of current address and the North-South relationship variable derived by using PCCF+#; North includes areas coded as “North” or "North transition"; South includes areas coded as "South" or "South transition"

*Asterisks indicate a wild-card character used as an alternative to listing all disease or drug codes within a section or subsection of the corresponding classification system.
†TIV, inactivated trivalent influenza vaccine; NMS, nonmelanoma skin cancer; COPD, chronic obstructive pulmonary disease.
‡Drugs were classified based according to their drug identification number and the Anatomical Therapeutic Chemical Classification System with Defined Daily Doses (www.who.int/classifications/atcddd/en/).
§Codes in parentheses are International Classification of Diseases (ICD)-10-CA codes for hospital admission data and ICD-9-CM codes for physician claims data.
¶Based on previously validated chronic disease identification algorithms with modifications.
#Automated Postal Code Conversion File Plus system, Statistics Canada (www.statcan.gc.ca/bsolc/olc-cel/olc-cel?lang=eng&catno=82F0086X).

detection varied by the sex, age, or place of residence of study participants; by the presence of chronic medical conditions in participants; or by the epidemic phase (Table 4). For example, the OR was 1.2 (95% CI 0.6–2.4) for participants who were ≥50 years of age, and the OR was 0.9 (95% CI 0.6–1.3) for younger participants ($p_{interaction}$ = 0.31). The OR was 0.9 (95% CI 0.6–1.3) among those who did not have a chronic disease and 1.3 (95% CI 0.7–2.3) for those who did ($p_{interaction}$ = 0.33).

Among participants with laboratory-confirmed influenza A(H1N1)pdm09 virus infection, the receipt of the 2008–09 TIV was associated with a statistically nonsignificant reduction in the risk for hospitalization (OR 0.6, 95% CI 0.3–1.3) (Table 5). On the other hand, having ever received PPV23 was associated with a statistically nonsignificant increase in risk for hospitalization. However, these analyses were limited by small numbers, which resulted in wide CIs and, likely, unstable estimates.

With 626 community case-patients and 2,479 community controls and by using a 2-sided 5% significance level, our study had ≈80% power to detect an OR as small as 1.3 (30% increase in risk), assuming 19% of controls received the 2008–09 TIV (*28*). With 205 hospitalized case-patients and 640 community case-patients, the study power

Table 2. Demographic and clinical characteristics of persons enrolled in a study to determine possible association between receipt of 2008–09 TIV and subsequent infection with influenza A(H1N1)pdm09 virus, Manitoba, Canada, 2009*

Characteristic	Community controls	Community case-patients	Hospitalized case-patients	Total
Total	2,479 (74.9)	626 (18.9)	205 (6.2)	3,310
Female sex	1,468 (59.2)	322 (51.4)	115 (56.1)	1,905
Age, y				
0.5–4	210 (8.5)	62 (9.9)	48 (23.4)	320
5–19	413 (16.7)	223 (35.6)	41 (20.0)	677
20–34	553 (22.3)	154 (24.6)	44 (21.5)	751
35–49	600 (24.2)	110 (17.6)	35 (17.1)	745
50–59	331 (13.4)	56 (8.9)	20 (9.8)	407
≥60	372 (15.0)	21 (3.4)	17 (8.3)	410
Age, y, median (Q1–Q3)	36 (19–51)	22 (10–39)	23 (5–43)	34 (16–52)
Residence				
Northern Manitoba	362 (14.6)	156 (24.9)	82 (40.0)	600
Urban area	1,406 (56.7)	341 (54.5)	88 (42.9)	1835
SEFI quintile†				
1	596 (24.0)	106 (16.9)	22 (10.7)	724
2	541 (21.8)	140 (22.4)	28 (13.7)	709
3	499 (20.1)	126 (20.1)	25 (12.2)	650
4	468 (18.9)	130 (20.8)	41 (20.0)	639
5	375 (15.1)	124 (19.8)	89 (43.4)	588
Physician visits in past year, median (Q1–Q3)	14 (5–32)	7 (3–19)	20 (10–51)	15 (6–34)
Hospitalizations in past 5 y, median (Q1–Q3)	0 (0–1)	0 (0–1)	3 (1–5)	1 (0–2)
Pregnant‡	85 (5.8)	17 (5.3)	25 (21.7)	127
Chronic disease	548 (22.1)	90 (14.4)	78 (38.0)	716
Diabetes	219 (8.8)	30 (4.8)	35 (17.1)	284
COPD	98 (4.0)	11 (1.8)	15 (7.3	124
Asthma	133 (5.4)	38 (6.1)	25 (12.2)	196
Ischemic heart disease	51 (2.1)	6 (1.0)	6 (2.9)	63
Chronic renal failure	43 (1.7)	5 (0.8)	10 (4.9)	58
Cancer, excluding NMS	95 (3.8)	7 (1.1)	10 (4.9)	112
Receipt of drug treatment				
Antiviral drug treatment	276 (11.1)	123 (19.6)	15 (7.3)	414
Antiviral prophylaxis	72 (2.9)	22 (3.5)	3 (1.5)	97
Antimicrobial drug treatment	1,035 (41.8)	219 (35.0)	106 (51.7)	1,360
Receipt of TIV				
2007–08	544 (21.9)	123 (19.6)	37 (18.0)	704
2008–09	564 (22.8)	109 (17.4)	39 (19.0)	712
No. TIVs received in past 5 years				
0	1,450 (58.5)	383 (61.2)	119 (58.0)	1,952
≥1	1,029 (41.5)	243 (38.8)	86 (42.0)	1,358
1–3	711 (28.7)	187 (29.9)	65 (31.7)	963
4–5	318 (12.8)	56 (8.9)	21 (10.2)	395
5	156 (6.3)	19 (3.0)	9 (4.4)	184
Ever received PPV23	343 (13.8)	41 (6.5)	32 (15.6)	416

*Values are no. (%) except as indicated. See Table 1 for definitions of variables. TIV, inactivated trivalent influenza vaccine; Q1–Q3, quartiles 1-3; SEFI, Socioeconomic Factor Index; COPD, chronic obstructive pulmonary disease; NMS, nonmelanoma skin cancer; PPV23, pneumococcal polysaccharide vaccine.
†SEFI quintiles are in order of worsening socioeconomic scale (*24*).
‡Of 5- to 49-year-old female participants.

Table 3. Association between receipt of seasonal influenza vaccine and subsequent infection with influenza A(H1N1)pdm09 virus, by vaccine type, Manitoba, Canada, 2009*

Variable	No. community controls, n = 2,479	No. community case-patients, n = 626	Odds ratio (95% CI)		
			Model A†	Model B‡	Model C§
Received TIV					
2007–08	544	123	0.9 (0.7–1.1)	1.3 (1.0–1.7)	1.4 (1.0–1.9)
2008–09	564	109	0.7 (0.6–0.9)	1.1 (0.8–1.4)	1.0 (0.7–1.4)
Vaccinated with					
None	1,728	466	Referent	Referent	Referent
2007–08 only	187	51	1.0 (0.7–1.4)	1.3 (0.9–1.8)	1.3 (0.9–1.9)
2008–09 only	207	37	0.7 (0.5–1.0)	0.9 (0.6–1.3)	0.9 (0.6–1.4)
Both	357	72	0.7 (0.6–1.0)	1.3 (0.9–1.8)	1.4 (1.0–2.0)
No. TIVs in past 5 y					
None	1,450	383	Referent	Referent	Referent
At least 1	1,029	243	0.9 (0.7–1.1)	1.2 (1.0–1.5)	1.2 (1.0–1.5)
1–3	711	187	1.0 (0.8–1.2)	1.2 (0.9–1.5)	1.2 (0.9–1.5)
4–5	318	56	0.7 (0.5–0.9)	1.3 (0.9–1.9)	1.4 (1.0–2.2)
5	156	19	0.5 (0.3–0.8)	0.8 (0.4–1.4)	0.8 (0.4–1.4)
Ever received PPV23	343	41	0.4 (0.3–0.6)	0.9 (0.6–1.4)	0.9 (0.6–1.5)
Vaccinated with					
None	1,796	499	Referent	Referent	Referent
2008–09 TIV only	340	86	0.9 (0.7–1.2)	1.1 (0.8–1.4)	1.0 (0.7–1.4)
PPV23 only	119	18	0.5 (0.3–0.9)	0.9 (0.5–1.5)	0.9 (0.5–1.6)
Both	224	23	0.4 (0.2–0.6)	1.0 (0.6–1.7)	1.0 (0.5–1.8)

*TIV, inactivated trivalent influenza vaccine; PPV23, pneumococcal polysaccharide vaccine.
†Unadjusted model.
‡Model adjusted for age, sex, region of residence, Socioeconomic Factor Index (*24*), and week of specimen collection.
§Model adjusted for all model B variables plus no. of hospital admissions and family physician visits in previous 12 mo, pregnancy, having a chronic or immunocompromising medical condition, and antiviral drug use. Model also included mutual adjustment for 2007–09 TIV and PPV23.

was considerably lower for the hospitalization analysis: the smallest detectable OR with 80% power was 1.7.

Discussion

We found no evidence that receipt of the 2008–09 TIV increased or decreased the risk for laboratory-confirmed influenza A(H1N1)pdm09 virus infections during the first wave of the pandemic in Manitoba. In analyses limited by small numbers, the 2008–09 TIV was associated with a statistically nonsignificant reduction in the risk for hospitalization.

These results are consistent with those in the bulk of the literature. Several studies using different designs (cohort as well as test-negative and conventional case–control studies) from Australia (*4*,*5*), England (*6*), Spain (*7*,*8*), and the United States (*9*–*11*) found that the 2008–09 TIV neither increased nor decreased the risk for influenza A(H1N1)pdm09 virus infection during the first wave of the pandemic.

The lack of protective effects against influenza A(H1N1)pdm09 virus is not surprising given the substantial antigenic divergence between the pandemic virus and recently circulating seasonal influenza A (H1N1) viruses among humans (*29*) and the lack of a cross-reactive antibody response to the pandemic strain in serologic studies of TIVs for humans and animals (*12*–*14*,*30*). However, 2 case–control studies from Mexico have indicated a protective effect (35%–74%), especially against severe infections (*2*,*3*). Concerns about possible selection bias and uncontrolled confounding were raised about both studies (*31*,*32*), although a reanalysis of the second study that attempted to address these concerns confirmed the initial results (*33*). Lower levels of seroconversion among TIV-vaccinated compared with unvaccinated persons were observed among nurses in a cohort study in Canada (*15*) and among military personnel in a cohort study in Singapore (*16*). However, it is unclear whether the results from these subpopulations are applicable to the general population. In the Singapore study, TIV was not protective against seroconversion among community participants. Similar reservations might be applicable to a US case–control study that reported a protective effect for the 2008–09 TIV among active-duty military service members (*34*).

On the other hand, increased risk for influenza A(H1N1)pdm09 virus infection with receipt of the 2008–09 TIV was reported for US military beneficiaries who sought care for influenza-like illness at Navy clinics in San Diego County, California, USA, during the first wave of the pandemic (*35*). However, the positive association with confirmed subtype H1N1 infection was seen only in univariate analyses restricted to active-duty members and was not observed for other study groups. In a small pilot study from Hong Kong, 31% of children who were randomly selected to receive TIV in November 2008 had serologically confirmed influenza A(H1N1)pdm09 virus infection, compared with 12% of the children who received a placebo (*30*). However, there were no significant differences between the 2 groups in rates of influenza-like illness, acute respiratory symptoms, or PCR-confirmed pandemic infections. Four studies from Canada, including

Table 4. Effect of receipt of 2008–09 TIV on risk for infection with influenza A(H1N1)pdm09 virus, Manitoba, Canada, 2009*

Data subsets, by demographic and clinical characteristic	No. community controls, n = 2,479	No. community case-patients, n = 626	Odds ratio (95% CI)	
			Model A†	Model C‡
Sex				
F	1,468	322	0.8 (0.6–1.1)	1.0 (0.7–1.5)
M	1,011	304	0.6 (0.4–0.9)	1.1 (0.7–1.8)
p for interaction			0.304	0.997
Age group, y				
0.5–49	1,776	549	0.9 (0.7–1.2)	0.9 (0.6–1.3)
≥50	703	77	1.0 (0.7–1.7)	1.2 (0.6–2.4)
p for interaction			0.563	0.308
Age, y				
0.5–4	210	62	1.1 (0.5–2.4)	1.3 (0.3–4.9)
5–19	413	223	1.1 (0.6–1.8)	1.6 (0.7–3.6)
20–34	553	154	0.9 (0.5–1.5)	0.9 (0.5–1.8)
35–49	600	110	1.0 (0.6–1.7)	0.9 (0.4–1.7)
50–59	331	56	1.4 (0.8–2.6)	1.7 (0.7–4.5)
≥60	372	21	1.4 (0.6–3.5)	0.4 (0.1–3.4)
p for interaction			0.882	0.916
Locality of residence				
Rural	1,073	285	0.8 (0.5–1.1)	1.0 (0.6–1.7)
Urban	1,406	341	0.7 (0.5–0.9)	1.0 (0.7–1.5)
p for interaction			0.632	0.628
Area of residence				
North	362	156	1.0 (0.6–1.7)	1.3 (0.6–2.8)
South	2,117	470	0.7 (0.5–0.9)	0.9 (0.7–1.3)
p for interaction			0.236	0.255
Epidemic phase, 2009				
Apr 27–Jun 20	1,071	423	0.7 (0.5–0.9)	0.9 (0.6–1.4)
Jun 21–Aug 21	1,408	203	0.8 (0.5–1.1)	1.1 (0.6–1.7)
p for interaction			0.586	0.482
Chronic disease				
No	1,931	536	0.8 (0.6–1.0)	0.9 (0.6–1.3)
Yes	548	90	1.0 (0.6–1.4)	1.3 (0.7–2.3)
p for interaction			0.402	0.330
Respiratory disease				
No	1,456	386	0.7 (0.5–1.0)	0.9 (0.6–1.3)
Yes	1,023	240	0.7 (0.5–1.0)	1.3 (0.8–2.0)
p for interaction			0.831	0.764

*TIV, inactivated trivalent influenza vaccine.
†Unadjusted model.
‡Model adjusted for age, sex, region of residence, Socioeconomic Factor Index (*24*), and week of specimen collection, no. hospital admissions and family physician visits in previous 12 mo, pregnancy, presence of a chronic or immunocompromising medical condition, and antiviral drug use. Model also included mutual adjustment for 2007–09 TIV and PPV23.

the aforementioned Ontario study, have also reported increased risk with TIV use, especially among younger persons (*1*). The inconsistency between our results and those of the Ontario study could be due to bias or residual confounding in either study.

Major strengths of our study include its population-based design and relatively large sample size. Because of the availability of accurate automated vaccination records (*23*), this study was less susceptible to recall bias and to misclassification of exposure status, issues that are common in observational studies in which vaccination information is self-reported. Misclassification of disease status was minimized by use of an accurate diagnostic test (RT-PCR) (*22*). However, it is well-known that viral RNA is occasionally not detectable by RT-PCR (e.g., because of delay in specimen collection), which means that some case-patients in our study might have been misclassified as controls. It is difficult to predict the direction of resulting bias. If the likelihood of false-negative results was not related to receipt of vaccine, our estimates would generally bias toward the null, masking any associations (*36*). If false-negative results were more likely among the unvaccinated persons (which could be the case if lack of vaccination and the delay in getting tested are caused by lack of timely access to primary care), our OR estimates could have been biased downwards, potentially masking any harmful effects of vaccination. We did not have information about testing delay, but we used proxies for access to health care (e.g., frequency of physician encounters) to adjust for factors that might be associated with promptness of testing. Stratifying the analysis by quintiles of the number of physician visits in the previous year did not result in any significant differences in the estimated ORs.

To further control for confounding by access to and propensity to seek health care, we employed a test-negative case–control design, in which all participants

Table 5. Association between receipt of seasonal influenza vaccine and subsequent risk for hospitalization among patients with influenza A(H1N1)pdm09 virus infection, Manitoba, Canada, 2009*

Variable	No. community case-patients, n = 626	No. hospitalized case-patients, n = 205	Odds ratio (95% CI) Model A†	Model B‡	Model C§
Received TIV					
2007–08	123	37	0.9 (0.6–1.4)	0.6 (0.3–1.0)	0.4 (0.2–0.8)
2008–09	109	39	1.1 (0.7–1.7)	0.8 (0.5–1.3)	0.6 (0.3–1.3)
Vaccinated with					
None	466	149	Reference	Reference	Reference
2007–08 only	51	17	1.0 (0.6–1.9)	0.7 (0.3–1.4)	0.5 (0.2–1.2)
2008–09 only	37	19	1.6 (0.9–2.9)	1.3 (0.6–2.6)	0.8 (0.4–1.8)
Both	72	20	0.9 (0.5–1.5)	0.5 (0.3–1.0)	0.2 (0.1–0.5)
No. TIVs in past 5 y					
None	383	119	Reference	Reference	Reference
≥1	243	86	1.1 (0.8–1.6)	0.9 (0.6–1.4)	0.8 (0.5–1.2)
1–3	187	65	1.1 (0.8–1.6)	1.0 (0.6–1.4)	0.8 (0.5–1.3)
4–5	56	21	1.2 (0.7–2.1)	0.8 (0.4–1.6)	0.5 (0.2–1.3)
5	19	9	1.5 (0.7–3.4)	1.1 (0.4–3.2)	0.6 (0.2–2.3)
Ever received PPV23	41	32	2.6 (1.6–4.3)	1.8 (1.0–3.6)	1.7 (0.8–3.8)
Vaccinated with					
None	499	153	Reference	Reference	Reference
2008–09 TIV only	86	20	0.8 (0.5–1.3)	0.6 (0.3–1.1)	0.5 (0.2–1.1)
PPV23 only	18	13	2.4 (1.1–4.9)	1.4 (0.6–3.4)	1.2 (0.4–3.1)
Both	23	19	2.7 (1.4–5.1)	1.9 (0.8–4.6)	1.6 (0.5–4.7)

*TIV, inactivated trivalent influenza vaccine; PPV23, pneumococcal polysaccharide vaccine.
†Unadjusted model.
‡Model adjusted for age, sex, region of residence, Socioeconomic Factor Index (*24*), and week of specimen collection.
§Model adjusted for all model B variables plus no. hospital admissions and family physician visits in previous 12 mo, pregnancy, presence of a chronic or immunocompromising medical condition, and antiviral drug use. Model also included mutual adjustment for 2007–09 TIV and PPV23.

were tested for influenza. We believe that using test-negative controls was the most practical way to sample controls from the population that gave rise to case-patients in our study (*37*). Had we sampled from the population at large, some of these controls would have been persons who would have never been tested for influenza if they had it (e.g., because of asymptomatic infection or because of lack of timely access to ambulatory care) and would have never appeared in our database as case-patients. The resulting bias could lead to underestimation of vaccine effectiveness if, as expected, receipt of the vaccine is positively associated with better access to ambulatory care and, therefore, to testing.

The controls in our study appeared to be representative of their respective age groups in the Manitoba population, and in general, they had characteristics similar to those for the control group in the Ontario case–control study (*1*) (Table 6). For instance, in our study the percentage of controls who received the 2008–09 TIV was ≈8% for participants 12–19 years of age, 14.5% for those 20–34 years of age, 19% for those 35–44 years of age, 28% for those 45–64 years of age, and 57% for those ≥65 years

Table 6. Demographic and clinical characteristics of case-patients and controls in a study of the association between the 2008–09 TIV and influenza A(H1N1)pdm09 virus infection, Manitoba, Canada, 2009*

Characteristic	No (%) study participants, by age group, y <12	12–19	20–34	35–44	45–64	≥65
Community controls						
Total	381 (15.4)	242 (9.8)	553 (22.3)	384 (15.5)	638 (25.7)	281 (11.3)
Female sex	185 (48.6)	138 (57.0)	353 (63.8)	237 (61.7)	385 (60.3)	170 (60.5)
Chronic disease	30 (7.9)	21 (8.7)	57 (10.3)	74 (19.3)	203 (31.8)	163 (58.0)
Receipt of 2007–08 TIV	34 (8.9)	19 (7.9)	76 (13.7)	67 (17.4)	187 (29.3)	161 (57.3)
Receipt of 2008–09 TIV	53 (13.9)	20 (8.3)	80 (14.5)	72 (18.8)	179 (28.1)	160 (56.9)
Community case-patients						
Total	180 (28.8)	105 (16.8)	154 (24.6)	76 (12.1)	102 (16.3)	9 (1.4)
Female sex	88 (48.9)	50 (47.6)	81 (52.6)	39 (51.3)	58 (56.9)	6 (66.7)
Chronic disease	9 (5.0)	15 (14.3)	17 (11.0)	13 (17.1)	29 (28.4)	7 (77.8)
Receipt of 2007–08 TIV	26 (14.4)	9 (8.6)	24 (15.6)	21 (27.6)	35 (34.3)	8 (88.9)
Receipt of 2008–09 TIV	24 (13.3)	10 (9.5)	20 (13.0)	15 (19.7)	34 (33.3)	6 (66.7)
Hospitalized case-patients						
Total	68 (33.2)	21 (10.2)	44 (21.5)	25 (12.2)	37 (18.0)	10 (4.9)
Female sex	23 (33.8)	13 (61.9)	32 (72.7)	12 (48.0)	27 (73.0)	8 (80.0)
Chronic disease	17 (25.0)	5 (23.8)	10 (22.7)	13 (52.0)	25 (67.6)	8 (80.0)
Receipt of 2007–08 TIV	6 (8.8)	0 (0.0)	4 (9.1)	9 (36.0)	12 (32.4)	6 (60.0)
Receipt of 2008–09 TIV	9 (13.2)	3 (14.3)	4 (9.1)	5 (20.0)	14 (37.8)	4 (40.0)

*TIV, inactivated trivalent influenza vaccine.

of age (Table 6). The corresponding percentages for the Manitoba population were 14%, 13%, 18%, 28%, and 67%, respectively (*38*). In the years leading to the pandemic, influenza vaccination policy in Manitoba was consistent with the recommendations of the Canadian National Advisory Committee on Immunization (*21*).

Information about several confounders was obtained from administrative databases. The completeness and accuracy of the MH database are well established, and these databases have been used extensively in studies of postmarketing surveillance of various drugs and vaccines (*39*). However, it is possible that there was a measurement error in some variables, which could result in residual confounding. In addition, the protective effects we observed against hospitalization might be related to confounding by factors that were not measured in this study, e.g., functional capacity (healthy vaccinee bias) (*40*).

Results from our study in Manitoba corroborate findings from studies outside Canada that the 2008–09 TIV neither increased nor decreased the risk for influenza A(H1N1)pdm09 virus infection. Additional epidemiologic and experimental investigations are needed to clarify the relationship between TIV use and infection with the pandemic strain.

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Use of Spatial Information to Predict Multidrug Resistance in Tuberculosis Patients, Peru

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To determine whether spatiotemporal information could help predict multidrug resistance at the time of tuberculosis diagnosis, we investigated tuberculosis patients who underwent drug susceptibility testing in Lima, Peru, during 2005–2007. We found that crude representation of spatial location at the level of the health center improved prediction of multidrug resistance.

In many locations where risk for tuberculosis (TB) is high, access to drug-susceptibility testing (DST) is limited. The detection of drug resistance in these instances usually requires the use of culture-based DST, but laboratory capacity in these areas is in short supply. As a result, DST is rationed, with patients at highest risk for drug resistance receiving priority. New rapid tests for resistance that circumvent some constraints are being implemented, and universal DST might eventually be available (*1*); however, most clinicians in high-risk areas will not have access to these tools for at least several years. Accordingly, improved prediction of risk for multidrug-resistant (MDR) TB, defined as resistance to at least isoniazid and rifampin, might reduce delay to appropriate diagnosis, improve treatment outcomes, and decrease the risk for MDR TB transmission.

Demographic and clinical characteristics that have been associated with increased risk for MDR TB among patients with incident TB are young age, previous TB treatment, and known contact with MDR TB (*2*,*3*). In the context of limited access to DST, these risk factors are often incorporated into diagnostic algorithms to help justify use of DST. We hypothesized that information about the location and time at which cases were detected might also improve prediction of MDR TB (*3*–*5*). We analyzed programmatic data collected in Lima, Peru, about TB patients who were receiving DST to assess whether predictive models that include information about time and location could improve prediction of risk for MDR TB.

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The Study

We selected our study population from among all 11,711 patients with reported cases of TB in 2 of Lima's 4 health districts, Lima Ciudad and contiguous catchment areas of Lima Este, during January 1, 2005–December 31, 2007. Demographic and clinical information about these patients was collected from routine TB program data. The home addresses of the patients were geocoded by using high-resolution maps created in Google Earth (Google Inc., Mountain View, CA, USA). In Peru, only a subset of TB patients determined to be at high risk for MDR TB receive sputum culture and DST; consistent with local guidelines, these patients are those who had previous TB treatment, known household contact with MDR TB patients, or lack of response to first-line TB treatment (*6*). We limited our analyses to patients who underwent DST and who had a definitive positive or negative result (n = 1,116); 346 of these patients had MDR TB (Figure 1). Additional study details are provided in Lin et al. (*7*).

To identify risk factors for MDR TB, we constructed a logistic regression model that included age, sex, sputum smear test result, previous TB treatment, known household contact with MDR TB patients, and HIV infection status as potential predictors. Univariable analyses showed that age at diagnosis, history of TB treatment, and sputum smear–negative disease were significantly associated with risk for MDR TB (Table). In the multivariable adjusted analysis, age at diagnosis, history of TB treatment, sputum smear–negative disease, and HIV-positive status were found to be independent predictors of MDR TB (Table).

To determine whether spatiotemporal information improved prediction of MDR TB, we further constructed 3 spatial regression models: 1) a health center model that combined demographic and clinical factors with health center information, modeled as random intercepts (*8*); 2) a spatial model that combined demographic and clinical factors with individual-level spatial information (i.e., patient residence), modeled as a smooth term using thin-plate regression splines (*9*); and 3) a spatiotemporal model that combined demographic and clinical factors with individual-level spatiotemporal information (i.e., patient residence and date of TB diagnosis), modeled as a smooth term using thin-plate regression splines (*10*). We compared

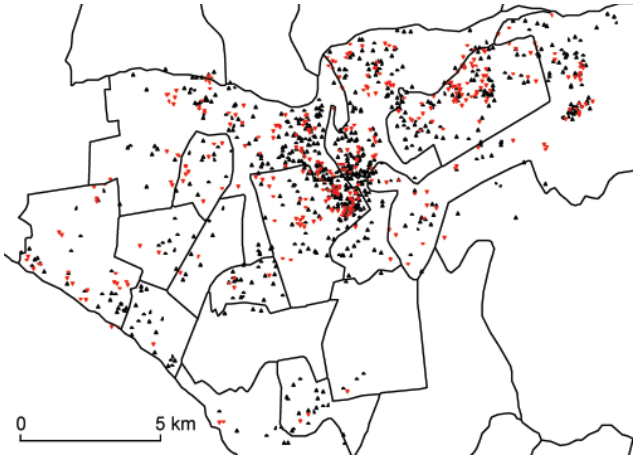

Figure 1. Spatial distribution of drug-sensitive (black triangles) and drug-resistant (red triangles) tuberculosis among patients who received drug susceptibility testing, Lima Ciudad and Lima Este, Peru, 2005–2007. A small random error was added to the spatial coordinates for each patient to protect confidentiality.

model performance of the 3 spatial models against a nonspatial model, which comprised only demographic and clinical factors.

To evaluate the accuracy of the models, we held out the last 50% of cases according to diagnosis date and used the first 50% of cases to fit the models. We then made predictions on the held-out cases by using the fitted models; receiver operating characteristic (ROC) analysis was used to estimate the area under the curve (AUC) for the held-out cases under each of the 4 models. We also computed the logistic regression likelihood (Bernoulli density) of the held-out data; the model with the largest logistic regression likelihood was judged to be most accurate (*11*).

The ROC analysis suggested that the addition of spatial information improved the performance of the nonspatial model (Figure 2). The AUC for the nonspatial model was 0.64 (95% CI 0.59–0.69, compared with 0.67 (95% CI 0.63–0.72) for the health center model (p = 0.02 for comparison with the nonspatial model); 0.67 (95% CI 0.62–0.72) for the spatial model (p = 0.06 for comparison with the nonspatial model); and 0.66 (95% CI 0.61–0.71) for the spatiotemporal model (p = 0.36 for comparison with the nonspatial model). The logarithm of logistic regression likelihood for the spatial model (−328.1) and the health center model (−327.0) were greater than that of the nonspatial model (−335.1), which suggests that the use of spatial information improved predictive power.

Conclusions

In locations where capacity is not available to provide DST for all patients with incident TB, improved methods to predict MDR TB at the time of diagnosis would be valuable. We found that information about location (represented as either the health center of diagnosis or the patient's residence location) improved prediction of MDR TB among those who received DST. Whereas the improvement in the models was either statistically significant (comparing health center and nonspatial models) or trending toward significance (comparing spatial and nonspatial models), the absolute differences in the AUCs from spatial and nonspatial models were modest. Despite the minor improvements, spatial and temporal information may be useful for targeting testing when access is limited. From a practical standpoint, these results suggest that adopting more lenient criteria for ordering DST for TB patients at individual health centers where risk for MDR TB is highest may be a rational approach while resources are limited.

Models with simple representations of space (i.e., identification of location only at the level of the health center) outperformed models that captured spatial risk in finer spatial resolution. This finding is consistent with an earlier analysis in which we found relative aggregation of new MDR TB at a spatial scale of 4–7 km (*7*). Together, these results suggest dispersed spatial risk for resistance in the study area, which indicates that, from a public health perspective, policies prioritizing the use of DST for patients originating from large administrative areas may be helpful.

Because we could include only patients who received DST, we can make inference only among this subgroup

Table. Risk for MDR TB among TB patients who received DST, by demographic and clinical characteristics, Lima Ciudad and Lima Este, Peru, 2005–2007*

Characteristic	Univariate OR† (95% CI)	p value	Multivariate OR† (95% CI)‡	p value
Age, per 10-y increase	0.89 (0.81–0.99)	0.034	0.90 (0.81–1.00)	0.046
Male sex	1.09 (0.81–1.46)	0.58	1.05 (0.78–1.43)	0.74
Negative sputum smear test results	2.05 (1.37–3.08)	<0.001	2.11 (1.40–3.19)	<0.001
HIV infection	0.59 (0.31–1.13)	0.11	0.52 (0.27–1.00)	0.049
History of TB treatment	2.38 (1.78–3.18)	<0.001	2.41 (1.80–3.23)	<0.001
Known household contact with persons with MDR TB	1.18 (0.89–1.57)	0.25	1.08 (0.81–1.44)	0.59

*MDR, multidrug resistant; TB, tuberculosis; DST, drug-susceptibility testing; OR, odds ratio.
†ORs represent risk for MDR TB conditional on receiving DST. For example, even if household contact with persons with MDR TB is marginally associated with increased risk for MDR TB, the observed association would likely be attenuated when the analysis is restricted to those who received DST because household contact with persons with MDR TB is an accepted criterion for ordering DST.
‡Adjusted for all other variables listed.

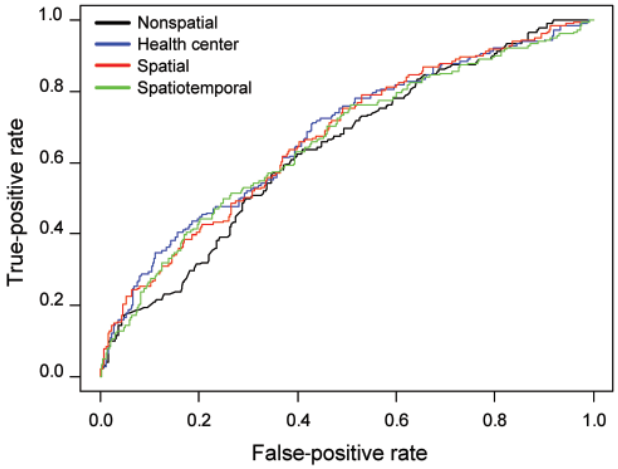

Figure 2. Receiver operating characteristic curves for the 4 prediction models for multidrug-resistant tuberculosis among patients who received drug susceptibility testing, Lima Ciudad and Lima Este, Peru, 2005–2007.

of patients. However, if use of DST were randomized throughout the study area (as earlier analysis suggests [*7*]), inference from this subgroup should be generalizable to all patients with incident TB. Use of historical data for spatial prediction relies on the assumption that the spatial patterns remain constant or change in a predictable manner. Temporal changes in spatial distribution of MDR TB would have reduced the predictive ability of the models, yet we found that spatial information improved our predictions. Further research is warranted to test this approach in settings where the spatial pattern of TB differs from that of Lima, preferably by using datasets in which DST has been conducted for all TB patients to prevent potential sampling bias.

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Influenza Virus A (H10N7) in Chickens and Poultry Abattoir Workers, Australia

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In March 2010, an outbreak of low pathogenicity avian influenza A (H10N7) occurred on a chicken farm in Australia. After processing clinically normal birds from the farm, 7 abattoir workers reported conjunctivitis and minor upper respiratory tract symptoms. Influenza virus A subtype H10 infection was detected in 2 workers.

Reported outbreaks of low pathogenicity avian influenza (LPAI) viruses of influenza A subtype H10 in poultry are uncommon but have occurred among turkeys and emus in the United States (*1*,*2*), farmed Pekin ducks in South Africa (*3*), and chickens in Canada (*4*). Isolation of influenza virus A (H10N7) was reported in Italy from smuggled poultry products from China (*5*). Transmission of LPAI viruses from birds to humans, resulting in symptomatic disease, has been reported for influenza virus A subtypes H9N2 in China and Hong Kong, H7N2 in North America and the United Kingdom, H7N3 in Canada, H7N7 in the United Kingdom (*6*), and H10N7 in Egypt (*7*).

The Study

In March 2010, an outbreak of LPAI A (H10N7) was identified in a biosecure intensive commercial poultry enterprise in New South Wales, Australia. For 8–14 days, 10–25 birds died each day, compared with the normal number of 2–6 birds per day. An egg production decrease of up to 15% was documented in the affected flocks. In contrast to other reported poultry outbreaks (*1*–*4*), respiratory signs were absent in the flock.

All cloacal and tracheal swabs from 10 dead and 10 live birds submitted for influenza virus exclusion were positive by an influenza A matrix gene quantitative real-time reverse transcription PCR (*8*), and virus was detected at various levels (cycle threshold [C_t] 15–37). The influenza A viruses were then subtyped from swabs by using a microarray assay (Clondiag, Jena, Germany) that enabled the rapid identification of influenza virus A (H10N7). The virus was readily cultured from swabs in embryonated chicken eggs and in MDCK cell cultures. Several viral genome segments were sequenced, which enabled confirmation of the virus as LPAI A (H10N7) and performance of phylogenetic analysis. A fluorescence-based neuraminidase inhibition assay showed the isolate to be sensitive to the antiviral drugs oseltamivir and zanamivir (mean 50% inhibitory concentration ± SD 0.5 ± 0.1 nmol/L and 1.8 ± 0.3 nmol/L, respectively) (*9*).

Serologic testing was conducted by using an influenza A nucleoprotein–based blocking ELISA and a subtype H10–specific hemagglutination inhibition test; results showed widespread infection in the affected flock, with 18 of 20 samples seropositive. Sampling across a 4 additional flocks on site showed that an additional 9 of 40 birds were seropositive for influenza A subtype H10.

Ten days after the outbreak was confirmed, 3 previously seronegative flocks from the site were sent to an abattoir; 1 day earlier, they had passed state government clinical inspection, including inspection and examination of production and mortality records. Within a week, 7 workers at the abattoir showed signs of conjunctivitis; 2 also reported rhinorrhea and 1 a sore throat.

Conjunctival swabs were collected from 6 of the workers and nose and throat swabs from all 7. Influenza A RNA was detected by PCR 4 days after abattoir exposure in conjunctival swabs from a worker who reported conjunctivitis, rhinorrhea, and sore throat (C_t 31.8) and 7 days after abattoir exposure from the nose/throat swab of another worker who reported only conjunctivitis with onset 2 days earlier (C_t 35). Partial sequence analysis of the hemagglutinin genes from both samples (GenBank accession nos. CY063325 and CY063326) confirmed the presence of influenza A subtype H10; the partial sequences were identical to the subtype H10 chicken isolate, although no virus was cultured from the workers.

The conjunctivitis and other reported symptoms among the 7 workers were mild and of short duration, and there was no evidence of seroconversion by

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hemagglutination inhibition or virus neutralization tests in any of the workers from whom convalescent-phase blood was collected, including the 2 with confirmed influenza A subtype H10 infection. These findings are consistent with the mild symptoms and lack of serologic evidence reported in humans after experimental infection with influenza A (H10N7), which may indicate the limited ability of the virus to multiply and stimulate a detectable immune response in humans (*10*). Other studies have reported no evidence of elevated subtype H10–specific antibody titers among poultry abattoir workers, although serologic evidence of subtype H10 infection was detected among turkey farmers in the absence of clinical symptoms (*11*).

Although 4 farm staff members from the site of the initial infections reported conjunctivitis and other symptoms to health care workers, influenza was not confirmed. The abattoir workers with laboratory-confirmed influenza A subtype H10 infection handled offal and giblets in a section of the abattoir where automated evisceration usually took place; however, because of the size of the birds, evisceration was manually assisted on the day that these flocks were slaughtered.

No obvious breach of biosecurity occurred on the farm. The water supply to the farm was chlorinated town water; no large dams were on site, only small paddock dams for cattle. The sheds were birdproof and protected by additional bird netting. A feed mill supplied the feed, which was delivered into silos through blow pipes from outside the perimeter fence. Litter (wood shavings) was delivered in enclosed bales. Workers showered on the way in and out of facilities; disinfectant foot baths were placed at the entrance of each shed, and staff were required to use the separate footwear provided inside the shed. Staff were not allowed to have birds or pigs at home.

Conclusions

During 2010, the number of wild waterfowl observed on the affected site was unusually low. Surveillance of poultry flocks within a 2-km radius of the affected farm did not detect any serologic or virologic evidence of subtype H10 infection. Ongoing surveillance of wild waterfowl in New South Wales reported influenza virus A (H10N7) in other areas in the previous year (K.E. Arzey, unpub. data); however, during 2007–2008, onsite surveillance detected no evidence of influenza A infection among wild waterfowl (G.G. Arzey, unpub. data).

The phylogenetic analysis of the full hemagglutinin sequence from the influenza A (H10N7) infections reported here in chickens showed a high degree of homology with North American avian influenza A subtype H10 viruses (Figure). This is an unusual finding, given that most avian influenza viruses detected in Australia are related to Eurasian avian influenza strains (*12*).

The finding of LPAI in commercial poultry in Australia is rare, with only 2 reports published (*13*). To our knowledge, no transmission to humans has been reported previously, including during the 5 reported HPAI outbreaks in Australia during 1976–1997 (*13*). The United Kingdom Advisory Committee on Dangerous Pathogens (*14*) acknowledged the occupational risk from slaughter of LPAI-infected birds and recommended that appropriate personal protective equipment (PPE) be used during handling of infected birds. However, because of the recent testing of the flock, the birds in this instance were assumed

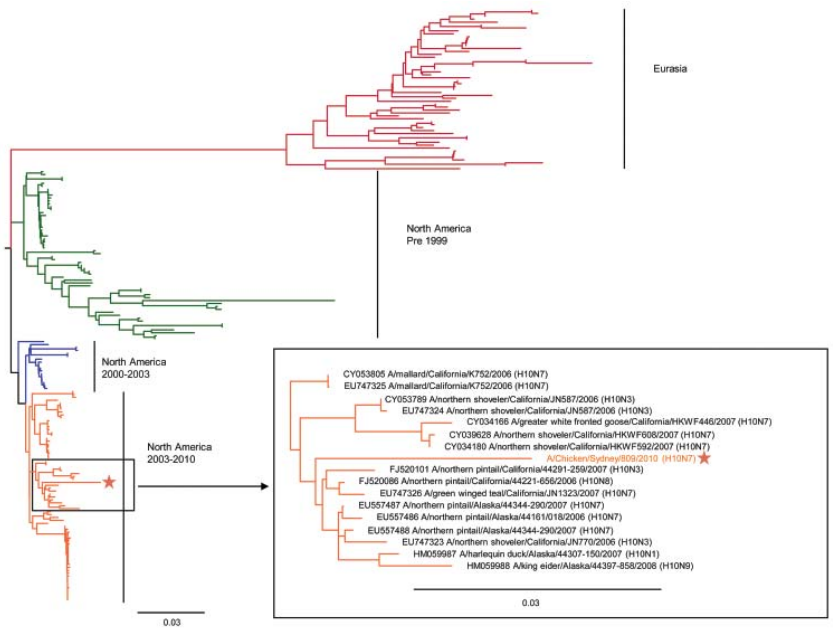

Figure. Phylogenetic analysis of avian influenza subtype H10 hemagglutinin (HA) sequences. HA sequences of all subtype H10 viruses deposited in GenBank were downloaded, and a neighbor-joining tree was created by using Jukes-Cantor as the genetic distance model on Geneious 5.14 software (Biomatters Ltd, Auckland, New Zealand) and a phylogenetic tree drawn by using FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). A representative HA sequence from the subtype H10N7 viruses detected from the New South Wales chicken farm outbreak (A/chicken/Sydney/809/2010) has been submitted to the Global Initiative on Sharing All Influenza Data (accession no. EPI339225) and is marked by the star in the tree. Scale bars indicate nucleotide substitutions per site.

to be free of infection, and workers did not wear goggles and face masks in the evisceration section on the day the flocks were processed. After this incident, use of full PPE, including the use of enclosed rim goggles and P2 (N95) face masks, was implemented in sections of the abattoir where exposure to birds and carcasses from the infected site was likely. Staff training, compliance with PPE use, and the decision to slaughter flocks from the site only after a sufficient number of birds were sampled to enable detection of a low infection prevalence and at a 99% confidence level resulted in no further reported infection of workers during the processing of all flocks from the site over 10 months. Analysis of preslaughter samples showed that the spread of infection stopped about 4 weeks after influenza virus A (H10N7) was first confirmed on the site.

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Influenza A and B Virus Attachment to Respiratory Tract in Marine Mammals

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Patterns of virus attachment to the respiratory tract of 4 marine mammal species were determined for avian and human influenza viruses. Attachment of avian influenza A viruses (H4N5) and (H7N7) and human influenza B viruses to trachea and bronchi of harbor seals is consistent with reported influenza outbreaks in this species.

Understanding is limited about factors determining the ability of influenza viruses to cross the species barrier and persist in a new host population (*1*,*2*). In marine mammals, several subtypes of avian influenza A virus have caused epidemics in harbor seals (*Phoca vitulina*) (*3*–*6*). Also, human influenza B virus has been detected in harbor seals (*7*). These observations indicate the ability of both viruses to cross the species barrier and persist in harbor seals. In other marine mammal species, outbreaks of avian influenza A virus or infection with human influenza B virus have not been reported.

Attachment of influenza virus to tissues in the respiratory tract is a major determinant of host susceptibility to infection, efficiency of transmission, and pathogenicity and has been studied only to a limited degree (*8*,*9*). Attachment is determined largely by the specificity with which influenza virus attaches to sialosaccharide receptors on the host cell surface. In general, human influenza viruses prefer sialosaccharides in which sialic acid is linked to galactose by an α-2,3 linkage (SA-α-2,3-Gal), and avian influenza viruses prefer those with an α-2,6 linkage (SA-α-2,6-Gal) (*10*).

To understand differences in these properties between harbor seals and other marine mammals, we determined patterns of attachment for influenza virus strains known to have infected the respiratory tract of harbor seals, gray seals (*Halichoerus grypus*), harbor porpoises (*Phocoena phocoena*), and bottlenose dolphins (*Tursiops truncatus*). We chose gray seals, porpoises, and dolphins because their ranges overlap those of harbor seals and they are commonly kept in captivity.

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The Study

We determined patterns of attachment to respiratory tract tissues of 4 sympatric marine mammal species for several influenza viruses. Avian influenza A virus subtypes H7N7 (A/Seal/Massachusetts/1/80) and H4N5 (A/Seal/Ma/47/83) were chosen because they had caused outbreaks in harbor seals (*4*,*5*). An influenza B virus strain (B/Seal/Netherlands/1/99) was chosen because it had been isolated from a harbor seal (*7*).

For each of these 3 viruses, we also included a closely related strain from the putative donor host species (H7N7 A/Mallard/Sweden/100/02, H4N5 A/Mallard/Netherlands/13/2008, and B/Harbin/7/94, respectively) to determine whether adaptation to the new host species was associated with a change in attachment. Influenza virus A(H1N1)pdm09 (A/Netherlands/164/09) and seasonal subtype (H3N2) virus (A/Netherlands/213/03) were chosen because they circulate endemically in humans and might have contact with captive marine mammals through their caretakers. All viruses were isolated as described (*11*–*13*).

We obtained respiratory tract specimens from marine mammals from archives of paraffin-embedded tissues. Trachea and lung (including bronchus, bronchiole, and pulmonary alveoli) from 3 animals per species were examined.

Attachment of influenza virus to tissues was visualized by histochemical analysis as described (*13*). A positive result by light microscopy was granular to diffuse red staining on the apical surface of epithelial cells in trachea, bronchi, and bronchioles and on alveolar cells. Staining was scored as the percentage of cells in a section showing virus attachment. We also evaluated virus attachment to submucosal glands.

Results of attachment differed between avian influenza A viruses, human influenza A viruses, and human influenza B viruses. First, attachment of avian influenza A viruses to tracheal and bronchial epithelium was moderate in seals (harbor seal and gray seal) and absent in cetaceans (harbor porpoise and bottlenose dolphin) (Figure). Attachment to bronchiolar epithelium was moderate in seals and scarce in cetaceans, and attachment to alveolar epithelium was scarce in all 4 species. There were a few exceptions for virus attachment (Table). The source of avian influenza viruses (mallard or harbor seals) did not have a consistent effect on virus attachment in respiratory tract epithelium of any evaluated species (Table).

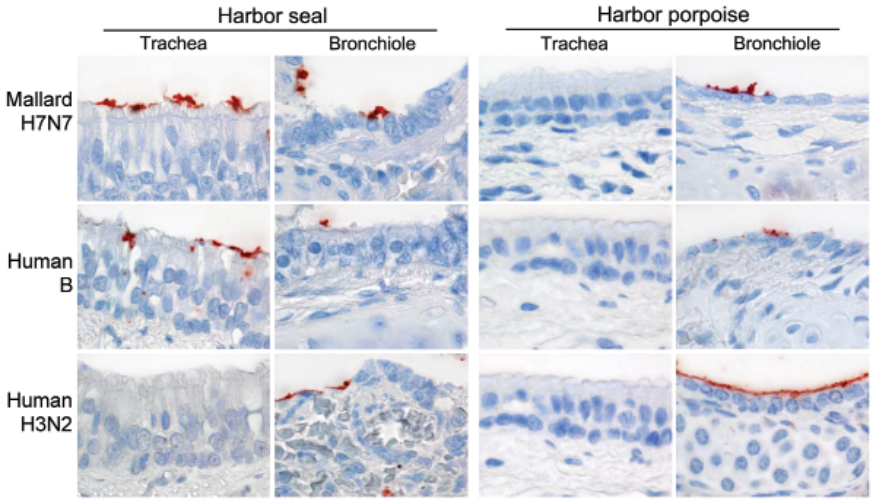

Figure. Attachment of 2 human influenza viruses and 1 avian influenza virus to trachea and bronchiole of harbor seal (*Phoca vitulina*) and harbor porpoise (*Phocoena phocoena*). Red staining indicates virus on the surface of epithelial cells (histochemical staining counterstained with hematoxylin); original magnification ×100).

Second, attachment of human influenza A viruses to tracheal and bronchial epithelium was absent in seals and scarce in cetaceans. Attachment to bronchiolar and alveolar epithelium was absent or scarce in seals and moderate to abundant in cetaceans (Table, Figure). We detected few differences between attachment of influenza virus A(H1N1) pdm09 and seasonal subtype (H3N2) virus to respiratory tract tissues of cetaceans (Table).

Third, attachment of influenza B viruses to respiratory tract epithelium at all levels was scarce to moderate in seals. Attachment was negative for tracheal and bronchial epithelium, scarce for bronchiolar epithelium, and moderate for alveolar epithelium in cetaceans (Table, Figure).

Conclusions

Attachment of avian influenza A viruses to the respiratory tract was generally consistent with reports, or lack thereof, of avian influenza in these 4 marine mammal species. Moderate attachment of avian influenza A viruses to the trachea and bronchi of harbor seals suggests high susceptibility to and efficient transmission of these viruses. This finding is consistent with reported outbreaks of avian influenza in harbor seals (*4*–*6*). Scarce attachment of avian influenza viruses to bronchioles and alveoli of harbor seals is consistent with low pathogenicity of these viruses for harbor seals during experimental infection (*4*–*6*).

Attachment of avian influenza A virus to the respiratory tract in gray seals strongly resembles attachment in harbor seals. However, infection or outbreaks of avian influenza A virus in gray seals have not been reported, probably because virus attachment is required but is not sufficient for infection. Lack of attachment of avian influenza A viruses to trachea and bronchi of harbor porpoises and bottlenose dolphins suggests low susceptibility and inefficient transmission. This finding is consistent with lack of reported avian influenza A virus infections in these species (*14*).

Absence or scarcity of attachment of human influenza A viruses to trachea and bronchi of any of the marine mammal species contrasts with that of humans (*13*), in whom trachea and bronchi mainly express SA-α-2,6-Gal (*15*). This finding suggests low susceptibility to infection and can explain the lack of reported human influenza A virus infections in these 4 marine mammal species (*14*), even though they are often kept in captivity and are therefore at risk for infection from humans.

Attachment of influenza B virus to the respiratory tract of the 4 marine mammal species resembled that of the avian influenza A viruses. Moderate attachment of influenza B virus to the respiratory tract of seals suggests high susceptibility and efficient transmission. This finding is consistent with isolation of influenza B virus from a harbor seal and serologic evidence of influenza B virus infection in gray seals (*7*). Lack of attachment of influenza B virus to trachea and bronchi of cetaceans is consistent with absence of reported influenza B virus infections in these species.

Source of virus strain had little effect on its attachment. In general, there was high similarity of attachment of avian influenza A viruses from harbor seals and mallards. These findings suggest that avian influenza viruses do not require a different pattern of attachment to infect and transmit efficiently among harbor seals and that harbor seals might be susceptible to a wider range of avian influenza viruses than reported.

In conclusion, we report extensive diversity in the pattern of attachment of influenza viruses to the respiratory tract of marine mammals, which was determined by virus strain and host species involved. Our results correspond to field observations of influenza in marine mammals, i.e.,

Table. Attachment of mammal and human avian influenza viruses to respiratory tracts of 4 marine mammals*

Host (species) and virus strain	Level and cell tropism of virus attachment							
	Trachea		Bronchus		Bronchiole		Alveolus	
	Score	Predominant cell type	Score	Predominant cell type	Score	Predominant cell type	Score	Predominant cell type
Harbor seal (*Phoca vitulina*)								
Seal (H4N5)	+	Cil	+	Cil†	+	Cil	±	ND
Seal (H7N7)	+	Cil	+	Cil†	±	Cil	±	ND
Mallard (H4N5)	+	Cil	+	Cil†	+	Cil	±	ND
Mallard (H7N7)	+	Cil	+	Cil†	±	Cil	±	ND
Human (H1N1)	–		–		±	Cil	±	ND
Human (H3N2)	–		–		±	Cil	±	ND
Human B	+	Cil	+	Cil†	+	Cil	±	ND
Seal B	±	Cil	±	Cil†	±	Cil	±	ND
Gray seal (*Halichoerus grypus*)								
Seal (H4N5)	+	Cil†	+	Cil†	+	Cil	±	ND
Seal (H7N7)	+	Cil†	+	Cil†	+	Cil	±	ND
Mallard (H4N5)	+	Cil†	+	Cil†	+	Cil	±	ND
Mallard (H7N7)	+	Cil†	+	Cil†	+	Cil	±	ND
Human (H1N1)	–		–		–		–	
Human (H3N2)	–		–		–		–	
Human B	+	Cil†	+	Cil†	+	Cil	+	I
Seal B	±	Cil†	±	Cil†	±	Cil	±	ND
Harbor porpoise (*Phocoena phocoena*)								
Seal (H4N5)	±	Cil†	++	Cil	++	Cil	±	ND
Seal (H7N7)	–		–		±	Cil	±	ND
Mallard (H4N5)	–		–		±	Cil	±	ND
Mallard (H7N7)	–		–		±	Cil	±	ND
Human (H1N1)	–		±	Cil	+	Cil	++	I and II
Human (H3N2)	–		±	Cil	++	Cil	++	I and II
Human B	–		–		±	Cil	+	I
Seal B	–		–		±	Cil	+	I
Bottlenose dolphin (*Tursiops truncatus*)								
Seal (H4N5)	–		±	Cil	+	Cil	++	I and II
Seal (H7N7)	–		±	Cil	±	Cil	±	ND
Mallard (H4N5)	–		±	Cil	+	Cil	++	I and II
Mallard (H7N7)	±	Cil†	±	Cil	±	Cil	±	ND
Human (H1N1)	±	Cil†	±	Cil	+	Cil	+	I
Human (H3N2)	±	Cil†	±	Cil	++	Cil	++	I and II
Human B	–		–		±	Cil	+	I
Seal B	–		–		+	Cil	+	I

*Mean abundance of cells to which virus attached was scored as follows:+, moderate (10%–50% cells positive); ±, scarce (≤10% cells positive); –, negative (no attachment); ++, abundant (>50% cells positive). Cil, ciliated epithelial cell; ND, not determined; I, type I pneumocytes; II, type II pneumocytes.
†Virus attachment to submucosal glands.

outbreaks of avian influenza A virus and human influenza B virus infection in harbor seals (*4*,*5*) and lack of evidence of human influenza A virus infection in marine mammals. These results suggest that, as in humans (*11*,*15*), attachment of influenza virus to the proximal part of the respiratory tract, which depends largely on appropriate sialic acid moieties, is critical for susceptibility and efficient transmission of influenza viruses in marine mammals.

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Coxsackievirus A21, Enterovirus 68, and Acute Respiratory Tract Infection, China

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During August 2006–April 2010, in Beijing, China, 2 rare human enterovirus serotypes, coxsackievirus A21 and enterovirus 68, were detected most frequently in human enterovirus–positive adults with acute respiratory tract infections. Thus, during some years, these 2 viruses cause a substantial proportion of enterovirus-associated adult acute respiratory tract infections.

Human enteroviruses (HEVs) are small, nonenveloped RNA viruses belonging to the family *Picornaviridae*. HEVs are classified into 4 species (HEV-A to -D) according to their molecular and antigenic properties (*1*). HEVs are associated with diverse clinical syndromes, ranging from mild upper respiratory tract illnesses to severe and potentially fatal conditions, such as aseptic meningitis, encephalitis, myocarditis, acute flaccid paralysis, and hand-foot-and-mouth disease (*1*).

Although HEV serotypes can cocirculate, spatial and temporal factors determine the predominant serotype. For instance, in France and Spain, echovirus 11 and echovirus 6 are the predominant serotypes in patients with enterovirus respiratory infections (*2*,*3*). Clusters of the rare serotype enterovirus 68 (EV68), which causes severe respiratory infections in children, have been recently reported in the Philippines (*4*) and Japan (*5*). To help clinicians and public health officials better understand the epidemiologic and clinical profiles of HEV respiratory infections, temporal and geographic patterns of circulation, especially the dynamics of HEV serotype shift, need to be determined. We report that in some years in Beijing, People's Republic of China, the rarely reported coxsackievirus A21 (CVA21) and EV68 are the predominant serotypes in adults with enterovirus-associated acute respiratory tract infection (ARTI).

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The Study

From August 2006 through April 2010, throat and nasal swabs were collected from 6,942 (3,158 male and 3,784 female) adult patients (≥15 years of age) at the time of admission to the Fever Clinic Department at the Peking Union Medical College Hospital for ARTI. The 6,942 participants were randomly selected by physicians, as described (*6*). To detect HEV, we amplified 350–400 bp of the viral protein 1 (VP1) gene by reverse transcription PCR (*7*) and verified the findings by sequence analysis (GenBank accession nos. JN168998–JN169120). According to results of BLAST analysis (www.ncbi.nlm.nih.gov), the HEV detected in a sample was assigned to the serotype with which the amplified sequence shared ≥75% nt or ≥88% aa sequence identity (*8*). All samples were simultaneously screened for influenza viruses (A, B, and C), human parainfluenza viruses (1–4), respiratory syncytial virus, human coronaviruses (229E, NL63, HKU1, and OC43), metapneumovirus, adenovirus, and rhinovirus (*6*).

HEV was detected in 130 (69 male and 61 female) patients 15–70 years of age (median 27.5 years, mean 29.6 years). We identified 21 HEV serotypes. The most frequently detected serotypes were 2 that are rarely reported: CVA21 and EV68. The only HEV-C serotype detected in this study, CVA21, was found in 34 (26.2%) patients. The only HEV-D serotype detected in this study, EV68, was found in 13 (10%) patients. We also found 7 HEV-A serotypes and 12 HEV-B serotypes (Figure 1, panel A).

We detected 21 cases of CVA21 during August–December 2006 and 8 cases during September–December 2008, which accounted for 41.2% and 26.7% of all cases of HEVs, respectively. A few CVA21-positive cases were also detected in March and during June–August 2007. EV68 was detected in only 11 samples collected during August–October 2006 and in 2 samples in August 2008 (Figure 1, panel B; Table 1). The peaks of CVA21 and EV68 overlapped during August–October 2006. We co-detected other respiratory viruses in only 1 CVA21-positive patient and only 1 EV68-positive patient, indicating that the ARTIs in these patients are primarily associated with CVA21 or EV68 (Table 2).

Major symptoms associated with HEV infection included pharyngeal congestion, headache, myalgia, chills, and sore throat but not increased respiratory rate or difficulty breathing. According to the Guidelines for Surveillance on Severe Acute Respiratory Infections at Sentinel Hospitals (2011) issued by the Chinese Ministry of Health (www.

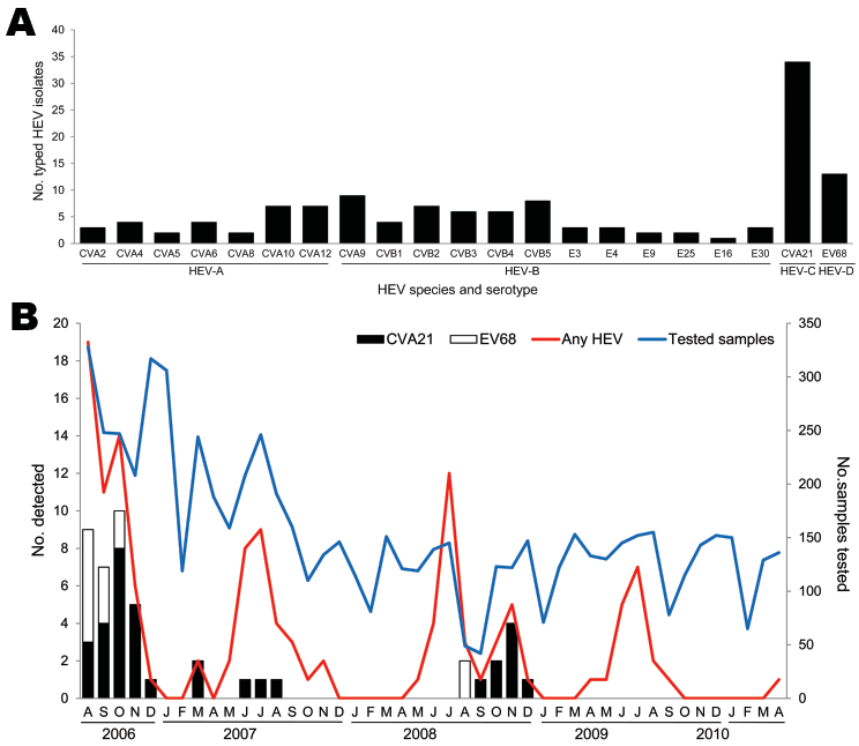

Figure 1. Frequency of human enterovirus (HEV) serotypes detected among adult patients by using sequence analysis of a partial viral protein 1 gene, in Beijing, People's Republic of China, August 2006–April 2010. A) Number of patients detected for each HEV serotype; B) Seasonal distribution of the HEVs in adults with acute respiratory tract infection. Numbers of samples tested in each month during the study period are shown on the right-side y-axis. CV, coxsackievirus; E, echovirus; EV, enterovirus.

moh.gov.cn/publicfiles/business/htmlfiles/mohjbyfkzj/s3577/201102/50590.htm), ARTI patients ≥5 years of age who had increased respiratory rate (≥25 breaths/min) or difficulty breathing were considered to have a severe ARTI. Our data showed that all CVA21- or EV68-positive patients identified in this study had mild ARTIs.

The VP1 nucleotide sequences of the CVA21 and EV68 strains identified showed high sequence identity and were located in the same cluster in their phylogenetic trees, HEV-C and HEV-D, respectively (Figure 2). Nucleotide identity among the CVA21 strains identified was 95.6%–100% and that among EV68 strains was 92.3%–99.7%. However, the CVA21 and EV68 strains had low nucleotide identity compared with their prototype strains (CVA21, GenBank accession no. AF546702, 87%–89.2%; EV68, GenBank accession no. AY426531, 76.6%–78.9%). Alignment of the putative VP1 amino acid sequences of HEV strains with their respective prototype strains revealed a limited divergence; only 2 substitutions were found in CVA21, and 9 substitutions and 1 deletion was found in EV68 (data not shown). These findings suggest that the CVA21 or EV68 strains were similar but are changing and diverging from their prototype strains.

Conclusions

The frequency of detecting CVA21 and EV68 among HEV-positive adults with ARTIs in Beijing, China, was high. The difference in the HEV serotypes we detected compared with serotypes detected by others (*2*,*3*) suggests that the spectrum of HEV serotypes associated with ARTIs differs among geographic regions.

Since isolation of these viruses, infections with CVA21 and EV68 have been rarely reported (*9*,*10*). In the United States, only 42 detections of CVA21 and 26 detections of EV68 have been reported during 36 years of HEV surveillance (*11*). However, EV68 was recently associated with ARTIs in France, the Netherlands, the United States, the Philippines, and Japan (*4*,*5*,*12*–*15*), indicating epidemics of EV68 are increasing in number and severity.

Table 1. Yearly distribution of human enterovirus infections in adults with acute respiratory tract infection, People's Republic of China, August 2006–April 2010

Date	Coxsackievirus A21, no. (%)	Enterovirus 68, no. (%)	Any human enterovirus, no.
2006 Aug–Dec	21 (41.2)	11 (21.6)	51
2007 Jan–Dec	5 (16.1)	0	31
2008 Jan–Dec	8 (26.7)	2 (6.7)	30
2009 Jan–Dec	0	0	17
2010 Jan–Apr	0	0	1
Total	34 (26.2)	13 (10.0)	130

Table 2. Demographic and clinical characteristics of sampled population with human enterovirus infections, People's Republic of China, August 2006–April 2010*

Characteristic	No. (%) persons		
	Coxsackievirus A21, n = 34	Enterovirus 68, n = 13	Any human enterovirus, n = 130
Sex			
M	18 (52.9)	7 (53.8)	69 (53.1)
F	16 (47.1)	6 (46.2)	61 (46.9)
Signs and symptoms			
Pharyngeal congestion	34 (100)	13 (100.0)	128 (98.5)
Headache	27 (79.4)	10 (76.9)	104 (80.0)
Myalgia	25 (73.5)	9 (69.2)	93 (71.5)
Chills	25 (73.5)	7 (53.8)	89 (68.5)
Sore throat	23 (67.6)	7 (53.8)	82 (63.1)
Rhinorrhea	17 (50.0)	5 (38.5)	50 (38.5)
Sneezing	14 (41.2)	3 (23.1)	44 (33.8)
Cough	7 (20.6)	3 (23.1)	19 (14.6)
Swelling of tonsils	6 (17.6)	1 (7.7)	16 (12.3)
Expectoration	4 (11.8)	0	6 (4.6)
Rigors	1 (2.9)	1 (7.7)	4 (3.1)
Preliminary clinical diagnosis			
Upper respiratory tract infection	33 (97.1)	13 (100.0)	126 (96.9)
Tonsillitis	0	0	2 (1.5)
Pulmonary infection	0	0	1 (0.8)
Other viruses co-detected	1 (2.9)†	1 (7.7)†	8 (6.2)‡

*Median age (range) of patients with coxsackievirus A21 infection, 22 (15–67) y; median age (range) of patients with enterovirus V68 infection, 34 (18–67) y; median age (range) of patients with any enterovirus infection, 27.5 (15–70) y. Median age of patients with coxsackievirus A21 infection was younger than that of patients with other human enterovirus infections (χ^2 14.7; p<0.01).
†Influenza virus A (n = 1).
‡Influenza virus A (n = 2), parainfluenza virus 3 (n = 2), rhinovirus (n = 2), metapneumovirus (n = 1), coronavirus OC43 (n = 1).

The reason for the high frequency of CVA21 and EV68 infections in Beijing is unclear. The EV68 strains identified in this study are probably novel strains or genetic variants to which the population is immune. Moreover, the deduced amino acid identity of the EV68 strains with strains detected in children in Japan (GenBank accession nos. AB601872–

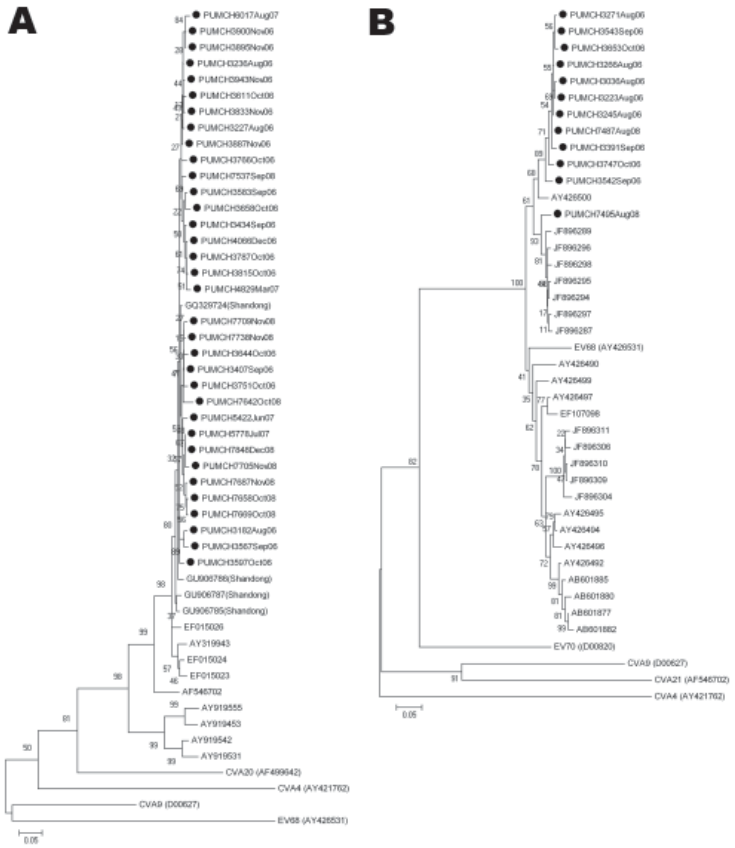

Figure 2. Phylogenetic analysis of human enteroviruses according to partial viral protein 1 (VP1) nucleotide sequences. The tree was generated with 1,000 bootstrap replicates. Neighbor-joining analysis of the targeted VP1 nucleotide sequence was performed by using the Kimura 2-parameter model with MEGA software version 4.0 (www.megasoftware.net). The scale bars indicate evolutionary distance. GenBank accession numbers for reference serotypes are indicated in parentheses. Each detected strain is indicated by black circles and a specific identification code followed by the patient number and the time of sampling. Strains detected by other research groups are indicated by a GenBank accession number. A) Phylogenetic tree of partial coxsackievirus (CV) A21 VP1 gene. The 375-bp fragments, which correspond to the locations of nt 2565–2939 of the CVA21 prototype strain (GenBank accession no. AF546702), were used to construct the tree. Enterovirus 68 (EV68), CVA9, CVA4, and CVA20 (GenBank accession nos. AY426531, D00627, AY421762, and AF499642, respectively) were used as outgroups. B) Phylogenetic tree of partial EV68 VP1 gene. The 390-bp fragments, which correspond to the locations of nt 2494–2883 of the EV68 prototype strain (GenBank accession no. AY426531), were used to construct the phylogenetic tree. CVA9, CVA4, CVA21, and EV70 (GenBank accession nos. D00627, AY421762, AF546702, and D00820, respectively) were used as outgroups.

AB601885) (*5*) was 89.4%–93.8%, indicating the possible co-existence of 2 distinct phylogenetic lineages of EV68 strains (*13*). The emergence of new genetic lineages might be the reason for increasing activity of EV68 (*5*,*13*). Because the median age of CVA21-positive patients was significantly lower than that of other HEV-positive patients (Table 2), we suspect that years of CVA21quiescence probably resulted in increased susceptibility of the younger population (*11*). Outbreaks of classical HEVs tend to occur in several-year cycles (*11*). We noted that CVA21 infections were concentrated in 2006 and 2008. However, because patients were not enrolled in our study after April 2010, our data on HEV circulation might be incomplete. Longer surveillance would be useful for determining the cycle of CVA21 and EV68 infections. Another limitation of this study is that samples were collected from outpatients but not hospitalized patients, which could bias the classification of illness toward being mild. However, this study presents evidence that CVA21 and EV68 cause mild disease and fills a void left by other studies that included only hospitalized patients.

In conclusion, we provide evidence that during some years in adults in Beijing, China, the rarely reported HEV serotypes CVA21 and EV68 are responsible for a substantial proportion of enterovirus-associated ARTIs. Our findings provide further insight into the pathogenesis of HEVs and increase our awareness of their clinical role. To predict possible outbreaks, global surveillance and investigation of HEV serotypes in patients with ARTIs should be strengthened.

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Unsuspected Rickettsioses among Patients with Acute Febrile Illness, Sri Lanka, 2007

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We studied rickettsioses in southern Sri Lanka. Of 883 febrile patients with paired serum samples, 156 (17.7%) had acute rickettsioses; rickettsioses were unsuspected at presentation. Additionally, 342 (38.7%) had exposure to spotted fever and/or typhus group rickettsioses and 121 (13.7%) scrub typhus. Increased awareness of rickettsioses and better tests are needed.

Globally, rickettsioses are increasingly recognized as causes of undifferentiated fever. Paired serum samples are infrequently obtained, but testing acute-phase serum alone is insensitive (IgG is initially absent) and nonspecific (IgG can persist for years, and IgM results represent cross-reactions).

Sentinel studies in Malaysia (*1*), Thailand (*2*), India (*3*), Laos (*4*), and Nepal (*5*) suggest that scrub and murine typhus are frequent and that misdiagnosis as enteric fever results in ineffective therapy (*5*). Unrecognized rickettsial species are likely present in Sri Lanka, an island connected to the southern tip of India by an underwater 30-km land bridge. Kularatne reported acute rickettsioses diagnosed by using only acute-phase serum IgM in 56 of 118 patients who had fever in hilly central Sri Lanka (*6*); another study in the Western Province confirmed few (5/31cases) of suspected rickettsioses (*7*). Both studies were limited by selective enrollment. To characterize rickettsioses among undifferentiated febrile illnesses in southern Sri Lanka, we prospectively studied patients who came to a large hospital.

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The Study

Consecutive patients ≥2 years of age with fever (≥38°C tympanic) who came to Teaching Hospital Karapitiya were enrolled (*8*). Standardized epidemiologic and clinical data and blood were obtained during acute illness and 2–4 weeks later. During the study (March–October 2007), the atmospheric temperature ranged from 27.5°C–32°C (high) to 24°C–26°C (low), and rainfall was variable (mean 301 mm/mo, range 36–657 mm/mo).

Because rickettsial species broadly cross-react within groups (*9*,*10*), paired serum samples were tested by using an IgG indirect immunofluorescence assay (IFA) and *Rickettsia rickettsii* and *R. typhi* antigens (Focus Diagnostics, Cypress, CA, USA) to identify infections with spotted fever group (SFGR) and typhus group (TGR) rickettsial infections. Serum samples reactive at a titer of 80 were considered potentially positive and were titered.

To identify scrub typhus (ST) infections, we tested paired serum samples using IgG ELISA as described (*11*), except for use of recombinant antigens (0.2 μg each of r56 Chimeric1, Gilliam, and Kato strains) to detect antibodies to *Orientia tsutsugamushi*. Comparative blind testing of 200 serum samples with an established (pooled-antigen) quantitative assay enabled validation (*12*).

Acute rickettsioses (SFGR, TGR, and ST) required a ≥4-fold rise in specific IgG titer or its equivalent; patients with equal SFGR and TGR convalescent-phase titers were SFGR/TGR group-indeterminate. IgG (titer ≥160) in acute-phase serum defined rickettsial exposure (seroprevalence). Stata IC version 11.0 (StataCorp LP, College Station, TX, USA) was used for analyses.

We analyzed paired serum samples for rickettsioses for 883 (81.9%) of 1,079 patients. Median acute–convalescent phase follow-up was 21 days (intraquartile range 15–33 days). Patients with and without paired serum samples were comparable (*8*). Acute rickettsioses were documented in 156 (17.7%) patients (Table 1). The increase in convalescent-phase geometric mean titer was 14-fold (845) for SFGR, 17-fold (920) for TGR, and 11-fold (951) for SFGR/TGR rickettsiae.

Acute rickettsioses were found in 19.7% of patients ≥18 years of age and 10.5% of patients <18 years of age ($p = 0.003$); patients with rickettisoses were older than those without rickettsioses (median age 34.5 vs. 28.0 years; $p = 0.005$) (Figure 1). Among patients <18 years of age, acute rickettsioses were more common in male than in female patients (14.6% vs. 5.8%; $p<0.05$). Patients with acute ST alone were older than those with other rickettsioses and those without rickettsioses (median 36.5 vs. 34.4 vs. 28.0 years; $p = 0.02$) and more likely to report rice paddy

Table 1. Clinical and laboratory characteristics of febrile patients with and without acute rickettsial infections, southern Sri Lanka, 2007*

Characteristic	Rickettsial infection						
	None, n = 727	SFGR, n = 86	TGR, n = 29	*Orientia tsutsugamushi*, n = 9	SFGR/TGR, n = 24	SFGR or TGR + *O. tsutsugamusi,* n = 8	p value†
Median age, y (IQR)	28 (18–47)	31 (22–46)	44 (21–59)	37 (19–58)	39 (22–48)	37 (22–56)	0.06
Male sex	86	8	2	0.3	2	1	0.10
Admitted to hospital	71	78	69	89	71	88	0.51
Symptoms							
Headache	78	79	79	67	79	63	0.86
Sore throat	29	28	21	22	29	13	0.82
Cough	60	49	54	56	30	63	0.048
Dyspnea	18	8	21	22	29	25	0.14
Joint pain	44	41	52	44	52	57	0.83
Muscle pain	48	52	57	56	42	43	0.83
Fatigue	66	69	66	56	71	63	0.96
Abdominal pain	19	19	21	33	30	0	0.43
Emesis	37	36	34	44	54	43	0.65
Diarrhea	12	9	7	22	13	0	0.66
Dysuria	13	23	7	22	25	13	0.07
Oliguria	8	17	3	11	21	13	0.02
Signs							
Conjunctivitis	13	21	24	22	13	50	0.008
Throat exudate	15	12	21	0	8	0	0.41
Lymphadenopathy	23	20	14	22	21	38	0.74
Jaundice	2	0	4	0	0	0	0.70
Pulmonary rales	15	5	14	22	8	0	0.09
Tender abdomen	10	8	10	0	17	0	0.63
Hepatomegaly	5	6	3	22	0	13	0.19
Rash‡	9	15	0	50	0	0	0.18
Laboratory measures, mean ± SD							
Leukocytes, cells/μL	8,893 ± 4,634	8,253 ± 3,774	10,341 ± 7,931	9,778 ± 4,800	9,129 ± 3,925	9,943 ± 4,527	0.96
ANC, cells/μL	6,325 ± 4,054	5,825 ± 3,263	7,114 ± 4,905	7,280 ± 3,890	6,494 ± 3,887	6,514 ± 3,762	0.57
ALC, cells/μL	2,317 ± 1,234	2,284 ± 1,378	2,822 ± 3,170	2,380 ± 1,291	2,460 ± 1,215	3,036 ± 1,526	0.33
Hemoglobin, g/dL	12.6 ± 1.7	12.6 ± 1.5	12.7 ± 1.6	13.0 ± 2.2	12.7 ± 1.6	12.4 ± 2.0	0.98
Platelets × 1,000/μL	249 ± 99	228 ± 84	237 ± 81	187 ± 74	219 ± 72	301 ± 119	0.05

*Values are % patients except as indicated. SFGR, spotted fever group rickettsiae; TGR, typhus group rickettsiae; IQR, intraquartile range; ANC, absolute neutrophil count; ALC, absolute lymphocyte count.
†Kruskal-Wallis test to compare proportions and non-normally distributed continuous variables. Analysis of variance test to compare normally distributed continuous variables.
‡n = 309; 6/6 with rickettsioses and rash had eschars, compared with 11/22 of those without rickettsioses (p<0.001).

exposure (44.4% vs. 15.1% vs. 9.3%; p = 0.001). More acute rickettsioses occurred during the July–October rains (70.6% of ST infections and 59.7% of other rickettsioses), whereas more nonrickettsial infections (67.0%) occurred during the drier period of March–June (p<0.0001) (Figure 2).

Acute rickettsioses were clinically similar to each other and to nonrickettsial febrile illnesses, except for frequency of cough, oliguria, and conjunctival suffusion (Table 1). Except for a higher temperature with SFGR than with TGR infection (mean 38.6 vs. 38.2°C; p = 0.006), no feature differentiated these rickettsial infections. Conjunctival suffusion was more common (p = 0.004) with ST (35.3%) than with SFGR/TGR (12.8%) or no rickettsiosis (12.8%).

Antecedent antimicrobial drug use was commonly reported in patients with (45/115 [39.1%]) and without (195/536 [36.4%]; p = 0.58) rickettsioses. Amoxicillin and cephalosporins were administered most frequently in both groups (16.8% and 20.3%; p = 0.40), but infrequent administration of doxycycline (0.9% and 1.1%; p = 0.82) was recorded for 813 patients with paired serum samples, including 139 with acute rickettsioses. Rickettsioses were rarely clinically identified when present (3/139, sensitivity 2.2%, 95% CI 0.447%– 6.2%). Rickettsioses were also infrequently confirmed when diagnosed clinically (3 SFGR rickettsioses among 9 with suspected scrub typhus; positive predictive value 33.3%, 95% CI 7.5– 70.1) and rarely treated appropriately (2/9 given doxycycline, 1 with SFGR rickettsiosis). Patients with rickettsioses were hospitalized longer than others (median 5 vs. 4 days; p = 0.01), although the proportion hospitalized was similar. No one with confirmed rickettsioses died, but 11 of 12 patients died before follow-up.

At enrollment, 292 (33.1%) patients had IgG-confirmed rickettsial exposure. However, only 59 (20.2%) had a 4-fold increase in titer, and 97 (62.2%) of 156 acute infections were identified as rickettsioses by seroconversion. Exposures to rodents and rice paddies were associated with TGR rickettsiae and *O. tsutsugamushi* IgG (Table 2). Farmers were more likely (p = 0.01) to have IgG against

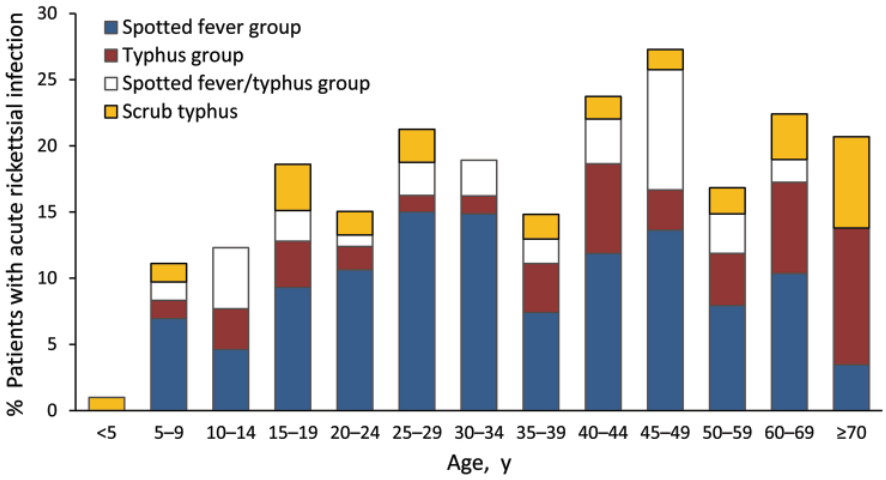

Figure 1. Proportion of febrile patients with acute rickettsial infections, by age group, southern Sri Lanka, 2007.

O. tsutsugamushi (5.7%) than SFGR/TGR rickettsiae IgG alone (4.3%) or no rickettsial IgG (1.5%). If IgG titer ≥160 at either sampling were used to denote rickettsial exposure (preexisting or acute), 342 (38.7%) patients had exposure to SFGR and/or TGR rickettsiae. If a lower titer (≥80) were used, 398 (45.1%) had such exposure. Similarly, 121 (13.7%) patients had either acute- or convalescent-phase IgG against *O. tsutsugamushi*.

Conclusions

We documented endemic rickettsioses as a major cause of acute febrile illness in southern Sri Lanka. Epidemiologic features could not differentiate acute infection from prior exposure. Rickettsioses were infrequently suspected and not treated empirically when suspected. Underrecognition of rickettsioses could reflect nonspecific clinical features, limited diagnostic tools, lack of awareness that rickettsioses occur or cause severe illness, and absence of evidence-based local algorithms for acute febrile illness.

Studies in neighboring regions have used flawed methods, including spectrum bias, small sample size, selective enrollment, and testing acute-phase serum only. In many instances, clinical features were not predictive (*5*,*13*); however, older age was reported with rickettsioses (*14*) and lymphadenopathy and rapid respiratory rate with ST compared with TGR rickettsiosis (*4*). Although part of the rickettsial triad, rash is often absent initially (*10*) and among unselected febrile patients (*5*,*14*). SFGR/TGR cross-reactions and apparent co-infections could also impair clinical differentiation of specific rickettsioses. Laboratory abnormalities associated with ST (*3*) could reflect disease severity, not etiology, and such testing is often unavailable. Divergent conclusions might reflect different study populations, diagnostic criteria, reference groups, features evaluated, or real geographic differences.

Our estimate of rickettsioses is conservative. Confirmation required follow-up (return or home visit); the US Centers for Disease Control and Prevention no

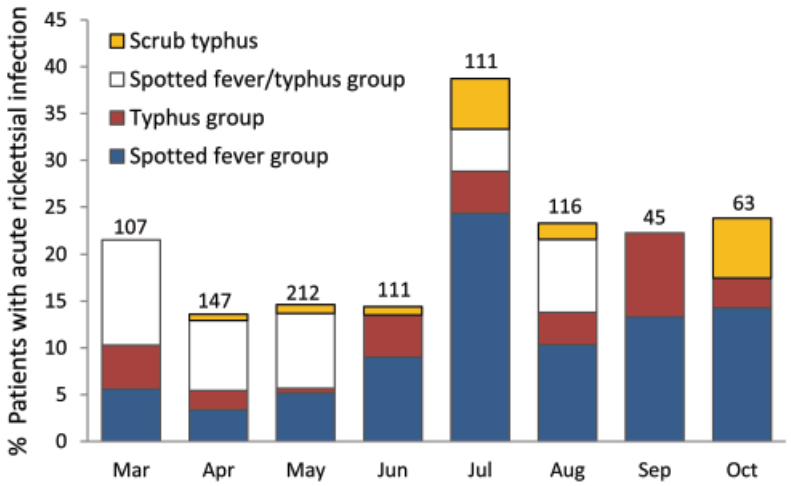

Figure 2. Proportion of febrile patients with acute rickettsial infections, by month, southern Sri Lanka, March–October 2007.

Table 2. Epidemiologic characteristics of patients with and without IgG evidence of rickettsial exposure at enrollment, southern Sri Lanka, 2007*

Characteristic	Negative rickettsial titer, n = 591	SFGR, n = 76	TGR, n = 12	*Orientia tsutsugamushi*,† n = 106	SFGR/TGR, n = 98	p value‡
Median age, y (IQR)	26 (16–44)	30 (23–48)	25 (19–48)	46 (34–56)	34 (23–55)	0.0001
Male sex	42	33	17	38	36	0.21
Residence						0.03
Urban	6	13	9	13	12	
Rural	94	87	91	87	88	
Type of work						<0.001
Homemaker	26	26	18	32	24	
Laborer	21	26	45	35	28	
Farmer	2	1	9	6	6	
Merchant	2	3	9	6	4	
Student	28	11	9	6	19	
Other	21	34	9	16	20	
Exposure						
Dog	57	46	50	57	44	0.10
Cat	35	26	25	30	30	0.43
Rodent	27	22	83	35	19	<0.001
Cow	5	4	17	5	6	0.48
Paddy field	10	8	0	18	8	0.07
Water source						0.03
Tap	29	45	17	29	35	
Boiled	13	1	8	8	4	
Well	57	54	75	65	60	
Other	1	0	0	1	1	

*Values are % patients unless otherwise indicated. SFGR, spotted fever group rickettsiae; TGR, typhus group rickettsiae; IQR, interquartile range.
†Includes 8 patients with apparent SFGR or TGR co-infections.
‡Kruskal-Wallis test to compare proportions across groups.

longer accepts a single high titer to confirm *R. rickettsii* infection (*10*). We required a 4-fold rise in titer even for seroconversions because IFA results are subjective, even among experts. We used *R. rickettsii* as a surrogate antigen, which could be less sensitive for detecting local SFGR. We chose ELISA for ST because a commercial IFA was not available and IFA as a standard for ST has been questioned (*15*).

Optimal clinical management of acute rickettsioses requires development of locally tested, evidence-based algorithms for acute febrile illness. Better diagnostic tests are needed to identify new species, elucidate vector–host relationships, and enable appropriate therapy. Molecular approaches hold promise but will require prospective validation.

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Origin of Human T-Lymphotropic Virus Type 1 in Rural Côte d'Ivoire

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Simian T-lymphotropic virus type 1 (STLV-1) strains occasionally infect humans. However, the frequency of such infections is unknown. We show that direct transmission of STLV-1 from nonhuman primates to humans may be responsible for a substantial proportion of human T-lymphotropic virus type 1 infections in rural Côte d'Ivoire, where primate hunting is common.

Human T-lymphotropic virus type 1 (HTLV-1) can induce adult T-cell leukemia or lymphoma and HTLV-1–associated myelopathy or tropical spastic paraparesis. These pathologies are a serious threat to the several million persons infected with HTLV-1 (*1*).

Although HTLV-1 has spread globally, its geographic distribution is not uniform. Most infected persons live in areas where the virus is endemic and seroprevalence is comparatively high (>1%) (*1*), i.e., in Japan, Melanesia, South America, the Caribbean, and sub-Saharan Africa. Phylogenetic analyses demonstrate that the geographic distribution of HTLV-1 genetic diversity is also not uniform. The most genetic diversity is seen in sub-Saharan Africa, where 6 of the 7 human molecular subtypes (HTLV-1A, B, D, E, F, and G) are found. Of those 6 subtypes, 5 are mainly found in or endemic to central Africa: HTLV-1B, D, E, F, and G (*1*).

Molecular HTLV-1 subtypes from humans in central Africa belong to composite clades that comprise HTLV-1 strains and simian T-lymphotropic virus type 1 (STLV-1) strains derived from nonhuman primates (*2*). Nonhuman primates in Africa are considered to be the source of recurrent zoonotic transmissions of STLV-1 to local human populations; virus transmission is believed to occur during the collection and consumption of nonhuman primate bushmeat. This belief is supported by the fact that self-reported nonhuman primate hunters in Cameroon were infected with viruses closely related to STLV-1 strains circulating among local nonhuman primate prey (*3*). However, because intrafamilial transmission of HTLV-1B and -1D was also documented among hunters-gatherers in Cameroon (*4*), it is impossible to sort out cases of direct zoonotic transmission of STLV-1 from cases of consecutive human-to-human spread of virus (evolutionary rates for HTLV-1/STLV-1 are very slow) (*5*).

However, HTLV-1 and STLV-1 strains from western African segregate clearly in phylogenetic analyses; most humans are infected with HTLV-1A, the only human-restricted molecular subtype (*6*–*9*). Therefore, in western compared with central Africa, human infections with viruses closely related to local STLV-1 strains are much more likely to reflect direct zoonotic transmission. This situation enabled us to investigate the frequency of such direct zoonotic transmissions in a rural region of Côte d'Ivoire neighboring the Taï National Park (Figure 1).

The Study

During 2006–2007, blood samples were obtained from 776 volunteers living in 18 villages bordering Taï National Park. All participants signed informed consent forms and completed questionnaires aimed at determining their exposure to nonhuman primate bushmeat through activities such as hunting of nonhuman primates or consumption of nonhuman primate bushmeat.

To determine effective exposure to HTLV-1/STLV-1, we used an HTLV-1/2 ELISA to test serum samples for reactivity to HTLV-1/2 antigens (*10*). Of the 776 serum samples, 16 were positive according to the ELISA manufacturer's criteria; an additional 15 samples had values just below the cutoff. We extracted DNA from all ELISA-reactive samples and performed a search for HTLV-1/STLV-1 sequences by using a *tax*-specific quantitative PCR (*8*). Of the 31 samples, 10 were positive and were analyzed by using a multiplex nested/seminested PCR targeting *env* and long terminal repeat (LTR) sequences (Table 1; online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1663-Techapp.pdf). To identify multiple infections with HTLV-1/STLV-1, this assay was applied on near endpoint dilutions of the 10 DNA extracts (2–6 starting template molecules per reaction; online Technical Appendix). For each person, 6–20 *env* and 2–20 LTR sequences (15–40 sequences per person) were determined by Sanger sequencing. No evidence of multiple infections was found.

Phylogenetic analyses were performed by using Bayesian and maximum likelihood methods on *env* and LTR datasets (online Technical Appendix). Both methods agreed on all essential features of the LTR tree topology

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Figure 1. Sampling zone in study of the origin of human T-lymphotropic virus type 1 in rural western Africa, 2006–2007. Taï National Park is indicated in white on the gray background of Côte d'Ivoire. The black rectangle overlapping Taï National Park defines a zone encompassing the 18 villages where study participants resided. Village names and the number of participants are as follows: Daobly (38), Djereoula (31), Djiboulay (40), Gahably (55), Gouléako (37), Goulégui-Béoué (55), Kéibly (90), Pauléoula (20), Ponan (21), Port-Gentil (47), Sakré (75), Sioblooula (35), Taï (26), Tieleoula (40), Zagné (17), Zaïpobly (37), Ziriglo (74).

(Figure 2) and *env* tree topology (online Technical Appendix Figure). Six of the newly determined HTLV-1 sequences were unambiguously related to HTLV-1A (bootstrap, 94; posterior probabilities, 1) (Figure 2), confirming the predominance of this molecular subtype in Côte d'Ivoire and in western Africa (*6*). Another 3 HTLV-1 sequences were closely related to STLV-1 sequences found in sooty mangabeys (*Cercocebus atys*) from Taï National Park (bootstrap, 83; posterior probabilities, 1) (Figure 2; online Technical Appendix Table 2), whereas the last 1 was related to STLV-1 sequences from red colobus monkeys (*Piliocolobus badius badius*) and chimpanzees (*Pan troglodytes verus*) from Taï National Park (bootstrap, 98; posterior probabilities, 1) (Figure 2; online Technical Appendix, Table 2) (*7*,*8*). Bayesian analyses were run under the assumption of a molecular clock and calibrated. However, reliable divergence dates could not been determined because most shallow nodes of the trees, including those of interest here, were not supported (online Technical Appendix). Observed divergences, however, seemed compatible with cross-species transmission events, particularly in the case of study participant Pau009 (divergence to closest STLV-1, 0% in LTR and 0.2% in *env*) (Table 1).

Conclusions

We investigated the frequency of direct zoonotic transmission of STLV-1 in a rural region of Côte d'Ivoire neighboring Taï National Park and found that only 2 of the STLV-1–related sequences would be compatible with a local human-to-human transmission (Gah050 and Kei005; Figure 2). Therefore, our data support the notion that direct zoonotic transmissions of STLV-1 represent a measurable proportion of HTLV-1 infections, at least in rural regions bordering nonhuman primate habitat. In addition, these results mirror observations made among adult chimpanzees from Taï National Park, which are often infected with retroviruses (i.e., simian foamy viruses and STLV-1) of their prey (Figure 2) (*7*,*11*).

Despite the high prevalence of STLV-1, simian foamy virus, and simian immunodeficiency virus infections among red colobus populations (*8*) and the fact that this nonhuman primate species is the one most frequently hunted by humans (*4*), most zoonotic transmissions of retroviruses in western Africa seem to originate from sooty mangabeys, as shown here for STLV-1 and previously described for simian immunodeficiency virus of sooty mangabeys, the precursor of HIV-2 (*12*). It remains to be determined whether these zoonotic transmissions from sooty mangabeys are favored as a result of molecular determinants (e.g., convergent

Table 1. Characteristics of persons positive for HTLV-1 or STLV-1 in a study of the origin of HTLV-1, rural western Africa, 2006–2007*

Study participant†, sex	Infecting subtype	Minimum observed distance to any STLV-1, %		Type of contact and nonhuman primate contacted			
		LTR	*env*	Hunting	Dismembering	Preparation or cooking	Eating
Gah050, M	STLV-1I/SM	0.6	0.4	None	Monkeys, chimp	Monkeys, chimp	Monkeys, chimp
Gul014, F	HTLV-1A	3.2	2.9	None	None	Monkey, chimp	Monkeys, chimp
Kei005, F	STLV-1I/SM	0.6	0.5	None	Monkeys	Monkeys	Monkeys
Kei025, M	STLV-1J	0.3	0.2	None	Monkeys	None	Monkeys
Kei075, F	HTLV-1A	3.0	3.0	None	None	Monkeys	Monkeys
Pau002, F	HTLV-1A	3.2	2.5	None	None	Monkeys	Monkeys
Pau009, M	STLV-1I/SM	0	0.2	Monkeys, chimp	Monkeys, chimp	None	Monkeys, chimp
Pon002, F	HTLV-1A	4.2	2.8	None	Monkeys	Monkeys	Monkeys
Tie005, F	HTLV-1A	4.0	2.5	None	Monkeys	Monkeys	Monkeys
Tie011, F	HTLV-1A	4.5	2.4	None	Monkeys	Monkeys	Monkeys

*Gray shading indicates infections with STLV-1–like HTLV-1 (as determined through phylogenetic analyses). Minimum distances were calculated by using the same datasets as for phylogenetic analyses (see online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1663-Techapp.pdf). HTLV-1, human T-lymphotropic virus type1; STLV-1, simian T-lymphotropic virus type1; LTR, long terminal repeat; chimp, chimpanzee(s).
†First 3 letters refer to the persons' village of residence.

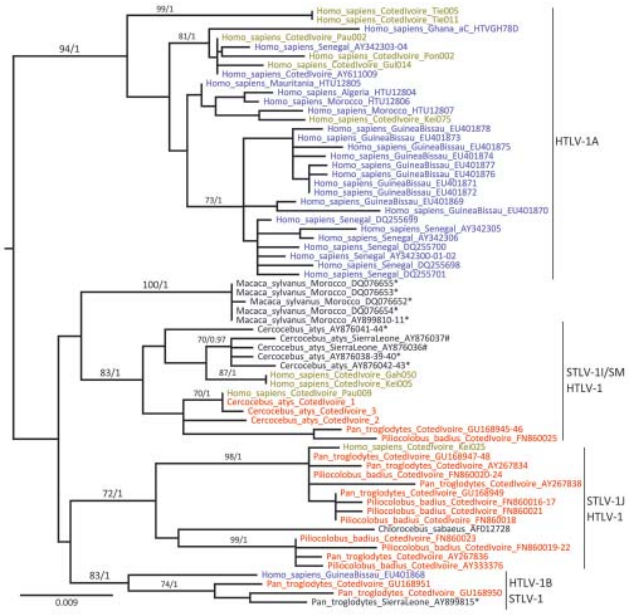

Figure 2. Maximum-likelihood tree based on the analysis of a long terminal repeat (853 bp) alignment in a study of the origin of human T-lymphotropic virus type 1 (HTLV-1) in rural western Africa, 2006–2007. Bayesian analyses supported similar topologies. After rooting, branches leading to outgroup sequences (HTVMEL5 and Z46900) were removed from the figure to increase its legibility. The HTLV-1 sequences determined from specimens from West and North Africa are shown in light blue; HTLV-1 sequences determined from persons living in the region of Taï National Park are shown in green; STLV-1 sequences determined from specimens from West and North Africa are shown in black; and STLV-1 sequences determined from persons living in the Taï National Park are shown in red. Sequence names are built as follows: [host species]_[country of origin]_[GenBank accession number]. Reference sequence names also include their molecular subtype assignation: [host species]_[country of origin]_[molecular subtype]_[accession number]. *Sequences determined from captive or semicaptive hosts; #sequences determined from bushmeat samples. Molecular subtypes were assigned on the basis of an analysis performed on an enlarged dataset including assigned reference sequences (data not shown). Bootstrap (Bp) and posterior probability (pp) values are indicated where Bp≥50.0 and pp≥0.95. Scale bar indicates nucleotide substitutions per site.

evolution of retroviral receptors) or behavioral determinants (e.g., increased aggressiveness).

Considering the human exposure to nonhuman primate bushmeat in this region (as illustrated by ≈150,000 kg sold per year in markets) (*13*) and given the high prevalence of STLV-1 among local nonhuman primates (*7*,*8*), the observation that zoonotic transmission events are, in absolute terms, exceedingly rare is striking. Yet, the accumulation of genetically distinct HTLV-1/STLV-1 over restricted geographic areas remains possible, as illustrated by the finding of 1 person infected with HTLV-1A and 2 persons infected with putative STLV-1 in a single village, Kéibly (Table 1; Figure 1). Such local accumulations add to the threat represented by direct transmissions of STLV-1 because they can provide an opportunity for recombinant viruses to emerge, even though HTLV-1/STLV-1 biology may be unfavorable to recombination (*14*).

The analysis of behavioral data reveals generalized exposure of local populations to cooked nonhuman primate bushmeat (Table 2). Exposure to fresh tissues, which can be expected to be more risky in terms of retroviral transmission, is less common (Table 2). Along a gradient of bushmeat freshness, going from hunting to preparation and cooking, a clear reversal of sex-related skew can be observed: only men are hunters, men and women are equally involved in dismembering, and women predominantly prepare and cook nonhuman primate bushmeat (Table 2). Hence, men likely constitute a population at risk. In our study, 75% of persons who were identified as infected with viruses closely related to STLV-1 were men, whereas all HTLV-1A–infected persons were women. Increased surveillance for zoonotic transmission of STLV-1 to humans in areas where such transmission is more likely and increased surveillance of nonhuman primate species with high transmission potential (like sooty mangabeys) will contribute to a better understanding of risk factors.

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Table 2. Type of nonhuman primate contact by participants in a study of the origin of HTLV-1 in rural western Africa, 2006–2007*

Variable	Activity resulting in contact			
	Hunting	Dismembering	Preparing or cooking	Eating
Women, n = 402	0%	62.4%	66.9%	81.3%
Men, n = 371	11.6%	63.6%	21.6%	90.8%
Relative exposure, men vs. women	NA	1.02	0.32	1.12

*Sex assignation was lost for 3 persons; thus, the total sampling size was 773 rather than 776, the total number included in the study. HTLV-1, human T-lymphotropic virus type1; NA, not applicable.

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Human Infections with Novel Reassortant Influenza A(H3N2)v Viruses, United States, 2011

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During July–December 2011, a variant virus, influenza A(H3N2)v, caused 12 human cases of influenza. The virus contained genes originating from swine, avian, and human viruses, including the M gene from influenza A(H1N1)pdm09 virus. Influenza A(H3N2)v viruses were antigenically distinct from seasonal influenza viruses and similar to proposed vaccine virus A/Minnesota/11/2010.

Around the world, cases of human infection with swine-origin influenza viruses have been reported sporadically (*1*–*5*). From 1990 through 2010, a total of 27 cases of human infection with these viruses were confirmed by the US Centers for Disease Control and Prevention (CDC) (*4*,*6*). Of these cases, 21 were caused by triple-reassortant influenza A viruses (13 subtype H1N1, 1 subtype H1N2, and 7 subtype H3N2), which have inherited genes from classical swine, avian, and human influenza viruses. The 2009 influenza pandemic, caused by a variant triple reassortant influenza virus, influenza A(H1N1)pdm09 virus (*7*,*8*), proved that swine influenza viruses (SIVs) can cause widespread infection among humans and result in substantial economic costs. In 2010, an increase in the number of human cases of swine-origin influenza (H3N2) virus infection prompted selection of a candidate vaccine virus of swine origin, A/Minnesota/11/2010 (H3N2)v (*9*).

Systematic surveillance and characterization of novel viruses infecting humans and SIVs in swine are critical for early detection of viruses with pandemic potential. Since 2009, CDC has provided public health laboratories with a real-time reverse transcription PCR (rRT-PCR)–based assay for diagnostic testing for influenza (*10*). This assay enables detection and discrimination of influenza A virus subtypes H1N1, H3N2, and H1N1pdm09 and preliminary identification of triple-reassortant viruses possessing the nucleoprotein gene originating from SIVs.

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The Study

In 2011, public health laboratories in 5 states detected 12 cases of human infection with a novel variant of influenza virus, influenza A(H3N2)v virus, by using the CDC rRT-PCR protocol. Respiratory specimens from these patients were sent to CDC for virus confirmation. History of direct or indirect contact with swine was confirmed for 6 patients. However, swine contact could not be verified for the other 6, suggesting that these infections might have been contracted through limited person-to-person transmission (*11*–*13*) (Table 1). All 12 patients recovered fully from their illness (*10*–*12*).

Genetic sequence analysis of RNA isolated from clinical respiratory specimens (Table 1) revealed that these influenza A(H3N2)v viruses possessed a combination of gene segments not previously found in humans (Figure 1). Of the 8 gene segments, 7 (hemagglutinin, neuraminidase, polymerase basic proteins 1 and 2, polymerase acidic protein, nucleoprotein, and nonstructural protein) were similar to those of triple-reassortant SIV A(H3N2) currently circulating in North America and to those from human triple-reassortant influenza A(H3N2) viruses isolated in 2010 from Pennsylvania, Minnesota, and Wisconsin (*4*), including the proposed vaccine virus of swine origin, A/Minnesota/11/2010 (*14*) (Figure 2, panel A; online Technical Appendix Figure, wwwnc.cdc.gov/EID/pdfs/11-1922-Techapp.pdf). However, the M genes of all 2011 influenza A(H3N2)v viruses were inherited from a pandemic (H1N1) 2009 virus (Figure 2, panel B). Although SIVs of subtypes A(H3N2) and A(H1N2) with the M gene of influenza A(H1N1)pdm09 virus have been detected in swine since 2009 (*15*), influenza A(H3N2)v virus possessing the M gene of influenza A(H1N1)pdm09 virus had not been detected in humans.

According to genetic analysis results, amino acid diversity among influenza A(H3N2)v hemagglutinins was low (0–3 aa) compared with that of influenza A/Minnesota/11/2010. In addition, there have been no conserved amino acid changes in the hemagglutinin when comparing 2011 influenza A(H3N2)v from humans with 2011 influenza A(H3N2) SIVs. In particular, the known receptor binding site of the hemagglutinin protein of influenza A(H3N2)v virus was typical of SIV A(H3N2) viruses recently isolated in North America.

Hemagglutinins of the influenza A(H3N2)v viruses differed substantially from the hemagglutinin of the 2011–12 human seasonal vaccine virus, A/Perth/16/2011

Table 1. Results of analysis of viral RNA isolated from original clinical samples from persons with influenza A(H3N2)v virus infection, United States, 2011*

Influenza virus strain	Contact with swine (*11–13*)	Specimen collection date	Specimen type	rRT-PCR–positive results†	Genes sequenced‡
A/Indiana/08/2011	No	Jul 24	NPS	InfA, H3, pdmInfA	Full genome
A/Pennsylvania/09/2011	Indirect	Aug 20	NPS	InfA, H3, pdmInfA	Full PB2, PB1, HA, NP, NA, M, NS, partial PA
A/Pennsylvania/10/2011	Direct	Aug 26	NPS	InfA	Full NS, partial HA, M, NA
A/Pennsylvania/11/2011	Indirect	Aug 25	NPS	InfA, H3, pdmInfA	Full PA, NP, NA, M NS, partial PB2, PB1, HA
A/Maine/06/2011	Direct	Oct 10	NPS	InfA, H3, pdmInfA	Full genome
A/Indiana/10/2011§	Direct	Oct 22	Cell culture	InfA, H3, pdmInfA	Full genome
A/Maine/07/2011	Direct	Oct 24	NPS	InfA	Partial HA, M, NS
A/Iowa/07/2011	No	Nov 14	NPW	InfA, H3, pdmInfA	Full PB2, PB1, PA, NP, NA, M, NS, partial HA
A/Iowa/08/2011	No	Nov 14	NS	InfA, H3, pdmInfA	Full genome
A/Iowa/09/2011	No	Nov 14	NS	InfA, H3, pdmInfA	Full genome
A/West Virginia/06/2011	No	Nov 21	NW	InfA, H3, pdmInfA	Full genome
A/West Virginia/07/2011	No	Dec 07	NPS	InfA	Partial HA, NA, M

*Influenza A(H3N2)v, influenza virus variant identified in humans; rRT-PCR, real-time reverse transcription PCR; NPS, nasopharyngeal swab; PB, polymerase basic protein; HA, hemagglutinin; NP nucleoprotein; NA, neuraminidase; M, matrix protein; NS, nonstructural protein; PA, polymerase acidic protein; NPW, nasopharyngeal wash; NS, nasal swab.
†Results obtained by using the Centers for Disease Control and Prevention Human Influenza Virus Real-Time RT-PCR Diagnostic Panel.
‡Sequences available from GenBank and the online Technical Appendix Table (wwwnc.cdc.gov/EID/pdfs/11-1922-Techapp.pdf).
§This patient was >18 years of age; all others were <18.

(58–60 aa), which resulted from divergent evolutionary paths for the H3 hemagglutinin in swine and human viruses. The effect of these substitutions on virus antigenicity was examined in the hemagglutination-inhibition assay by using a panel of reference ferret antiserum. Hemagglutination-inhibition analysis of 6 available influenza A(H3N2)v virus isolates revealed no measureable inhibition by antiserum against the current human seasonal influenza A(H3N2) vaccine virus, A/Perth/16/2009 (Table 2), indicating that influenza A(H3N2)v virus is antigenically distinct from influenza A(H3N2) viruses currently circulating among humans.

All influenza A(H3N2)v viruses tested were antigenically similar, demonstrating hemagglutination-inhibition titers with only a 2-fold difference from antiserum against other influenza A(H3N2)v viruses. These viruses were also antigenically closely related to earlier human triple-reassortant virus isolates that contained the M gene from classical SIVs (A/Wisconsin/12/2010, A/Pennsylvania/14/2010, and A/Minnesota/11/2010). All influenza A(H3N2)v viruses tested were also antigenically closely related to the proposed vaccine reassortant X-203 (*13*) between triple-reassortant A/Minnesota/11/2010 (H3N2) and A/PR/8/34 (H1N1) (Table 2).

The level of cross-protective immunity against influenza A(H3N2)v in humans previously vaccinated and/or exposed to previously circulated seasonal influenza A(H3N2) viruses is unknown. The antigenic

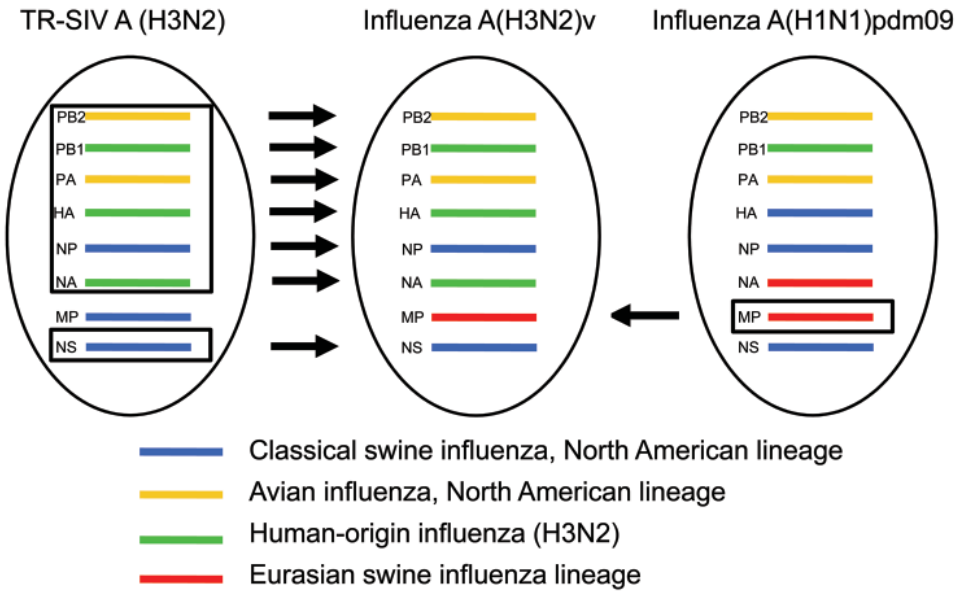

Figure 1. Derivation of genes segments of novel influenza A(H3N2) viruses isolated from humans, United States, 1990–2011. TR-SIV, triple reassortment swine influenza virus.

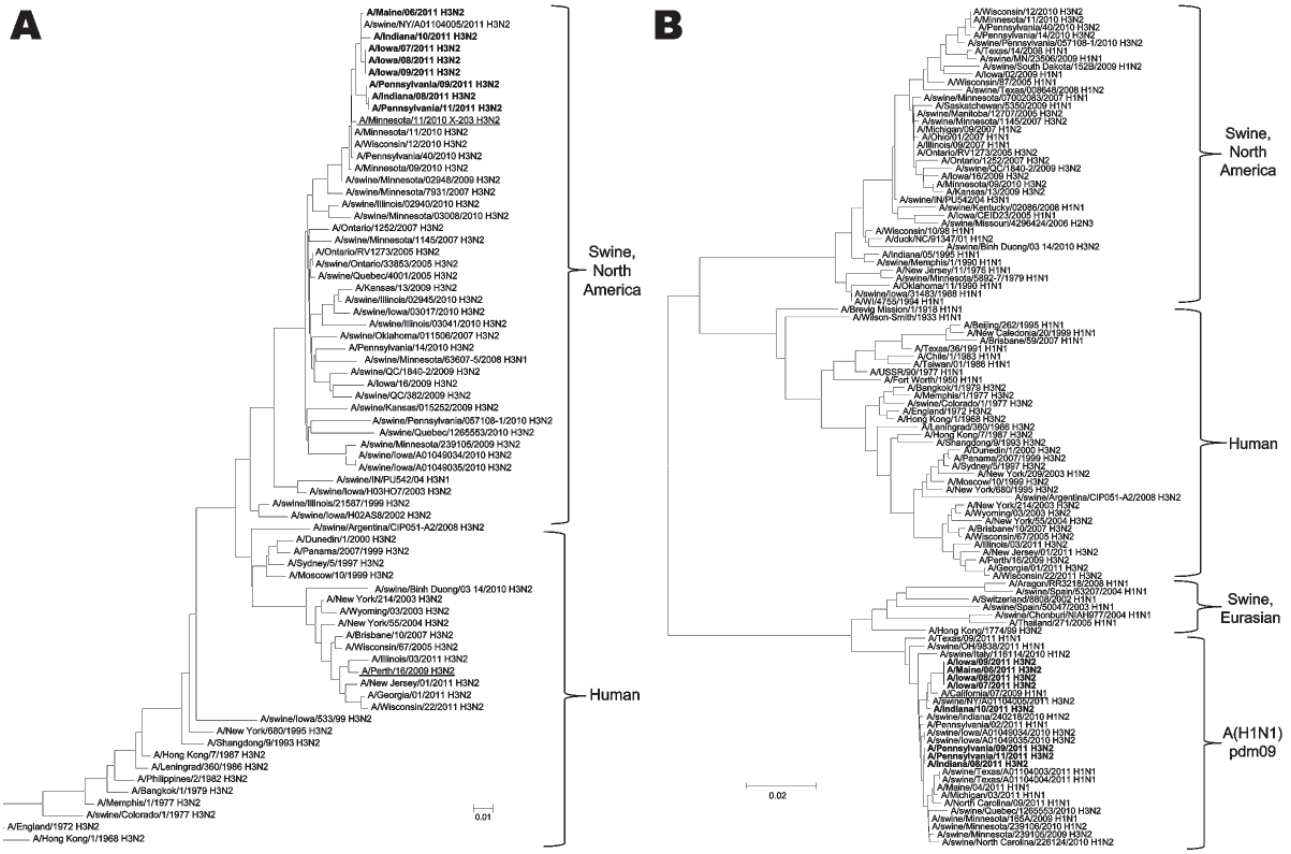

Figure 2. Phylogenetic analysis of the A) hemagglutinin and B) matrix genes of influenza A(H3N2)v viruses. Sequences obtained from human A(H3N2)v isolates in the United States during 2011 are shown in **boldface**; sequences of proposed vaccine virus are underlined. Scale bars indicate number of base substitutions per site.

characterization described here demonstrates that vaccination with the current trivalent influenza vaccine might not provide immune protection against influenza A(H3N2)v virus. A vaccine containing a contemporary influenza A(H3N2)v or an antigenically similar virus (such as A/Minnesota/11/2010) might be needed to elicit protective immunity.

Functional neuraminidase inhibition assays indicated that 6 influenza A(H3N2)v virus isolates were sensitive to the neuraminidase inhibitors oseltamivir and zanamivir. No genetic markers known to decrease sensitivity to neuraminidase inhibitors were found in the neuraminidase genes of all 12 influenza A(H3N2)v viruses. Similar to pandemic (H1N1) 2009 viruses, influenza A(H3N2)v

Table 2. Hemagglutinin-inhibition assay results, including the 6 available influenza A(H3N2)v viruses isolated in 2011, United States*

Influenza virus strain (culture method)	Titers to reference ferret antiserum								Specimen collection date
	PER/16	KS/13	WI/12	PA/14	MN/11	X203	IN/08	IN/10	
A/Perth/16/2009 (egg)†	1,280	<10	<10	<10	20	20	<10	<10	2009 Apr 7
A/Kansas/13/2009 (MDCK cells)	<10	640	80	160	40	40	40	80	2009 Jul 29
A/Wisconsin/12/2010 (MDCK cells)	<10	40	1,280	320	640	320	640	1,280	2010 Sep 10
A/Pennsylvania/14/2010 (egg)	<10	160	320	640	320	320	640	640	2010 Oct 26
A/Minnesota/11/2010 (egg)	<10	<10	320	160	640	1,280	320	160	2010 Nov 26
A/Minnesota/11/2010 X-203 (egg)‡	10	<10	80	40	320	640	160	80	Not applicable
A/Indiana/08/2011 (MDCK cells)*	<10	10	1,280	640	640	320	1,280	1,280	2011 Jul 24
A/Indiana/10/2011 (MDCK cells)*	<10	40	1,280	320	1,280	640	1,280	1,280	2011 Oct 22
A/Indiana/10/2011 (egg)*	<10	10	1,280	320	640	320	1,280	1,280	2011 Oct 22
A/Iowa/07/2011 (MDCK cells)*	<10	10	1,280	640	1,280	640	1,280	2,560	2011 Nov 14
A/Iowa/08/2011 (MDCK cells)*	<10	40	1,280	640	640	640	1,280	2,560	2011 Nov 14
A/Iowa/09/2011 (MDCK cells)*	<10	40	1,280	640	1,280	640	2560	2,560	2011 Nov 14

*Influenza A(H3N2)v, virus variant identified in humans, United States, 2011. Gray shading indicates antigenically similar viruses.
†Current seasonal influenza A(H3N2) vaccine virus.
‡Reassortant virus possessing the hemagglutinin and neuraminidase genes of A/Minnesota/11/2010 and the remaining 6 genes of A/PR/8/34.

viruses have genetic markers (V27A, S31N) in the M2 protein that confer resistance to the antiviral medications amantadine and rimantadine.

Conclusions

The detection of multiple cases of human infection with influenza A(H3N2)v virus within a 5-month period in 5 US states, coupled with possible human-to-human transmission, underscores the need for continued influenza surveillance at the swine–human interface. Coordinated surveillance of human and animal influenza viruses enables rapid detection of human infections with novel influenza viruses and timely identification of new virus variants in swine. As was evident during the 2009 influenza pandemic, this information is vital for development of resources that might be needed to effectively respond to the emergence and spread of a novel influenza virus in humans.

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Dr Lindstrom is the team lead of the Diagnostics Development Team of the Virus Surveillance and Diagnosis Branch, Influenza Division, at the Centers for Disease Control and Prevention. His research interests are development and qualification of molecular diagnostic testing procedures for influenza viruses, confirmatory diagnostic testing and reporting of human cases of influenza, including infections potentially caused by novel influenza viruses.

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Pigs as Natural Hosts of *Dientamoeba fragilis* Genotypes Found in Humans

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Dientamoeba fragilis is a common intestinal parasite in humans. Transmission routes and natural host range are unknown. To determine whether pigs are hosts, we analyzed 152 fecal samples by microscopy and molecular methods. We confirmed that pigs are a natural host and harbor genotypes found in humans, suggesting zoonotic potential.

The flagellated protozoan *Dientamoeba fragilis* is one of the most common parasites in the intestinal tract of humans (*1*). Infection is highly prevalent in economically developing regions and in industrialized countries (*1*,*2*). Infected persons often show no symptoms, but a pathogenic role for this parasite has been reported recently in humans and gorillas (*2*–*4*). Little is known about transmission routes of this parasite, and a transmissible stage (e.g., a cyst) has not been described (*1*,*5*). Molecular characterization of human isolates based on sequence analysis of ribosomal genes revealed 2 genotypes (1 and 2), with genotype 1 predominating worldwide (*6*,*7*).

Other than humans, few animal hosts of *D. fragilis* have been reported. Surveys of mammals and birds have identified only nonhuman primates (gorillas, macaques, and baboons) as natural hosts (*8*,*9*). Recently, however, a high prevalence of infection (43.8%) has been reported in pigs in Italy (*10*). To determine whether pigs are a host of *D. fragilis*, we analyzed fecal samples from 152 pigs in Italy by microscopy and molecular methods.

The Study

During June–August 2010, a total of 152 fecal samples were collected from the rectums of piglets (age 1–3 months; weight 6–24 kg), fattening pigs (age 3–4 months; weight 25–50 kg), and sows (age 1–2 years; weight 180–250 kg). The pigs were raised in 6 farrow-to-finish farms, 2 fattening farms, and 1 weaner indoor farm of central Italy (7 farms in the Umbria region and 2 farms in the Marche region). Pig fecal samples from 7 of the 9 farms were available for molecular analysis. Fecal samples from 21 pig farmers were collected from 5 of the 9 farms, 17 of which were available for molecular analysis.

Microscopic diagnosis of *D. fragilis* was based on visualization of pleomorphic trophozoites, ranging in size from 4 μm to 20 μm, with fragmented chromatin and pale gray-blue finely vacuolated cytoplasm after Giemsa staining (Figure 1). DNA was extracted directly from 200 mg of feces by using the QIAamp DNA stool minikit (QIAGEN, Hilden, Germany). Reference *D. fragilis* DNA of genotype 1 (strains 379 and 1085) was used as a positive control.

A TaqMan real-time PCR that targets the 5.8 S ribosomal locus was performed in a LightCycler 480 apparatus (Roche Diagnostics GmbH, Mannheim, Germany) as described (*11*). For the 18S rRNA gene, a published assay (*12*) was used to amplify a 662-bp fragment, followed by amplification of a 366-bp fragment with newly designed primers DF322For (5′-GAGAAGGCGCCTGAGAGATA-3′) and DF687Rev (5′-TTCATACTGCGCTAAATCATT-3′). For the internal transcribed spacer 1 (ITS1) region, a nested PCR protocol was developed. In the primary reaction, the forward primer ssu2 (*13*) and the reverse primer Df-ITSRev (5′-GCGGGTCTTCCTATATAAACAAGAACC-3′) were used, whereas the forward primer Df-ITSnesFor (5′-ATA CGTCCCTGCCCTTTGTA-3′) and the reverse primer Df-ITSnesRev (5′-GCAATGTGCATTCAAAGATCGAAC-3′) were used in the nested PCR. PCR products were purified and sequenced on both strands. The sequences were assembled by using SeqMan II (DNASTAR, Madison, WI, USA) and compared with those available in public databases by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences from this study were submitted to GenBank under accession nos. JQ677147–JQ677168.

The microscopic examination showed that 52 of the 74 piglets, 11 of the 14 fattening pigs, and 8 of the 64 sows were positive for *D. fragilis* (Table 1). More trophozoites were observed in fecal samples from piglets, suggesting a higher susceptibility of young animals to infection (data not shown). The microscopic analysis also showed *Blastocystis* spp. (in 42% of pigs), *Endolimax nana* protozoa (32%), *Iodoamoeba buetschli* protozoa (25%), and other flagellates (4.5%). Of the 21 samples from pig farmers, 4 from farmers working on 2 farms were positive for *D. fragilis* by microscopy (Table 1).

Molecular techniques were applied to 38 pig fecal samples, specifically 24 samples positive by microscopy from 6 farms and 14 samples negative by microscopy

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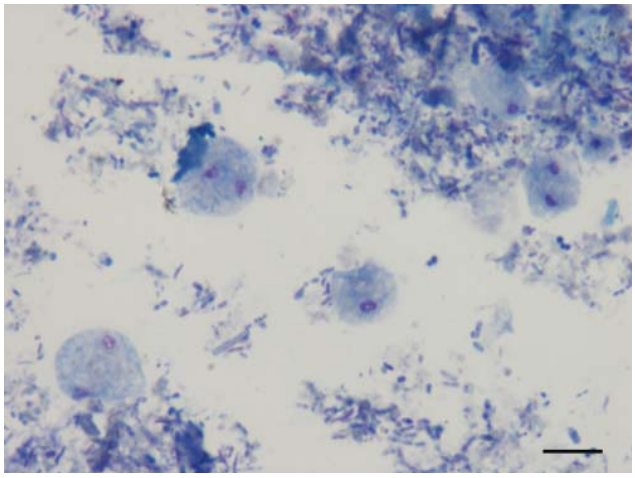

Figure 1. *Dientamoeba fragilis* trophozoites in a smear of pig feces after Giemsa staining, Italy, 2010–2011. Scale bar = 10 µm.

from 2 farms, and to all 17 human fecal samples. A comparison of human and pig samples collected from the same farm was possible for farms 2, 3, and 5 (Table 2). Using real-time PCR, all 24 positive pig samples were amplified, with cycle threshold values ranging from 30 to 34, whereas none of the 14 negative samples were positive to this assay (Table 2). However, because no inhibition controls were run, false-negative results cannot be ruled out. Of the 17 human fecal samples, 13 were positive with cycle threshold values of 29–40. The sequence analysis of 15 amplified products (11 from pigs and 4 from humans) showed 100% homology with *D. fragilis* genotype 1 (Table 2). Genotype 2 was not found in any of the samples from pigs or humans.

Next, a 366-bp fragment of the 18S rRNA gene was analyzed. In this fragment, genotypes 1 and 2 can be distinguished by 8 substitutions or insertions or deletions (Figure 2), which were further confirmed by sequencing the entire 18S rRNA gene from 2 reference isolates and 2 human isolates from this study. Amplification was obtained from 6 of the 24 positive pig samples and from 8 of the 17 human samples. Genotype 1 was identified in all samples (Figure 2). One human isolate (H7) showed a single nucleotide substitution in the fragment sequenced (Figure 2). Sequences from 3 microscopically negative pig samples (all from farm 1) had a high homology (96%) with *Trichomitus batrachorum*, a flagellate of reptiles, although the sequence could originate from *T. rotunda*, a flagellate of pigs that has not been described at the molecular level.

Last, we studied the more variable ITS1 locus. Amplification was obtained from 11 of the 24 pig samples (Table 2), but only 2 sequences could be clearly identified as *D. fragilis*. Four sequences showed homology (80%) with flagellates from different vertebrate classes whereas the remaining 5 sequences were excluded because of insufficient quality. The 2 *D. fragilis* sequences from pigs showed 100% homology with sequences from human isolates from the United Kingdom (Table 2), further supporting the presence of genotype 1 in these 2 hosts. A direct comparison of ITS1 sequences from humans and pigs from a single farm in Italy was not possible because *D. fragilis* was amplified from only 2 human samples from 2 farms from which no pig samples were available. The analysis of ITS1 from the 2 human isolates showed full identity to human isolates from the Netherlands and the United Kingdom (Table 2).

Conclusions

Considering the size of the world's pig population (>1 billion), the close contact between pigs and humans in many parts of the world, and the difficulties in the proper management of pig fecal waste, the role of these animals as reservoirs of zoonotic pathogens must be carefully evaluated. We demonstrated that pigs are hosts of *D. fragilis*, on the basis of molecular analysis of 3 fragments in the ribosomal cluster. Sequence analyses of fragments of the 18S and 5.8S rRNA genes showed genotype 1 in isolates collected in the same farm from humans and pigs, suggesting the potential for zoonotic transmission

Table 1. Prevalence of *Dientamoeba fragilis* protozoa in pig and human fecal samples after microscopy and Giemsa staining, Italy, 2010–2011

		No. samples positive/no. tested*			
Farm	Herd type	Piglet	Fattening pig	Sow	Human
1	Weaner production	10/10	NA	1/10	NA
2	Farrow-to-finish	9/10	NA	3/10	0/4
3	Farrow-to-finish	10/10	7/10	0/10	2/8
4	Farrow-to-finish	1/10	NA	0/10	NA
5	Farrow-to-finish	4/10	NA	0/10	0/2
6	Farrow-to-finish	4/10	NA	1/10	NA
7	Fattening	NA	NA	3/4	NA
8	Fattening	10/10	NA	NA	2/3
9	Farrow-to-finish	4/4	4/4	NA	0/4
	Total	52/74	11/14	8/64	4/21

*NA, sample not available.

of this parasite. If a transmissible cyst stage exists, then environmental contamination with pig feces should be considered a key factor in the transmission of this parasite. Pigs also are a fascinating animal model to elucidate the life cycle of this elusive parasite.

Table 2. Results of the molecular tests for *Dientamoeba fragilis* applied to pig and human fecal samples, Italy, 2010–2011*

			5.8S		18S		ITS1	
Sample	Farm	Microscopy	Real-time PCR	Sequence	PCR	Sequence	PCR	Sequence
P21	2	+	+	ND	–	ND	–	ND
P26	2	+	+	*D. fragilis*	+	*D. fragilis*†	+	*D. fragilis*‡
P27	2	+	+	*D. fragilis*	–	ND	+	unclassified
P37	2	+	+	ND	–	ND	–	ND
P42	3	+	+	*D. fragilis*	+	*D. fragilis*†	+	*D. fragilis*§
P44	3	+	+	*D. fragilis*	–	ND	+	flagellate
P45	3	+	+	ND	–	ND	–	ND
P50	3	+	+	ND	–	ND	–	ND
P52	3	+	+	*D. fragilis*	–	ND	+	unclassified
P54	3	+	+	*D. fragilis*	–	ND	+	flagellate
P56	3	+	+	*D. fragilis*	+	*D. fragilis*†	+	unclassified
P59	3	+	+	*D. fragilis*	+	*D. fragilis*†	+	unclassified
P60	3	+	+	ND	–	ND	–	ND
P71	4	+	+	ND	–	ND	–	ND
P75	4	+	+	ND	–	ND	–	ND
P91	5	+	+	ND	–	ND	–	ND
P93	5	+	+	*D. fragilis*	+	*D. fragilis*†	+	unclassified
P 97	5	+	+	*D. fragilis*	+	*D. fragilis*†	+	flagellate
P111	6	+	+	ND	–	ND	–	ND
P113	6	+	+	ND	–	ND	–	ND
P116	6	+	+	ND	–	ND	–	ND
P122	6	+	+	*D. fragilis*	–	ND	+	flagellate
P131	6	+	+	ND	–	ND	–	ND
P133	7	+	+	ND	–	ND	–	ND
Pig 1	1	–	–	ND	+	*Trichomitus*¶	–	ND
Pig 2	1	–	–	ND	–	ND	–	ND
Pig 3	1	–	–	ND	+	*Trichomitus*¶	–	ND
Pig 4	1	–	–	ND	–	ND	–	ND
Pig 5	1	–	–	ND	+	*Trichomitus*¶	–	ND
Pig 6	1	–	–	ND	–	ND	–	ND
Pig 7	1	–	–	ND	–	ND	–	ND
Pig 8	1	–	–	ND	–	ND	–	ND
Pig 9	1	–	–	ND	–	ND	–	ND
Pig 10	1	–	–	ND	–	ND	–	ND
DF-P1	6	–	–	ND	–	ND	–	ND
DF-P2	6	–	–	ND	–	ND	–	ND
DF-P3	6	–	–	ND	–	ND	–	ND
DF-P4	6	–	–	ND	–	ND	–	ND
H1	2	–	+	*D. fragilis*	+	*D. fragilis*†	–	ND
H2	2	–	–	ND	–	ND	–	ND
H3	2	–	+	ND	–	ND	–	ND
H4	2	–	+	ND	+	*D. fragilis*†	–	ND
H5	5	–	+	ND	–	ND	–	ND
H6	5	–	+	ND	+	*D. fragilis*†	–	ND
H7	3	–	+	ND	+	*D. fragilis*†	–	ND
H8	3	–	+	ND	–	ND	–	ND
H9	3	–	–	ND	–	ND	–	ND
H10	3	+	+	ND	+	*D. fragilis*†	–	ND
H11	3	+	+	*D. fragilis*	+	*D. fragilis*†	–	ND
H12	3	–	+	ND	–	ND	–	ND
H13	3	–	+	ND	–	ND	–	ND
H14	3	–	–	ND	–	ND	–	ND
H15	8	+	+	*D. fragilis*	+	*D. fragilis*†	+	*D. fragilis*#
H16	8	+	–	ND	–	ND	–	ND
H17	9	–	+	*D. fragilis*	+	*D. fragilis*†	+	*D. fragilis*#

*ITS, internal transcribed spacer; ND, not done.
†100% identity to AY730405.
‡100% identity to DQ223443, DQ223447, and DQ223455.
§100% identity to DQ223448 and DQ223453.
¶96% identity to AF124610.
#100% identity to DQ223442, DQ223450, DQ223452, DQ223454, DQ223456, and DQ167586.

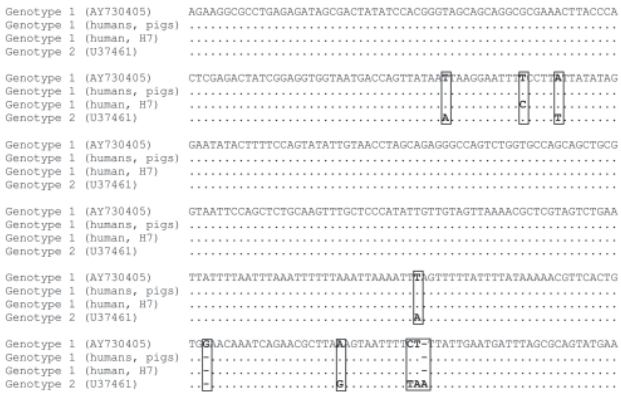

Figure 2. Multiple alignment of the 366-bp fragment of the 18S rRNA gene from *Dientamoeba fragilis* genotypes 1 and 2. Dot indicates identical nucleotides. Dashes indicate insertion or deletion. Nucleotide differences are presented in boxes.

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Plasmodium vivax Malaria–associated Acute Kidney Injury, India, 2010–2011

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Plasmodium vivax is causing increasingly more cases of severe malaria worldwide. Among 25 cases in India during 2010–2011, associated conditions were renal failure, thrombocytopenia, jaundice, severe anemia, acute respiratory distress syndrome, shock, cerebral malaria, hypoglycemia, and death. Further studies are needed to determine why *P. vivax* malaria is becoming more severe.

India is a major contributor to the worldwide distribution of *Plasmodium vivax* malaria (*1*). Severe and complicated malaria is usually caused by the *P. falciparum* parasite, but *P. vivax*, usually considered a benign parasite that causes disease resulting in low case-fatality rates, can also occasionally cause severe disease. Reported severe manifestations of *P. vivax* include cerebral malaria, liver dysfunction, acute kidney injury, severe anemia, acute respiratory distress syndrome, shock, abnormal bleeding, and multiple organ failure (*2–10*). The mechanism of *P. vivax*–associated acute kidney injury and its effective management remain unclear. In addition, little information is found in the literature to explain the recent increase in incidence of acute kidney injury and the shift toward multiple complications, specifically in India (*11*). This scarcity of data prompted us to review cases at the Institute of Kidney Diseases and Research Centre–Institute of Transplantation Sciences, Civil Hospital, Ahmedabad, India.

The Study

We conducted a prospective study during 2010–2011 to describe clinical characteristics, laboratory parameters, prognostic factors, and outcomes for 25 Civil Hospital patients who required hemodialysis for acute kidney injury associated with *P. vivax* monoinfection. *P. vivax* monoinfection was diagnosed by direct visualization of the parasite in Giemsa-stained peripheral blood films (Figure 1) and rapid diagnostic test results (negative for histidine-rich protein 2 of *P. falciparum* and positive for *P. vivax*–specific lactate dehydrogenase). The severity of illness was assessed by using the Acute Physiology and Chronic Health Evaluation (APACHE II), Sequential Organ Failure Assessment (SOFA), Multiple Organ Dysfunction Score (MODS), and Glasgow Coma Scale. Smear evaluation included reading 200–500 fields with the oil immersion objective lens for ≥20 minutes by an expert. If the initial smear examination was negative for *P. falciparum*, additional smears were reviewed within 6–24 hours to rule out mixed infection. Patients with other concurrent illness or *P. falciparum* mixed infections were excluded from the study. After a diagnosis was established

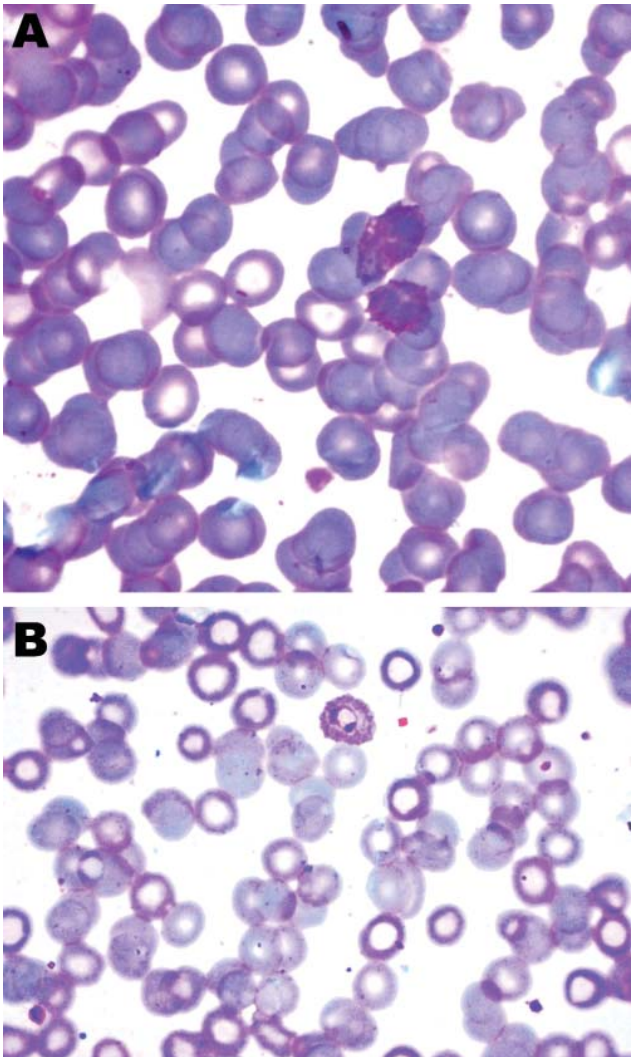

Figure 1. Schizonts (A) and ring form (B) of *Plasmodium vivax*, Ahmedabad, India, 2010–2011. Original magnification ×1,000.

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and treatment was initiated, the parasitologic response was monitored. Other causes of acute kidney injury, fever, and jaundice (i.e., dengue viral infection, leptospirosis, sepsis, typhus, enteric fever, and viral hepatitis and drug reactions) were excluded by history and relevant investigations.

Acute kidney injury was defined as serum creatinine >3 mg/dL and urine output <400 mL/24 hours with normal kidney size according to ultrasonography. The patients were treated with antimalarial drugs (artesunate and doxycycline [n = 25], quinine [n = 2]); fluid replacement and supportive measures were instituted as needed. Intravenous ceftriaxone was given initially until a bacterial infection (e.g., *Salmonella* infection) was excluded. Renal replacement therapy was initiated before overt symptoms and signs of acute kidney injury developed (*10*,*11*). Hemodialysis was initiated for fluid overload, hyperkalemia, clinical evidence of uremia, metabolic acidosis, blood urea nitrogen >100 mg/dL, and creatinine >4–5 mg/dL or rapidly increasing. Intermittent hemodialysis (n = 24) was provided on alternate days through a temporary dialysis catheter, and continuous renal replacement therapy was provided for 1 hemodynamically unstable patient. Renal replacement therapy continued until the patient's kidney function improved (increase in urine output or progressive decline in creatinine).

Blood transfusion was reserved for patients with hemoglobin ≤7 g/dL. Exchange transfusion was performed for patients with parasite density >10%, end-stage organ failure complications, and serum bilirubin >25 mg/dL. Renal biopsy was performed when oliguria, heavy proteinuria, or hematuria persisted for ≥3 weeks.

Of the 202 malaria patients in whom acute kidney injury was treated, 177 (87.7%) cases of total malaria with acute kidney injury were caused by *P. falciparum* and 25 (12.3%) by *P. vivax*. Baseline characteristics of the study patients are shown in Table 1. Comparison of patients who survived or died is shown in Table 2. Initial clinical features were fever (100%), nausea or vomiting (84%), oliguria (60%), abdominal pain or tenderness (40%), jaundice (40%), dyspnea (32%), diarrhea (12%), altered sensorium (4%), and convulsions (4%). The time between onset of symptoms and admission to the hospital for acute kidney injury was 7–15 days (median 11 days). At admission, cultures of blood, sputum, and urine were negative for microorganisms for all patients. The following complications were observed: renal failure (100%), thrombocytopenia (64%), thrombocytopenia <50,000 cells/μL (40%), hyponatremia (52%) (usually asymptomatic), jaundice (bilirubin >3 mg/dL) (40%), severe anemia (hemoglobin ≤7 gm/dL) (40%), acute respiratory distress syndrome (16%), shock (12%), cerebral malaria (4%), hypoglycemia (4%), and death (12%).

Urinalysis showed muddy brown granular casts or epithelial cell casts; trace protein; and absence of dysmorphic red cells, heavy protein, and leukocytes. None of the patients were deficient in glucose 6-phosphate dehydrogenase activity in erythrocytes. The mean APACHE II, SOFA, MODS, and Glasgow Coma scores were higher for patients who died than those who survived (Table 2). SOFA and MODS scores <12 were associated with a low mortality rate (22 patients, 0 deaths), whereas scores ≥12 indicated a worse outcome (3 patients, 3 deaths). APACHE II score <24 was associated with a low mortality rate (22 patients, 0 deaths), whereas score ≥24 indicated a worse outcome (3 patients, 3 deaths) ($p<0.008$).

Table 1. Baseline values for 25 *Plasmodium vivax* patients with acute kidney injury, Ahmedabad, India, 2010–2011*

Characteristic	Value
Age, y	30.1 ± 12.3
Hypotension not resolving after receipt of fluid, no. (%)	3 (12)
Mechanical ventilation, no. (%)	4 (16)
Lactate dehydrogenase, U/L	1267 ± 462
APACHE 2 score	17.4 ± 3.8 (9–27)
SOFA score	8.4 ± 2.8 (4–14)
MODS score	7.9 ± 2.8 (4–14)
GCS score	14.3 ± 1.14 (12–15)
Hemoglobin, gm/L	8.02 ± 1.6
Hematocrit, %	24.6 ± 4.9
Leukocytes, cells/mm^3	9,002.8 ± 3,098
Platelets, cells/μL	108,680 ± 91,606
Total bilirubin, mg/dL	5.6 ± 7.6
Direct bilirubin, mg/dL	4.1 ± 6.2
Indirect bilirubin, mg/dL	1.5 ± 2.1
Alanine aminotransferase, U/L	66 ± 45.5
Sodium, mmol/L	130 ± 6.8
Creatinine, mg/dL	7.37 ± 2.6
Blood urea, mg/dL	140.4 ± 59
No. hemodialysis sessions	5.1 ± 3.5

*Values are mean ± SD (range) except as indicated. APACHE, Acute Physiology and Chronic Health Evaluation; SOFA, Sequential Organ Failure Assessment; MODS, Multiple Organ Dysfunction Score; GCS, Glasgow Coma Scale.

Cerebral malaria occurred in 1 patient, who died. Blood component transfusion was given to 7 (28%) patients. Exchange transfusion was given to 1 (4%), who survived. For all patients, initial rehydration failed to lead to renal recovery. Most patients no longer needed dialysis after 2 weeks, and renal function returned in 3 weeks. Renal biopsies, performed for 4 patients, detected patchy cortical necrosis in 3 (Figure 2) and acute tubular necrosis in 1. A total of 19 (76%) patients completely recovered with normal renal function (creatinine <1.4 mg/dL [reference 0.7–1.4 mg/dL]), 3 (12%) did not recover completely (creatinine 1.5–3 mg/dL) and continued to receive conservative treatment, and 3 (12%) died. Factors related to acute kidney injury were heavy parasitemia (48%), hyperbilirubinemia (40%), volume depletion (40%), intravascular hemolysis (100%), and sepsis (12%).

Conclusions

Our study highlights the possibility that *P. vivax* can cause acute kidney injury. There is no direct pathogenic

Table 2. Comparison of 25 *Plasmodium vivax* patients with acute kidney injury, Ahmedabad, India, 2010–2011*

Characteristic	Survived, n = 22	Died, n = 3	p value
Age, y	27.7 ± 9.9	47.3 ± 17.5	0.058
Hypotension not resolving after receipt of fluid, no. (%)	0	3 (100)	0.0001
Mechanical ventilation, no. (%)	1 (4.5)	3 (100)	0.0001
Lactate dehydrogenase, U/L	1126.3 ± 243.2	2300 ± 360.5	0.001
APACHE 2 score	16.4 ± 2.61 (9–23)	25.3 ± 1.52 (24–27)	0.001
SOFA score	7.73 ± 2.3 (4–11)	13.3 ± 1.15 (12–14)	0.001
MODS score	7.23 ± 2.26 (4–10)	13 ± 1.0 (12–14)	0.001
GCS score	14.5 ± 1.01 (12–15)	12.3 ± 0.57 (12–13)	0.013
Hemoglobin, gm/L	8.0 ± 1.71	8.13 ± 0.70	0.96
Hematocrit, %	24.5 ± 5.2	25.6 ± 2.3	0.78
Leuckocytes, cells/mm^3	9,175.9 ± 3,110	7,733.3 ± 3,300	0.49
Platelets, cells/μL	113,409 ± 96,618	74,000 ± 2,5159	0.90
Total bilirubin, mg/dL	6.1 ± 8	2.56 ± 0.75	0.72
Direct bilirubin, mg/dL	4.46 ± 6.58	1.76 ± 0.40	0.60
Indirect bilirubin, mg/dL	1.64 ± 2.23	0.80 ± 0.34	0.90
Alanine aminotransferase, U/L	66.9 ± 47.8	59.3 ± 29.1	0.96
Sodium, mmol/L	130.5 ± 7.17	126.6 ± 2.3	0.23
Creatinine, mg/dL	7.36 ± 2.7	7.5 ± 2.17	0.66
Blood urea, mg/dL	147.7 ± 57.5	87 ± 48.8	0.08
No. hemodialysis sessions	5.3 ± 3.7	3.33 ± 1.15	0.23

*Values are mean ± SD (range) except as indicated. APACHE, Acute Physiology and Chronic Health Evaluation; SOFA ,Sequential Organ Failure Assessment; MODS, Multiple Organ Dysfunction Score; GCS, Glasgow Coma Scale.

linkage between *P. vivax* and acute kidney injury. Hence, despite the association, cause-and-effect relationships remain doubtful. However, the following can contribute to acute kidney injury: heavy parasitemia, volume depletion, hyperbilirubinemia, intravascular hemolysis, renal ischemia, sepsis, disseminated intravascular coagulation, cytoadherence to endothelial cells, and microvascular sequestration (*2*,*10*,*11*,*12*,*13*). Bilirubin and hemoglobin were useful for predicting *P. vivax*–induced nephropathy (*9*). Because co-infection with *P. vivax* and *P. falciparum* protects patients from severe malaria, mixed infection is unlikely in patients with severe malaria (*2*,*9*,*14*).

In our study, APACHE II score ≥24 and SOFA and MODS scores ≥12 were associated with higher mortality rates. These scores can be used for prognosis for patients with malaria and acute kidney injury and can help us better categorize patients for better management and improved outcomes. One limitation of our study is that PCR confirming *P. vivax* monoinfection was not performed routinely.

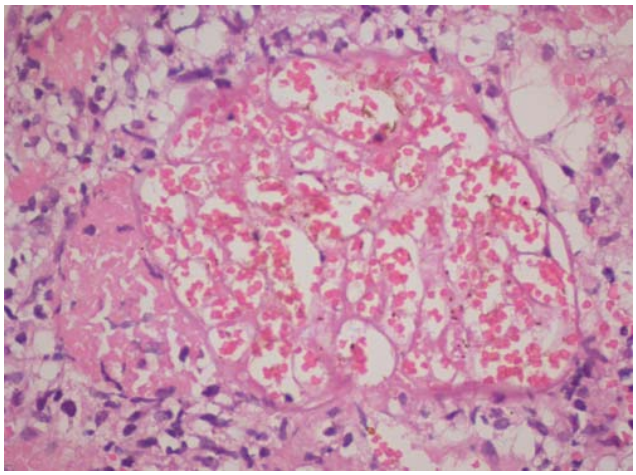

Figure 2. Renal biopsy sample showing patchy cortical necrosis, hematoxylin and eosin staining, Ahmedabad, India, 2010–2011 Original magnification ×400.

Acute cortical necrosis is rare in patients with acute kidney injury from malaria (*15*). The possible pathogenic factors are renal damage through renal hypoperfusion or endothelial injury through release of various circulating substances (intravascular hemolysis and sepsis). *P. vivax* should be suspected in patients with acute kidney injury who have prolonged oligoanuria. *P. vivax* can lead to serious, potentially life-threatening complications, such as acute kidney injury. Further studies are needed to determine why *P. vivax* infections are becoming more severe.

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Novel Human Adenovirus Strain, Bangladesh

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We report a novel human adenovirus D (HAdV-65) isolated from feces of 4 children in Bangladesh who had acute gastroenteritis. Corresponding genes of HAdV-65 were related to a hexon gene of HAdV-10, penton base genes of HAdV-37 and HAdV-58, and a fiber gene of HAdV-9. This novel virus may be a serious threat to public health.

Human adenoviruses (HAdVs) are common pathogens that cause several diseases, such as pneumonia, acute gastroenteritis, and epidemic keratoconjunctivitis (*1*). HAdV infection is also associated with a serious adenovirus syndrome in immunocompromised patients after stem cell transplantation (*2*). Acute gastroenteritis causes illness and death in humans worldwide. Illness is associated with infection of enteric viruses, including rotavirus, astrovirus, norovirus, sapovirus, and adenovirus.

HAdVs are divided into 7 species (HAdV-A–G) on the basis of DNA genome homology. Most acute gastroenteritis related to HAdVs is caused by HAdV-F species (HAdV-40 and HAdV-41 (*3*,*4*). Recently, we detected HAdV-D in feces of children with diarrhea in Bangladesh (*5*). Other HAdV-D strains have also been associated with diarrhea in Kenya and Brazil (*6*,*7*).

We report a novel HAdV-D (HAdV-65) strain detected in feces of 4 children with acute gastroenteritis during October 2004–March 2005 in Bangladesh (*5*) and results of hexon, penton base, and fiber gene sequence analyses. We also report the full genome sequence of this virus, whose corresponding genes are closely related to the hexon gene of HAdV-10, penton base genes of HAdV-37 and HAdV-58, and the fiber gene of HAdV-9.

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The Study

Cloned virus (3 plaque purifications) was propagated in an A549 cell line, which was maintained in minimal essential medium supplemented with 1% fetal bovine serum (Cansera International Inc., Toronto, Ontario, Canada). Cultures were observed for 3–4 weeks for a cytopathic effect. After a cytopathic effect was observed, cell lysates were centrifuged at 1,430 × *g* for 20 min at 4°C. Supernatants were centrifuged at 72,000 × *g* for 3 h at 4°C. Pellets were resuspended in sterile water and treated with 10 μL (20 mg/mL) of proteinase K. DNA was extracted by using the phenol:chloroform:isoamyl alcohol (25:24:1) method (Invitrogen, Carlsbad, CA, USA) and precipitated with isopropyl alcohol.

PCR was performed in a total volume of 50 μL containing 20 pmol/μL of each primer, 2.5 mmol/L of dNTP, 1.25 units of GXL DNA polymerase (Takara, Shiga, Japan), and 5 μL of DNA template. After PCR products were purified by using the MinElute PCR Purification Kit (QIAGEN, Hilden, Germany), cycle sequencing was conducted by using the Genome Lab DTCS Quick Start Kit (Beckman Coulter Inc., Fullerton, CA, USA).

The complete genome of HAdV-65 was sequenced by using the primer walking method. The 5′ terminus of full-length DNA was phosphorylated with 20 units of T4 polynucleotide kinase and 0.5 μL of 100 mmol/L ATP, and ligated to a blunt *Eco*RI-*Not*I-*Bam*HI adaptor (1 pmol/μL) for 3 h at 8°C by using the DNA Ligation Mighty Mix Kit (Takara), PCRs with primer pairs containing adaptor sequences were conducted as described (*8*). DNA sequences were assembled by using the CEQ 2000XL DNA Analysis System version 4.3.9 (Beckman Coulter Inc.).

The genome of HAdV-65 was 35,172 bp. It had a GC content of 56.9%, and the inverted terminal repeat sequence of this virus was 150 bp. Phylogenetic trees were generated by using the maximum-likelihood method with MEGA5 (www.megasoftware.net) after alignment was performed by using ClustalW (www.clustal.org).

Complete genome analysis showed that HAdV-65 was ≥5.0% distant from any other HAdV-D reference strains (online Appendix Figure 1, panel A, wwwnc.cdc.gov/EID/article/18/5/11-1584-FA1.htm). On the basis of hypervariable loop 1 and loop 2, which encode the neutralization epitope of HAdVs, this virus clustered with HAdV-10 (online Appendix Figure 1, panels B and C). However, HAdV-65 was closely related to HAdV-37 and HAdV-58 in the hypervariable loop 1 and the Arg-Gly-Asp (RGD) loop of the penton base gene, respectively (online Appendix Figure 1, panels D and E). Phylogenetic analysis also showed that this novel virus clustered with HAdV-9 on the basis of the fiber gene sequence (online Appendix Figure 1, panel F). Sequences of HAdV-65 and 3 other strains (DC 11, 253, and 303) isolated from infants in this

study had identical hexon, penton base, and fiber genes.

Potential recombination in HAdV-65 was investigated by using the SimPlot program (http://sray.med.som.jhmi.edu/RaySoft/simplot_old/Version1/SimPlot_Doc_v13.html). DNA sequence alignments were created by using DNASIS Pro (Hitachi Solutions, Tokyo, Japan). SimPlot analysis showed no potential recombination in the hexon and fiber genes (online Appendix Figure 2, panels A and C, wwwnc.cdc.gov/EID/article/18/5/11-1584-FA2.htm), and recombination between the hypervariable loop 1 and the RGD loop was predicted in the penton base gene (online Appendix Figure 2, panel B). HAdV-65 had nucleotide identities of 97.9% to HAdV-10 in the hexon gene, 92.3% and 96.7% to HAdV-37 and HAdV-58, respectively, in the penton base gene, and 98.2% to HAdV-9 in the fiber gene. The GenBank accession number for HAdV-65 is AP012285.

Conclusions

We report the complete genome of HAdV-65, a novel human adenovirus isolated from children with gastroenteritis. Recombination is an essential feature for viral evolution and immune escape. Recombination can be facilitated by antiviral immune pressure and co-infection with different HAdV strains of the same species (*9*). Recently, newly identified HAdVs appeared to originate by recombination among ≥2 viruses. HAdV-53 was reported as a novel recombinant HAdV with a close genetic relationship to loop 1 and loop 2 of HAdV-22, the penton base gene of HAdV-37, and the fiber gene of HAdV-8. HAdV-56 had a loop 1 and loop 2 highly similar to those of HAdV-15 (*10*,*11*). All other regions of the genome were genetically related to HAdV-9.

HAdV-58 was recently characterized as a novel HAdV with unique hexon and fiber genes of HAdV-25 and HAdV-29 (*12*). In the present study, we demonstrated that hexon and fiber coding regions of HAdV-65 were formed by recombination in regions around these genes but not by potential recombination within these genes. The potential recombination site within the penton base gene is located at the central position between hypervariable loop 1 and the RGD loop.

These findings suggest that the most conserved sequences around the hexon and fiber genes and in the penton base gene may play a major role in recombination. In addition, this recombination mechanism may be more efficient in enabling new processes of infection and immune escape for maintaining HAdVs than individual small mutations, such as insertions, substitutions, and deletions.

Most recombinant HAdVs have been found in AIDS patients. (*9*,*13*). The RGD loop of the penton base protein can be digested by trypsin secreted in the intestines, which results in inhibition of proliferation of HAdV except for HAdV-F types in the intestines (*14*). In this study, HAdV-65 was isolated from infants who had lower immunocompetence and secretion rates of digestive enzymes than adults. These results indicate that emergence of HAdV-65 might have been caused by long coexistence of multiple HAdV-D types and depending on a decrease in immunity, as observed in AIDS patients, and decreased digestive capacity in the intestines.

We detected this type of recombination not only in HAdV-65, but also in 3 other HAdV strains that had a genome sequence identical with that of HAdV-65 from children in Bangladesh during 2004–2005. This finding indicates that this virus might be a newly emerging HAdV, which might be a serious threat to public health.

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Rhabdomyolysis Associated with Antimicrobial Drug–Resistant *Mycoplasma pneumoniae*

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We describe a case of rhabdomyolysis in a patient infected with antimicrobial drug–resistant *Mycoplasma pneumoniae* The patient's acute-phase serum levels of interleukin-18 and tumor necrosis factor–α were high, which suggests a pathogenic role for *M. pneumoniae*. In an era of increasing antimicrobial drug resistance, a system for rapidly identifying resistant *M. pneumoniae* would be beneficial.

Mycoplasma pneumoniae, one of the major causes of community-acquired pneumonia in children, can cause a variety of extrapulmonary manifestations. Antimicrobial drug–resistant strains of *M. pneumoniae* have been isolated from children in Japan; however, to our knowledge, extrapulmonary manifestations caused by antimicrobial drug–resistant *M. pneumoniae* have not been reported. We describe a case of rhabdomyolysis, the rapid breakdown of striated muscle, in a 7-year-old girl in Japan who had antimicrobial drug–resistant *M. pneumoniae* infection, and we discuss the possible pathomechanisms for rhabdomyolysis.

Case Report

A 7-year-old girl had been healthy until 7 days before she was admitted to Niigata Prefectural Hospital, Niigata, Japan, on June 21, 2010, for cough and prolonged fever. On day 1 of her illness, the girl had visited her primary care physician and was prescribed azithromycin, a macrolide antimicrobial drug, for a lower respiratory tract infection. On day 7 of her illness, the girl's condition worsened acutely, with increased cough and fever, and she again visited her primary care physician. A chest radiograph showed pulmonary infiltrates in the left upper lung, and the patient was referred to our hospital on day 8 of her illness. The girl's history and family history were unremarkable.

On hospital admission, the patient was alert and oriented. Her temperature was 38.9°C, heart rate was 101 beats/min, and oxygen saturation was 97%. Chest auscultation was unremarkable. The girl did not describe symptoms of myalgia, and physical examination did not show signs of erythema, hepatosplenomegaly, neurologic abnormalities, muscle weakness, or muscle atrophy.

Results of the initial laboratory test were as follows: leukocyte count, 6.2×10^9 cells/L (reference $3.0–8.6 \times 10^9$ cells/L); hemoglobin, 1.95 mmol/L (reference 1.67–2.31 mmol/L); platelet count, 23.3×10^9/L (reference $15.0–36.1 \times 10^9$/L); C-reactive protein, 27 mg/L (reference <3.0 mg/L); aspartate aminotransferase, 161 IU/L (reference 13–31 IU/L); alanine aminotransferase, 83 IU/L (reference 6–27 IU/L); lactate dehydrogenase, 691 IU/L (reference119–229 IU/L); blood urea nitrogen, 3.2 mmol urea/L (reference 2.9–7.2 mmol urea/L); creatinine, 31.8 μmol/L (reference 44.2–70.6 μmol/L); sodium, 135 mmol/L (reference 138–146 mmol/L); potassium, 4.1 mmol/L (reference 3.6–4.9 mmol/L); and chloride 96 mmol/L (reference 99–109 mmol/L). A venous blood gas determination on room air showed a pH of 7.464 (reference 7.35–7.45kPa) and carbon dioxide partial pressure of 4.9 kPa (reference 4.7–6.0 kPa). Levels of serum glucose, albumin, calcium, amylase, and bilirubin were normal (references 70–109 mg/dL, 4.1–5.0 g/dL, 8.7–10.0 mg/dL, 39–108 U/mL, and 0.3–0.9 mg/dL, respectively). Creatine phosphokinase was elevated to 12,159 ng/mL (reference 45–163 ng/mL). Urinalysis showed blood 3+, but analysis of urine sediment by microscopy showed no erythrocytes. The urine myoglobin level was 39,900 μg/L (reference <10 μg/L). No antinuclear factor or circulating immune complex was detected. The serum concentration of cytokine interleukin (IL)-18 on admission was 612 pg/mL (reference <260 pg/mL), and the concentration of tumor necrosis factor–α (TNF-α) was 3.48 pg/mL (reference <1.79 pg/mL).

The girl's fever did not respond to treatment with azithromycin, and she was given a tentative diagnosis of antimicrobial drug–resistant *M. pneumoniae* infection, which was prevalent in the region. Because the patient was <8 years of age, she was started on treatment with tosufloxacin, a fluoroquinolone, granules for children (12 mg/kg/d) and steroid therapy (methylprednisolone, 1 mg/kg/d). The patient's fever resolved the next day, and her urine output was maintained with intravenous hydration. No signs or symptoms of muscle involvement developed during the patient's hospital stay.

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On day 16 after the patient was admitted to the hospital, results of laboratory testing showed improved values for creatine phosphokinase (1,855 ng/mL), aspartate aminotransferase (101 IU/L), alanine aminotransferase (162 IU/L), lactate dehydrogenase (294 IU/L), and urine myoglobulin (10 μg/L). Pulmonary infiltrates seen on a chest radiograph had decreased substantially by day 16, and the patient was discharged from the hospital. On day 8 after discharge, her abnormal test results returned to normal, and her illness showed no signs of relapse.

Culture results for a respiratory sample obtained during hospitalization revealed normal bacterial flora, and the results for rapid diagnostic tests for influenza virus, adenovirus, and respiratory syncytial virus were negative at admission. The *M. pneumoniae* antibody titer by the particle agglutination test was 1,280 at admission, and 2 days later, the antibody titer had increased to 10,240. At hospital admission, with permission from the girl and her parents, a pharyngeal swab specimen was obtained to test for *M. pneumoniae*. The sample was sent to the laboratory of the Kanagawa Prefectural Institute of Public Health, Chigasaki, Japan, where PCR and restriction fragment length polymorphism analysis were performed as described (*1*). A macrolide-resistant *M. pneumoniae* strain with an A→G transition at position 2063 of the 23S rRNA gene (designated A2063G) was detected. Laboratory test results from admission to 8 days after discharge are summarized in the Table.

Conclusions

This case of rhabdomyolysis in a 7-year-old girl is an unusual extrapulmonary manifestation of antimicrobial drug–resistant *M. pneumoniae* infection. Rhabdomyolysis is characterized by rupture and necrosis of muscle fibers, resulting in the release of cell breakdown products into the bloodstream and extracellular space. Direct muscle injury is the most common cause of rhabdomyolysis, but a number of other causes are possible: hereditary enzyme disorders, drugs, toxins, endocrinopathies, malignant hyperthermia, neuroleptic malignant syndrome, heatstroke, hypothermia, electrolyte alterations, diabetic ketoacidosis and nonketotic hyperosmolar coma, severe hypothyroidism or hyperthyroidism, and bacterial or viral infections (*2*). Bacterial and viral infections account for ≈5% of rhabdomyolysis cases in adults (*3*).

Because pathomechanisms other than infection can cause rhabdomyolysis (*4*,*5*), we cannot say with certainty that *M. pneumoniae* infection caused this syndrome in the patient reported here. One possible mechanism of rhabdomyolysis is induction of inflammatory cytokines, such as TNF-α and IL-1. These cytokines can cause acute proteolysis in a variety of organs, including skeletal muscles (*6*,*7*), and *M. pneumoniae* can induce these cytokines (*8*). Our patient had high levels of TNF-α and IL-18 during the acute phase of *M. pneumoniae* infection, and it is highly possible that these *M. pneumoniae*–induced cytokines were involved in the pathomechanism of rhabdomyolysis. No other apparent cause, such as trauma, endocrine disorder, or infection, other than *M. pneumoniae*, was found for the development of rhabdomyolysis in this patient.

A confounding factor in this case was that the extrapulmonary manifestation of *M. pneumoniae* infection was caused by an antimicrobial drug–resistant strain of *M. pneumoniae*. The implicated strain, A2063G, is the dominant type of antimicrobial drug–resistant *M. pneumoniae* in Japan (*1*,*9*,*10*). Approximately 15% of *M. pneumoniae* strains isolated from patients in Japan are resistant to antimicrobial drugs (*1*,*9*,*10*), which may explain why extrapulmonary manifestations of antimicrobial drug–resistant *M. pneumoniae* have not been frequently reported. However, the proportion of antimicrobial drug–resistance in Japan is increasing, so extrapulmonary manifestations of antimicrobial drug–resistant *M. pneumoniae* infection might also increase.

In conclusion, this case of rhabdomyolysis was associated with and, in the absence of any other apparent cause, appears to be attributable to infection with antimicrobial drug–resistant *M. pneumoniae*. The

Table. Laboratory test results for a 7-year-old patient with rhabdomyolysis associated with antimicrobial drug–resistant *Mycoplasma pneumonia*e, Japan, June 22–July 23, 2010*

Laboratory test	Laboratory value, by days after onset of fever					
	7 d	9 d	13 d	17 d	23 d	31 d
Leukocytes, $\times 10^9$ cells/L	6.2	8.0	11.8	7.3	5.9	4.7
C-reactive protein, mg/L	27	7	4	1	<1	<1
Creatine phosphokinase, mg/L	12,159	12,918	10,937	5,839	1,855	130
Aspartate aminotransferase, IU/L	161	203	169	94	101	24
Alanine aminotransferase, IU/L	83	205	284	209	162	37
Lactate dehydrogenase, IU/L	691	553	451	358	294	234
Creatinine, μmol/L	31.8	23.0	30.1	32.7	30.9	29.2
Urine myoglobin, μg/L	39,900	NT	2,500	NT	10	<10
Urine erythrocytes	3+	3+	±	–	–	–
Serum interleukin-18, pg/mL	612	519	NT	NT	367	232
Serum tumor necrosis factor–α, pg/mL	3.48	3.38	NT	NT	2.03	1.64
M. pneumoniae titer	1,280	10,240	NT	NT	NT	NT

*NT, not tested.

development of a system that can be used in routine clinical practice to rapidly identify antimicrobial drug–resistant *M. pneumoniae* would be highly beneficial in this era of increasing antimicrobial drug resistance.

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Human Adenovirus Type 7 Outbreak in Police Training Center, Malaysia, 2011

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In March 2011, an outbreak of acute respiratory disease was reported at the Kuala Lumpur (Malaysia) Police Training Centre. Approximately 100 trainees were hospitalized and 5 were admitted to the intensive care unit. Three of these 5 trainees died. Human adenovirus type 7 was identified as the etiologic agent.

Human adenoviruses (HAdVs) consist of nonenveloped, double-stranded DNA and belong to the family *Adenoviridae*, genus *Mastadenovirus*. The 51 recognized serotypes of human adenoviruses have been placed in 7 human adenovirus species, A–G (*1*). These viruses cause infections ranging from mild syndromes to severe, life-threatening disease.

Depending on the species, these viruses may infect respiratory, conjunctival, gastrointestinal, and genitourinary sites. They have been recognized for decades as the primary causes of acute respiratory disease (ARD), gastrointestinal infection, and fever (*2*). Outbreaks of adenoviruses associated with respiratory disease have been reported worldwide (*3*,*4*) and commonly occur among the military trainees (*5*,*6*). These cases of ARD are most frequently associated with a strain of HAdV-B, HAdV-7 (*7*).

We describe the emergence of HAdV-7 in Malaysia. The outbreak occurred during March–April 2011 and involved new police recruits in the Kuala Lumpur Police Training Centre. Approximately 100 trainees were admitted to the Kuala Lumpur Hospital, and 4 more were treated in the intensive care unit. This outbreak affected 851 police trainees and claimed 3 lives.

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The Study

In April 2011, the virology unit at the Institute for Medical Research, Kuala Lumpur, received respiratory samples from police trainees admitted to Kuala Lumpur Hospital with ARD and tissue samples from 2 of the 3 patients with fatal cases. The postmortem specimens consisted of cerebrospinal, pericardial effusion, and pleural effusion fluids; lung, liver, spleen, and kidney tissues; skin; and bone marrow aspirate.

Viral nucleic acid was extracted from the clinical samples by using Roche High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany). Of the initial samples, 10 were screened for respiratory syncytial viruses, influenza viruses, parainfluenza viruses, human metapneumoviruses, coxsackieviruses, echoviruses, rhinoviruses, coronaviruses, adenoviruses, and bocavirus by multiplex PCR. All samples were then subjected to adenovirus nucleic acid detection by PCR.

Partial HAdV hexon gene was amplified by PCR (*8*). The SeqMan and Megalign software modules in the Lasergene suite of programs (DNASTAR, Madison, WI, USA) were used to format the nucleotide sequences. A phylogenetic tree was constructed by using the neighbor-joining method from the MEGA4 software (www.megasoftware.net).

A total of 33 clinical specimens, including respiratory and fecal samples as well as postmortem samples, from 21 trainees admitted to Kuala Lumpur Hospital were collected. Of these, only 31 samples from 19 trainees were sufficient for analysis. PCR or reverse transcription PCR was performed on 10 of the 31 samples by using primer sets specific for respiratory viruses with the ResPlex II Panel (QIAGEN, Valencia, CA, USA). The 10 samples contained HAdV-B species.

On the basis of this finding, partial hexon genes of adenovirus were amplified from all 31 samples by using PCR with HAdV-specific primers (*8*). The results (Table) indicated that 53% (10/19) of the samples tested were positive for adenovirus. An antemortem tracheal aspirate sample, received from the first case-patient who died, was positive for adenovirus. Subsequently, postmortem lung and spleen tissue samples tested by PCR were also positive for adenovirus. In the second fatal case, adenovirus was detected from postmortem samples consisting of pleural effusion fluid, pericardial effusion fluid, lung tissue, and serum, but other tissues, such as heart, spleen, and liver, and cerebrospinal fluid were negative (Figure 1).

All positive samples were sequenced, and BLAST sequencing analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that their sequences were similar to human adenovirus type 7 strain 0901H2/Shix/CHN/2009 isolated in People's Republic of China in 2009 (*9*). The phylogenetic tree, constructed on the basis of partial hexon gene (160)

Table. Patient information and adenovirus PCR results on samples from police trainees with acute respiratory disease from Kuala Lumpur, Malaysia, 2011*

Patient	Age, y†	Sample type	PCR results
RP301/11‡	22	T/A	Pos
RP302/11‡	25	T/A, antemortem	Pos
		Lung, spleen	Pos
		Kidney, liver, skin	Neg
		CSF, BMA	Neg
RP303/11	21	T/S	Pos
RP304/11	23	T/S	Pos
RP305/11	22	T/S	Pos
RP306/11	22	T/S	Neg
RP307/11	23	T/S	Neg
RP308/11	22	T/S	Neg
RP309/11	26	T/S	Neg
RP310/11	24	T/S	Pos
RP311/11	24	T/S	Pos
RP312/11	22	T/S	Pos
RP313/11	23	T/S	Neg
RP314/11	26	Feces	Neg
RP315/11	20	Feces	Neg
RP316/11	25	Feces	Neg
RP317/11	23	R/S	Neg
RP318/11	22	R/S	Pos
RP319/11	23	Feces	ND
RP320/11	20	Feces	ND
RP381/11‡	25	Lung, PF, PE, serum	Pos
		CSF, heart, spleen	Neg
		Liver	Neg

*T/A, tracheal aspirate; pos, positive; neg, negative; CSF, cerebrospinal fluid; BMA, bone marrow aspirate; T/S, throat swab; R/S, rectal swab; ND, test not done; PF, pericardial fluid; PE, pleural effusion.
†All patients were male.
‡Fatal case.

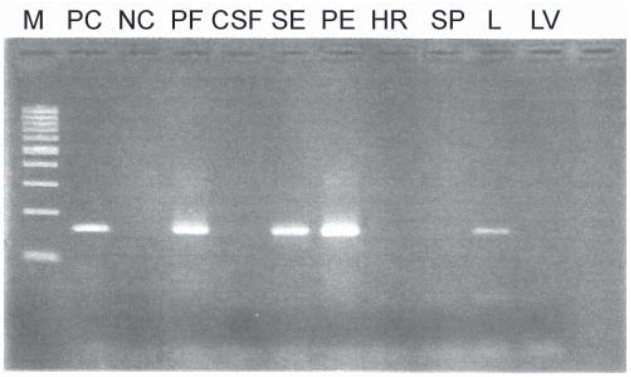

Figure 1. PCR products of postmortem samples RP381/11 on 3% agarose gel in study of human adenovirus type 7 outbreak in a police training center, Malaysia, 2011. M, 100-bp ladder; PC, positive control; NC, negative control; PF, pericardial fluid; CSF, cerebrospinal fluid; SR, serum; PE, pleural effusion; HR, heart; SP, spleen; L, lung tissue, LV, liver.

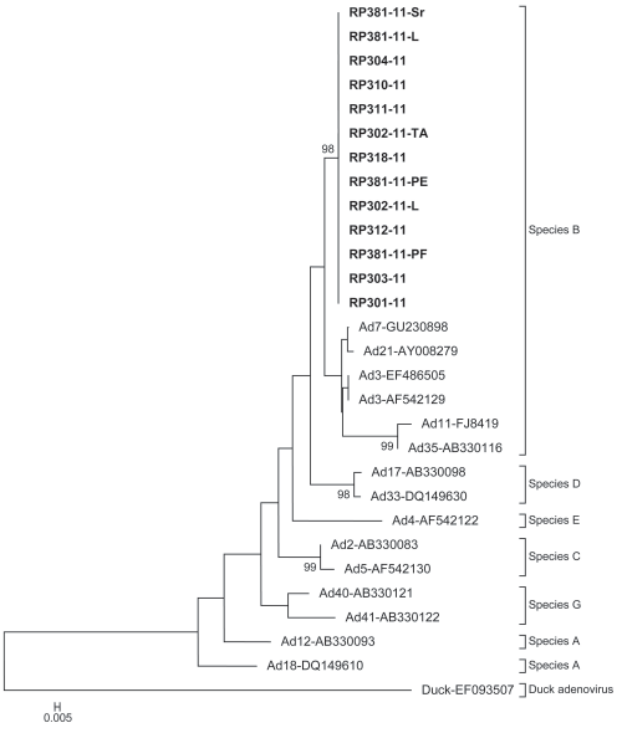

Figure 2. Phylogenetic tree of partial hexon gene sequences (160 bp) of human adenovirus inferred by using the neighbor-joining method from the MEGA4 software (www.megasoftware.net). Study was in a police training center, Kuala Lumpur, Malaysia, 2011. The evolutionary distances were computed by using the maximum composite likelihood method. Species A–F are indicated by square brackets with duck adenovirus A as an outgroup. Thirteen human adenovirus isolates from the police training center outbreak are indicated in **boldface**. Representative strains of each species obtained from GenBank are labeled by using the adenovirus species and accession number. Bootstrap values (>75%) for 1,000 pseudoreplicate datasets are indicated at branch nodes. Scale bar indicates nucleotide substitutions per site.

nucleotides (Figure 2), revealed that all positive samples by PCR belong to the species *Human adenovirus B* and are in the same cluster with adenovirus 7.

Conclusions

The outbreak of ARD, caused by HAdV-7, in the Kuala Lumpur Police Training Centre started in early March 2011. The police trainees had signs and symptoms of ARD such as fever, cough, and loss of appetite. The disease rapidly spread among the trainees, and community-acquired pneumonia was the initial diagnosis early in the outbreak. Other etiologic agents that recently caused ARD, including seasonal influenza virus and pandemic (H1N1) 2009 virus, were excluded because the reverse transcription PCR for influenza viruses was negative.

The source of the infection is not known. There is a strong possibility that one of the trainees was infected with HAdV-7 in the community outside the training center and then spread the infection to others once training resumed. Transmission occurs through respiratory droplets and close contact, which leads to rapid and widespread dissemination. Similar events have been seen in military camps (*4*), high schools, and day care centers (*10*). All affected trainees were 20–26 years old. Risk factors such as overcrowding, increased physical activities, and psychological stress

could possibly increase susceptibility to ARD. These risk factors associated with outbreaks of HAdV-caused ARD, which are prevalent in military and police training centers, have been reported in previous HAdV outbreaks in military recruits (*11*).

In Malaysia, 2 researchers (*12*,*13*) described the role of HAdV in causing ARD (*12*). They analyzed 27 HAdV isolates from patients with ARD who sought consultation and treatment at University of Malaysia Medical Center during 1999–2005. Among the 27 isolates, the following species were represented: 19 (70%) belonged to HAdV-C, 6 (22%) belonged to HAdV-B, and 2 (7%) belonged to HAdV-F. Among the HAdV-B species isolates, 5 had the HAdV-3 serotype and only 1 had the HAdV-7 serotype. An earlier analysis of HAdV isolates in Malaysia (*13*) revealed that HAdV-21 was associated with acute flaccid paralysis during the outbreak of hand, foot, and mouth disease in Sarawak, but none of the isolates were HAdV-7.

Pneumonia caused by HAdV-7, commonly associated with lower respiratory tract infection, can lead to severe disease and death in some infants and immunocompromised persons (*9*,*14*). Outbreaks of pneumonia caused by HAdV-7 have been reported among hospitalized children in South Korea and in the United States (*15*). This outbreak demonstrated the potential of ARD caused by HAdV-7 to produce illness and death in police and army recruit camps and in institutional settings. HAdV-7 infection also is fatal in children.

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Lymphocytic Choriomeningitis Virus–associated Meningitis, Southern Spain

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Lymphocytic choriomeningitis virus (LCMV) was detected in 2 patients with acute meningitis in southern Spain within a 3-year period. Although the prevalence of LCMV infection was low (2 [1.3%] of 159 meningitis patients), it represents 2.9% of all pathogens detected. LCMV is a noteworthy agent of neurologic illness in immunocompetent persons.

Lymphocytic choriomeningitis virus (LCMV; family *Arenaviridae*) is a rodent-borne pathogen; its main reservoir is the common house mouse (*Mus musculus*), but it has also been detected in pets, research rodents, and wild mice (*1*,*2*). Presumed transmission routes to humans are ingestion or inhalation of contaminated rodent feces, urine, or both. Although LCMV usually produces a self-limited mild or asymptomatic infection, it can cause aseptic meningoencephalitis (AME) with teratogenic effects in infants (*3*). Although LCMV was one of the earliest viruses to be associated with human AME, it is now rarely reported as an etiologic agent (*4*).

Historically, in Spain, LCMV was detected in 1 person with encephalitis (*5*) and 4 persons with meningitis (*6*). A similar virus, characterized as LCMV lineage IV, was identified in rodents (*2*).

During 2008–2010, a multicenter project was conducted to investigate viral causes of AME in Spain. The following viruses were considered: human enterovirus (HEV), herpesviruses, Toscana virus (TOSV), mumps virus, phleboviruses, flaviviruses, arenaviruses, and adenoviruses. We report 2 LCMV meningitis case-patients who lived 1,200 meters apart within Granada Province in southern Spain.

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The Study

The study period was January 2008–June 2010. The population included patients at Hospital Virgen de las Nieves (Granada, Spain) who had suspected AME. Routine virologic investigation included reverse transcription PCR (RT-PCR) of cerebrospinal fluid (CSF) samples for detection of HEV (Xpert EV system, Cepheid, Sunnyvale, CA, USA), TOSV (*7*), and mumps virus (*8*), and PCR of herpes simplex virus (HSV) and varicella-zoster virus (LC VHS 1/2 Qual and LC VZV systems, respectively; Roche Diagnostics, Mannheim, Germany). Negative samples were subsequently tested for Epstein-Barr virus, cytomegalovirus, human herpes 6 virus (*9*), flavivirus (*10*), arenavirus (*2*), adenovirus (*11*), and phlebovirus (*12*). Specific LCMV RT-PCR was conducted by using a previously described protocol (*2*). Finally, CSF specimens from PCR-negative case-patients were inoculated in Vero and MRC-5 cell lines for viral culture.

CSF and acute-phase serum samples were also tested for IgG and/or IgM against TOSV (EIA Enzywell Toscana virus IgG/IgM, Diesse, Siena, Italy), West Nile virus (ELISA IgG and IgM-capture ELISA; Focus Diagnostics, Cypress, CA, USA), and LCMV by indirect fluorescent assay (IFA) with further confirmation by Western blot (*2*).

We studied 159 CSF samples by using PCRs for the presence of HEV, HSV, varicella-zoster virus, TOSV, and mumps virus, yielding 68 positive cases. The remaining viruses were further investigated in the 91 negative samples. A viral agent was detected in 70 (44%) cases: HEV accounted for 44 (63%) of positive cases, followed by varicella-zoster virus in 11 (16%), HSV-1 in 8 (11%), TOSV in 4 (6%), LCMV in 2 (3%), and HSV-2 in 1 (1%).

Case-patient 1, a 21-year-old woman, came to the hospital's emergency unit in April 2008 exhibiting headache, chills, fever (38.9°C), confusion, nausea, vomiting, and slight nuchal rigidity. Aseptic meningitis was suspected, and samples of CSF and serum were obtained. Laboratory results were 415 leukocytes/mm^3 (100% mononuclear cells), 43 mg/100 dL glucose level (reference 35–65 mg/dL), and 128 mg/dL protein level (reference 15–45 mg/dL) in the CSF. Results of a computed tomographic scan of the brain were normal. IgG and IgM titers of 640 and 128 against LCMV were detected in the serum sample by IFA and titers of 400 and 200 by Western blot assay, respectively. Results of RT-PCR for arenavirus and LCMV were negative. Viral culture of the CSF in Vero cells revealed no cytopathic effect after 1 month of incubation. Cell culture supernatants from several passages were subjected to specific RT-PCR. LCMV PCR was positive at a dilution of 10^{-4} at the third passage. Viral

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isolate (EEB-7) was used for genetic characterization. To sequence the genome, degenerate and specific primers were designed on the basis of an alignment of all complete LCMV large (L), glycoprotein complex and nucleocapsid protein sequences (Table). Terminal sequences were generated by using a universal arenavirus primer, targeting the conserved viral termini (5′-CGC ACM GDG GAT CCT AGG C-3′), combined with 4 specific primers positioned near the ends of each segment. Amplification products were size-fractionated by electrophoresis in 1% agarose gels, purified (MinElute, QIAGEN, Valencia, CA, USA), and sequenced in both directions on an ABI PRISM 3700 DNA analyzer (PE Applied Biosystems, Foster City, CA, USA). To determine the evolutionary history of isolate EEB-7, we performed Bayesian phylogenetic analysis of all available complete genome sequences of LCMV using BEAST, BEAUti, and Tracer analysis software packages (*13*). The analysis showed that EEB-7 (GenBank accession nos. JN872494–5) belonged to LCMV lineage I (Figures 1, 2). Clinical diagnosis was acute meningitis and the patient was discharged from hospital on day 9.

Case-patient 2, a 39-year-old man, sought treatment in May 2010 with headache, nausea, vomiting, increased perspiration, and a temperature of 37.5°C. Aseptic meningitis was suspected, and CSF and serum samples were collected. CSF analysis demonstrated 1,715 leukocytes/mm^3 (95% mononuclear cells), normal glucose level (68 mg/dL), and elevated protein levels (240 mg/dL). Results of a cranial computed tomographic scan were normal. Further virologic investigation detected LCMV RNA in the CSF and an IgG titer of 640 by IFA and IgM antibodies against LCMV in the serum sample. Serum amount was insufficient to conduct IgM titration and Western blot assay. No CSF sample was available to attempt viral isolation. The sequence of a 194-nt PCR product (nucleocapsid protein gene) obtained was most closely related to sequences of the lineage I. Sequence homology among the LCMV amplicon from case-patient 2 and lineage I strains was >87% versus 77%–79% sequence homology among case-patient 2 and strains from lineages II–IV. Clinical diagnosis was subacute meningitis, and the patient was discharged on day 16.

PCR has become the reference standard for identifying common viruses involved in AME (*6*). However, no commercial tests are available for LCMV; and in-house PCRs have to be optimized according to the natural genetic diversity of the virus (*14*). Serologic testing has been found useful in detecting LCMV. Neurologic LCMV infection in Spain has been diagnosed primarily by serologic tests (*5*,*6*). The diagnosis in case-patients 1 and 2 in the current study was achieved by serologic testing as well. Nonetheless, PCR was useful because it allowed genetic characterization of the LCMV strain from case-patient 2. Furthermore, the viral isolate of case-patient 1 was evident only by detection of LCMV RNA in the cell culture supernatant because no cytopathic effect was observed.

Table. Consensus primers used to identify LCMV sequences, southern Spain, 2008–2010*

Name	Sequence, 5′ → 3′
LCMV_L_1F	ATAAARTGYTTTGARAARTTYTTTGA
LCMV_L_1R	TCAAARAAYTTYTCAAARCAYTTTAT
LCMV_L_2R	CATYTTCAWRCAWGARAACCAATC
LCMV_L_3R	CCWGARTABGMRTTNGCATTCAT
LCMV_L_2F	AGYAARTGGGGNCCVATGATGTG
LCMV_L_3F	GARGTHCCHTTYCCTGTTGT
LCMV_L_4F	TCYAGYCTYATTGAYATGGG
LCMV_L_5F	AARTTYACHAGRGGNGCRCAGAA
LCMV_L_4R	GTRTARTGYTCRTCYCTYTTCCA
LCMV_L_5R	CCRTCYTCDGANGGCATCAT
LCMV_L_6F	GGNTWYGGDTGGTTYTCTTA
LCMV_L_6R	GGSACDGGBTCCCABTCAGG
LCMV_GPC_1R	GARCARGARGCVGAYAAYATGAT
LCMV_GPC_1F	TARTTRCARTANGGYACNCCCAT
LCMV_GPC_2F	CCNCCRAARGCWGTYCTRAACAT
LCMV_GPC_2R	AGRGGNAGRGTBYTRGAYATGTT
LCMV_GPC_3R	TCYCAYCAYTAYATHAGYATGGG
LCMV_NP_1F	ATGCCVAGYYTRACHATGGC
LCMV_NP_1R	GCCATDGTYARRCTBGGCAT
LCMV_NP_2F	GAYGTYGTRCARGCVCTMACAGA
LCMV_NP_2R	CCHACYTGRTCNGADACRAACAT

*LCMV, lymphocytic choriomeningitis virus; GPC, glycoprotein comlex; NP, nucleocapsid protein.

Isolates from both LCMV case-patients belonged to the classical lineage I, which has been detected elsewhere in Europe. Lineage I is usually associated with human disease (as are lineages II and III) and is linked to the common house mouse as its reservoir (*14*). Lineage IV viruses were previously detected in Spanish wood mice (*2*) and have not been associated with human disease.

Although no further epidemiologic studies could be conducted to search for infected reservoirs, the common genetic lineage and the fact that both case-patients resided

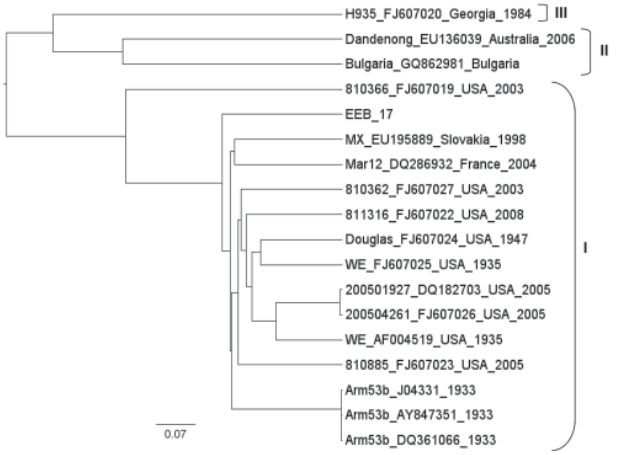

Figure 1. Phylogenetic tree showing genetic lymphocytic choriomeningitis virus sequences relationship within the large segment. The name of the strain is followed by GenBank accession number, country, and year of detection. Clusters grouped in brackets depict the lymphocytic choriomeningitis virus lineage. Scale bar indicates nucleotide substitutions per site.

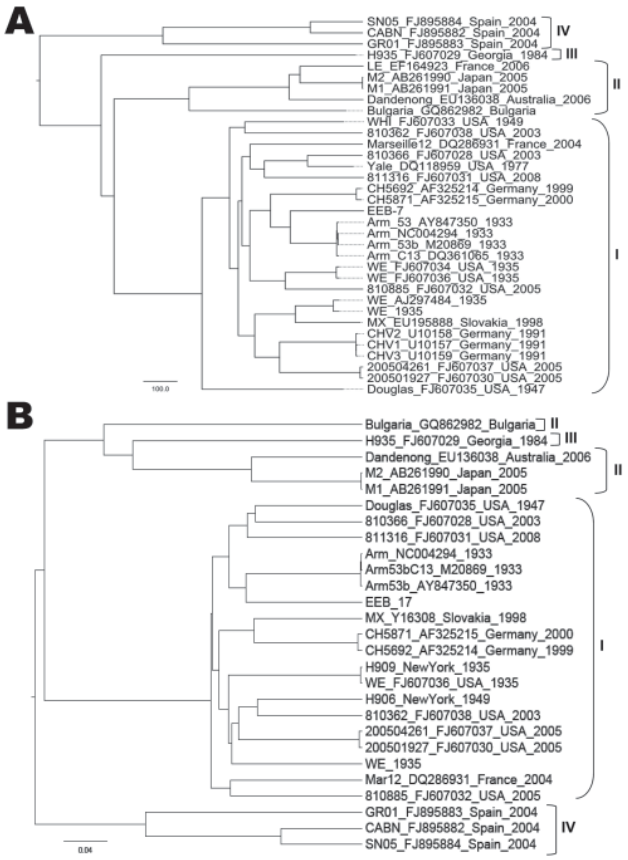

Figure 2. Phylogenetic tree showing genetic lymphocytic choriomeningitis virus sequences relationship within the small segment coding for the glycoprotein complex (A) and nucleocapsid proteins (B). The name of the strain is followed by GenBank accession number, country, and year of detection. Clusters grouped in brackets depict the lymphocytic choriomeningitis virus lineage. Scale bar indicates nucleotide substitutions per site.

within the same community might suggest transmission of LCMV by a common vector. Previous seroprevalence studies have associated human LCMV infection with high exposure to the common house mouse (*15*).

Conclusions

Human LCMV infections might be underdiagnosed because the clinical characteristics of LCMV meningitis are similar to those of other viral meningitis; no commercial tests are available for serologic or molecular diagnostic assays, and usually no clear epidemiologic clue is available at the moment of diagnosis. Thus, epidemiologic and virologic surveillance might ascertain that the true incidence of LCMV AME is more frequent than reported.

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Diversity of Parvovirus 4–like Viruses in Humans, Chimpanzees, and Monkeys in Hunter–Prey Relationships

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During 2010–2011, we investigated interspecies transmission of partetraviruses between predators (humans and chimpanzees) and their prey (colobus monkeys) in Côte d'Ivoire. Despite widespread infection in all species investigated, no interspecies transmission could be detected by PCR and genome analysis. All sequences identified formed species- or subspecies *(*chimpanzee)-specific clusters, which supports a co-evolution hypothesis.

Since 2005, new parvoviruses have been discovered in the following groups: humans (parvovirus 4 [PARV4]), bats (*Eidolon helvum* parvovirus 1), and other mammals (cows, pigs, wild boars, and sheep; Hong Kong virus) (*1*–*5*). Phylogenetic analysis suggests that these parvoviruses form a separate novel genus, with the proposed name of Partetravirus, within the subfamily *Parvovirinae*. Globally, 3 genotypes of PARV4 have been found to infect humans (*6*,*7*). Recently, PARV4-like viruses have also been described in chimpanzees and gorillas (*8*). Researchers have suggested that partetraviruses have co-diverged with their hosts during mammalian evolution. Strains described so far have shown restricted sequence diversity within their host-specific clusters. However, the highly restricted sequence diversity of circulating variants of PARV4 also suggests that the virus has emerged and spread in the human population relatively recently. To clarify whether interspecies transmission is possible for primate PARV4-like viruses, as has been shown for other parvoviruses (*9*), we investigated samples in a setting where transmission of certain simian viruses between these species has been documented (*10*,*11*). We analyzed samples from wild chimpanzees (*Pan troglodytes verus*) in the Taï National Park, Côte d'Ivoire; their prey, red colobus monkeys (*Piliocolobus badius*) and black-and-white colobus monkeys (*Colobus polykomos*); and humans who hunt colobus monkeys in the same region.

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The Study

Chimpanzee and monkey samples were obtained as described from 2002–2007 (*10*,*11*). Human volunteers, recruited during a broad study of primate-borne zoonoses (samples collected between June 2006 and January 2007), ranged in age from 7 to 95 years, and all lived adjacent to the primate habitat. All human participants acknowledged eating bushmeat; most (74%) also reported butchering animals, and a small group (8%) admitted hunting bushmeat. Written informed consent forms were signed by all participants. Ethical approval was obtained from the Institut Pasteur Côte d'Ivoire and the Ministère de la Santé of Côte d'Ivoire. Cross-contamination of samples was avoided by using disposal materials and maintaining a strict safety regime for sampling humans and animals. Samples from different species were handled separately throughout the process, from sampling to analysis.

DNA from 17 chimpanzees (lung, spleen, or liver), 30 red colobus monkeys (buffy coat, blood, bone marrow, intestine, spleen, liver, lung, or muscle), and 15 black-and-white colobus monkeys (buffy coat, liver, intestine, or muscle) and 700 humans (blood), was prepared by using commercial DNA extraction kits (QIAGEN, Hilden, Germany). Partetravirus generic quantitative real-time PCR (qPCR) was used to screen the samples as described (*1*). Viral DNA from positive samples was amplified by using primers for conserved regions spanning human variants and other partetraviruses described in pigs and cows (*2*).

PARV4-like viruses were detected by qPCR in 7 (41%) of 17 chimpanzees tested. Seven (23%) of 30 red colobus and 2 (13%) of 15 black-and-white colobus monkeys were positive for PARV4-like viruses. The availability of only 1 sample per individual animal limited the analysis of viral tissue distribution (Table).

In a recent study of 91 Old World monkeys tested, none exhibited PARV4-like virus seroreactivity, whereas 63% of chimpanzees (*P.t. troglodytes*) and 18% of

[1]These authors contributed equally to this article.

Table. Results of testing analyzed samples for PARV4 and PARV4-like viruses, Côte d'Ivoire*

Sample origin	Sample material, no. PCR positive/no. tested (%)							
	Blood	Bone marrow	Intestine	Spleen	Muscle	Liver	Lung	Total
Red colobus monkey (*Piliocolobus badius*)	1/14 (7)†	2/6 (33)	0/1	0/3	2/2 (100)	1/2 (50)	1/2 (50)	7/30 (23)
Black and white colobus monkey (*Colobus polykomos*)	2/10 (20)‡	–	0/1	–	0/3	0/1	–	2/15 (13)
Wild chimpanzee (*Pan troglodytes verus*)	0/1‡	–	–	5/13 (38)	–	–	2/3 (67)	7/17 (41)
Human§	12/700 (2)	–	–	–	–	–	–	12/700 (2)

*Each sample represents 1 animal or person; PARV4, parvovirus 4; –, no sample.
†Blood or buffy coat.
‡Buffy coat.
§Dried blood spot on filter paper.

gorillas (*Gorilla gorilla*) were reactive (*8*). The Old World monkeys belonged to the family *Cercopithecinae*, whereas the PARV4-positive *Colobus* and *Piliocolobus* species described herein belong to the *Colobinae* branch of family *Ceropithecidae*. The serologic assay performed by Sharp et al. is based on a human PARV4 ELISA; therefore, as suggested by the study's authors, PARV4-like viruses that infect members of the *Ceropithecinae* family might be too divergent to cross-react. The percentage (43%) of *P.t. verus* chimpanzees that tested positive for PARV4-like DNA in our study is comparable to the proportion of seropositive *P.t. troglodytes* chimpanzees in Cameroon (63%), although only 1 animal was positive by PCR in Cameroon. The low rate of PCR-positive results from serum samples in the study by Sharp et al. (*8*), compared with the rate in the current study, is not unexpected: PARV4 can be found in tissues of nonviremic animals and humans because of its persistence in tissue after resolution of acute infections.

In 12 (1.7%) of the 700 human blood samples, PARV4 genotype 3 was amplified. The median age of infected persons was 11 years (mean 20.8 years, range 7–75 years). As discussed previously (*12*), the broad age range suggests that PARV4 genotype 3 in Africa has alternative routes of infection from genotypes 1 and 2 that are found in Europe.

Near full-length nucleotide sequences from viral genomes were generated from samples from 4 persons (GenBank accession nos. JN798193–196), 1 chimpanzee (JN798203), and 1 black-and-white colobus monkey (JN798211). Partial sequences were derived from 7 persons (JN798192, JN798197–201), 3 chimpanzees (JN798204–206), and 4 red colobus monkeys (JN798207–210).

On the basis of phylogenetic analyses of the 20 sequences obtained, we could not detect any interspecies transmission; all sequences formed host species–specific clusters (Figure). Sequence diversity of genotype 3 viruses from humans in the defined region in our study was 0.9% over 1,423 nt positions, which is in accordance with what has been published (*12*). One additional near full-length genotype 3 sequence from Africa was included in the phylogenetic analysis; it clustered with human sequences found in this study. Compared with sequences from human PARV4 (AY622943) isolates, sequences from isolates from chimpanzees differed by 19% over 4,771 nt and formed a distinct cluster, together with sequences derived from chimpanzees of other subspecies. PARV4-like virus from *P.t. verus* (JN798203) from this study and *P.t. troglodytes* (HQ113143) from Cameroon (*8*) differed by 10%, suggesting subspecies-specific PARV4-like viruses within chimpanzees of 2 different subspecies from distinct habitats. Black-and-white colobus monkey viruses exhibited a closer relationship to viruses from humans than did PARV4-like viruses from red colobus monkeys. This finding corresponds to a previous phylogenetic analysis in which the genomic relationship of different species showed that colobus monkeys separated earlier from piliocolobus monkeys during evolution (*13*). The distance between PARV4-like viruses from piliocolobus monkeys to those of colobus monkeys supports these data.

To evaluate whether underlying infections were present in the hunters (chimpanzees and humans) originating from the prey, a red colobus PARV4-like virus–specific qPCR was designed. Samples from humans, chimpanzees, and black-and-white colobus monkeys, which previously tested positive in the generic partetravirus qPCR, were retested.

Although chimpanzees consumed immense quantities of red colobus meat and organs (≈45 kg/year for adult males [*14*]), we could not detect red colobus PARV4-like virus in any of the 17 chimpanzees analyzed. Similarly, no red colobus PARV4-like virus DNA was discovered in humans, although in this particular region the red colobus monkey is the most hunted and eaten primate (*15*). These results support the hypothesis that PARV4-like viruses are species specific, notwithstanding constant high exposure to infectious materials.

Conclusions

We demonstrated that partetravirus infection is widespread in monkeys, chimpanzees, and humans in West Africa. However, the PARV4-like viruses seem to remain species specific, despite continuous opportunities for interspecies transmission. The data presented here suggest that the risk for zoonotic transmission of PARV4-

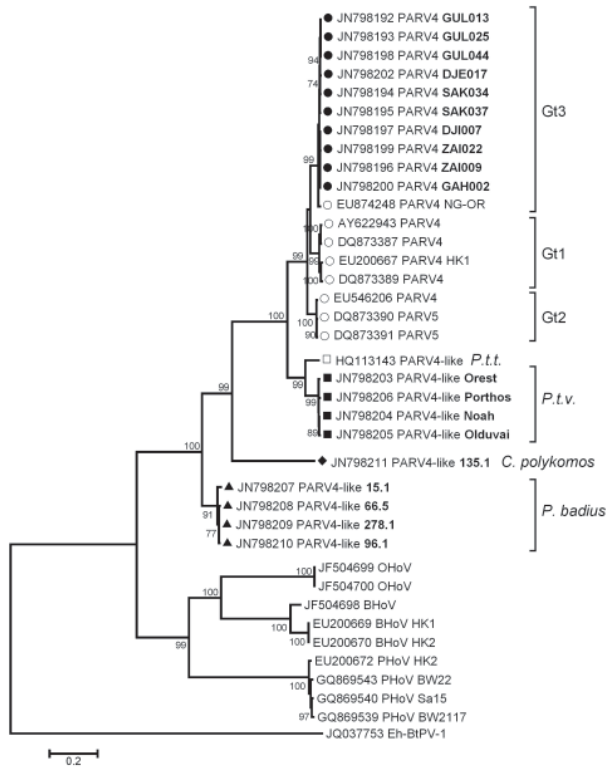

Figure. Phylogenetic tree of near full-length and partial sequences (open reading frame 2) of parvovirus 4 (PARV4), PARV4-like viruses, and Hong Kong virus (HoV) created by using MEGA5.05 (www.megasoftware.net) with the maximum likelihood-method (GTR+G+I) and bootstrap analysis of 1,000 resamplings. Sequence origin was indicated as follows: circle, from humans; square, from chimpanzees; triangle, from red colobus monkeys; and tetragon, from black-and-white colobus monkeys. New sequences from Côte d'Ivoire are shown with filled symbols. *P.t.t.*, *Pan troglodytes troglodytes*; *P.t.v.*, *Pan troglodytes verus*; *P. badius*, *Piliocolobus badius*; *C. polykomos*, *Colobus polykomos*; BHoV, bovine HoV; PHoV, porcine HoV; OHoV, ovine HoV; Eh-BtPV-1, *Eidolon helvum* parvovirus 1; Gt, genotype of human PARV4. Scale bar indicates nucleotide substitutions per site.

like viruses from nonhuman primates in West Africa is low. Nonetheless, parvovirus evolution has pointed toward occasional cross-species transmissions (*9*). This observation, coupled with the frequent intimate contact between bushmeat hunters and their prey, compels the continued vigilance for cross-species transmission of these viruses and others with the intention of mitigating the risk posed by novel introductions of viral zoonoses.

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GenBank accession numbers of the generated sequences: human PARV4, JN798192–JN798202; *P.t. verus* PARV4-like, JN798203–JN798206; *P. badius* PARV4-like, JN798207–JN798210; *C. polykomos* PARV4-like, JN798211.

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Emergency Department Visits for Influenza A(H1N1)pdm09, Davidson County, Tennessee, USA

Wesley H. Self, Carlos G. Grijalva, Yuwei Zhu, H. Keipp Talbot, Astride Jules, Kyle E. Widmer, Kathryn M. Edwards, John V. Williams, David K. Shay, and Marie R. Griffin

To determine the number of emergency department visits attributable to influenza A(H1N1)pdm09 in Davidson County, Tennessee, USA, we used active, population-based surveillance and laboratory-confirmed influenza data. We estimated ≈10 visits per 1,000 residents during the pandemic period. This estimate should help emergency departments prepare for future pandemics.

The 2009 pandemic influenza (H1N1) strain, hereafter referred to as influenza A(H1N1)pdm09, had the potential to substantially increase visits to emergency departments, many of which operate at or near capacity (*1*–*5*). Surges in emergency department patient volume cause treatment delays, low quality care, and increased risk for medical error (*6*). Understanding the number of visits associated with influenza A(H1N1)pdm09 should help emergency departments prepare for future influenza epidemics. We therefore estimated population-based emergency department visit rates attributable to influenza A(H1N1)pdm09 during the first year it circulated in Davidson County, Tennessee, USA. The Vanderbilt University Institutional Review Board approved this study.

The Study

As part of the Influenza Vaccine Effectiveness network (Flu-VE) (*7*), we conducted active, prospective, population-based influenza surveillance among residents of Davidson County. We included those who had visited Vanderbilt University adult or pediatric emergency departments for acute respiratory infection (ARI) or fever/feverishness for <14 days during May 1, 2009–March 31, 2010. Nasal and throat swabs were tested for influenza with reverse transcription PCR (RT-PCR) by using primers and probes provided by the Centers for Disease Control and Prevention (Atlanta, GA, USA) (*8*). Specimens were classified as A(H1N1)pdm09 virus if results were positive on both pandemic subtyping assays (pandemic A and pandemic H1) or positive for influenza A, negative for seasonal subtypes H1 and H3, and positive on 1 pandemic subtyping assay.

We obtained the number of emergency department visits associated with ARI or fever (International Classification of Diseases, Ninth Revision, Clinical Modification, codes 381–382, 460–466, 480–487, 490–493, 786, and 780.6) from the Tennessee Hospital Discharge Data System (HDDS) (*9*), which is required to include a record of every hospital-based health care encounter. We combined data from Flu-VE RT-PCRs, influenza test results obtained clinically in the surveillance emergency departments, and HDDS discharge diagnoses to calculate age-specific visit rates attributable to influenza A(H1N1)pdm09. We used 2 epidemiologic methods: surveillance sampling and capture–recapture.

For surveillance sampling, we enrolled 826 (52%) of 1,589 eligible patients in the Flu-VE study who had visited surveillance emergency departments; 88 (11%) had positive RT-PCR results for A(H1N1)pdm09 virus (Figure). We divided the pandemic period into 3 intervals according to prevalence of A(H1N1)pdm09 among Flu-VE participants: prepeak (May–July 2009), peak (August–November 2009), and postpeak (December 2009–March 2010). Within each period, we assumed that the proportion of ARI- or fever-associated visits caused by A(H1N1)pdm09 virus among enrolled county residents was the same as that for such emergency department visits among all county residents. Estimated influenza A(H1N1)pdm09–associated emergency department visits were thus calculated by multiplying age- and time- specific counts of total county ARI- or fever-associated emergency department visits by these proportions (Table 1). We divided age-specific counts by age-specific county population estimates for July 2009 (*10*) and calculated rates per 1,000 residents (Table 2). We used the binomial Wilson method to calculate 95% CIs for the proportions of ARI- or fever-associated emergency department visits caused by A(H1N1)pdm09 virus.

We developed a capture–recapture model (*11*) by linking 2 independent data sources for influenza testing from the same population: the Flu-VE RT-PCRs, performed in a research laboratory and not reported to patients or clinicians, and influenza tests performed as routine care in the surveillance emergency departments. Unlike the research laboratory tests, not all clinical tests included

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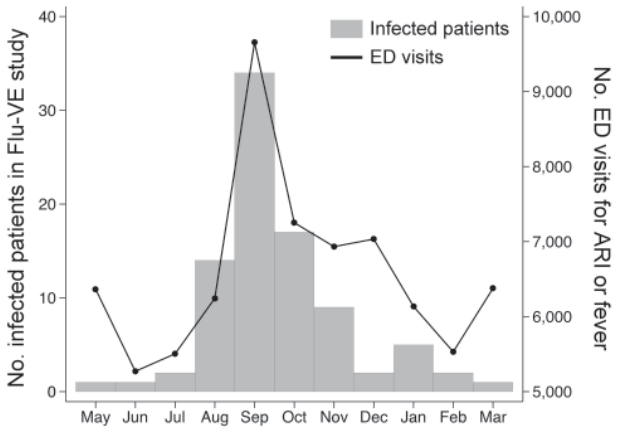

Figure. Number of patients enrolled in the Influenza Vaccine Effectiveness study at Vanderbilt University (Nashville, Tennessee, USA) who had laboratory-confirmed influenza A(H1N1)pdm09 virus infection (bars) and number of emergency department (ED) visits associated with a discharge diagnosis of acute respiratory illness (ARI) or fever (line) among all residents of Davidson County, Tennessee, May 1, 2009–March 31, 2010.

influenza A subtyping. However, all positive influenza A results were assumed to be A(H1N1)pdm09 virus because that strain circulated almost exclusively during the study period (*12*). To calculate the total number of influenza A(H1N1)pdm09–associated visits in surveillance emergency departments, we summed the following: the number of such emergency department visits detected by Flu-VE and clinical laboratory testing (a), the number detected by Flu-VE alone (b), the number detected by clinical testing alone (c), and the number missed by both systems (d). For each age group, we estimated the number of emergency department visits for influenza A(H1N1) pdm09 missed by both surveillance systems by using the nearly unbiased estimator equation, a modification of the Petersen estimator that performs well with rare outcomes: $d = bc / (a + 1)$ (*11*,*13*).

RT-PCR identified 88 persons with influenza A(H1N1) pdm09; 541 patients had positive influenza A results by clinical tests: 506 BinaxNOW influenza rapid antigen tests (Alerei Inc., Waltham, MA, USA), 19 clinical RT-PCRs, and 16 viral cultures. Influenza A(H1N1)pdm09 virus was detected by clinical and research laboratory testing ("a" in the formula) for only 13 patients; age groups were <5 years (3 patients), 5–17 years (3), 18–49 years (7), and ≥50 (0). Using the nearly unbiased estimator equation, we calculated 572, 1,000, 528, and 90 surveillance emergency department visits for influenza A(H1N1)pdm09 for each age group, respectively. HDDS data indicated that 62.3%, 48.4%, 18.3%, and 14.2% of ARI- or fever-associated emergency department visits among county residents <5, 5–17, 18–49, and ≥50 years of age, respectively, occurred in surveillance emergency departments. We calculated the total number of influenza A(H1N1)pdm09–associated emergency department visits by county residents by dividing the number of influenza A(H1N1)pdm09–associated visits to surveillance emergency departments by the age-specific proportions above. To estimate rates, we divided estimated influenza A(H1N1)pdm09 visits by age-specific county populations for July 2009 (*10*) and multiplied by 1,000, yielding rates comparable to those obtained by the surveillance sampling method (Table 2). We calculated 95% CIs for capture–recapture estimates by using a bias-corrected bootstrap method (*14*). Because no persons ≥50 years of age were identified by both surveillance systems, 95% CIs for this group and the entire population could not be calculated.

Conclusions

Using 2 epidemiologic techniques for calculating rates, we found that ≈1% of the Davidson County, Tennessee, population had visited an emergency department for influenza A(H1N1)pdm09 during the first year of virus circulation. The study has several limitations. The reported rates are dependent on the sensitivity and specificity of influenza tests. Delays in seeking care could have resulted in some influenza A(H1N1)pdm09 cases being undetectable, and if so, rates reported here would underestimate true rates of influenza A(H1N1)pdm09–attributable emergency department visits. Because active surveillance activities did not influence the possibility of influenza identification through routine emergency department care, the independence of these systems was assumed for capture–recapture calculations. However, this assumption could have been violated in some instances, for example

Table 1. Estimated total number of emergency department visits for influenza A(H1N1)pdm09, calculated by surveillance sampling method, Davidson County, Tennessee, USA, May 1, 2009–March 31, 2010*

Patient age, y	No. ARI or fever visits countywide†			% ARI or fever visits to surveillance emergency departments for influenza A(H1N1)pdm09‡			Estimated no. visits for influenza A(H1N1)pdm09 countywide
	Prepeak	Peak	Postpeak	Prepeak	Peak	Postpeak	
<5	3,135	6,461	5,219	0	19.0	0	1,249
5–17	1,599	5,269	2,324	15.0	24.0	0	1,528
18–49	7,322	11,523	10,486	2.4	23.0	6.3	3,455
≥50	5,085	6,832	7,046	0	6.2	1.5	503

*ARI, acute respiratory infection.
†Data from Tennessee Hospital Discharge Data System (*9*).
‡Data from Influenza Vaccine Effectiveness Study (Vanderbilt University, Nashville, TN, USA).

Table 2. Estimated emergency department visits for influenza A(H1N1)pdm09, Davidson County, Tennessee, USA, May 1, 2009–March 31, 2010*

Patient age, y	County population	No. visits/1,000 population (95% CI) Surveillance sampling estimates	Capture–recapture estimates
<5	47,446	26.3 (15.2–42.7)	19.3 (11.0–38.3)
5–17	93,710	16.3 (8.90–27.9)	22.0 (12.0–46.5)
18–49	318,006	10.9 (7.48–17.2)	9.08 (5.67–22.6)
≥50	176,548	2.85 (1.05–7.77)	3.60 (NC)
Total	635,710	10.6 (6.48–18.0)	10.2 (NC)

*NC, not calculated because of no matched cases in patients ≥50 years of age.

if influenza viral load varied substantially among persons and higher viral loads increased the likelihood of detection by both systems. In this scenario, our method would underestimate the true number of emergency department visits (by increasing the number of matched cases). The proportions of ARI- and fever-associated emergency department visits for A(H1N1)pdm09 virus infection were extrapolated from surveillance emergency departments to the entire county population. If this proportion were higher (or lower) in the surveillance emergency departments than in other emergency departments, our rates would overestimate (or underestimate) true rates. Additionally, the small number of cases detected in adults ≥50 years of age precluded further age stratification among older adults. Because this study was conducted in an urban US county with high accessibility to emergency departments, we advise caution when extrapolating our estimates directly to other populations.

A modern influenza pandemic of mild severity can quickly cause large surges in emergency department visits. To minimize emergency department overcrowding and to maximize efficient use of resources, long-term preparation for these surges is vital. The high number of emergency department visits during the pandemic also illustrates the large effect a novel influenza stain can have on an unvaccinated, susceptible population and highlights the need for continued influenza vaccine development and use.

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Screening for Influenza A(H1N1)pdm09, Auckland International Airport, New Zealand

Michael J. Hale, Richard S. Hoskins, and Michael G. Baker

Entry screening for influenza A(H1N1)pdm09 at Auckland International Airport, New Zealand, detected 4 cases, which were later confirmed, among 456,518 passengers arriving April 27–June 22, 2009. On the basis of national influenza surveillance data, which suggest that ≈69 infected travelers passed through the airport, sensitivity for screening was only 5.8%.

The virus that caused the 2009 influenza pandemic, hereafter referred to as influenza A(H1N1)pdm09, is mainly spread internationally by air travel (*1*). To prevent or delay such spread, during the pandemic many countries initiated screening of air travelers arriving at airports, even though these measures have not been recommended by the World Health Organization (*2*). On April 25, 2009, New Zealand was one of the first countries outside the Americas to confirm influenza A(H1N1)pdm09 in arriving airline passengers (*3*). During April 27–June 22, 2009, at the direction of the Ministry of Health, the Auckland Regional Public Health Service began a screening program at Auckland International Airport.

Protocols for border screening were updated throughout the pandemic and evolved as new information became available. Screening was initially applied to all passengers arriving or transferring on flights from countries where community transmission of influenza A(H1N1)pdm09 virus was occurring. The screening program included the following (Figure):

- All flights notified New Zealand of the health of passengers and crew on board before landing; if indicated, the aircraft was met by public health officials who triaged these travelers.
- Cabin crew announced a scripted health message requesting passengers to identify themselves if symptomatic; after disembarkation, all passengers passed through a public health checkpoint where signage encouraged ill travelers to seek screening.
- Information about influenza A(H1N1)pdm09 was available, those with symptoms could self-declare, and public health officials visually inspected arriving passengers and approached those with apparent symptoms.
- Neither thermal scanning nor active screening of every arriving passenger was used.

Each unwell passenger and crew member was screened for influenza-like illness by a nurse and assessed by a medical officer if illness met the definition of a suspected case. Those whose illness met the case definition had nasopharyngeal swabs taken, were offered oseltamivir, and were sent home or to a facility for isolation. Reverse transcription PCR (RT-PCR) was used to confirm infection. Screening was escalated on April 29 to include all passengers arriving from other countries and stopped on June 22.

The Study

We quantified the results of entry screening for influenza A(H1N1)pdm09 at Auckland International Airport. Using the information generated during screening, we retrospectively estimated the number of infected travelers who actually passed through the airport. To estimate the sensitivity of screening, we then compared screening findings with the expected number of infected travelers who passed through the airport. Ethical approval was received from the Northern X Regional Ethics Committee of the New Zealand Ministry of Health.

The number of screened passengers was obtained from airport records. The numbers of crew members on inbound international aircraft were estimated by using averages for flights into Auckland. The number of travelers detected at each step and referred to the next step of the screening process was obtained from Auckland Regional Public Health Service records. Virologic test results were extracted from laboratory information systems. A confirmed case was one that met the current case definition (as published on the Ministry of Health website, www.health.govt.nz) and one for which RT-PCR result was positive.

We estimated the number of infected travelers screened as the total number of confirmed cases in New Zealand during this period, multiplied by the proportion of overseas-acquired cases, and the proportion of international travelers arriving at the airport. On April 30, 2009, nonseasonal influenza A (H1N1) was made notifiable,

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and these data were collated on the national surveillance database (EpiSurv) (www.surv.esr.cri.nz/episurv). The proportion of infected travelers who acquired the infection overseas was extrapolated from Ministry of Health records of the first 100 cases of pandemic (H1N1) 2009 because this information was not collected for all travelers with confirmed infection. The proportion of travelers who passed through the airport was determined from Statistics New Zealand (www.stats.govt.nz) arrivals records. Confidence intervals were calculated by using the online calculator for screening on Open Epi (*4*).

During the screening period, 456,518 international travelers were screened; 406 (0.09%) of these were referred for medical assessment. Of those, 109 (27%) met the case definition and received virologic testing. RT-PCR results were located for 89 (82%), among which 4 were positive. A total of 312 cases were confirmed. The proportion of travelers who acquired the infection overseas was 32%. The proportion who passed through the airport was 69%. The expected number of infected travelers estimated to have passed through the border during the screening program was therefore 69, giving an estimated sensitivity of 5.8% (95% CI 2.3%–14.0%).

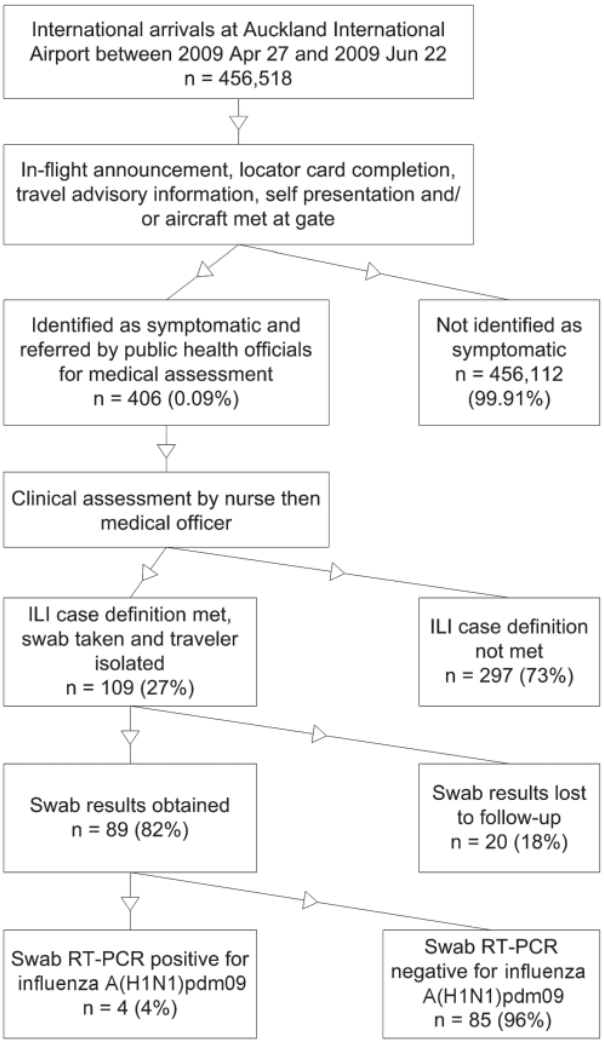

Figure. Process used to screen arriving international passengers for influenza A(H1N1)pdm09, Auckland International Airport, New Zealand, April 27–June 22, 2009. ILI, influenza-like illness; RT-PCR, reverse transcription PCR.

Conclusions

The influenza A(H1N1)pdm09 screening program at Auckland International Airport had low sensitivity. This form of border screening is therefore unlikely to have substantially delayed spread of the pandemic into New Zealand in 2009.

Limitations of influenza screening include the high proportion of asymptomatic infected travelers (*5*), incubation of infections acquired before or during a flight (*3*), reliance on self-identification, limitations of case definitions, and limitations of thermal scanning (*6*). Modeling data have shown that the ability of border screening to delay global pandemic influenza is closely linked to the effectiveness of the screening process or travel restriction used. To delay influenza spread by 1.5 weeks, border restrictions need to reduce imported infections by 90% (*7*). The entry screening program we describe does not meet these standards.

The potential effectiveness of screening arriving travelers to prevent or delay influenza epidemics has been debated. Mathematical models and literature reviews have argued for (*7*,*8*) and against (*9*–*11*) this approach. Some authors have found that entry screening for respiratory conditions or influenza A(H1N1)pdm09 is insensitive and not cost-effective (*12*). Border screening did not substantially delay local transmission of influenza A(H1N1) pdm09 (*13*).

This study has several limitations, particularly with regard to estimating the number of infected travelers who would have passed through the airport during the screening period. Most cases of illness acquired overseas would probably not have been notified, particularly those in patients with mild illness who did not see a doctor or who saw a doctor but did not receive a diagnosis. The estimated proportion of overseas-acquired cases was based on data from the first 100 cases and would have decreased as the pandemic progressed. The net effect of these factors is unknown, but they would probably have increased the estimated number of undetected infected travelers passing through screening, thereby further reducing the estimated sensitivity of screening.

Border screening might be conducted for reasons other than preventing or delaying an epidemic. It might

provide public assurance and confidence that something is being done (*14*). The communication of health information and advice on how to seek treatment is consistently recommended as a pandemic prevention strategy (*12*,*15*) and is usually delivered as part of border screening programs. These benefits need to be balanced against the considerable resources used, opportunity cost (resources used for this activity and thereby unavailable for other activities), uncertain effectiveness, and inconvenience of border screening.

To delay or prevent influenza entry at borders, influenza screening needs to be considerably more effective than the mostly passive program described here. We hope that during this interepidemic period, a major international review of the role of international air travel in the dissemination of emerging infectious diseases will be conducted to identify effective interventions. Such a review should consider systemwide approaches, including exit screening, standardized health declarations, active screening of individual passengers (including use of rapid laboratory tests and thermal scanning), passenger tracking, policies and practices that support sick travelers wishing to defer travel, and circumstances where airline travel should be suspended entirely.

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Hepatitis E Virus Infection among Solid Organ Transplant Recipients, the Netherlands

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We screened 1,200 living heart, lung, liver, and kidney transplant recipients for hepatitis E virus infection by reverse transcription PCR. In 12 (1%) patients, hepatitis E virus infection was identified; in 11 patients, chronic infection developed. This immunocompromised population is at risk for hepatitis E virus infection.

Hepatitis E virus (HEV) can cause acute or chronic infection in humans. Four genotypes have been identified in humans. HEV genotype 3 predominantly infects pigs and deer, but is also recognized as a zoonotic agent. As awareness increases, more reports of HEV infection among humans, especially immunocompromised persons, have been published (*1*,*2*).

Analysis of exposure histories of persons with HEV genotype 3 infections has demonstrated its underdiagnosis, and a source was not identified for most cases (*3*). Because HEV has been reported as a cause of liver disease in solid organ transplant (SOT) recipients (*4*), we screened all living recipients of SOTs during 2000–2011 at Erasmus Medical Center, the largest SOT center in the Netherlands, for HEV RNA. This study was designed to identify SOT recipients with acute or chronic HEV infection.

The Study

A cross-sectional study was performed of all living adult SOT recipients for whom serum or EDTA-plasma samples were available in the Erasmus Medical Center biobank (stored at –20°C and –80°C, respectively, and collected during previous routine visits to the outpatient clinic; complete methods are described in detail in the online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1712-Techapp.pdf). Some recipients eventually had been referred to peripheral hospitals. A Laboratory Information Management System database search was performed for availability of the most recent follow-up sample. Thirty-nine HEV RNA–positive samples in the center's biobank from non-SOT patients were genotyped and used as reference for phylogenetic analysis. Samples were screened for HEV RNA by using real-time reverse transcription PCR (RT-PCR) (*5*) with primers detecting all 4 genotypes and validated according to International Standards Organization guidelines 9001 and 15189 (www.iso.org/iso/search.htm). HEV IgM and IgG were detected by using the PE2 HEV-IgM and IgG ELISA (Wantai Biological Pharmacy Enterprise Co., Ltd., Beijing, People's Republic of China). A case of HEV infection was defined by the following criteria: an HEV RNA–positive sample, confirmed either by presence of HEV IgM or IgG or HEV RNA in sequential samples. Chronic infection was diagnosed by retrospective testing of stored samples and defined as HEV RNA positive for >6 months. We retrospectively tested samples from HEV RNA–positive patients so the antibody kinetics and viremia levels could be studied. For calculating phylogenetic relationships, HEV open reading frame (ORF) 1 sequences were generated with primer set MJ-C (*6*). All viral sequences were deposited into GenBank (accession nos. JQ015399–JQ015448).

The 1,200 SOT recipients consisted of 259 heart transplant (HTX), 53 lung transplant (lungTX), 300 liver transplant (LTX), 574 kidney transplant (NTX), and 14 multiple SOT recipients (4 HTX–NTX, 1 lungTX–NTX, and 9 LTX–NTX). Twelve HEV-infected patients were identified: 5 HTX, 1 lungTX, 3 LTX, and 1 NTX recipients and 2 multiple SOT-recipients (1 HTX–NTX and 1 LTX–NTX). For 11 patients, HEV infection was chronic (Table 1). The median age of the HEV-infected patients was 56.9 years (range 19.9–63.5 years); 9 (75%) were men. In 10 HEV patients, immunosuppression was achieved by using prednisolone and tacrolimus, combined with mycophenolate mofetil (n = 3) or everolimus (n = 2). Two patients received regimens of cyclosporine and prednisolone or mycophenolate mofetil and prednisolone.

Table 1. Overview of HEV infections among SOT recipients, the Netherlands, 2000–2011*

SOT group	No. recipients	HEV infections, no. (%)	
		Confirmed	Chronic
HTX	259	5 (1.9)	5 (1.9)
LungTX	53	1 (1.9)	1 (1.9)
LTX	300	3 (1.0)	3 (1.0)
NTX	574	1 (0.2)	1 (0.2)
Multiple SOT†	14	2 (14.3)	1 (7.1)
Total	1,200	12 (1.0)	11 (0.9)

*HEV, hepatitis E virus; SOT, solid organ transplant; HTX, heart transplant; lungTX, lung transplant; LTX, liver transplant; NTX, kidney transplant.
†9 NTX–LTX, 4 NTX–HTX, and 1 NTX–lungTx.

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All patients who had chronic HEV infection had elevated liver enzyme levels; bilirubin levels were elevated in 45.5% of the patients (Table 2). Although it proved difficult to identify abnormal liver functions uniquely related to the HEV infection, HEV RNA detection always coincided with or was followed by an increase in alanine aminotransferase. Apparently no overt clinical symptoms were associated with infection; however, such symptoms are difficult to recognize in immunosuppressed SOT recipients. Inflammation compatible with viral hepatitis was shown in 8 of 9 patients with chronic infection for whom liver biopsy specimens were available. Other findings were F0–F2 fibrosis, steatosis 1–2 (Brunt classification), cholestasis, and Councilman bodies.

Samples from all 12 HEV patients were tested for HEV RNA and HEV IgM and IgG. One infection was traced to 2003 (lungTX), 1 to 2008 (NTX), 1 to 2009 (multiple SOT recipient, NTX–HTX), 7 to 2010 (5 HTX, 1 LTX and 1 multiple SOT recipient, NTX–LTX) and 3 to 2011 (all LTX). Among the patients, 1 LTX recipient had an acute HEV infection and cleared the virus within 6 days. Because HEV IgM and IgG were detected 4 years before HEV RNA detection, both reactivation and reinfection should be considered. The median span of HEV RNA-positive time period of chronic HEV cases was 16 months (range 6–55) with a median peak cycle threshold value of 20.0 (range 16.7–26.6). HEV RNA was detected during viremia (median cycle threshold value 19.9, range 15.5–28.3) in feces from 8 patients with chronic illness.

To assess the value of diagnostic techniques for detection of HEV infection in SOT recipients, we studied antibody kinetics (HEV IgM and IgG) and viremia. The median time from RNA positivity to IgM detection was 32 days (range 0–826 days). Five patients had detectable HEV IgM at the time of HEV RNA positivity. In 1 case, no HEV IgM was detected. HEV IgG titers were detectable an average of 124 days later than HEV RNA (range 0–826 days). HEV IgG was absent in 2 samples, and in 4 samples, HEV IgG was detectable when HEV RNA was detected. The median time between transplantation and first HEV RNA-positive result was –0.3 to 20.0 years (median 1.99 years).

Viruses isolated from samples from 11 HEV-infected patients were all within the genotype 3 group. Because no ORF1b sequences from the Netherlands were available in GenBank, ORF1b sequences were determined from samples from non-SOT HEV-infected patients in the Netherlands (Figure). No indications for a common or nosocomial source of HEV transmission were found.

Conclusions

Recent HEV infections in SOT recipients (*4*,*7*–*9*) prompted us to perform a survey among SOT recipients admitted to the largest transplantation center in the Netherlands. Our findings showed that they are at risk for HEV infection. Nine of 12 case-patients were treated postoperatively with a tacrolimus-based regimen, which has been associated with increased risk for HEV infection (*9*).

The cross-sectional RT-PCR screening detected 12 HEV infections but could not provide information about previously acquired and cleared HEV infections. Real-time RT-PCR screening was performed for 2 reasons. First, because a patient received immunosuppressive drugs, specific antibodies against HEV might be absent. Second, ELISAs have been developed to detect antibodies to genotypes 1 (Myanmar) and 2 (Mexico) and might not be sensitive enough to detect antibodies to genotype 3 or 4 (*10*). Information about results of serologic assays to validate HEV genotype 3 is limited, and seroprevalence measured can vary with the assays used (*11*–*13*). Furthermore, independent studies found that sensitivity and specificity of HEV RNA assays from laboratories in the Netherlands (S.D. Pas and B. Hogema, unpub. data) and other European countries (*14*) differ greatly. Therefore, international standardization should be encouraged.

Although the observed 1% of HEV-infected SOT recipients may seem low, HEV infection may be life threatening in immunocompromised patients. Misdiagnosis of HEV infection as drug-induced liver injury or autoimmune hepatitis has been reported (*15*); empirical treatment of these misdiagnoses by raising immune suppression would exacerbate the condition. Temporary reduction of immunosuppression resulted in immune-mediated control and clearance of HEV in 30% of cases (*9*).

Table 2. Parameters in chronic HEV infections among SOT recipients, the Netherlands, 2000–2011*

Parameter	Median	Range	ULN (F/M)
Peak alanine aminotransferase, U/L	301	81–909	30/40
Peak aspartate aminotransferase, U/L	172	66–1016	30/36
Peak gamma-glutamil transferase, U/L	299	72–1740	34/49
Peak bilirubin, μmol/L	16	5–100	16/16
Peak HEV RNA, cycle threshold values	20.0	16.7–26.6	NA
Period of HEV RNA positivity, mo	16	6–55	NA
Time between SOT and first HEV RNA–positive result, mo	2.0	–0.3 to 20.1	NA
Time of HEV RNA positivity before HEV IgM positive, d	32	0–826	NA
Time of HEV RNA positivity before HEV IgG positive, d	124	0–826	NA

*HEV, hepatitis E virus; SOT, solid organ transplant; ULN (F/M), upper limit of normal (female/male); NA, not applicable.

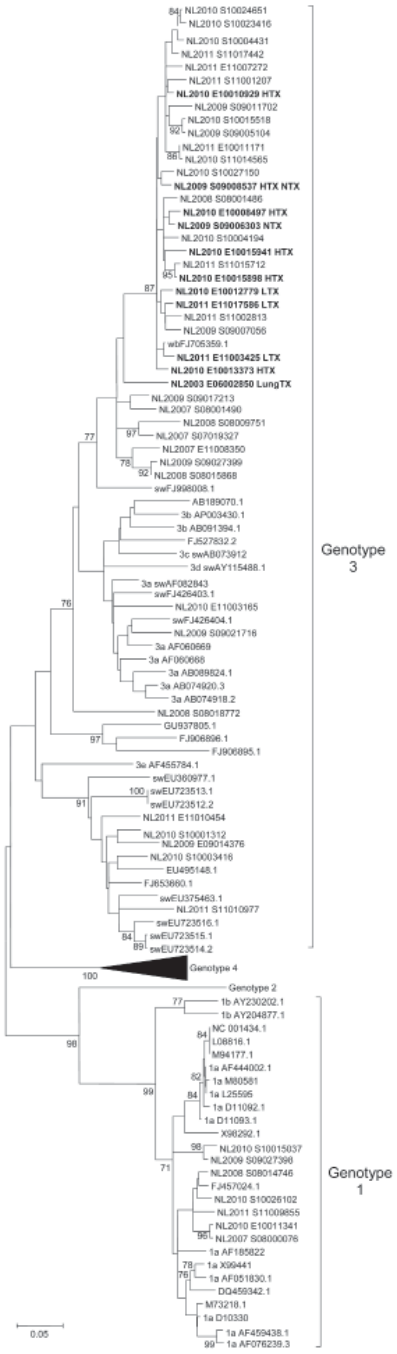

Figure. Phylogenetic tree of hepatitis E virus (HEV) open reading frame (ORF) 1 sequences, including HEV infections, the Netherlands, 2000–2011. Phylogenetic relation of a 306-bp ORF1 region was calculated by using maximum-likelihood, Kimura 2-parameter analysis with bootstrapping (n = 1,000). HEV sequences originating in the Netherlands are indicated as NL with year of isolation and isolate number (GenBank accession nos. JQ015399–JQ015448). **Boldface** indicates virus strains of chronic HEV-infected solid organ transplant recipients identified in this study. Scale bar indicates number of nucleotide substitutions per site. HTX, heart transplant; NTX, kidney transplant; LTX, liver transplant; lungTX, lung transplant.

This study also found that in patients with chronic HEV infection, HEV RNA was detected an average of 32–124 days before HEV IgM and IgG, respectively. Therefore, in SOT recipients with elevated liver enzymes (alanine aminotransferase), the diagnosis of HEV infection should be considered and verified by detection of HEV RNA.

This systematic survey of HEV infections among SOT recipients in a major transplant center shows that this population is at risk for HEV infection. Given the consequences of HEV infection, SOT recipients with liver function impairment of unknown etiology should be tested for HEV RNA.

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Sapovirus Outbreaks in Long-Term Care Facilities, Oregon and Minnesota, USA, 2002–2009

Lore E. Lee, Elizabeth A. Cebelinski, Candace Fuller, William E. Keene, Kirk Smith, Jan Vinjé, and John M. Besser

We tested fecal samples from 93 norovirus-negative gastroenteritis outbreaks; 21 outbreaks were caused by sapovirus. Of these, 71% were caused by sapovirus genogroup IV and 66% occurred in long-term care facilities. Future investigation of gastroenteritis outbreaks should include multi-organism testing.

Viral gastroenteritis outbreaks are associated with illness and death when they occur in institutional settings, notably in long-term care facilities (LTCFs) for the elderly (*1*). Although most reported outbreaks in LTCFs are caused by norovirus (*2*), some have similar epidemiologic characteristics but are norovirus-negative after ≥2 fecal samples are tested by real-time reverse transcription PCR (RT-PCR). Epidemiologically, these norovirus-like gastroenteritis outbreaks are characterized by 24–48-hour incubation periods, if known; vomiting in ≥50% of affected persons; and 12–60-hour median illness durations (*3*).

Norovirus and sapovirus are separate genera of the family *Caliciviridae*. Sapovirus was first detected in 1977 as the cause of a gastroenteritis outbreak in a home for infants in Sapporo, Japan (*4*), and was thereafter reported primarily among young children with acute gastroenteritis (*5*). After sapovirus RT-PCR was developed (*6*), sapovirus outbreaks were discovered in LTCFs and other settings populated by adults (*7*–*9*). Sapovirus genogroups I, II, IV, and V (GI, GII, GIV, and GV, respectively) infect humans (*10*). This report describes sapovirus outbreaks in Oregon and Minnesota, USA, during 2002–2009.

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The Study

The Oregon and Minnesota state public health departments investigated 2,161 gastroenteritis outbreaks reported during 2002–2009. Of these, 1,119 (52%) were caused by laboratory-confirmed norovirus (defined as ≥2 norovirus-positive fecal samples by RT-PCR); 466 (22%) were caused by bacteria, parasites, and other agents; 403 (19%) had no fecal samples to analyze; 142 (7%) were norovirus negative (defined as ≥2 norovirus-negative fecal samples by RT-PCR) and, when tested, were negative for enteric bacterial pathogens; and 31 (<1%) had a single norovirus-negative stool sample. Outbreak-related fecal samples were archived when any specimen remained after analysis, creating a convenience sample of feces for this and other studies.

The Minnesota Public Health Laboratory tested feces from 93 (66%) of the 142 norovirus-negative outbreaks with RT-PCRs for astrovirus, adenovirus, rotavirus, norovirus, and sapovirus (*6*). Sapoviruses were genotyped by sequence analysis of the capsid gene (*11*).

Defining a sapovirus outbreak in this study as ≥1 sapovirus-positive fecal sample, 21 (23%) of the 93 norovirus-negative outbreaks were found to be caused by sapovirus. Adenovirus or norovirus were also identified in 4 (19%) of the 21 sapovirus outbreaks (Table 1). The unexpected norovirus finding is likely due to slight variations in testing methods between state public health laboratories and viral loads nearing the detection level of the RT-PCR.

Of 21 sapovirus outbreaks, LTCFs accounted for 12 (66%); grade schools for 2 (10%); and a prison, a large psychiatric hospital, a cruise ship, a restaurant, and a bed and breakfast for 5 (24%). During 2007, 10 outbreaks (48%) occurred; 14 outbreaks (67%) occurred during the colder months (November–March) of each observed year. Person-to-person transmission accounted for 18 (86%) of 21 outbreaks. On the basis of the outbreak setting, foodborne transmission was suspected, but not confirmed, in 3 (14%) of 21 sapovirus outbreaks; food items were not implicated. Outbreaks involved 5–44 persons (median 34 persons) per outbreak and lasted 1–28 days (median 15 days) (Table 2).

Clinical data were available for 141–269 patients from 14 sapovirus outbreaks in which neither adenovirus nor norovirus were identified. Of 141 patients, 32 (23%) had fevers. Of 269 patients, 132 (49%) had vomiting, and 238 (88%) had diarrhea (Table 2). In Oregon, 1 person with sapovirus was hospitalized and 1 died; no hospitalizations or deaths occurred in Minnesota among persons with sapovirus. Symptoms lasted 24–105 hours (median 48 hours) (data not shown).

Four (19%) of 21 sapovirus outbreaks were caused by sapovirus GI, 1 (5%) by sapovirus GII, 15 (71%) by

Table 1. Microbiology of 21 sapovirus outbreaks, Oregon and Minnesota, USA, 2003–2009*

State	Outbreak no.	Fecal samples, no.		Genotype	Results
		Sapovirus positive	Tested		
MN	2002–438	1	4	IV	Sapovirus only
MN	2002–439	3	5	IV	Sapovirus only
MN	2003–644	1	2	II	Sapovirus only
OR	2004–066	1	2	V	Sapovirus only
MN	2006–924	2	3	IV	Sapovirus only
OR	2007–001	3	8†	IV	Sapovirus, norovirus GI
OR	2007–013	3	3	IV	Sapovirus only
OR	2007–023	3	7	IV	Sapovirus only
OR	2007–028	4	6	IV	Sapovirus only
OR	2007–039	3	4	IV	Sapovirus only
OR	2007–046	4	4	IV	Sapovirus only
OR	2007–086	4	5	IV	Sapovirus only
OR	2007–091	4	6‡	IV	Sapovirus, adenovirus
OR	2007–221	1§	2	I	Sapovirus, norovirus GII
OR	2007–228	1	1	IV	Sapovirus only
OR	2008–109	1	6	I	Sapovirus only
OR	2008–128	3	5¶	I	Sapovirus, adenovirus
MN	2008–1308	3	3	I	Sapovirus only
MN	2008–1327	3	3	IV	Sapovirus only
OR	2009–146	3	3	IV	Sapovirus only
OR	2009–167	2	2	IV	Sapovirus only

*MN, Minnesota; OR, Oregon; G, genogroup.
†One norovirus GI–positive sample.
‡One adenovirus-positive sample.
§Norovirus GII co-infection.
¶Two adenovirus-positive samples.

Table 2. Descriptive epidemiology of 21 sapovirus outbreaks, Oregon and Minnesota, USA 2002–2009*

Infection and state	Outbreak no.	Setting	Transmission	Outbreak features		No. cases‡	Symptoms, no. patients		
				Date	No. days†		Vomiting§	Diarrhea§	Fever¶
Sapovirus only									
MN	2002–438	Grade school	Person-to-person	2002 Apr	11	15	NA	NA	NA
MN	2002–439	Long-term care	Person-to-person	2002 Apr	1	34	NA	NA	NA
MN	2003–644	Grade school	Person-to-person	2003 Dec	8	17	NA	NA	NA
OR	2004–066	Long-term care	Person-to-person	2003 Mar	17	44	23	44	8
MN	2006–924	Long-term care	Person-to-person	2006 Feb	13	24	9	24	11
OR	2007–013	Long-term care	Person-to-person	2007 Jan	15	12	7	9	NA
OR	2007–023	Long-term care	Person-to-person	2007 Jan	7	35	16	33	3
OR	2007–028	Long-term care	Person-to-person	2007 Jan	9	12	7	5	6
OR	2007–039	Long-term care	Person-to-person	2007 Jan	22	14	8	12	2
OR	2007–046	Long-term care	Person-to-person	2007 Jan	5	15	11	15	0
OR	2007–086	Long-term care	Person-to-person	2007 Feb	13	8	6	7	NA
OR	2007–228	Long-term care	Person-to-person	2007 Nov	10	34	14	27	NA
OR	2008–109	Long-term care	Person-to-person	2008 Apr	28	24	10	21	NA
MN	2008–1308	Cruise ship	Foodborne suspected	2008 Aug	1	5	3	5	NA
MN	2008–1327	Bed and breakfast	Foodborne suspected	2008 Nov	3	7	2	7	2
OR	2009–146	Psychiatric hospital	Person-to-person	2009 Jul	9	13	9	11	NA
OR	2009–167	Long-term care	Person-to-person	2009 Aug	11	22	7	18	NA
Sapovirus and norovirus									
OR	2007–001	Prison	Person-to-person	2006 Dec	23	154	70	119	1
OR	2007–221	Long-term care	Person-to-person	2007 Nov	16	34	8	29	NA
Sapovirus and adenovirus									
OR	2007–091	Long-term care	Person-to-person	2007 Feb	13	25	15	25	NA
OR	2008–128	Restaurant	Foodborne suspected	2008 Apr	4	26	10	25	NA

*MN, Minnesota; OR, Oregon; NA, data were not collected or could not be analyzed.
†Median no. days: 15 (range 1–28 days).
‡Laboratory-confirmed and epilinked cases with systematically collected symptoms; these are not complete case counts. Median no. cases: 34 (range 5–44 cases).
§Of 269 patients, vomiting was reported for 132 (49%) and diarrhea for 238 (88%).
¶Of 141 patients, fever was reported for 32 (23%).

sapovirus GIV, and 1 (5%) by sapovirus GV (Table 1). The genogroup-specific differences between outbreak settings and between the proportions of vomiting, diarrhea, and fever were not statistically significant. Seventy-three percent of sapovirus GIV outbreaks occurred in 2007. A representative sequence from each outbreak was placed in the phylogenic tree (Figure). Of 14 sapovirus outbreaks with ≥2 sapovirus-positive samples, sequences from 12 were identical within the outbreaks, and 2 had ≈2 different sequences (Figure).

Conclusions

In this study, the high (66%) proportion of sapovirus outbreaks in LTCFs among 21 outbreaks of previously unknown etiologies is likely to be an artifact of legally mandated outbreak reporting by health care facilities rather than the true distribution of sapovirus outbreaks in Oregon and Minnesota. Still, elderly residents of LTCFs are especially vulnerable to rapid transmission of viral enteric pathogens and serious complications from infection with these agents (*12*), and therefore merit the attention of public health.

Our data, together with a recent study in Canada (*7*), demonstrate that sapovirus has been circulating among the institutionalized elderly since at least 2002 and that sapovirus outbreaks increased in 2007 as part of a worldwide surge in gastroenteritis outbreaks (*2*,*7*,*9*). Before these retrospective studies, sapovirus infections among adults ≥65 years old had been reported as single cases at a low (3%) rate in 2002 (*13*) and as nosocomial outbreaks in 2010 and 2005 (*8*,*14*). In 2010, Svraka et al. reported an age distribution shift from younger to older persons (*9*).

Sapovirus outbreaks occurred in the same settings and had the same seasonal distribution as norovirus outbreaks (*2*,*15*). Our study adds clinical details to information provided by studies in Canada and Europe (*7*,*9*). The clinical profile of sapovirus outbreaks in this study (49% vomiting, 88% diarrhea, and 23% fever, plus a median duration of 48 hours) approximates the criteria of Kaplan et al. (*3*), which are still used to evaluate norovirus outbreaks when laboratory resources are limited. We found, however, that sapovirus and norovirus outbreaks are clinically and epidemiologically similar enough to be indistinguishable without laboratory testing.

This study has at least 3 limitations. First, testing a convenience sample of fecal specimens from norovirus-negative outbreaks might have introduced selection bias, the impact of which is uncertain. Second, because outbreak reporting from institutions other than LTCFs is not legally mandated, outbreaks in these settings are underreported. Third, feces from norovirus-positive outbreaks were not assayed for sapovirus. Previously undetected norovirus GI and GII discovered among 21 sapovirus outbreaks indicates that outbreaks might have had >1 etiology. It is therefore likely that the number of sapovirus outbreaks was underestimated.

In summary, gastroenteritis outbreaks in LTCFs should be investigated by public health departments in conjunction

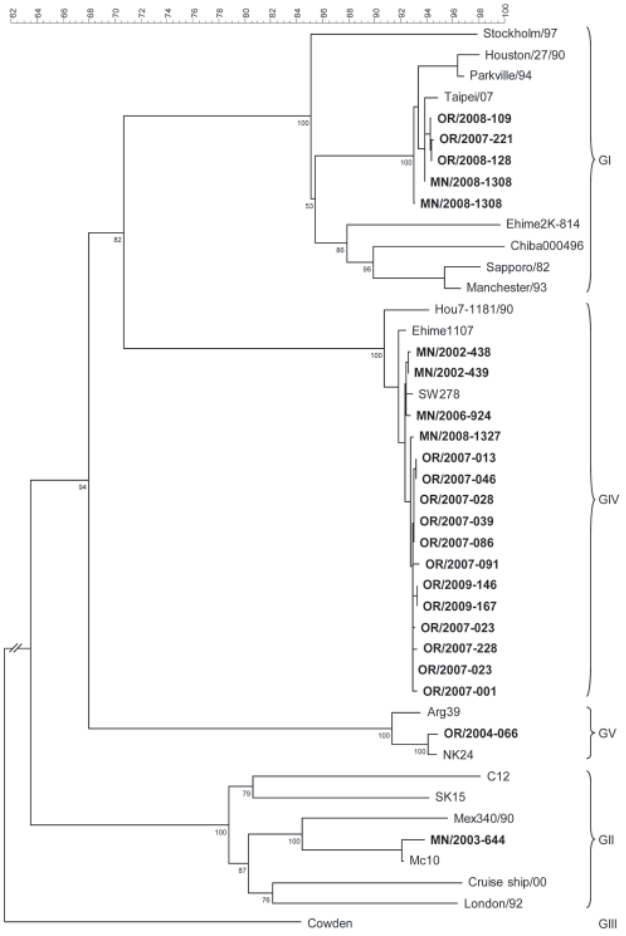

Figure. Phylogenetic tree of sapovirus sequences from outbreaks of acute gastroenteritis reported to state public health departments in Oregon and Minnesota, 2002–2009, on the basis of partial capsid nucleotide sequences. Reference strains [GenBank accession numbers] include Sapporo/1982/JP [U65427], Parkville/1994/US [U73124], Stockholm318/1997/SE [AF194182], Chiba000496/2000/JP [AJ606693], Ehime2K-814/2000/JP [AJ606698], London/1992/UK[U95645], Mex340/1990/MX [AF435812], cruise ship/2000/US [AY289804], PEC-Cowden/1980/US [AF182760], Hou7-1181/1990/US [AF435814], and Argentina39/AR [AY289803]. **Boldface** indicates state-assigned outbreak identification numbers. Scale bar represents percent genetic similarity between sequence types. Genogroups are indicated on the right. For genogrouping, GenBank sequences of well-characterized genogroups were aligned with outbreak sequences, and a phylogenetic tree was created by the neighbor-joining method by using BioNumerics (Applied Maths, Austin, TX, USA). Genotypes were assigned on the basis of >95% similarity to reference strains. Outbreak strain sequences were deposited in GenBank under accession nos. HM800902–HM800920.

with testing of fecal specimens. Public health laboratories should archive fecal samples from all gastroenteritis outbreaks until a cause is established. As in this study, testing with assays for sapovirus, astrovirus, adenovirus, and rotavirus, should be conducted when standard methods for norovirus and enteric bacterial pathogens fail to identify a causative agent.

In keeping with recent recommendations, at minimum, adding sapovirus to routine diagnostics of infections that occur in any setting and by any mode of transmission will establish etiologies of some norovirus-negative outbreaks and help define the disease impact and clinical characteristics of sapovirus infections (*9*,*10*,*13*). These data can in turn be used to develop and evaluate sapovirus disease management guidelines and sapovirus outbreak prevention and control measures.

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Possible Nosocomial Transmission of *Pneumocystis jirovecii*

To the Editor: Diversity of genotypes among *Pneumocystis jirovecii* (human-specific *Pneumocystis* species) isolates mainly involves internal transcribed spacer (ITS) loci (*1*). Type Eg, one of the most frequently detected ITS genotypes, has been found worldwide (*2*). The locus of dihydropteroate synthase (DHPS) is also of interest because DHPS is the target of sulfonamides, the main drugs used to treat *Pneumocystis* pneumonia (PCP). Studies of the DHPS locus have found mutations at positions 165 and 171, which confer potentially lower sensitivity to sulfonamides to mutant *P. jirovecii* organisms (*3*).

Airborne transmission of *Pneumocystis* ssp. has been demonstrated among animals and probably occurs among humans (*4*). Reports of clusters of PCP cases in hospitals (*4,5*) provide a rationale for considering the possibility of nosocomial *P. jirovecii* infections. Moreover, we recently quantified *P. jirovecii* in the air surrounding patients with PCP (*6*). Our findings suggested that the fungus is exhaled from infected patients and then spreads into their surrounding air.

Because matches of *P. jirovecii* genotypes between pulmonary and air samples would strengthen these findings, we conducted DHPS and ITS typing of *P. jirovecii* isolates from PCP patients and from the air in their close environment. We assayed *P. jirovecii* DNA that we had previously detected in pulmonary samples (bronchoalveolar lavage and induced sputum) from 15 PCP patients and in 15 air samples collected 1 meter from each patient's head (*6*).

ITS genotyping was based on sequence analysis of ITS 1 and 2 regions after amplification with a nested PCR, cloning, and sequencing, as described (*7*). ITS alleles were identified by using the typing system by Lee et al. (*2*). DHPS genotyping was based on a PCR restriction fragment-length polymorphism assay that enables detection of mutations at positions 165 and 171, as described (*8*).

Among the 15 pulmonary samples, ITS genotyping was successful for all 15; among these, 8 ITS genotypes were identified (Table). Type Eg was most frequently identified. Mixed infections, which correspond to detection of >1 genotype in a given sample, were detected in 5 samples. DHPS genotyping was successful for all 15 pulmonary samples. A wild genotype was identified in 9 samples, a 165 mutant genotype in 1 sample, and a 171 mutant genotype in 2 samples. Mixed infections were identified in the 3 remaining samples.

Among the 15 room air samples, ITS genotyping was successful for 7; among these, 4 ITS genotypes were identified (Table). Type Eg was again most frequently identified. A mixed infection was detected in 1 of the 7 samples. These results enabled us to compare ITS genotypes for 7 pairs of pulmonary and air samples. A full match was found for 4 (57.1%) pairs of samples, and a partial match, defined as at least 1 common genotype for pulmonary and air samples in mixed infections, was found for 2 (28.6%) pairs. No matches were found for the remaining pair of samples. DHPS genotyping was successful for 6 of the 15 air samples. A wild genotype was identified in 4 samples, a 165 mutant genotype was identified in 1 sample, and a 171 mutant genotype

Table. Genotyping of *Pneumocystis jirovecii* in pairs of pulmonary and air samples from 15 patients with *Pneumocystis* pneumonia*

Patient no.†	No. days between pulmonary and air sampling	ITS genotype (no. sequenced clones)‡		DHPS genotype§	
		Pulmonary sample	Air sample	Pulmonary sample	Air sample
1	6	Gg, Fg (3)¶	ND	Wild	ND
2	1	Ih (3)	Eg (3)	Wild	ND
4	0	Gg (3)	Gg (3)	Wild	Wild
5	0	Eg (3)	Ec, Eg (3)¶	Wild	Wild
6	0	Eg (3)	Eg (3)	Mutant 171#	Mutant 171
7	1	Eg (3)	ND	Wild	Wild
8	0	Eg (2)	Eg (1)	Wild	ND
10	0	Eg (3)	ND	Mutant 171	ND
11	0	Be, Ec (3¶)	Ec (3)	Mutant 165**	Mutant 165
13	2	Eg (3)	ND	Wild	ND
15	0	Eg, Fg (3)¶	ND	Wild + mutant 171	ND
16	0	Eg (3)	ND	Wild	Wild
17	1	Ie, Ih (2)	ND	Wild	ND
18	1	BI (3)	BI (3)	Wild + mutant 165	ND
19	0	Eg, BI (3¶)	ND	Wild + mutant 165	ND

*ITS, internal transcribed spacers; DHPS, dihydropteroate synthase; ND, not determined.
†Patients are numbered as described in (*6*). Pulmonary samples were bronchoalveloar lavage specimens for patients 2, 4, 6, 7, 8, 10, 11, 18, and 19 and induced sputum specimens for patients 1, 5, 13, 15, 16, and 17.
‡*P. jirovecii* ITS genotype identification using sequence analysis with a prior cloning step and applying the score by Lee et al. (*2*).
§*P. jirovecii* DHPS genotype identification using a PCR restriction fragment length polymorphism assay (*8*).
¶Major ITS genotype, as identified in 2 of 3 clones.
#Mutant genotype with the mutation at position 171.
**Mutant genotype with the mutation at position 165.

was identified in 1 sample. These results enabled us to compare DHPS genotypes for 6 pairs of samples. A full match was found for these 6 pairs. DHPS and ITS genotype matches were found for 4 pairs.

Several lines of evidence suggest that *P. jirovecii* is exhaled by infected patients and transmitted by the airborne route to susceptible persons (*4*). In the study reported here, ITS or DHPS genotype matches between pairs of pulmonary and air samples are consistent with the possibility that *P. jirovecii* organisms in the air originated from patients. DHPS mutants were detected in 6 (40%) of the 15 pulmonary samples; none of the15 patients had received sulfonamide treatment at the time of PCP diagnosis. These results were not unexpected because frequency of finding DHPS mutants in PCP patients in Paris who had no prior sulfonamide treatment is high (*8*). The exhalation of DHPS mutants from infected patients can spread potentially sulfonamide-resistant organisms.

Matches of *P. jirovecii* genotypes in pairs of pulmonary and room air samples argue in favor of *P. jirovecii* exhalation by infected patients. The exhalation of *P. jirovecii* organisms emphasizes the risk for their nosocomial transmission. Our data provide additional arguments in favor of the application of measures to prevent the airborne transmission of *P. jirovecii* in hospitals.

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Fatal Human Co-infection with *Leptospira* spp. and Dengue Virus, Puerto Rico, 2010

To the Editor: Leptospirosis, caused by *Leptospira* spp. bacteria, and dengue, caused by dengue viruses (DENVs), are potentially fatal acute febrile illnesses (AFI) endemic to the tropics (*1*,*2*). Because their clinical manifestations are similar (*3*), leptospirosis may be misidentified as dengue (*4*). We report a fatal case of co-infection with *Leptospira* spp. and DENV-1 in a man in Puerto Rico.

On May 23, 2010, a 42-year-old unemployed male carpenter came to an outpatient clinic in Puerto Rico reporting a 4-day history of fever, headache, generalized myalgia, anorexia, nausea, and vomiting. He was being treated for chronic hypertension and had been released from jail 2 weeks before illness onset. On evaluation, he was febrile, hypertensive, and tachycardic; laboratory results showed thrombocytopenia and leukocytosis with a predominance of neutrophils. Viral syndrome was diagnosed, and the patient was given acetaminophen, solumedrol, and ketoprofen.

The patient returned to the clinic on May 25 with continued fever, myalgia, worsening headache, and bilateral calf pain; he was afebrile and tachycardic and appeared acutely ill. He had no rash, jaundice, icteric sclera, cardiac murmurs, or organomegaly, and his

lungs were clear on auscultation. He was given intravenous (IV) saline, and results of laboratory tests performed afterward showed leukocytosis with a predominance of neutrophils, thrombocytopenia, increased blood urea nitrogen (BUN)–to-creatinine ratio, hyponatremia, hyperglycemia, and elevated aspartate aminotransferase. He was given IV ampicillin, meperidine, and promethazine and was transferred to a local hospital for admission, with a presumptive diagnosis of pre–renal azotemia and leptospirosis.

On arrival at the emergency department on the same day, the patient was febrile, tachycardic, and hypotensive, with cold, clammy skin. Results of an electrocardiogram showed sinus tachycardia; cardiac enzymes were not elevated. He was given repeat IV saline and piperacillin/tazobactam. New laboratory findings included anemia, prolonged prothrombin time, elevated creatinine kinase, hematuria, and a further increase in BUN-to-creatine ratio. Chest radiograph showed cardiomegaly with increased pulmonary vascularity and perihilar alveolar densities. Arterial blood gas (ABG) results showed compensated metabolic acidosis, with low oxygen partial pressure (pO_2). He was given IV saline again, and vancomycin and ceftriaxone were added to his medication regimen.

On admission to the intensive care unit, the patient continued to be hypotensive and was again given IV saline. Although ABG results on the morning of May 26 were somewhat improved, the patient was started on respiratory treatments for new-onset cough and increasing respiratory rate. Laboratory test results showed a large drop in hematocrit, worsening thrombocytopenia and leukocytosis, hypocalcemia, and hypoalbuminemia; he was given an infusion of 25% albumin.

The patient's condition continued to worsen, with ABG results showing further decline in pO_2. Severe respiratory distress developed, and he was placed on mechanical ventilation and given IV saline. Repeat ABG results showed severe respiratory acidosis and metabolic acidosis. Soon after, generalized edema developed, and the patient became cyanotic, with no measurable pulse; despite aggressive resuscitation efforts, he died on March 26.

All results of bacterial cultures were negative, as was detection of anti-*Leptospira* IgM. Postmortem examination showed rash and pleural effusion, and blood and tissue specimens were taken for diagnostic testing. Liver sections showed bile stasis, dilated sinusoidal space, and pericentral hepatocellular necrosis (Figure, panel A); lung sections showed intraalveolar hemorrhage, edema, and focal inflammatory infiltrates (Figure, panel B). Heart sections showed perivascular edema, and kidney sections showed evidence of interstitial inflammatory infiltrates and acute tubular necrosis (Figure, panel C). Immunohistochemical analysis of kidney (Figure, panel D), liver, lung, and heart sections showed *Leptospira* antigen. Dengue virus nonstructural (NS) protein 1 was detected in whole blood, and flavivirus NS5 gene was amplified from RNA extracted from the liver; sequencing showed 98% homology with DENV-1.

This case report demonstrates the need for antigen-based rapid diagnostic tests (RDT) for AFI patients. All available leptospirosis RDTs detect anti-*Leptospira* IgM (*5*), which was not detectable in this patient's blood on the seventh day of illness, although *Leptospira* antigen was detected in postmortem analysis. Therefore, it is unlikely that any available leptospirosis RDT would have been clinically useful when leptospirosis signs first were recorded on the fourth day of illness. Because the incidence of both dengue and leptospirosis is increasing worldwide (*6,7*), physicians should have access

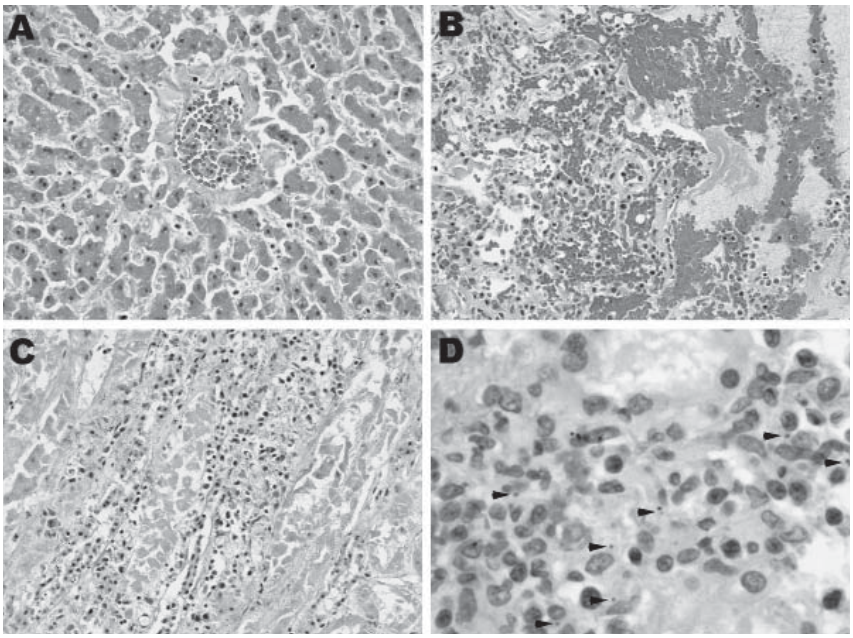

Figure. Histopathologic evaluation of tissue samples collected postmortem from a person co-infected with *Leptospira* spp. and dengue virus 1. Tissue specimens were taken from the liver (A), lung (B) and kidney (C and D) and stained with hemotoxylin-eosin (A, B, C; original magnification ×20) or probed with polyclonal anti-*Leptospira* antibody for immunohistochemical detection of *Leptospira* antigen (D; arrowheads indicate antigen; original magnification ×63). A color figure of this figure is available online (wwwnc.cdc.gov/EID/article/18/5/11-1555-F1.htm).

to antigen-based RDT to make timely and thorough diagnoses.

Nonetheless, even if leptospirosis had been diagnosed in this patient, dengue virus infection would likely still have been overlooked. Therefore, clinicians in areas where both *Leptospira* spp. and DENVs are endemic should include both pathogens in the differential diagnosis when evaluating AFI patients and should consider the possibility of co-infection. Early administration of doxycycline and penicillin G to treat mild and severe leptospirosis, respectively, may reduce the duration and severity of illness (*8*). For cases of severe dengue, packed red blood cells should be given in response to severe anemia. For patients with either dengue or leptospirosis, intravenous fluid administration should be closely monitored to prevent fluid overload.

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Serologic Evidence of West Nile Virus Infection among Humans, Morocco

To the Editor: West Nile virus (WNV) infections were reported in horses in Morocco in 1996, 2003 (*1*), and 2010 (*2*). The isolates from 1996 and 2003 belong to WNV lineage 1, clade 1a (*1*). In 1996, WNV infection was reported in a human in Morocco (*3*), and in 2008, a serosurvey of wild birds confirmed the circulation of WNV in native birds (*4*). To our knowledge, there are no seroprevalence data for WNV antibodies in humans in Morocco. Thus, we evaluated the prevalence of WNV neutralizing bodies in serum samples collected during March–April 2011 from 499 healthy persons living in the vicinities of Meknes, Rabat, or Kenitra. All persons consented to study participation.

The participants were divided into 3 cohorts, A, B, and C. Cohort A consisted of 150 persons from the Meknes area, where no WNV infections among horses have been reported. The mean age of persons in cohort A was 52 years (SD ± 15 years), and 31% were male. Cohort B consisted of 200 persons living in the region of Rabat (median age 49 years [SD ± 12 years]; 38% male), where the WNV outbreaks among horses were described in 1996 (*3*) and 2010 (*2*). Cohort C consisted of 149 participants living in the region of Kenitra (median age 48 years [SD ± 17 years]; 43% male), which was affected by the WNV outbreaks among horses in 1996, 2003, and 2010.

Serum was stored at –20°C until tested. Just before testing, serum samples were heated at 56°C for 30 minutes. The samples were screened for neutralizing antibody against the equine WNV strain, Morocco 96–111 (*3*), by using a micro virus-neutralization test in 96-well plates and

an adaptation of a described method (*5*). Dilutions of test serum (50 µL) were incubated with one hundred 50% tissue culture infectious doses of the virus in the same volume (50 µL) for 1 hour at 37°C in Dulbecco minimum Eagle medium. We then added 150 µL (10^5 cells/mL) of a Vero cell suspension with 5% fetal calf serum. This mixture was incubated for 5–6 days at 37°C until cytopathic effects were observed in a negative control well containing a 50% tissue culture infectious dose of virus. Serum samples were screened in duplicate at dilutions of 1:6, 1:18, and 1:54. Samples that neutralized the virus, characterized by absence of cytopathic effects, at 1 of the dilutions tested were retested in 4 replicates to confirm the result. We then titrated the samples by testing 6 serial dilutions ranging from 1:6 to 1:1,458. Titers were calculated by using the Spearman-Kärber method (*6*). Titers ≥18 were considered positive.

Of the 499 participants, 59 (11.8%) had WNV neutralizing bodies (7 of 150 in cohort A, 24 of 200 in cohort B, and 28 of 149 in cohort C). Titers determined by the micro virus-neutralization test ranged from 18 to 2,630 (Table).The prevalence of WNV neutralizing bodies was significantly higher in cohort B and C participants than in cohort A participants ($p<0.01$). A significant correlation was not observed between the presence of WNV neutralizing bodies and the age or sex of participants.

The low prevalence of WNV neutralizing antibodies (4.7%; median ± SD titer 54 ± 42.5) in persons from Meknes (cohort A) suggests a low level of WNV circulation in the area. This finding is likely related to the ecosystem of this region (arid and semi-arid, with an average altitude of 500 m), which is unfavorable for survival of the vectors. Infections are more likely the result of travel to areas where WNV is endemic.

In persons from Rabat (cohort B), the medium prevalence reported (12%; median ± SD titer 54 ± 31) confirms human infection with epidemic WNV strains found in horses during WNV epidemics of 1996 and 2010. However, this prevalence is lower than that for persons in Kenitra (cohort C) (18.8%; median titer 95 ± 72). High titers were obtained in cohort C, which is located in an extremely humid area that includes several wetlands and rice fields flooded through regulated channels from the Sebou River. In addition, this region includes 2 natural bird reserves along a principal migratory Europe–sub-Saharan route. These conditions are favorable for WNV circulation and are likely related to the high prevalence of WNV infection registered in this region.

We found evidence for local circulation of WNV in Morocco. The Moroccan WNV strains most often cause mild and self-limiting illnesses. These illnesses are difficult to distinguish from many other febrile illnesses, making it less likely that viral testing would be performed for WNV. Our results show that WNV is an emerging disease in Morocco, and a national response plan should be implemented by public health authorities.

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Table. West Nile virus neutralizing antibody titers in human serum samples obtained during March–April 2011, Morocco

Cohort, location*	No. samples positive/no. tested (%)	Median ± SD	Titer (%)		
			Weak, 18–80	Intermediate, 81–320	High, >320
A, Meknes	7/150 (4.7)	54 ± 42.5	5 (71.4)	1 (14.3)	1 (14.3)
B, Rabat	24/200 (12)	54 ± 31	22 (91.7)	1 (4.2)	1 (4.2)
C, Kenitra	28/149 (18.8)	95 ± 72	19 (67.9)	3 (10.7)	6 (21.4)
Total	59/499 (11.8)	72 ± 41	46 (78)	5 (8.5)	9 (13.5)

*Study participants were divided into 3 cohorts according to the vicinity from which they were recruited.

Enterovirus 104 Infection in Adult, Japan, 2011

To the Editor: Human enterovirus (HEV) C (family *Picornaviridae*, genus *Enterovirus*) consists of 3 types of poliovirus (1, 2, and 3), 9 types of coxsackievirus A (CV-A1, 11, 13, 17, 19, 20, 21, 22, and 24), and 9 types of enterovirus (EV) (95, 96, 99, 102, 104, 105, 109, 113, and 116) (www.picornaviridae.com/enterovirus/hev-c/hev-c.htm). EV-104 was first identified in 2009 in Switzerland in 8 children who had pneumonia or acute otitis media (*1*). To our knowledge, there has been only 1 other report of EV-104, detected in Italy in 3 adults and 2 children who had upper respiratory tract infection (RTI) (*2*). We report the detection of a novel EV-104 strain in an adult with upper RTI in Japan.

In February 2011, a nasal swab specimen was collected from a 36-year-old immunocompetent man in Japan who had rhinorrhea, cough, pharyngitis, and fever (38.3°C). The sample underwent viral nucleic acid extraction and cDNA synthesis (*3*) and was PCR screened for HEV and human rhinovirus [HRV] by using primers EVP4 and OL68-1, which detect viral protein (VP) 4/VP2 gene in HEV and HRV as amplicons of ≈650 and 530 bp, respectively (*4*). Unexpectedly, an amplicon of ≈600 bp was generated.

To identify this amplicon, sequencing and BLAST analysis (www.ncbi.nlm.nih.gov) was conducted and yielded a 522-nt sequence with sequence similarity to EV-104 (94.4% identity with the prototype strain CL-12310945; 7,229 nt [GenBank accession no. EU840733]). The sequence similarity corresponded to nt 633–1154 of the novel strain, which was designated AK11 (7,408 nt; GenBank accession no. AB686524).

The complete genome sequence of AK11 was determined as follows: cDNA was synthesized by using sequence-specific primers and amplified as 4 fragments (nt 1–494, 65–3852, 1975–3852, and 3284–7408 with poly A). End-specific nucleotide sequences were determined by using the 5′ RACE system (Rapid Amplification of cDNA Ends; Invitrogen, Life Technologies Corp., Carlsbad, CA, USA) and 3′ RACE by using primer TX30SXN (*5*).

The genome and predicted amino acid sequences of AK11 were compared with those of CL-12310945, the only EV-104 strain for which a large part of the genome sequence is available. This analysis showed that CL-12310945 is shorter than AK11 at both termini. Specifically, nt 1–64 (corresponding to the 5′ untranslated region [UTR]) and nt 7291–7408 (corresponding to part of the 3D gene and the 3′ UTR) of AK11 were not sequenced from CL-12310945.

The identities between the strains were calculated by using BioEdit version 7.09 (www.mbio.ncsu.edu/bioedit/bioedit.html), with results as follows: 5′ UTR (partial sequence 95.0% nt identity; amino acid identity not applicable), VP4 (95.2% nt, 100% aa), VP2 (95.6% nt, 96.3%), VP3 (95.2% nt, 99.6% aa), VP1 (96.2% nt, 99.0% aa), 2A (96.2% nt, 99.3% aa), 2B (94.8% nt, 100% aa), 2C (92.0% nt, 99.1% aa), 3A (83.3% nt, 94.3% aa), 3B (84.8% nt, 90.9% aa), 3C (84.5% nt, 94.5% aa), and 3D (partial sequence 84.7% nt, 93.3% aa). The 3′ UTR was not analyzed.

Phylogenetic analysis of VP1 sequences among HEV-C viruses showed that AK11 clusters with CL-12310945 and is genetically close to CV-A1, CV-A19, CV-A22, and EV-109 (Figure). These results are consistent with reported results (*6*). Virus isolation, attempted by

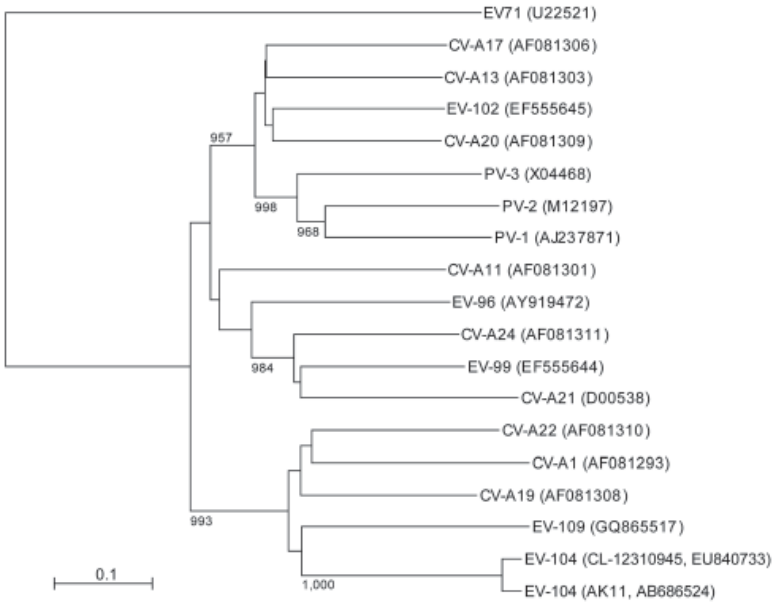

Figure. Phylogenetic tree of human enterovirus C and viral protein (VP) 1 sequences constructed by using the neighbor-joining method. Complete VP1 gene sequences in enterovirus (EV) 104 (888 nt, corresponding to nt 2461–3348 of novel strain AK11) and other human enterovirus C viruses were aligned by using ClustalX version 2 (www.clustal.org). Sequences in EV-95, EV-105, EV-113, and EV-116 were not available from the database. Genetic distances between sequences were calculated by using the Kimura 2-parameter method. Bootstrap values >950 from 1,000 replicates are shown at the nodes. GenBank accession numbers for strains used in this analysis are shown in parentheses. Scale bar indicates nucleotide substitutions per site. CV-A, coxsackievirus A; PV, poliovirus. EV-71 was used as outgroup.

using Vero and RD-18S cells, was unsuccessful. This result is consistent with previous EV-104 reports, wherein the virus could not be grown or isolated (*1,2*).

To determine the presence of other respiratory viruses in this patient, the EV-104–positive specimen was tested by using real-time PCR for any of 17 other viruses (human metapneumovirus, respiratory syncytial virus, human parainfluenza virus types 1–4, human bocavirus, human coronavirus [229E, OC43, HKU1, NL63], influenza virus [A, pandemic (H1N1) 2009, B, C], human adenovirus, and HRV). No other viruses were detected (data not shown). This result indicates that EV-104 was associated with upper RTI in this patient. During the 2 months in which the EV-104–positive sample was collected, influenza A virus, HRV, and respiratory syncytial virus were most frequently detected in other patients, and no enterovirus was observed in other specimens from persons with RTI.

EV-104 detection is rare (5/1,500 [0.3%] for a 1-year study in Italy [*1*]; 8/1,592 [0.5%] for a 10-year study in Switzerland [*2*]). As part of a virus surveillance program in Osaka City, Japan, during November 2010–October 2011, a total of 645 respiratory tract specimens were collected from children with RTI (360 male, 285 female; age 0–59 months, mean ± SD 18.9 ± 13.8 months) and subjected to PCR by using EVP4 and OL68-1 primers. No EV-104 was detected. In 2 previous studies in Japan, we detected no EV-104 in 764 specimens from patients with RTI during November 2008 and October 2010 (*3,7*); therefore, we have found EV-104 in only 1 (0.07%) of 1,410 samples tested.

Infrequent detection and insensitivity to cell culture contribute to the rarity of EV-104 identification. However, given the lack of contact between EV-104–positive patients in Italy and Switzerland, more RTI patients might actually be carrying EV-104 than testing has indicated. The collection of additional EV-104 strains and associated epidemiologic and virologic information will help clarify the role of this virus in RTI.

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Clonal Spread of *Geomyces destructans* among Bats, Midwestern and Southern United States

To the Editor: Bat geomycosis (white nose syndrome) is caused by the psychrophilic fungus *Geomyces destructans*, which has rapidly spread in the United States and Canada since it was first reported from Albany, New York (*1*,*2*). In 2011, a single genotype of *G. destructans* was found in bats with geomycosis in different parts of New York (*3*). The findings raised the possibility of clonal spread of a new pathogen with serious implications for the survival of the affected bat populations (*4*). To provide information for devising conservation measures, we explored whether this emerging infectious disease is caused by a novel pathogen (*5*). To do so, we genotyped *G. destructans* isolates from the midwestern and southern United States.

During 2010 and 2011, a total of 11 cultures of *G. destructans* were isolated and identified: 1 each from Pennsylvania and Ohio, 3 from North Carolina, and 6 from West Virginia

(Figure). The cultures came from 8 little brown bats (*Myotis lucifugus*) and 3 tricolored bats (*Perimyotis subflavus*). Two recent *G. destructans* isolates from New York and 1 *G. pannorum* isolate were included as controls. Genomic DNA was prepared from fungal growth by the conventional glass bead treatment, phenol–chloroform extraction, and ethanol precipitation. PCR amplifications of 8 *G. destructans* gene fragments (*ALR*, *Bpntase*, *DHC1*, *GPHN*, *PCS*, *POB3*, *SRP72*, and *VPS13*) were performed as described (*3*). The amplicons were sequenced and nucleotides were aligned by Sequencher 4.8 (www.genecodes.com); phylogenetic analyses were done using PAUP*4.0 software (www.sinauer.com).

A total of 4,722 nt sequences were obtained from 8 gene fragments of 13 *G. destructans* isolates (GenBank accession nos. JQ029780–JQ029883) and 1 *G. pannorum* isolate (GenBank accession nos. HQ834330, HQ834347, HQ834364, HQ834381, HQ834398, HQ834415, HQ834432, and HQ834449). Multiple alignments of these sequences showed 100% identity, and the aligned nucleotides matched perfectly with those of earlier *G. destructans* sequences for the same gene fragments analyzed from New York isolates (*3*). The nucleotide alignments of 8 sequences showed differences from those obtained from the closely related fungus, *G. pannorum*. Maximum-parsimony trees were generated by using sequences from each gene fragment. These trees showed a single clade of *G. destructans* strains distinct from *G. pannorum*; similar topologies were obtained when different phylogenetics methods were used for analysis (details not shown). A consensus maximum-parsimony tree derived from the 8 concatenated gene fragments also showed a single clade of *G. destructans* isolates from New York and the midwestern and southern United States (Figure).

The data obtained in this study strongly indicate further clonal spread of *G. destructans* from its origin near Albany, New York. The locations in which *G. destructans* was detected in the current study were spread across 5 states, which were >800 miles from Albany. The test isolates were compared with a New York isolate from 2008, which provided a 4-year temporal variation in our sampling. Bats of 2 species were positive for *G. desctructans* in the current samples, and they yielded the same *G. destructans* genotype. Thus, there is evidence for host-independent spread of a single clone of *G. destructans*.

These data would support the novel-pathogen hypothesis for the origin of bat geomycosis (*5*). However, these conclusions are based on limited sampling because isolations of *G. destructans* from affected bats are uncommon. The demonstration of pure fungal culture in the affected animals is still not the standard for geomycosis diagnostics, and most geomycosis is confirmed by bat morphologic appearance or histopathologic examination. Additionally, our phylogenetics analyses were limited to ≈5 kbp of fungal genomes, which could lead to sampling bias (*3*). Ideally, a large number of *G. destructans* isolates, including isolates from Europe, and additional polymorphic markers would be needed to determine the novel or local origin of this pathogen (*6,7*).

The environmental factors that led to introduction or reemergence of *G. destructans* in mines and caves remain unknown, and their contribution in the spread of the fungus through air, water, and soil is yet to be determined (*8*). Although no direct evidence has emerged, a role for anthropomorphic activities (occupational or recreational) in this spread is a distinct possibility (*9*). We provide genetic evidence for further spread of a single genotype of *G. destructans* from Albany, New York, to locations in the midwestern and southern United States. Experimental transmission of geomycosis from infected bats to healthy bats by direct contact has recently been confirmed (*10*). Therefore, *G. destructans* might

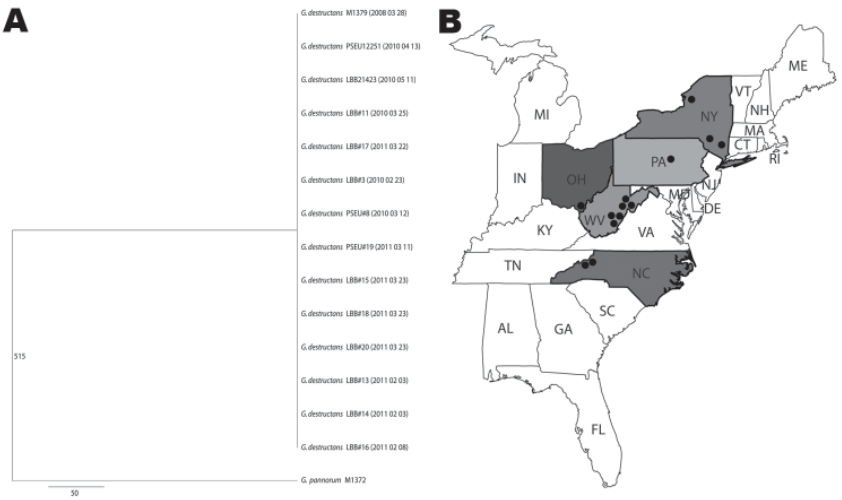

Figure. A) Consensus maximum-parsimony tree of 8 concatenated gene fragments of *Geomyces destructans*. Data were derived from 13 *G. destructans* test isolates. *G. destructans* M1379 and *G. pannorum* M1372 were used as controls in this study; they were described in an earlier report (*3*). The number 515 on the branch indicates the total number of variable nucleotide positions (of 4,722 nt) separating *G. pannorum* M1372 from the clonal genotype of *G. destructans*. Isolation dates are shown in parentheses (YYYY MM DD). Scale bar indicates nucleotide substitutions per site. B) States where *G. destructans* isolates were found; dots indicate locations of positive test results. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/5/11-1711-F1.htm).

be rapidly spreading along summer and winter migration routes of bats, which present ample opportunities for mixing of healthy and diseased animals.

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Electronic School Absenteeism Monitoring and Influenza Surveillance, Hong Kong

To the Editor: Potentially useful public health interventions, such as school closure, need to be introduced in a timely manner during the evolution of an ongoing epidemic to substantially affect community transmission (*1*,*2*). In most traditional surveillance systems that include health care use data, however, considerable delays occur between data collection and feedback, which leads to suboptimal and untimely information for guiding evidence-based public health decisions. Newer syndromic surveillance approaches have been attempted to improve timeliness by targeting earlier events in the health-seeking pathway and by promoting real-time collection and processing of surveillance data by using modern information technology (*3*,*4*). Building on an existing platform of an electronic school management system, we developed an automated school absenteeism surveillance system for influenza-like illness (ILI) in Hong Kong and evaluated its performance using data collected from March 2008 through June 2011. The Institutional Review Board of the University of Hong Kong/Hospital Authority, Hong Kong West Cluster, approved the study.

We collaborated with a commercial vendor that develops and provides online learning platforms and management systems for educational institutions, including 337 primary and secondary schools in Hong Kong, attended by children 6–18 years of age. Invitations to participate in the new absenteeism system were sent to all schools subscribing to the electronic school management system, and 62

schools throughout Hong Kong were recruited in phases during the study period. We began with 18 schools (17,255 students) from February through June 2008, then expanded to 45 schools (37,087 students) in 2008–09, to 50 schools (41,765 students) in 2009–10, and to 62 schools (49,425 students) in 2010–11.

The absenteeism system worked as follows. A student identification smart card was issued to each student in all participating schools, and students were required to swipe their cards over a sensor at the school entrance as an electronic record of attendance, which replaced a traditional paper-based roll call. Reasons for absence, including ILI, were asked in telephone calls in a subset (37%) of schools, and answers were manually entered into the system by teachers. Daily aggregated data, including the total number of students and number of absentees in each grade (stratified by reason for absence), were compiled each afternoon. Individual children were not identifiable. Data cleaning, aggregation, and analysis and generation of reports were automated with R version 2.12.1 (R Foundation for Statistical Computing, Vienna, Austria). Weekly overall absenteeism rates were calculated as the total number of absentees divided by the total number of students at all schools. Regular weekly and ad hoc reports of absenteeism patterns, with an interpretation of the overall influenza disease activity in the community, were distributed to all participating schools through the same school management system and disseminated to the general public through an existing influenza surveillance dashboard (*5*).

The school absenteeism rates and reference data from 2 existing traditional surveillance systems in Hong Kong during March 2008–June 2011 covered a total of 7 influenza seasons (Figure). Data from both systems were from all age groups in the entire territory. The laboratory virus isolation rate as a reference standard highlighted the typical seasonality of influenza in Hong Kong, which peaked around February during winter and around August during summer, in each year (Figure, panel D) (*6*). Clear and sharp peaks were detectable from both the overall and ILI-specific data during most of these influenza seasons (Figure, panels A, B), which generally occurred 1–3 weeks ahead of the peaks in the laboratory data (range 1 to 5 and −1 to 4 weeks; median 3 and 0.5 weeks for overall and ILI data, respectively). The data generally showed much sharper peaks than the outpatient sentinel data, possibly related to better coverage of disease activity in the community, in contrast to the sentinel data, which captured only episodes leading to visits to outpatient clinics (*7*). Limitations of our school absenteeism data included relatively lower perceived sensitivity of the ILI-specific absenteeism rate (because data are provided only by 37% of participating schools) and the presence of data gaps during school holidays or school closure as a result of public health measures to mitigate seasonal influenza in March 2008 (*1*) and pandemic influenza in June 2009 (*2*).

This study demonstrated the feasibility and potential benefit of using automatically captured school absenteeism data as a complementary data stream for influenza surveillance. Real-time monitoring of school absenteeism, an early event in the health care–seeking pathway, can improve situational awareness and help inform appropriate public health decisions and interventions in a more timely and evidence-based manner. Electronically capturing data from preexisting smart card systems is an attractive and cost-effective option that does not require substantial additional resources, systems, or labor in contrast to some other approaches (*8–10*). The increasing popularity of smart card technology in various situations might also provide potential opportunities for innovative surveillance systems.

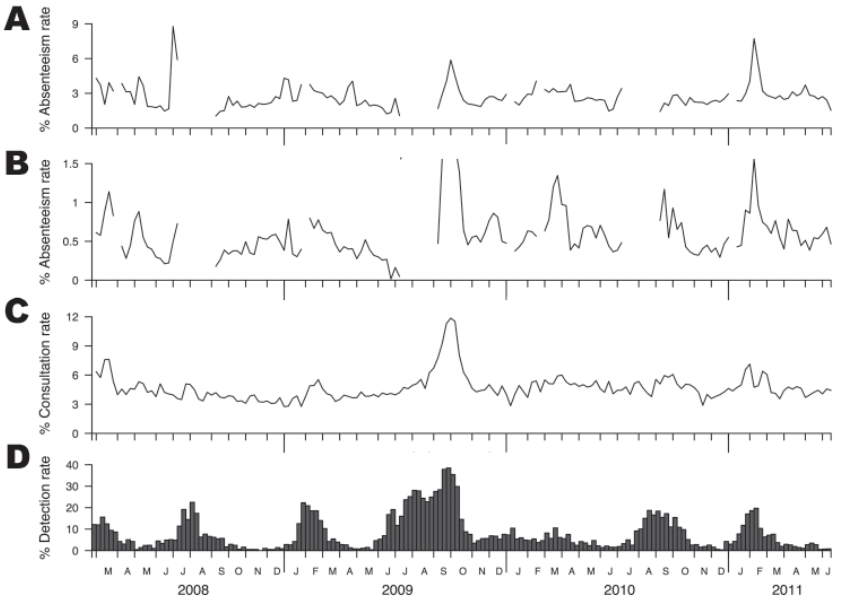

Figure. Influenza surveillance data, Hong Kong, February 23, 2008–June 18, 2011. A) Weekly overall school absenteeism rate. B) Weekly influenza-like illness (ILI)–specific school absenteeism rate. C) Weekly ILI (defined as fever plus cough or sore throat) consultation rates in sentinel networks of outpatient clinics in the private sector. D) Proportion of influenza A and B virus isolations (by date of collection) among all specimens submitted to the reference laboratory for Hong Kong Island at Queen Mary Hospital.

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Epidemic Genotype of *Coxiella burnetii* among Goats, Sheep, and Humans in the Netherlands

To the Editor: The 2007–2010 Q fever epidemic among humans in the Netherlands was among the largest reported in magnitude and duration (*1*). The increase in human Q fever cases coincided with an increase in spontaneous abortions among dairy goats in the southeastern part of the Netherlands, an area that is densely populated with goat farms (*1*). Genotypic analyses of the involved isolates could confirm the possible link between the human and animal Q fever cases.

In previous studies, genotypic investigations of human and animal samples in the Netherlands were performed by using a 3-locus multilocus variable-number tandem repeats analysis (MLVA) panel and single-nucleotide polymorphism genotyping, respectively (*2,3*). The first study, performed on relatively few samples from a minor part of the affected area, showed that farm animals and humans in the Netherlands were infected by different but apparently closely related genotypes. More recently, genotyping by using a 10-locus MLVA panel provided additional information about the genotypic diversity of *Coxiella burnetii* among ruminants in the Netherlands: 1 dominant MLVA genotype was identified among goats and sheep throughout the entire affected Q fever area (*4*). A different panel of MLVA markers was applied to human samples (*5*). Four markers that are shared by both panels showed identical alleles in human and animal samples, again implicating goats and sheep as possible sources of the outbreak.

MLVA, which is based on relatively unstable repetitive DNA elements, is sometimes criticized for producing results that are too discriminatory or difficult to reproduce in different settings (*6*). Because of their instability, use of tandem repeats as genotyping targets can lead to problems with data interpretation and to overestimation of genotypic diversity by showing small variations in MLVA genotypes in isolates of otherwise identical background.

We used a more stable, sequence-based typing method, multispacer sequence typing (MST), on samples from humans and a group of ruminant animals (goats, sheep, and cattle) to establish a firmer correlation between Q fever cases in humans and animals (*7*). We identified MST genotypes using a Web-based MST database (http://ifr48.timone.univ-mrs.fr/MST_Coxiella/mst) containing genotypes from several countries in Europe. Ultimately, this study could answer the question of whether the current outbreak situation could have been caused by a specific *C. burnetii* strain in the ruminant population in the Netherlands.

Real-time PCR-positive specimens from 10 humans and 9 Q fever–positive specimens from goats and sheep collected from various locations throughout the affected area were used (*8*). We also included Q fever-positive specimens from cattle to rule out cattle as a possible source of Q fever infection. Five samples of cow's milk and 1 bovine vaginal swab sample were analyzed (online Appendix Table, wwwnc.cdc.gov/EID/article/18/5/11-1907-TA1.htm). MST33 was identified in 9 of 10 tested human samples and in the remaining 8 of 9 clinical samples from goats and sheep (online Appendix Table). MST33 has been isolated incidentally in nonoutbreak situations in human clinical samples obtained in France during 1996, 1998, and 1999 and from a placenta of an asymptomatic ewe in Germany during 1992. All samples from cattle in the Netherlands, 1 goat, and cow's milk contained genotype MST20. Genotype MST20 has also been identified in human clinical samples from France, in a cow's placenta from Germany isolated in 1992 and in rodents from the United States isolated in 1958. In 1 human bronchoalveolar lavage sample, a novel (partial) MST genotype was found. This may be an incidental Q fever case unrelated to the outbreak situation. Because no historical genotyping data for the period before the outbreak of Q fever in the Netherlands are available, this explanation needs further research.

MST genotyping shows the presence of genotype MST33 in clinical samples from humans, goats and sheep. These results confirm that goats and sheep are the source of human Q fever in the Netherlands. Few worldwide genotyping studies have been conducted, and therefore information about a possible global persistence of this genotype is lacking. This study also indicates that the outbreak among humans is not linked to *C. burnetii* in cattle, although the infection is widespread among dairy herds in the Netherlands (*10*), exemplifying that most outbreaks are related to goats and sheep rather than to cattle. In conclusion, the increase in the number of Q fever cases in the Netherlands among humans most likely results from MST33 in the goat population in the Netherlands and could have been facilitated by intensive goat farming in the affected area and its proximity to the human population.

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High Anti–Phenolic Glycolipid-I IgM Titers and Hidden Leprosy Cases, Amazon Region

To the Editor: Leprosy remains a serious public health issue. Although the World Health Organization elimination target was achieved in 2000, with a prevalence of <1 case/10,000 persons, despite progress since introduction of multidrug therapy (*1*), large pockets of poverty remain in which the disease is hyperendemic and underdiagnosed. In fact, in highly disease-endemic areas, the prevalence of previously undiagnosed leprosy cases in the general population has been reported to be 6× higher than the registered prevalence (*2*).

Most leprosy patients are in India and Brazil. In Brazil, new cases are concentrated in the Northeast, Midwest, and Amazon regions (from state capitals to the inner counties). Access to the health system is poor in these regions because of severe inequalities in the public health system of Brazil (*3*),

A total of 34,894 new cases were registered in Brazil during 2010 (*4*), corresponding to an incidence rate of 18.22 cases per 100,000 population. Pará State accounted for 10.2% of cases (3,562 cases), an incidence rate of 46.93 per 100,000 population. When only children <15 years of age were considered, Pará registered 389 new cases of leprosy in 2010, representing 10.9% of all cases, an incidence rate of 16.52 per 100,000 population. In Oriximiná, a county with 62,794 inhabitants in northwestern Pará, ≈800 km from Belém, Pará's capital, a mean of 13.8 cases per year were registered for the past 5 years.

In 2010, in Oriximiná, we collected plasma samples from 138 students 8–18 years of age, from 35 leprosy patients who received a diagnosis during 2004–2009, and from 126 contacts of these patients (Federal University of Pará Research Ethics Committee protocol no. 197/07). We tested all of these samples for anti–phenolic glycolipid-I (PGL-I) IgM; 42% of students, 54.3% of case-patients, and 45% of case-patient contacts were seropositive. In addition to collecting samples, we clinically examined the leprosy patients and their contacts, among whom we identified 3 new leprosy cases. We did not examine students at that time. Contacts were persons from the same household or neighborhood whom the index case-patient described as a person with whom he or she had a close relationship. Leprosy cases were diagnosed in the field on the basis of clinical signs, loss of sensation on the skin lesions, and presence of enlarged nerves. For operational reasons, skin smears were not performed. All cases were diagnosed by 2 leprologists. We used the Ridley-Jopling classification, associated with the indeterminate clinical type, as defined by the Madrid classification. The ELISA cutoff for positive results was arbitrarily established as an optical density of 0.295 based on the average plus 3× the SD of the test results from 14 healthy persons from the Amazon region (*5*).

Because studies of the seroprevalence among contacts have reported a proportion of seropositive persons ranging from ≈1.9% to 18.4% (*6*), we returned to Oriximiná 16 months after the first visit. We examined 2 groups of students and their contacts; 1 group was positive for anti–PGL-I, and the other group was negative for anti–PGL-I. We visited 44 households in 1 week. From the 35 leprosy patients encountered during the first visit, we selected 25 households to survey (14 with an anti–PGL-I–positive contact in the household and 11 without), and among students with results of anti–PGL-I serology, we selected 19 households (11 positive with an anti–PGL-I–positive contact in the household and 8 without). During our visits to all of these households, we examined 222 persons (Table).

When we arrived in Oriximiná, only 2 cases had been registered in the national notifiable diseases information system. By using our approach, 23 new cases were found after we investigated households that had a person positive for anti–PGL-I (15 multibacillary, 8 paucibacillary); we found only 7 new cases in households where residents were negative for anti–PGL-I (4 multibacillary, 3 paucibacillary) (Table). For comparison, during the last traditional leprosy campaign in Oriximiná in 2008, eight new cases were detected. Furthermore, by using our strategy, the local public health service detected 9 additional new cases during the 4 months after our departure from Oriximiná.

These data emphasize that contact examination is crucial for

Table. New leprosy cases detected among selected households, Oriximiná, Pará State, Brazilian Amazon, 2010*

Group	Household contact anti–PGL-I IgM ELISA result	No. households visited	No. persons examined	No. new cases		
				Among persons previously tested	Among contacts of persons previously tested	Total†
Leprosy patients	Positive	14	43	9	4	13
	Negative	11	42	1	1	2
Students	Positive	11	84	5	5	10
	Negative	8	53	1	4	5
Total		44	222	16	14	30

*Households were selected from among 35 leprosy patients encountered during the first visit (25 households, 14 with an anti–PGL-I–positive contact in the household and 11 without) and among students with results of anti–PGL-I serology (19 households, 11 with an anti–PGL-I–positive contact in the household and 8 without). PGL-I, phenolic glycolipid-I.
†Fisher exact test comparing case-patients and non–case-patients among those positive or negative for anti-PGL-I IgM revealed a statistically significant difference (p = 0.0280).

identifying new cases (*7*) and that such investigation must be conducted periodically. Our data also indicate that subclinical infections are highly prevalent among public school students in the Amazon region and that identifying students with positive anti–PGL-I test results can lead to discovery of new leprosy cases among students' household contacts.

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C.G.S. designed and coordinated the study; clinically examined, diagnosed, and classified the subjects; statistically analyzed the data; and wrote the manuscript. D.V.G.F. collected and processed the samples, performed laboratory assays, and statistically analyzed the data. M.A.C.F. clinically examined, diagnosed leprosy, and classified the subjects. L.S.G. evaluated the functional statuses of the subjects. M.B.S. performed laboratory assays. J.G.B. designed the study and interviewed the participants. All authors participated in the interpretation of the data and read and approved the final manuscript.

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Novel Prion Protein in BSE-affected Cattle, Switzerland

To the Editor: In a recent issue of Emerging Infectious Diseases, Seuberlich et al. (*1*) reported a novel prion protein in cattle with bovine spongiform encephalopathy (BSE). Two cows in Switzerland, 8 and 15 years of age, tested positive

in 2 approved screening tests, the PrioSTRIP test and the Prionics Check WESTERN (Prionics, Zurich, Switzerland). According to World Organisation for Animal Health guidelines, the 2 cattle are considered BSE positive. Histopathologic and immunohistochemical results were inconclusive because the tissues were severely autolyzed. Clinical signs were absent or the clinical history was not known.

After further analysis of brain tissues by using several monoclonal antibodies in a Western blot (WB), the authors concluded that they had identified an N-terminal truncated protease-resistant prion protein (PrP^{res}) fragment that differs from the PrP^{res} fragments in 3 known types of BSE. No reference was made to the existence of N-truncated fragments, such as C1, of the normal prion protein PrP^{C}, which have been reported for humans (*2*,*3*), mice (*4*), and cattle and other ruminants (*5*). The pattern in the WB of the novel prion protein (*1*) appears similar to that of the fragment C1 of the normal prion protein (*2*–*5*). The C1 fragment is more protease resistant than the intact PrP^{C} fragment because the protein part is more protected by the polysaccharide residues. Could it be that in the case of the severely autolyzed tissues of the cows in Switzerland, the proteinase K might already have been weakened or inhibited and when combined with the higher protease resistance of the C1 fragment, the digestion was incomplete?

Ten years ago, I looked at nonspecific, unusual samples from fallen stock cattle in New Zealand. Samples from these cattle had been confirmed as negative by paraffin-embedded tissue blot (University of Göttingen, Göttingen, Germany), sodium phosphotungstic acid precipitation, followed by WB (European Union Reference Laboratory for Transmissible Spongiform Encephalopathies, Veterinary Laboratories Agency, New Haw, UK), Prionics WB (Prionics AG, Zurich, Switzerland), histopathologic examination, and immunohistochemical testing (Veterinary Laboratories Agency). We became aware of such samples when the proteinase K digestion did not work properly (Figure). Unusual samples 1 and 2 contained increased amounts of a truncated fragment of normal PrP^{C}, which was digested completely after the proteinase K concentration was increased.

I am convinced that the novel PrP^{res} described in the article by Seuberlich et al. (*1*) is indeed a truncated fragment of the normal bovine PrP^{C} protein. Therefore, I would like to ask the editor to address the following issues with the authors: Why were no references to truncated fragments of PrP^{C} made in their article? Why was no WB analysis performed in which the novel PrP^{res} was shown next to normal, undigested PrP^{C} for band-size comparison? Why were no WB analyses shown in which the proteinase K concentration was increased?

It is laudable that in vivo transmission studies using transgenic mouse models and cattle are under way, which will sort out these findings conclusively. I expect that no disease development will be shown. Meanwhile, announcing new types of BSE is purely speculation.

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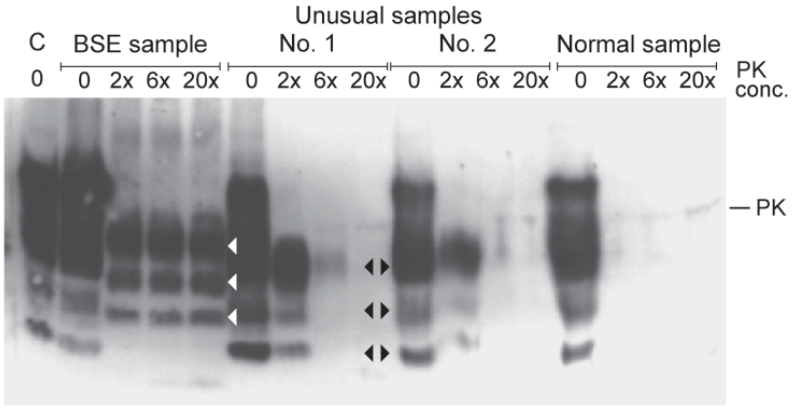

Figure. Western blot analysis of proteinase K (PK) digested brain stem samples with increasing concentrations of PK (relative to concentration used in the Prionics Check WESTERN (Prionics, Zurich, Switzerland). C, kit control, normal bovine brain homogenate; BSE, bovine spongiform encephalopathy sample from cow from Switzerland; unusual samples 1 and 2 and normal sample are from New Zealand cattle and had been confirmed as negative by several test methods (see text). Unusual samples 1 and 2 show a higher concentration of a truncated fragment of the normal prion protein, which was completely digested at increased PK concentrations. It is typical for this fragment that bands are identical in size to lower bands of the normal prion protein in the undigested samples of normal brain homogenates (black arrowheads). White arrowheads show major bands of PK-digested BSE prion protein.

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In Response: Dr Kittelberger comments on our recent report of 2 cows in Switzerland that were classified as positive for bovine spongiform encephalopathy (BSE), according to the established criteria (*1*,*2*). He raises concerns that the unusual prion protein signature in Western blot (WB) in these cows represents a physiologic prion protein (PrP^C) fragment, inefficiently degraded by proteinase K (PK), termed C1. Certainly the effects of tissue autolysis on PK activity and the molecular prion protein signature are of particular concern and deserve full consideration in data interpretation. In our study, molecular mass comparisons between PrP^C in non-PK–treated brain tissue of healthy cattle and the prion protein in samples from the 2 aberrant cows with BSE in WB were considerably hindered by overlapping C1- and full-length PrP^C bands in the non-PK–treated samples and did not allow for a robust conclusion (T. Seuberlich, unpub. data). It is noteworthy that the Prionics Check WESTERN (Prionics, Zurich, Switzerland) test has been extensively validated in terms of the diagnostic specificity, also on severely autolytic specimens (*3*–*5*). In none of these studies was a similar prion protein signature observed. We therefore considered it unlikely that the findings in the cases from Switzerland resulted from tissue autolysis.

Dr Kittelberger provides data from New Zealand cattle that revealed a similar prion protein signature in WB. He assumes that these animals had a negative BSE status and that the PK digestion in the WB did not work properly, which is supported by results from other diagnostic techniques. However, information about the degree of autolysis of these samples is missing, and, most notably, whether these findings are correlated with prion infectivity is not known. Strikingly, in contrast to the results for the samples from Switzerland, the samples from New Zealand are reported to be negative in the Prionics Check WESTERN. It would be fascinating to perform a side-by-side analysis of the samples from Switzerland and from New Zealand to determine whether the banding characteristics in both groups are identical. Studies are under way in our laboratory to further investigate the effect of tissue autolysis on PK activity and PrP^C degradation under experimental conditions. If our findings turn out to be the result of inhibited PK activity in BSE-negative cattle samples, the current diagnostic criteria might require revision. As long as the results of these experiments and the ongoing transmission studies are not available, we can neither confirm nor reject a novel type of BSE.

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Correction, Vol. 18 No. 1

Author Henry J.C. de Vries' initials were listed incorrectly in Cutaneous Leishmaniasis Acquired in Jura, France (W.R. Faber et al.). The article has been corrected online (wwwnc.cdc.gov/eid/article/18/1/11-0408_article.htm).

DOI: http://doi.dx.org/10.3201/eid1805.C11805

Correction, Vol. 18 No. 2

Author Richard Njouom's surname was misspelled in High Seroprevalence of Enterovirus Infections in Apes and Old World Monkeys (H. Harvala et al.). The article has been corrected online (wwwnc.cdc.gov/eid/article/18/2/11-1363_article.htm).

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Salvador Dalí (1904–1989) *Daddy Longlegs of the Evening—Hope!* (1940) Oil on canvas (25.4 cm × 50.8 cm) Fundación Gala-Salvador Dalí, (Artists Rights Society), 2011. Collection of the Salvador Dalí Museum, Inc., St. Petersburg, FL, USA, 2011

Health and the Myrmidons

Polyxeni Potter

"I'll be a genius, and the world will admire me," Salvador Dalí wrote in his diary at age 15. These confident words marked a journey of greatness started during childhood, in Figueres, Spain. In this rural town in Catalonia, steeped in artistic heritage, he started to read Voltaire, Nietzsche, Kant, Spinoza, and Descartes, and these philosophers' notions on the nature of reality created in his mind an abiding sense of purpose.

Much of Dalí's boyhood was spent in his parents' home at the coastal village of Cadaqués near Port Lligat, where he later made his own home. Rocks from the local beach found their way into many of his works. His early art education benefited from frequent visits with the family of artist Ramón Pichot and from studies at the Municipal School of Drawing in Figueres under engraver Juan Nuñez. Dalí's earliest surviving works date from this period, during which he also wrote copiously and showed interest in cubism. Soon he entered the famed Academy of San Fernando in Madrid, where he met poet Federico García Lorca, who later published an ode in his honor, "O Salvador Dalí of the olive-colored voice / I do not praise your halting adolescent brush / or your pigments that flirt with the pigments of your times / but I laud your longing for eternity with limits."

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As a student in Madrid, Dalí got to know the Prado Museum and the cubist works of Picasso, Georges Braque, Juan Gris, and the metaphysical paintings of Giorgio de Chirico. Encouraged by the attention of his peers and association with Lorca, he ventured outside the restrictions and requirements of the school. Increasingly disenchanted, he was suspended and later expelled by the academy, as he was at some point to be expelled from the surrealist movement, again for unwillingness to color inside the lines.

In a long period of experimentation also marked by personal notoriety, Dalí took his work in many directions, from a strictly academic style to cubism. An astonishing draftsman, he drew from nature, the imagination, or classical tradition as he searched for a distinctive style. He traveled to Paris where he eagerly embraced surrealist concepts. A solo exhibition there in 1929 brought a glowing review from poet André Breton, psychiatrist and head of the surrealist group.

Surrealists, Dalí among them, wanted to shake up comfortable middle class values, preferring to depict life as lived by the mind. This approach, with its probing of the unconscious, dreams, memories, and psychological associations, formed the foundation of new art rooted in paradox and contradictions rather than mimesis.

Daddy Longlegs of the Evening—Hope! on this month's cover, was inspired by war, the carnage of which Dalí experienced from close up. The Spanish Civil War took him to Paris. Shortly after his departure, the family

home in Cadaqués was bombed, his own place at Port Lligat ransacked and destroyed. His friend Lorca was executed; his sister, imprisoned and tortured. Chased to Italy and then again to Paris, he lived there until the outbreak of World War II, when once more he had to flee. His work of this time grew dark and intense, evoking tragedy, melancholy, and confusion. In 1940, he emigrated to the United States, where he was to live for 8 years.

In *Daddy Longlegs of the Evening—Hope!* the landscape is denuded. On the right side, an olive tree, the perennial emblem of peace, is bare of leaves. On the horizon, two human figures are engaged in some macabre dance, their elongated shapes and ballooned sleeves mocking similar forms in the foreground. Evening shadows cast a mournful look over the scene. In the center, Dalí places his own image, a melting face drawn in the outline of his familiar beach rocks and joined to Creativity, a hollow figure draped over the tree that once nourished it.

Literary and music references, two inkwells and a prominent cello, complete the hollow figure, offering clues about Dali's state of mind. On the upper left corner, the cannon from Giorgio de Chirico's painting *The Philosopher's Conquest* (1914) is held by a crutch, the cannonball melting into distorted body parts, lifeless fluids, a spent sperm-like form. Out of this unlikely background emerges a bandaged *Nike of Samothrace*, ill-defined and threatened by a decayed horse of the apocalypse leaping from the cannon. In the lower left corner, a winged putto, messenger of love and artistic pursuits, weeps at the spectacle. But despite the devastation, a symbol of hope appears on the artist's head: daddy longlegs, a token of good luck, amidst swarming ants.

The Myrmidons, ant-men (from *μύρμηξ* [*murmex*] "ant") mentioned in The Iliad and described in some detail by Ovid in the Metamorphoses, were a prolific warrior race patterned after ants from whom they allegedly descended by divine transformation. The term, which has survived with various connotations, still at times denotes mindless masses or "hired ruffians," who make up for unquestioning loyalty with sheer numbers. The term also still evokes somehow the ants of origin—the same plentiful arthropods Dalí sprinkled liberally in his paintings, along with flies and other insects—their glistening bodies clustered around the edges, foreshadowing decomposition.

"Beautiful as the chance meeting on a dissecting table of a sewing machine and an umbrella," is how revelatory juxtapositions were viewed by the surrealists. And the marvelous was to be detected in the everyday, the discarded, the coincidental, and the unnoticed. These ideas drove surrealism and, no longer incongruous or farfetched, now find their way in all aspects of life and no less in science.

Among health threats, viruses are like the Myrmidons in their sheer numbers. Even though several thousand have been identified, the large masses remain at large. With each new identification, the public health burden increases. And this is where Dalí would insert daddy longlegs. For with each identification, the opportunity also arises for new vaccines or other prevention strategies and effective treatments, as in the case of many known viruses, including the formidable HIV.

Like a surrealist painting, emergence of viruses around the globe features realities that by all appearances have nothing to link them, often in settings that by all appearances are not linked. In this issue alone, a new strain of Andes virus associated with fatal human infection was found in central Bolivia; and a new human adenovirus, in Bangladesh. Adenovirus type 7 is emerging in Malaysia. A variant West Nile virus strain, most related to the indigenous Kunjin, was characterized in Australia. Lymphocytic choriomeningitis virus–associated meningitis is reported in southern Spain; and hepatitis E virus infection, in solid organ transplant recipients in the Netherlands. The chance meeting on a "dissecting table" still applies, and the marvelous resides in identifying and controlling the Myrmidons, one by one.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Iatrogenic Creutzfeldt-Jakob Disease

Lessons Learned During Rabies Outbreaks, Flagstaff, Arizona

Trichomonas vaginalis Antimicrobial Drug Resistance in 6 US Cities, 2009–2010

Trends in Invasive Infection with Methicillin-Resistant *Staphylococcus aureus*, Connecticut

Geographically Dispersed *Vibrio cholerae* O1, Kenya, January 2009–May 2010

Intrafamilial Circulation of *Tropheryma whipplei*, France

Wild Boars as Reservoirs of Human-pathogenic *Anaplasma phagocytophilum* Variants

Bartonella vinsonii subsp. *arupensis* in Humans, Thailand

Molecular Epidemiology of the Hantavirus Laguna Negra in Mato Grosso State, Brazil

Local Transmission of Imported Endemic Syphilis, Canada

Macrolide-Resistant *Bordetella pertussis* in Newborn, France

Genome Analysis of Rift Valley Fever Virus, Mayotte

Accuracy of ICD-10 Codes for Surveillance of *Clostridium difficile* Infections, France

Rickettsia parkeri Infection in Domestic Dogs, Southern Louisiana

Clostridium difficile Infection, Colorado and Northwestern United States, 2007

Pneumococcal Serotype–specific Unresponsiveness in Vaccinated Child with Cochlear Implant

Recognition and Diagnosis of *Cryptococcus gatti* Infections in the United States

Coccidioidal Endophthalmitis in Immunocompetent Person, California

Human MRSA Isolates with Novel Genetic Homolog, Germany

Novel Human Paramyxovirus Origins Isolated from Rats, China

Rickettsia conorii Indian Tick Typhus Strain and *R. slovaca* in Humans, Sicily

Complete list of articles in the June issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

May 6–9, 2012
8th International Symposium on Shiga Toxin (Verocytotoxin) Producing *Escherichia coli* Infections
Amsterdam, the Netherlands
http://www.vtec2012.org

May 9–13, 2012
8th International Congress on Autoimmunity 2012
Granada, Spain
http://www2.kenes.com/autoimmunity/pages/home.aspx

June 13–16, 2012
15th International Congress on Infectious Diseases (ICID)
Bangkok, Thailand
http://www.isid.org/15th_icid

July 22–27, 2012
XIX International AIDS Conference (AIDS 2012)
Washington, DC, USA
http://www.aids2012.org/

August 25–29, 2012
2012 Infectious Disease Board Review Course
Ritz-Carlton, Tysons Corner
McLean, VA, USA
http://www.IDBoardReview.com

September 9–14, 2012
XVIIIth International Pathogenic Neisseria Conference (IPNC) 2012
Maritim Hotel, Würzburg, Germany
http://www.ipnc2012.de

Announcements

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Announcements may be posted on the journal Web page only, depending on the event date.

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Article Title

Risk Factors for Intestinal Invasive Amebiasis in Japan, 2003–2009

CME Questions

1. Based on the Japanese study by Dr. Nagata and colleagues, which of the following statements about prevalence of amebic colitis is most likely correct?

A. Overall, there was no significant increase in prevalence during the study period
B. During the study period, 0.5% of patients selected for analysis had developed amebic colitis
C. Prevalence among HIV-negative patients remained stable from 2003 to 2009
D. Among HIV-positive patients, the prevalence in 2009 increased 7-fold compared to 2003

2. You are a public health official asked to consult regarding an increase in prevalence of amebic colitis in Japan. Based on the study by Dr. Nagata and colleagues, which of the following statements about risk factors for amebic colitis is most likely to appear in your report?

A. Female sex is an independent risk factor
B. Age over 40 years is an independent risk factor
C. History of syphilitic infection is an independent risk factor
D. In multivariate analysis, HIV infection was associated with twice the risk for amebic colitis

3. Based on the study by Dr. Nagata and colleagues, which of the following statements about differences in risk factors for amebic colitis between HIV-positive and -negative patients would most likely appear in your report?

A. The risk factor profile was significantly different between HIV-positive and -negative patients
B. Contact with commercial sex workers (CSWs) was not a risk factor among HIV-negative patients
C. Immunosuppressed status was a significant independent risk factor in HIV-positive patients
D. Homosexual intercourse appeared to be a risk factor in HIV-positive patients

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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To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to **www.medscape.org/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Invasive *Haemophilus influenzae* Serotype e and f Disease, England and Wales

CME Questions

1. Which of the following statements regarding the overall epidemiology of *Haemophilus influenzae* f (Hif) and *Haemophilus influenzae* e (Hie) in the current study is most accurate?

A. There was a progressive increase in the incidence of Hif
B. Rates of Hie rose between 2001 and 2006, and then fell significantly
C. The annual incidence of invasive Hie disease was higher than the rate of invasive Hif disease
D. Older adults were much more likely to have Hif vs Hie infection

2. Which of the following statements regarding mortality related to Hif and Hie in the current study is most accurate?

A. The overall case fatality ratio was higher for Hif vs Hie
B. After adjustment, the risk of dying was higher with Hie vs Hif
C. Hif pneumonia was particularly associated with a higher risk of mortality compared with Hie pneumonia
D. Most fatal cases of invasive Hif and Hie were due to septicemia

3. What should you consider regarding infection with Hif and Hie among children and adolescents in the current study?

A. Children accounted for more than half of all cases
B. Nearly all infections among children less than one year old were clustered in the age range of less than 3 months of age
C. Most cases among infants involved meningitis
D. Cases were evenly distributed between early and late childhood

4. What should you consider regarding infection with Hif and Hie among older adults in the current study?

A. Older adults accounted for half of all cases
B. Only 30% of cases had comorbidity
C. Septicemia was the most common presentation of illness
D. They demonstrated infection with a wide variety of phylogenetic types of Hif and Hie

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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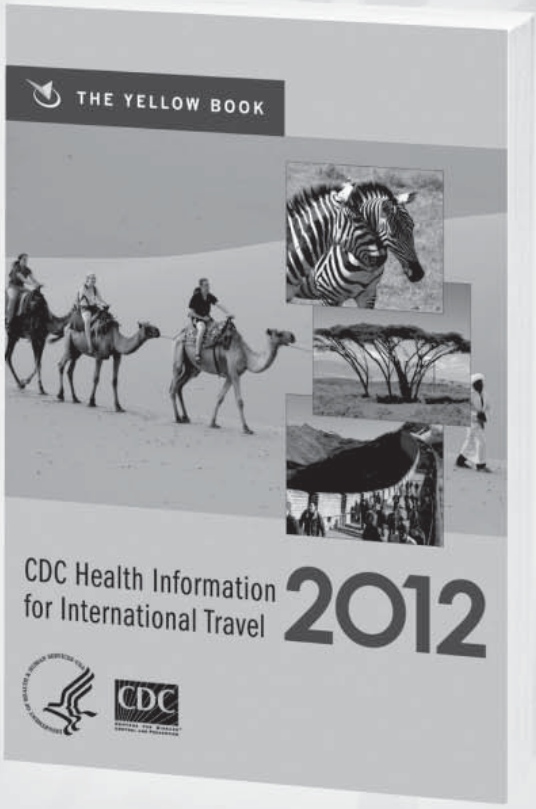
The book's features include clear and easy-to-read disease risk maps, information on where to find health care during travel, specific health information and itineraries for popular tourist destinations, detailed country-specific information for yellow fever and malaria, advice for those traveling with infants and children, and a comprehensive catalog of diseases, their clinical pictures, and their epidemiologies. The Yellow Book addresses the pre-travel consult and provides post-travel clinical guidance on ways to approach common syndromes of returned travelers who are ill.



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EMERGING INFECTIOUS DISEASES

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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

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Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

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Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

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Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

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Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit figures as separate files, in the native format when possible (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpg or .tif files. Other file formats may be acceptable; contact fue7@cdc.gov for guidance. Figures should not be embedded in the manuscript file. Use color only as needed. Use Arial font for figure lettering. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced to print size. Large figures may be made available online only. Place figure keys within the figure; figure legends should be provided at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

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

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.