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Jan Davidsz. de Heem, 1606 - c.1683/84, Antwerp. Still Life with Parrots, late 1640s, Oil on canvas (150.5 x 117.5 cm)
John and Mable Ringling Museum of Art, The State Art Museum of Florida, a division of Florida State University, USA.

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Severe Fever with Thrombocytopenia Syndrome, Shandong Province, China, 2011

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Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease in China. The incidence and clinical and laboratory characteristics of SFTS are not clearly defined. During May 22–October 2, 2011, a total of 24 patients with fever, thrombocytopenia, and leukopenia were clinically diagnosed as having SFTS in Yiyuan County, Shandong Province, China. We conducted laboratory tests for these SFTS patients. SFTS virus (SFTSV) infection was confirmed in 22 patients by using reverse transcription PCR and ELISA by acute-phase and convalescent-phase serum samples. Clinical and laboratory manifestations included fever (100%), gastrointestinal symptoms (91%), myalgia (55%), chills (41%), thrombocytopenia (100%), and leukopenia (95%).

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease that was identified in 2009 in rural areas in China. This disease is caused by SFTS virus (SFTSV), a novel bunyavirus in the family *Bunyaviridae*, genus *Phlebovirus*. (*1*). Fatal cases of infection with SFTSV have been recently reported in Japan and South Korea (*2*,*3*).

SFTS is a severe disease and has had a case-fatality rate of 12%–30% in China (*1*). The major manifestations of SFTS are fever, thrombocytopenia, leukopenia, and increased serum levels of hepatic aminotransferases. SFTSV has been detected in ticks and might be transmitted by them (*1*,*4*). Occasionally, the disease can also be transmitted from person to person through contact with infected blood or mucus (*5*–*9*).

The epidemiologic and clinical characteristics of SFTSV infection are not well defined. Approximately 30% of clinically diagnosed cases of SFTS cannot be confirmed by laboratory tests (*1*,*10*), and clinicians may confuse this disease with diseases caused by other pathogens. Therefore, to obtain information on clinical and laboratory characteristics of this disease, with a focus on diagnosis, we used acute-phase and convalescent-phase serum samples from 24 patients given a clinical diagnosis of SFTS in Yiyuan County, Shandong Province, China, an area to which SFTSV is endemic (*11*),

Study Site

Yiyuan County is located in Shandong Province in eastern China (35°55′–36°23′N, 117°48′–118 °31′E (Figure 1). It has a population of ≈550,000 persons, of whom 85% live in rural areas.

Clinical Case Definition and Blood Collection

We defined a clinically diagnosed case-patient with SFTS as a patient who had fever, leukopenia, or thrombocytopenia without another known acute infectious disease. We did not have data on what other infectious diseases were ruled out. We defined a laboratory-confirmed case of SFTS as a clinically diagnosed case with a positive antibody or reverse transcription PCR (RT-PCR) result for SFTSV. Acute-phase serum samples, clinical information, and laboratory data for all patients given a diagnosis of SFTS in 2011 in Yiyuan County were

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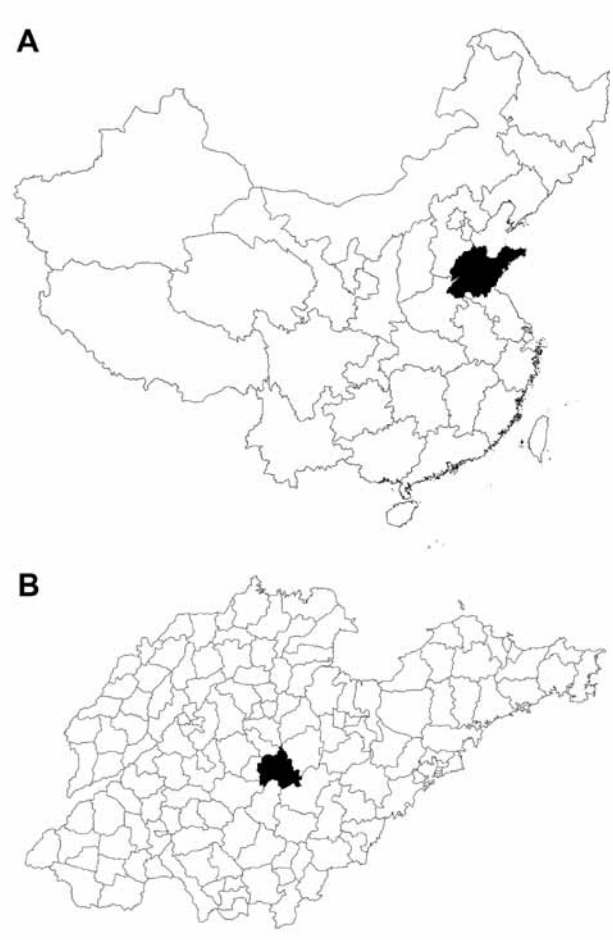

Figure 1. A) Shandong Province, China (black area) where severe fever with thrombocytopenia syndrome was studied, 2011. B) Yiyuan County (black area) in Shandong Province.

submitted to the Yiyuan County Centers for Disease Control and Prevention.

Acute-phase serum samples were obtained 4–13 days after onset of illness. Sixteen samples were obtained during the first week, and the remaining samples were obtained during the second week. Convalescent-phase serum samples were obtained 3–6 months after patients had recovered from the disease. All patients were admitted to Yiyuan County People's Hospital, the only hospital in Yiyuan County. Thus, we believe that we enrolled all SFTS patients from Yiyuan County during 2011. The research protocol was approved by the human bioethics committee of Shandong University, and all participants provided written informed consent.

Detection of Virus RNA in Acute-phase Serum Samples

Total RNA was extracted from blood by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). RNA was used as template for RT-PCR to amplify SFTSV RNA by using primers derived from large (L) and small (S) RNA segments of the virus (Table 1). RT-PCR was performed by using the One-Step PCR Kit (QIAGEN), and the RT-PCR product was used as template for nested PCR. Nested PCR products were sequenced to confirm SFTSV sequences.

ELISA

Serum samples were tested for antibodies (IgG and IgM) against SFTSV by using a double-antigen sandwich ELISA kit (Shanghai Zhengshuo Biotech Company, Shanghai, China) (*12*,*13*). The ELISA kit used recombinant nucleoprotein (NP) of SFTSV as antigen for coating plates. In initial screening, an undiluted serum sample was used to determine whether the sample was positive for antibodies against SFTSV. Positive serum samples were further diluted in 2-fold increments starting at 1:2. Each sample (50 mL) was added to wells of antigen-coated plates, and plates were incubated for 30 min at 37°C to enable SFTSV antibodies to bind to NP of SFTSV. The plates were washed, horseradish peroxidase–labeled recombinant SFTSV NP was added, and reactivity was detected by using substrates for horseradish peroxidase. Absorbance was read at 450 nm. A serum samples was considered to contain antibody to SFTSV when absorbance of the sample was ≥2.1 fold greater than that of a negative control (provided by the manufacturer), which was 3 SD above the mean optical density 450 nm for the person sampled. The ELISA had similar specificity and sensitivity as a microneutralization assay and showed

Table 1. Primers for RT-PCR and nested PCR testing for severe fever with thrombocytopenia syndrome virus, Shandong Province, China, 2011*

Type of primer	Primer name	Sequence, 5′→3′	Virus segment
Primary	$F1_S$	CAGCCACTTTACCCGAACAT	Small
	$R1_S$	GGAAAGACGCAAAGGAGTGA	
Nested	$F2_S$	CTGGTCTCTGCCCTCTCAAC	
	$R2_S$	GGATTGCAGTGGAGTTTGGTG	
Primary	$F1_L$	GGCAGCAAACCAGAAGAAAG	Large
	$R1_L$	CATTTCTCCGAGGGCATTTA	
Nested	$F2_L$	GGGTCTCCTGCTTAGCACAGG	
	$R2_L$	TCAGAFAAFACCCTGCCAGT	

*RT-PCR, reverse transcription PCR; F, forward; R, reverse.

no cross-reactivity with antibodies against hantavirus or dengue virus (*12*,*13*).

Patients

Twenty-four patients were given a diagnosis of SFTS according to our case definition. We considered that 22 of these 24 patients were confirmed SFTS cases because blood samples from these patients were PCR positive for SFTSV RNA and/or had antibodies positive to SFTSV. Major clinical manifestations of laboratory-confirmed case-patients are shown in Table 2. The frequency of major clinical signs and symptoms in these patients was 100% (22/22) for fever, 91% (20/22) for gastrointestinal symptoms (nausea, vomiting, diarrhea, abdominal pain), 55% (12/22) for myalgia, and 46% (10/22) for lymphadenopathy. Convalescent-phase blood samples were obtained from 21 patients; 95% (20/21) had leukopenia (leukocyte count $<4 \times 10^9$cells/L) and 100% (21/21) had thrombocytopenia (platelet count $<150 \times 10^9$/L). All patients recovered from their illness.

RT-PCR

SFTSV RNA L and S segments were amplified by 1-step RT-PCR and then by a nested PCR that produced a 900-bp fragment and a 600-bp fragment, respectively. The PCR detection rate was 50% (12/24) for the S segment (Table 3) and 17% for the L segment. All L segment–positive patients were also positive for S segment. PCR products were confirmed to be SFTSV RNA by DNA sequencing.

Table 2. Clinical parameters for 22 patients with confirmed severe fever with thrombocytopenia syndrome, Shandong Province, China, 2011

Clinical parameter	No. (%) patients
Sign or symptom	
Fever	22 (100)
Gastrointestinal	20 (91)
Myalgia	12 (55)
Lymphadenopathy	10 (45)
Chills	9 (41)
Headache	7 (32)
Flank pain	7 (32)
Laboratory test*	
Thrombocytopenia	21 (100)
Leukopenia	20 (95)

*Blood samples were obtained from 21 patients.

Detection of Antibodies against SFTSV

ELISA showed that 54% (13/24) of acute-phase serum samples and 86% (18/21) of convalescent-phase serum samples contained antibodies against SFTSV (Table 3). Although a 4-fold increase in antibody titer was not a criterion for ELISA, we diluted serum samples to determine whether serum antibody titer changed in the patients. Results indicated that 8/21 samples showed increased antibody titers and 1/21 showed a decreased titer.

Table 3. Detection of SFTSV RNA and virus-specific antibody in serum samples of 24 patients, Shandong Province, China, 2011*

Patient no. or parameter	Day of blood collection after disease onset	Acute-phase serum samples, n = 24		Convalescent-phase serum samples, n = 21	RT-PCR– or ELISA–confirmed SFTSV cases, n = 24
		RT-PCR, S RNA segment	ELISA titer	ELISA titer	
1	5	–	2	0	+
2	10	+	64	ND	+
3	6	–	0	64	+
4	4	–	0	0	–
5	9	–	256	≥512	+
6	7	+	0	≥512	+
7	4	+†	0	128	+
8	13	–	0	128	+
9	5	–	0	0	–
10	7	+†	0	256	+
11	6	–	0	256	+
12	6	+	64	≥512	+
13	7	+†	0	≥512	+
14	5	–	256	≥512	+
15	4	+†	0	≥512	+
16	10	+	0	ND	+
17	6	+	32	128	+
18	13	–	512	NA	+
19	10	–	64	256	+
20	10	–	256	256	+
21	7	+	512	256	+
22	6	+	32	≥512	+
23	8	+	32	32	+
24	6	–	64	256	+
SFTSV positivity rate, %	NA	50	54	86	92
Sensitivity, %	NA	55	59	95	NA

* SFTSV positivity rate was calculated by using patients given a clinical diagnosis (n = 24) as the denominator. Sensitivity was calculated by using laboratory-confirmed cases (n = 22) as the denominator. SFTSV, severe fever with thrombocytopenia syndrome virus; RT-PCR, reverse transcription PCR; S, small; –, negative; +, positive; ND, not determined; NA, not applicable.
†Positive by RT-PCR for S and large segments.

Sensitivity of PCR and ELISA for Diagnosis of SFTSV Infection

The sensitivities of RT-PCR and ELISA were calculated by using laboratory-confirmed cases as the denominator. Sensitivities were 55% (12/22) for RT-PCR and 59% (13/22) for ELISA. By combining RT-PCR and ELISA results for acute-phase serum samples, we found that 86% (19/22) of patients were given a diagnosis of SFTSV (Table 4). Of the 22 patients infected with SFTSV, 14 had acute-phase serum samples obtained during the first week after onset of illness; 9 (64%) of 14 had SFTSV RNA detected by RT-PCR and 7 (50%) of 14 had antibodies against SFTSV detected by ELISA. Of the 8 patients who had acute-phase serum samples obtained during the second week after onset of illness, 3 (38%) of 8 had SFTSV detected by RT-PCR and 6 (75%) of 8 had antibodies against SFTSV detected by ELISA. The difference in assay performance by week of illness onset was not significant ($p>0.05$, by χ^2 test).

Epidemiology

The 22 confirmed case-patients (9 women and 13 men) ranged in age from 40 to 78 years (median age 63.5 years). Patients were hospitalized during days 1–7 after onset of illness (median 4 days). The first case of SFTS occurred on May 22, and the last case occurred on October 2; 71% of the cases occurring during July–August (Figure 2). We confirmed 22 cases of SFTSV infection in Yiyuan County in 2011.

Conclusions

Our results showed that the RT-PCR detection rate for SFTSV is higher for blood samples obtained in the first week (64%) than for those obtained in the second week (38%) after illness onset; the SFTSV antibody detection rate showed a reverse pattern. We did not observe a difference in RT-PCR detection rate of SFTSV or antibody detection rate for serum samples collected during the first and second weeks of illness. Nonetheless, our findings support use of RT-PCR with serum samples for confirming a diagnosis of SFTS during the first week after illness onset and serum ELISA for diagnosis during the second week.

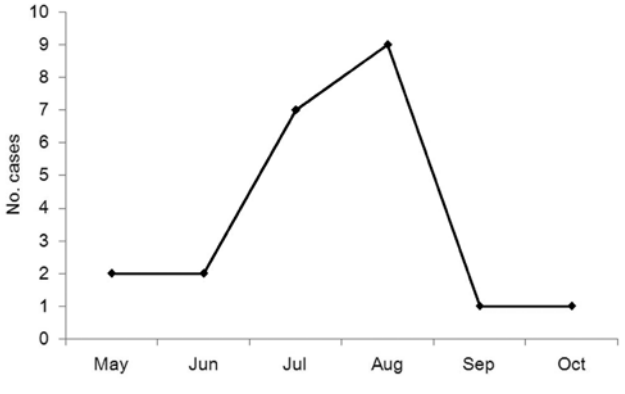

Figure 2. Cases of severe fever with thrombocytopenia syndrome, by month of illness onset, Shandong Province, China, 2011.

Table 4. Test results for severe fever with thrombocytopenia syndrome virus–infected patients, Shandong Province, China, 2011*

Acquisition time after illness onset, wk	RT-PCR +	RT-PCR –	ELISA +	ELISA –	No. patients
<1	9	5	7	7	14
1–2	3	5	6	2	8

*RT- PCR, reverse transcription PCR; +, positive; –, negative.

There are 2 possible explanations for 2 patients having negative results for both laboratory tests. The first explanation is that levels of SFTSV virus and antibody were below detection thresholds. The second explanation is that patients were truly negative for SFTS and were ill because of infections with other pathogens.

Our study had 2 limitations. First, our sample size of reported clinical cases was small, which may have limited our ability to detect a difference between PCR and antibody detection rates during the first and second weeks of illness. Second, our clinical case definition might not have been sensitive or specific because we had access only to data that were reported by local clinicians (suspect cases reported to the Yiyuan County Centers for Disease Control and Prevention), and we did not know what specific infections were ruled out.

We obtained acute-phase serum samples from 24 patients given a clinical diagnosis of SFTS and analyzed these samples SFTSV RNA by using RT-PCR and for SFTSV antibodies by using ELISA. Cases of SFTS were confirmed for 19 (86%) of these patients when acute-phase serum samples were analyzed. Previous reports for SFTS indicated that when acute-phase serum samples were tested, ≈70% of patients with SFTS had detectable virus or virus antibodies by similar methods (*1*,*10*). Among convalescent-phase serum samples from 21 patients, 8 showed seroconversion for SFTSV. Among these 8 patients, 5 had detectable SFTSV in acute-phase serum samples. However, convalescent-phase serum samples were needed for 3/22 patients to confirm the diagnosis. It was unlikely that patients who showed seroconversion were infected after hospital discharge because of low seroprevalence of SFTSV in the study region (*11*).

Our results suggest that RT-PCR or ELISA alone is insufficiently sensitive for diagnosis of SFTSV infection in the early stage of SFTS. However, combining RT-PCR and ELISA results can increase sensitivity to 86%. RT-PCR with primers for virus S segment was more sensitive than that with primers for virus L segment, which was likely caused by primer length. We conclude that in areas to which SFTSV is endemic, patients with clinically compatible SFTS (fever, thrombocytopenia, or leukopenia without another known infectious disease) should be treated with early supportive therapy, even before laboratory confirmation is available.

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A Historical Perspective of Influenza A(H1N2) Virus

Naomi Komadina, Jodie McVernon, Robert Hall, and Karin Leder

The emergence and transition to pandemic status of the influenza A(H1N1)A(H1N1)pdm09) virus in 2009 illustrated the potential for previously circulating human viruses to re-emerge in humans and cause a pandemic after decades of circulating among animals. Within a short time of the initial emergence of A(H1N1)pdm09 virus, novel reassortants were isolated from swine. In late 2011, a variant (v) H3N2 subtype was isolated from humans, and by 2012, the number of persons infected began to increase with limited person-to-person transmission. During 2012 in the United States, an A(H1N2)v virus was transmitted to humans from swine. During the same year, Australia recorded its first H1N2 subtype infection among swine. The A(H3N2)v and A(H1N2)v viruses contained the matrix protein from the A(H1N1)pdm09 virus, raising the possibility of increased transmissibility among humans and underscoring the potential for influenza pandemics of novel swine-origin viruses. We report on the differing histories of A(H1N2) viruses among humans and animals.

During 2009, emergence of influenza A(H1N1)pdm09 as a pandemic virus heightened public awareness of the potential for human influenza viruses to mutate. The viruses had been transmitted to animal reservoirs decades earlier, evolved, and were reintroduced to human populations as novel reassortant viruses (*1*). Reinforcing this concept, during 2012, >300 human cases of swine-origin influenza A(H3N2) variant (v) viruses were reported in the United States, predominantly acquired through close contact with pigs at agricultural shows, leading to 11 hospitalizations. The virus had limited person-to-person spread during 2012; a seed vaccine virus was developed for response to the H3N2v strain should the virus become readily transmissible among humans (*2*).

A swine-origin influenza subtype variant, A(H1N2)v, which was lesser known than H3N2v, infected 4 persons attending agricultural shows during the final days of the agricultural show season (*3*). Late emergence of this virus may have limited its spread. Although there was no evidence of human-to-human transmission, there was concern that the presence of the matrix protein derived from the A(H1N1)pdm09 virus, which had been circulating widely in the human population since 2009, could potentially confer the A(H1N2)v virus with increased transmissibility among humans (*3*).

Novel influenza viruses can arise among humans either by direct transmission from mammalian or avian sources or through genetic reassortment. The segmented nature of the influenza viral genome allows reassortment to occur in a host that is simultaneously infected with ≥2 subtypes of influenza A viruses. Although influenza viruses exhibit some host specificity, swine are susceptible to infection with viruses of avian and mammalian lineages, facilitated by the presence of receptors for both lineages in the respiratory tract. Swine can therefore serve as "mixing vessels" for different lineages, providing an opportunity for novel reassortants to arise. The reassortant viruses may acquire mammalian adaption characteristics, thereby allowing infection of humans to occur (*4*).

A(H1N2) viruses have been described among avian, swine, and human populations. Like A(H3N2) and A(H1N1) viruses, A(H1N2) viruses have become established in swine herds in many regions. In contrast, A(H3N2) and A(H1N1)pdm09 were the only type A viruses documented as circulating among humans as of 2009. Worldwide, 1 case of a human-origin reassortant was reported between 2003 and the events of 2012 in the United States (*5*). Here, we document the distinct lineages of swine and human influenza H1N2 subtypes, cross-species reassortment, and transmission events that result in the emergence of novel viruses.

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Evolution of Influenza A(H1N2) in Swine

Influenza was first recognized as a disease of swine during the 1918 pandemic, when it was observed that families infected with the pandemic virus often saw that their swine herds were also infected (*6*). Although the 1918 A(H1N1) pandemic virus appeared in swine and human populations around the same time, it is not known if the first viruses infected swine and were transmitted to humans, if human and swine populations were infected at the same time, or if the pandemic virus was transmitted from humans to swine (*7*). Once established, the virus evolved along independent evolutionary pathways in both populations (*8*,*9*).

The first A(H1N1) viruses isolated from swine in the United States during 1930 are known as classical swine influenza A(H1N1) viruses (*8*). A(H3N2) viruses were first identified in swine in 1970 during an influenza surveillance study in Taiwan. This study followed the emergence of the A(H3N2) pandemic virus in humans during mid–1968 known as the Hong Kong flu (*10*). Since the initial introduction of human A(H1N1) and A(H3N2) viruses into swine populations, multiple reassortants with differing genetic compositions have arisen (*8*,*9*).

During 1977, large numbers of A(H1N1) viruses were isolated from the swine population of Japan, indicating that the virus had become widespread. These viruses had a high degree of similarity to the classical swine A(H1N1) lineage, and it was postulated that this virus had been imported into Japan by swine from the United States (*11*). After identification of the A(H3N2) virus in humans, this human virus and its variants were isolated from swine in Europe and Asia (*9*). During 1980, Japan reported a period of high incidence of A(H3N2) viruses in swine and high prevalence of the A(H1N1) virus in the swine population, which provided the opportunity for mixed infection to occur; the first A(H1N2) virus reported in swine was in Japan during1980. This reassortant A(H1N2) virus was a classical swine A(H1N1) virus that had gained the neuraminidase (NA) from human A(H3N2) viruses (*11*).

Human influenza viruses were also identified in swine in Europe: the A(H1N1) virus was isolated circa 1938, and A(H3N2) viruses were identified by serologic surveillance of swine during 1968–1970 (*10*). The classical swine A(H1N1) virus was detected in Europe during 1950; however, the virus was not isolated until 1976 (*10*). During 1980, swine A(H1N1) viruses were isolated in France for the first time, and in 1981, A(H3N2) viruses were also isolated from swine. Both subtypes circulated either separately or jointly in the same geographic areas. During 1987 in Brittany, France, when these subtypes were co-circulating among swine in the surrounding region, A(H1N2) viruses were isolated (*12*). These viruses were reassortants of classical swine A(H1N1) and human-like swine A(H3N2) viruses that had circulated since the 1980s (*12*). In 1994, A(H1N2) viruses were isolated from swine in Great Britain for the first time. Unlike the A(H1N2) viruses previously circulating in Asia and Europe, these A(H1N2) viruses appeared to have undergone triple reassortment, inheriting genes from 3 parent sources: the hemagglutinin (HA) from human A(H1N1) viruses that circulated during 1980–1986, the NA from swine A(H3N2) viruses (a reassortant virus), and the avian-like swine A(H1N1) viruses that had emerged in swine in Europe during 1979 (*13*). Whereas the A(H1N2) variants that emerged in France during the 1980s remained localized, the A(H1N2) virus found in the United Kingdom subsequently spread to mainland Europe and became endemic among European swine (*13*,*14*).

In North America, until the late 1990s, influenza in swine was almost exclusively caused by the classical swine A(H1N1)-like virus. Toward the end of the 1990s, initial A(H3N2) viruses were reported (*15*,*16*). These A(H3N2) viruses had 2 genotypes. One reassortant inherited 3 genes (HA, NA, basic polymerase protein [PB] 1) from human seasonal H3N2 viruses; the remaining genes originated from the classical swine A(H1N1) viruses. The second genotype was a triple reassortant; its genes originated from human A(H3N2) (HA, NA, PB1), classical swine A(H1N1) matrix, nucleoprotein, and nonstructural (M, NP, NS) genes, and an avian virus (PB2, PA gene); this genotype became the established A(H3N2) swine virus in the United States after its emergence in 1998 (*16*).

In 1999, A(H1N2) virus was initially reported in the United States from pigs in Indiana (*15*). This virus was identified as a second-generation reassortant with the HA from the classical swine A(H1N1) virus and the remainder from the triple reassortant A(H3N2) virus (*15*). In 2005, a second lineage of A(H1N2) viruses was isolated in swine; these viruses had acquired the HA gene from seasonal human A(H1N1) viruses and maintained the triple reassortant A(H3N2) virus genes (*16*).

Shortly after the emergence of A(H1N1)pdm09 virus in the human population, the virus was noted to have reentered the swine population. In the United States, 9 H1N1/H1N1pdm09 reassortant virus subtypes with various gene constellations were isolated from swine a short time after the first reports of swine infections with the A(H1N1) pdm09 virus. These reassortant viruses contained the matrix gene from the A(H1N1)pdm09 virus. Reassortant H3N2/H1N1pdm09 subtypes containing the matrix gene were also isolated from swine in the United States. In 2010, the first A(H1N2) virus to have reassorted with the with the A(H1N1)pdm09 virus, gaining the A(H1N1)pdm09 matrix gene, was isolated from pigs in Ohio (*17*).

During 2012 in Australia, circulation of novel A(H1N2) reassortant viruses were reported in 2 widely geographically separated swine populations in Queensland,

and Western Australia (*18*). Two distinct reassortants were isolated. One, a triple reassortant, contained the HA derived from human A(H1N1) viruses, the NA from human A(H3N2) viruses, and the remainder of genes from the A(H1N1)pdm09 viruses. The triple reassortant viruses were isolated from both pig farms; however, they were distinct from each other and appear to have emerged independently (*18*). The other strain was essentially the A(H1N1) pdm09 virus, which had gained the NA from human A(H3N2) viruses and was isolated only at the Queensland pig farm. The A(H1N2) reassortant viruses circulating in Australia were distinct from A(H1N2) viruses circulating among swine in other countries (*18*). Little is known about influenza viruses in circulation among pigs in Australia because influenza surveillance is not routine; nevertheless, in 2009, A(H1N1)pdm09 viruses were isolated from several pig farms in Australia (*19*). A timeline of the introduction of A(H1N2) viruses into swine is shown in the Figure.

A(H1N2) influenza in swine is associated with respiratory illness and can cause sudden unexpected deaths in piglets. Modern farming systems have a higher potential than traditional farming for pig-to-pig transmission of virus to occur, because of the confined operation of intensive pig farms, where crowding results in more frequent and prolonged contact with infected swine. On farms that use traditional farming methods, influenza in swine is a seasonal illness; however, on farms that practice intensive swine farming, swine influenza infections occur year-round, peaking in the colder months (*20*). Influenza usually appears in a herd with the introduction of infected animals, either from movement between farms or by mixing infected pigs with susceptible pigs (*21*). The constant influx of influenza-naive piglets into a herd also contributes to the year-round occurrence of influenza, making the disease difficult to eradicate. Close contact among swine enhances the transmission of virus from swine-to-swine through the nasopharyngeal route by nasal secretions and aerosol droplets. Weather and environmental factors, along with swine husbandry practices of crowding, contribute to the persistence of the virus in the swine herds (*20*,*21*). Influenza has not been isolated from wild boar, although serologic studies have shown that wild boar have been in contact with

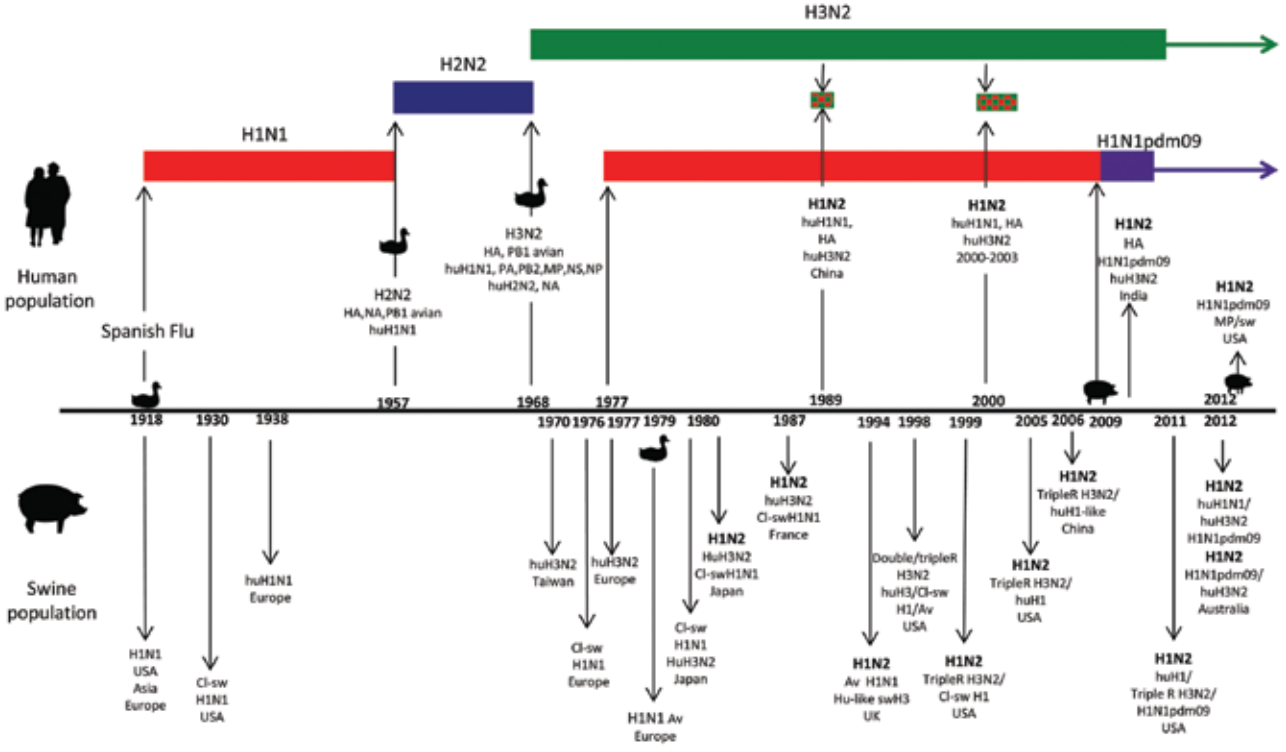

Figure. Significant points in the history of influenza viruses that have contributed to the emergence of influenza A(H1N2) viruses in human and swine populations. The bird and swine symbols on the timeline indicate when transmission appeared to occur directly from either avian or swine source into the relevant population. The bird symbols on the 1957 and 1968 time-points indicate that the circulating viruses of the time reassorted with viruses of an avian source resulting in novel subtypes. Significant events leading to the emergence of A(H1N2) in the human population are above the timeline and in swine below the timeline. A(H1N2) viruses appearing in both human and swine populations are indicated in boldface. Genotypes of A(H1N2) emerging in the human population are: 1989 (China), human A(H3N2) virus with hemagglutinin (HA) from human A(H1N1); 2000 (worldwide), same genotype as 1989 virus; 2009 (India), human A(H3N2) virus with HA from A(H1N1pdm09); 2012 (United States), human-like H1, A(H1N1pdm09) matrix, remainder swine H3N2 triple reassortant. Virus origins: Cl-sw, classical swine; hu, human; sw, swine; av, avian; hu-like, human like; double, double reassortant, tripleR, triple reassortant.

influenza viruses (*22*). The A(H1N2) viruses, along with H3N2 and H1N1 subtypes, have become enzootic in swine worldwide in areas that have intensive pig farming (*22*).

The availability of large numbers of susceptible piglets and short overall life span of pigs raised for meat production also ensures that the host immune pressure on the virus is less marked in pigs than in humans. Therefore, less antigenic drift occurs in swine influenza viruses than is seen in human viruses (*21*). Currently circulating A(H1N2) swine influenza viruses carry genes originating from humans related to prior reassortment events; however, the decreased immune pressure and slower rate of antigenic evolution in swine could facilitate swine acting as a reservoir for these previously circulating human strains. Populations exposed during the years of prevalence of prior strains would possess long-term immunity to antigenically similar strains, but there may be potential risks to human populations born after the years of prevalence of the older viruses (*22*).

Swine-origin viruses have also infected turkeys, in particular flocks of turkeys that are in close proximity to swine herds (*23*). A(H1N2) virus in turkeys has been associated with respiratory illness and sudden reduction in egg production (*23*). However, unlike in swine, the virus remains a seasonal infection because turkeys are frequently sent to market, which interrupts the infection cycle and prevents the virus from becoming endemic in farmed turkey flocks (*24*). Avian A(H1N2) viruses have also been isolated from wild ducks; however, A(H1) viruses in ducks are considered to be less common (*25*).

Evolution of Influenza A(H1N2) in Humans

The A(H1N2) viruses isolated from humans during 1989 and 2000–2003 were not of swine origin. Unlike the A(H1N2) viruses, which underwent several reassortment events in swine and became enzootic, the history of A(H1N2) virus in humans differs. The A(H1N1) influenza virus, which emerged in the human population in 1918, was an avian-descended virus, which appeared to have undergone adaptation to humans by unknown mechanisms (*26*). Today, all influenza viruses circulating in the human population carry several gene segments that are direct descendants of the avian-like 1918 A(H1N1) pandemic virus. In 1957, the Asian pandemic virus arose as a result of a reassortment event between the circulating A(H1N1) virus and avian virus to produce a progeny A(H2N2) virus, which retained 5 genes from the A(H1N1) virus and gained 3 novel genes from the avian source. The novel A(H2N2) virus replaced the previous A(H1N1) virus from circulation and continued to circulate until 1968. This new virus then also underwent reassortment, and the 1968 Hong Kong A(H3N2) pandemic virus emerged among the human population (*27*). The new progeny A(H3N2) virus inherited the same 5 genes retained in the 1957 reassortment event. The NA gene was retained from the A(H2N2) reassortment virus, with the HA and PB1 genes gained from an avian source. Descendants of the 1968 A(H3N2) virus continue to circulate in the human population.

In 1977, after a 20-year absence, A(H1N1) influenza virus re-emerged in the human population, causing worldwide epidemics but primarily affecting those under 25 years of age (*28*). This virus, which had ceased circulating in 1957 after the A(H2N2) virus emerged, was antigenically and genetically identical to A(H1N1) viruses that had been isolated in the 1950s (*29*). During the winter of 1978–79, the reappearance of the A(H1N1) virus coincided in some countries with epidemics of A(H3N2), and several instances of co-infection were reported in the United States and Japan. The A(H1N1) and A(H3N2) viruses, and a recombinant A(H3N1) virus, were subsequently isolated and characterized from single samples in both regions (*30*,*31*). Evidence of reassortment between the co-circulating viruses was also found when a reassortant A(H1N1) virus was isolated and found to have HA, NA, the M-gene segment, and NS gene inherited from the A(H1N1) virus and remaining genes from the A(H3N2) subtype (*32*). This virus circulated only in the Northern Hemisphere for 1 season.

Influenza A(H1N1) and A(H3N2) viruses continued to co-circulate among humans, with the predominant circulating subtype changing during following seasons. During 1988, A(H1N2) viruses were reported to be circulating sporadically in China for ≈4 months, although there were no reports of associated influenza outbreaks. This virus was an A(H1N1) and A(H3N2) human influenza reassortant, and it co-circulated with the parent viruses during the winter. There was no reported spread of the A(H1N2) virus to other countries, and after the initial cases in 1989, no further cases were documented in China (*33*).

In 2000, a second reassortant A(H1N2) virus began to circulate; however, this time it did not remain localized but became widespread during the 2001–02 Northern Hemisphere winter. The earliest A(H1N2) viruses isolated were from Thailand during 2000. A small number of A(H1N2) viruses were then detected in Singapore, then Malaysia and Indonesia (2001–02). Two A(H1N2) viruses were isolated in Australia; none were reported from New Zealand or the Pacific region (*34*). In contrast, the emergence of the A(H1N2) virus was associated with substantial outbreaks in the United Kingdom, where it was by far the predominant A(H1) virus during the 2001–02 influenza season. The A(H1N2) virus was first identified in the United Kingdom in September 2001, and it continued to be reported until the end of March 2002. In the United Kingdom, A(H1N2) viruses co-circulated with A(H3N2) viruses in relatively equal proportions, in addition to a small number of A(H1N1) viruses (*35*). Influenza surveillance identified A(H1N2) viruses circulating in Europe

and sporadically in Asia, the Middle East, and North and South America (*36*).

A comprehensive study of subjects participating in a vaccine trial conducted in 20 countries on 4 continents identified 65 A(H1N2) viruses from 228 influenza A(H1) viruses isolated (*37*). Most of these A(H1N2) viruses were isolated in South Africa, where A(H1N2) viruses accounted for >90% of the A(H1) cases documented in the trial, which took place during the 2001–2002 influenza season (*37*). Although sporadic circulation of A(H1N2) viruses was reported across the globe, the greatest effect was in the United Kingdom, where A(H1N2) accounted for 54% of the 420 influenza A viruses isolated during the 2001–2002 season (*35*).

The A(H1N2) viruses circulating during 2000–2003 were found to have a similar genetic make-up to those that had circulated sporadically in China during 1988–89 in that they were essentially an A(H3N2) virus where the HA had been replaced with the HA from the A(H1N1) virus (*33*,*38*). The antigenic characterization of these viruses also indicated that the HA of the A(H1N2) viruses were related antigenically to the A(H1N1) virus circulating at the time, including the vaccine strain in use, A/New Caledonia/20/99(H1N1). Further antigenic characterization showed that the NA of the A(H1N2) viruses were closely related to those of the A(H3N2) viruses that were co-circulating (*38*). Genetic characterization indicated that the HA genes of the A(H1N2) viruses were all broadly genetically similar to A/New Caledonia/20/99-like virus, with 2 signature amino acid changes when compared with the A/New Caledonia/20/99 HA gene. Genetic analysis of the remainder of the genome indicated that the other 7 genes were closely related to the H3N2 A/Moscow/10/99-like viruses, which had been circulating in the population at the time of the reassortment event (*35*,*38*). The 2 earliest viruses identified as A(H1N2) from Thailand, however, did not contain these signature changes in the HA gene, suggesting that these A(H1N2) viruses may have arisen from another reassortment event that did not persist (*37*). An A(H1N2) virus from Singapore, isolated from a child who was 3 months of age in October 2000, was the oldest virus of the A(H1N2) viruses containing the signature changes (*34*). Because all the viruses isolated from that point on were found to have <2% divergence, the A(H1N2) viruses were most likely to have originated from a single reassortment. This presumably occurred in 1999 or 2000 in Asia, with the virus subsequently spreading to Europe, the Middle East, Africa, and the Americas (*38*).

In Egypt, Israel, and the United Kingdom, infected persons were mainly 5–15 years of age (*38*), whereas in South Africa, children and the elderly were infected (*37*). In the United Kingdom, which had the greatest number of reported influenza A(H1N2) cases, it is notable that few adults became infected, that only a small number of viruses were isolated from adults >65 years of age, and that 75% of the viruses isolated were from children <15 years of age. Because a similar number of children <15 years of age also became infected with the A(H3N2) virus in the United Kingdom during the same period, it was considered that these children possibly had a primary infection (*35*). Adults and vaccinated persons >60 years of age appeared to have acquired protective immunity to the new subtype, presumably because a substantial proportion of the population had developed immunity either from previous infections by A(H1N1) or A(H3N2) viruses or by vaccination (*35*). The vaccine in use at the time was expected to provide protection against the H1N2 subtype because it contained the H1 from A/New Caledonia/20/99 and the N2 from A/Moscow/10/99-like viruses, which were both genetically and antigenically related to the novel A(H1N2) viruses (*36*).

By early 2003, A(H1N2) viruses were no longer being isolated from human samples. In 2006, an A(H1N2) virus that was a triple reassortant-like virus and, with the exception of the matrix gene, genetically similar to A(H1N2) pdm09 viruses, was isolated from swine in China (*41*). In late 2009, a novel A(H1N2) virus was isolated from a human in India (*5*). This H1N2 virus was a reassortant of A(H1N1)pdm09 and A(H3N2) viruses co-circulating in the population. Although this virus had a similar genetic make-up to previously observed A(H1N2) viruses, the source of the HA component differed and was derived from the A(H1N1)pdm09 virus (*5*). In 2012, swine-origin A(H1N2) v viruses were isolated from 4 humans in the United States. The A(H1N2)v viruses were reassortants of the triple reassortant A(H1N2) viruses viruses circulating in swine in the U.S and A(H1N1)pdm09 viruses (*16*). All persons with cases of A(H1N2)v viruses were linked to the Minnesota state fair and were isolated from humans who had been in close contact with swine. Because these A(H1N2) viruses contained the matrix protein from the A(H1N1)pdm09 virus, there was concern that this virus could transmit more readily in humans (*3*).

Conclusions

In swine, multiple A(H1N2) virus reassortments have included genetic material from avian, swine, and human influenza viruses and have formed multiple A(H1N2) reassortant viruses with differing genetic compositions over time (*10*). In humans, the A(H1N2) virus has also arisen as a result of the reassortment of human A(H1N1) and A(H3N2) strains, leading to circulation of A(H1N2) viruses that have a similar genetic composition circulating in China in 1989 and worldwide during 2000–2003 (*33*,*34*).

Direct cross-species transfer of swine A(H1N2) is rare and until recently was restricted to reports of single cases from the Philippines (2004) (*39*) and from Michigan and

Minnesota in 2007 and 2011, respectively (*3*). Detection of a cluster of 4 swine-origin human A(H1N2)v cases during the final days of the Minnesota agricultural fair in 2012 was therefore a noteworthy event. The rise of H1N2 reassortants containing genes from the H1N1pdm09 virus, in particular the matrix gene, which has been associated with high transmission efficiency (*40*), underscores the fact that influenza reassortment is an ongoing process, that humans can become infected with novel viruses caused by reassortment or transmission of swine origin viruses, and that these novel viruses may have the potential to cause human pandemics.

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Genomic Epidemiology of *Vibrio cholerae* O1 Associated with Floods, Pakistan, 2010

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In August 2010, Pakistan experienced major floods and a subsequent cholera epidemic. To clarify the population dynamics and transmission of *Vibrio cholerae* in Pakistan, we sequenced the genomes of all *V. cholerae* O1 El Tor isolates and compared the sequences to a global collection of 146 *V. cholerae* strains. Within the global phylogeny, all isolates from Pakistan formed 2 new subclades (PSC-1 and PSC-2), lying in the third transmission wave of the seventh-pandemic lineage that could be distinguished by signature deletions and their antimicrobial susceptibilities. Geographically, PSC-1 isolates originated from the coast, whereas PSC-2 isolates originated from inland areas flooded by the Indus River. Single-nucleotide polymorphism accumulation analysis correlated river flow direction with the spread of PSC-2. We found at least 2 sources of cholera in Pakistan during the 2010 epidemic and illustrate the value of a global genomic data bank in contextualizing cholera outbreaks.

In 2010, a surge in cholera cases seriously threatened public health across Pakistan, where previously sporadic cases of cholera had been reported (*1*–*3*). In late July and August 2010, record monsoon rainfall and the simultaneous glacier melt resulted in the worst flooding in the recorded history of Pakistan, affecting an area of 61,776 square miles and displacing >20 million persons (*4*). A cholera outbreak ensued, and the World Health Organization (WHO) reported 164 laboratory-confirmed cases with the help of National Institute of Health and other allied departments in Pakistan (*5*).

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Despite the paucity of data on the impact of cholera in Pakistan before 2010, seasonal epidemics are known to have occurred every year since then. Cholera is endemic to South Asia (*6*) and the Bay of Bengal (*7*,*8*) and is spread through contaminated food and water, often after civil unrest or natural disasters. Pakistan is particularly at risk for waterborne disease because it is an agricultural economy with one of the most expansive water distribution systems in the world. This vast irrigation system depends largely on the Indus River, which originates on the northern slopes of the Kailash mountain range in India and runs north to south through the entire length of Pakistan with many tributaries, including the Zaskar, the Shyok, the Nubra, and the Hunza, converging in the northern region and flowing through the provinces of Ladakh, Baltistan, and Gilgit.

Not all *Vibrio cholerae* strains cause major disease outbreaks. Although *V. cholerae* has >200 serogroups, only serogroups O1 and O139 are associated with epidemics. Serogroup O1 isolates can be assigned to 2 biotypes, classical and El Tor; the latter is responsible for the current seventh pandemic that has spread in global radiations, or waves, originating in the Bay of Bengal (*8*). The clinical severity of cholera is associated with the production of cholera enterotoxin (CT), which is encoded by a gene on the 6.9-kb CTX prophage integrated within chromosome 1 of all pandemic *V. cholerae* O1 cholera strains (*9*). Historically, CTX prophages have been categorized as $CTX^{classical}$ or $CTX^{El\ Tor}$ on the basis of DNA sequence of the *rstR* and the sequence variation in *ctx*B gene. During the last 2 decades, new variants of El Tor biotypes have emerged and have been used to differentiate *V. cholerae* isolates (*10*). However, such approaches do not have the resolution required to stratify the highly clonal *V. cholerae* O1 isolates of the seventh pandemic sufficiently to

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understand their precise phylogeny and relate that to geographic distribution and spread.

The application of whole-genome sequence analyses has revolutionized our ability to resolve the *V. cholera*e O1 El Tor populations and more precisely determine the patterns of spread of cholera within the worst affected countries of the world. Clarifying the routes of spread of cholera in Pakistan provides the unprecedented opportunity to inform public health provision. This study showed that the 2010 cholera outbreak was, in fact, an epidemic within an epidemic explained by 2 independent introductions of cholera in the country, 1 from the south and 1 following the flood water as it moved from north to south along the Indus River.

Materials and Methods

Strain Collection

To determine whole-genome sequence type and single-nucleotide polymorphism (SNP)–based phylogeny analysis of *V. cholerae* following the 2010 floods in Pakistan, 38 *V. cholerae* O1 El Tor were isolated from fecal samples/rectal swabs of cholera patients during August–October in the flood-affected and -unaffected districts of Sindh, Khyber Pakhtunkhwa (KPK), and Punjab Provinces. Identification, serogroup, and biotype were determined by standard biochemical methods (*11*–*13*).

Antimicrobial Susceptibility Test

The susceptibility of *V. cholerae* O1 El Tor to different antimicrobial drugs was tested by disk diffusion on MH agar. The antimicrobial drugs tested were ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), cefotaxime (30 μg), ceftazidime (30 μg), erythromycin (15 μg), nalidixic acid (30 μg), ofloxacin (5 μg), streptomycin (10 μg), tetracycline (30 μg), trimethoprim (25 μg), and trimethoprim/sulfamethoxazole (1.25/23.71 μg). *Escherichia coli* ATCC25922 was used as quality control according to Clinical Laboratory Standards Institute guidelines (*14*). To interpret the results, we followed these guidelines. All antimicrobial drugs used during this study were purchased from Oxoid Limited (Hampshire, UK).

Genome Sequencing

Unique index-tagged libraries, with 250-bp insertion size, were created and loaded on the 8 lanes in Illumina HiSeq cell (Illumina, San Diego, CA, USA) to perform 72-bp end sequencing of 96 separate libraries in each lane. After sequencing the index tags and libraries separately, the tag sequence information was used for assigning reads to the individual samples (*8*). All the samples achieved an average coverage of 200× in the regions where SNPs were called. All the data have been submitted to the European Nucleotide Archive with the accession codes.

Whole-Genome Alignment and Detection of SNPs in the Core Genome

The 72-bp end read data obtained were mapped to El Tor reference strain N16961 (GenBank accession nos. AE003852 and AE003853) by using SMALT (www.sanger.ac.uk/resources/software/smalt) to obtain whole-genome alignment for all the strains in this study, and SNPs were called by using methods described by Harris et al. (*15*). Any unmapped reads and the sequences that were absent from N16961 reference genome were excluded from the core genome; thus, SNPs from these regions were not called. The SNPs called were filtered to remove the sites with a SNP quality score <30. SNPs that were absent in at least 75% of the reads at any heterogeneous mapped sites were excluded, and high-density SNP clusters or the possible recombination sites were excluded as described by Croucher et al. (*16*).

Phylogenetic Analysis, Comparative Genomics, and Linear Regression Analysis

Default settings of RAxML version 0.7.4 (*17*) were used to estimate the phylogenetic trees on the basis of all the SNPs called from the genome as explained above. To calculate the number of SNPs on each branch, we reconstructed all the polymorphic events on the tree using PAML (*18*). M66, a pre–seventh-pandemic strain (accession nos. CP001233 and CP001234), was used as an outgroup to root the phylogenetic tree (*5*). The tree was visualized and ordered by using phylogenetic tree reading software, Figtree (httpp://tree.bio.ed.ac.uk/software/figtree/). The paired end reads were assembled by using a de novo genome assembly program Velvet version 0.7.03 (*19*), and a multicontig draft genome was generated for each sample. The parameters were set to give the best *k*-mer size and at least 20× *k*-mer coverage. To take advantage of the high similarity of the seventh-pandemic *V. cholerae* at the core genome, contigs were ordered by using Abacas with N16961 El Tor strain as reference (*20*). To each ordered draft genome, annotation transfer was made from the reference strain. Finally a genome comparison file was generated by TBLASTX (*21*) against N16961 FASTA sequence as a database to be used in Artemis Comparison Tool for manual comparison of the genomes (*22*). The final phylogenetic tree was opened by using Path-O-Gen version 1.3 (http://tree.bio.ed.ac.uk/software/pathogen), and the root-to-tip distance data for each strain were exported to Excel (Microsoft, Richmond, WA, USA). These data were used to plot a linear regression curve against date of isolation of the strain. The R^2 correlation, slope, and p values were determined by using the inbuilt regression package of R statistical environment (www.r-project.org).

Results

Sample Collection

A total of 319 fecal samples were collected from patients who had acute diarrhea who reported in the KPK, Sindh, and Punjab Provinces during August–October 2010. Of these, 219 (69%) and 100 (31%) were from flood-affected and non–flood-affected regions, respectively (Table; online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/20/1/13-0428-Techapp1.pdf). A total of 38 *V. cholerae* O1 El Tor biotype (22 from flood-affected and 16 from non–flood-affected regions) were isolated and mapped to locations across Pakistan (Figure 1). The *V. cholerae* isolates were serogrouped and verified by PCR amplification of the *ompW* (*11*) and *rfb*O1/*rfb*O139 genes (*12*) (online Technical Appendix Table 1). To interpret the progression of the outbreak, we used WHO data (year 2010, weeks 33–48 of the WHO epidemiologic reports in Pakistan) and compiled acute diarrhea incidence in Pakistan and its major provinces. These data demonstrated a dramatic increase in acute diarrhea cases in week 33, peaking in week 34 (fourth week of August) with ≈200,000 documented cases.

Antimicrobial Susceptibility Patterns

Antimicrobial susceptibility testing by disk diffusion showed that all *V. cholerae* isolates were resistant to streptomycin, trimethoprim, trimethoprim/sulfamethoxazole, and nalidixic acid. All isolates from the flood-affected patients were susceptible to ciprofloxacin, ofloxacin, chloramphenicol, ampicillin, cefotaxime, ceftazidime, and tetracycline. However, 6 of 8 isolates from Karachi (a non–flood-affected coastal city) and 1 of 8 isolates from Hyderabad (located at the bank of Indus River) were tetracycline resistant. Five (63%) isolates from Karachi and 1 (13%) isolate from Hyderabad were also resistant to ceftazidime (online Technical Appendix Table 2).

Whole-Genome Phylogenetic Analysis

Whole-genome sequences of the 38 *V. cholerae* O1 strains were determined by using the Illumina sequencing platform. On the basis of genomewide SNPs, we constructed a high-resolution maximum-likelihood phylogenetic tree using previously described methods (*15*). Sequence reads were mapped to the reference genome sequence of *V. cholerae* O1 El Tor strain N16961 (isolated in Bangladesh in 1975, accession no. AE003852–3) and compared with 146 globally and temporally representative *V. cholerae* O1 El Tor strains (*8*).

The consensus tree showed that all Pakistan strains fell within 2 contemporary subclades (PSC-1 and PSC-2), both of which branched from different positions within the third transmission wave of the seventh-pandemic lineage (Figures 2, 3). The PSC-1 isolates were derived from cases located in the coastal city of Karachi (6/7) and Hyderabad (1/8), whereas the PSC-2 isolates originated from a wide geographic region comprising flood-affected and non–flood-affected inland regions (30/31) and 1 case from Karachi (Figure 1). After removal of genomic recombination sites, 1,826 variable genomic sites defined variation in the El Tor global phylogeny, and PSC-1 and PSC-2 had only 12 and 22 distinguishing SNPs from their third-wave ancestors, respectively (Figure 2). Within each subclade, the strains were very closely related, with only 4 SNPs within the PSC-1 isolates and 76 SNPs within the PSC-2 isolates.

We previously showed that genomic variation occurs in the seventh-pandemic El Tor *V. cholerae* at a clock-like rate (*8*). Consequently, we plotted root-to-tip distances of the PSC-1 and PSC-2 isolates against isolation date and geographic location. We observed a strong and statistically

Table. Isolation of *Vibrio cholerae* O1 El Tor from flood-affected and non–flood-affected patients, Pakistan, 2010

Province, district	Flood status	Acute diarrhea samples collected, no. (%)	*V. cholerae* O1 El Tor isolates, no. (%)
Khyber Pakhtunkhwa			
Dera Ismail Khan	Affected	55	7 (13)
Nowshera	Affected	20	1 (5)
Charsada	Affected	40	0
Peshawar	Unaffected	50	4 (8)
Sindh			
Khairpur	Affected	36	1 (3)
Jamshoro	Affected	10	5 (50)
Sukkur	Affected	6	0
Shikarpur	Affected	6	0
Karachi	Unaffected	25	8 (32)
Hyderabad	Affected	19	8 (42)
Punjab			
Dera Ghazi Khan	Affected	2	0
Muzzaffargarh	Affected	25	0
Rawalpindi	Unaffected	25	4 (16)
Total, N = 319	Affected	219 (68)	22 (10)
	Unaffected	100 (32)	16 (7)

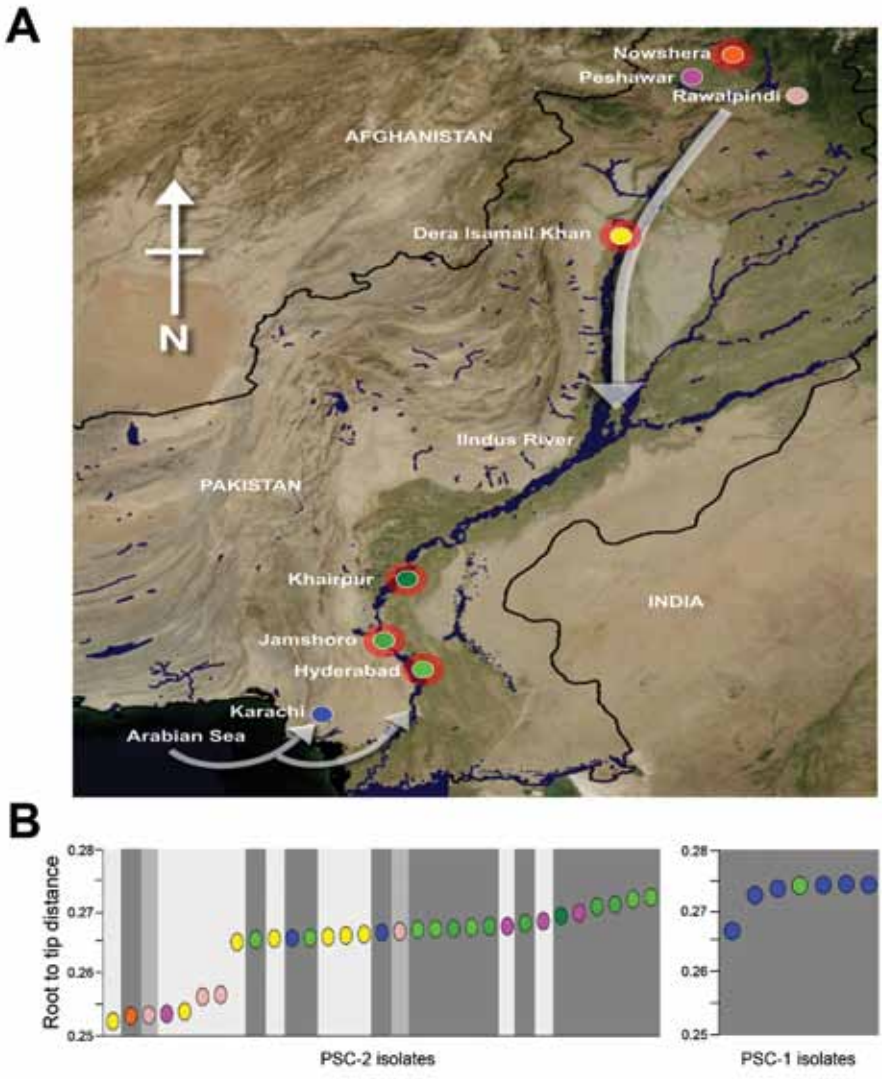

Figure 1. Cholera during the floods, Pakistan, 2010. A) North-oriented map of Pakistan indicating the 8 locations of *Vibrio cholerae* O1 El Tor isolation (shown by individual circles; red outer shading indicates the 5 locations that had flooding during the study period). White arrows indicate the hypothesized directions of spread of the various subclades of *V. cholerae* O1 El Tor. B) Cumulative root-to-tip distances of subclade 2 and subclade 1 *V. cholerae* O1 El Tor isolates. Each colored circle corresponds with an individual *V. cholerae* O1 El Tor isolate; colors correspond with the locations shown in panel A. Shading corresponds with the month of *V. cholerae* O1 El Tor isolation: light gray, August; medium gray, September; dark gray, October.

significant phylogeographic correlation between mutation rate and isolation date, recognizing that the PSC-1 and PSC-2 strains isolated earlier were closer to the root of the Pakistan clades, whereas those collected later were further away ($R^2 = 0.27$, $p<0.001$). The SNP acquisition rate in the Pakistan isolates occurred at 0.288 SNPs/month (3.4 SNPs/year) and was in accordance with our estimations inferred from a global seventh-pandemic strain collection (3.3 SNPs/year) (online Technical Appendix Figure 1).

Furthermore, we observed that the earlier isolates of PSC-2 clade, displaying shorter root-to-tip distances, were in closer proximity to the source of the Indus River and were mainly isolated from Peshawar, Nowshera, Rawalpindi, and Dera Ismail Khan in the north of Pakistan. Conversely, isolates in October tended to be from the southern regions of Khairpur, Jamshoro, Hyderabad, and Karachi (Figure 1, panel B). A root-to-tip phylogenetic tree distance plot of the PSC-2 subclade against distance from the source of the Indus River confirmed this association ($R^2 = 0.35$, $p<0.001$; online Technical Appendix Figure 2). The observed pattern is consistent with the origins and progression of the floods, which began in Peshawar in late July and followed the course of the Indus River southwest, passing Nowshera, Dera Ismail Khan, Khairpur, Jamshoro, and Hyderabad in August.

Subclade Signature Deletions

The Pakistan subclades could be distinguished from other El Tor *V. cholerae* by subclade-specific deletions in the DNA sequences, particularly in the 2, Vibrio pathogenicity island–1 and Vibrio seventh pandemic-2 (VPI-1 and VSP-2), that impact on the relative transmissibility and virulence of the respective subclades. All the PSC-1 isolates had a unique 3-gene deletion in the VPI-1 (VC_0819–0821) and a 4-gene deletion within the VSP-2 (VC_0495–0498), which was previously identified in El Tor strains responsible

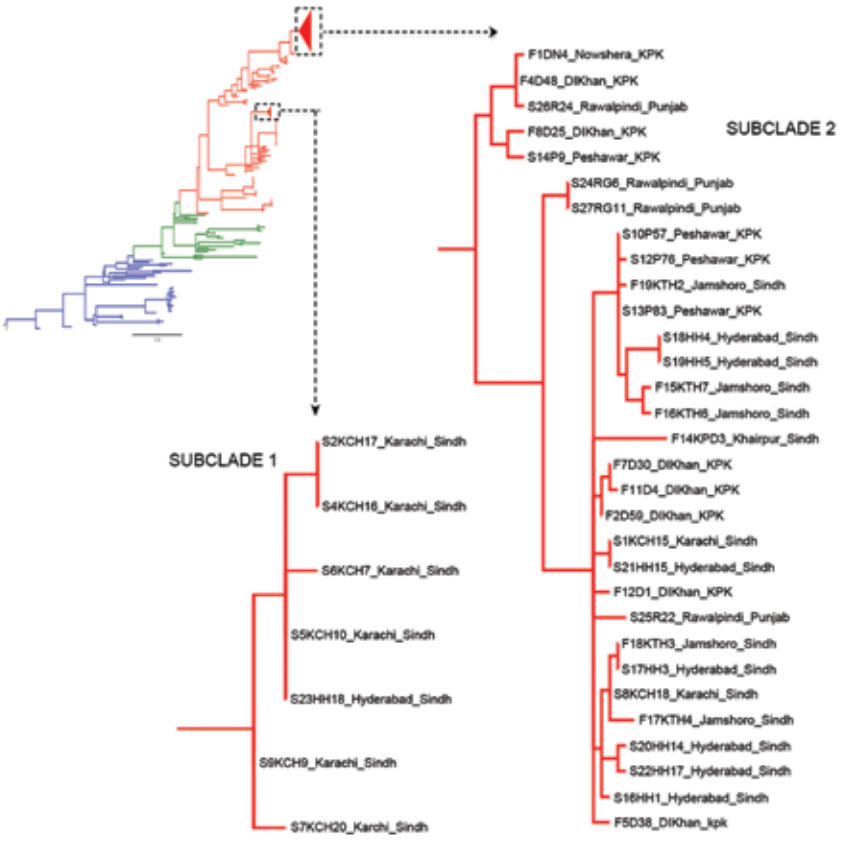

Figure 2. Phylogenetic tree showing the relative position of *Vibrio cholerae* O1 El Tor from Pakistan in wave 3 of the seventh-pandemic lineage, based on single-nucleotide polymorphism differences. The blue, green, and red branches represent waves 1, 2, and 3 respectively. The colors of the subclade 1 and 2 isolates match the colors assigned to the strain isolation locations in Figure 1, panel A.

for outbreaks in Bangladesh in 2008 (*23*). In contrast, the PSC-2 isolates had an 18-gene deletion (VC_0495–0512) in VSP-2 comparable to some of the most recently characterized strains of El Tor *V. cholerae* O1, including those from the Haiti outbreak in 2010 (*23*). VP1-I is intact in PSC-2 isolates except for a frame-shift mutation in the accessory colonization factor gene, *acfC* (VC_0841).

Discussion

We used whole-genome SNP-based analyses of *V. cholerae* from areas of Pakistan affected by the major floods of 2010 to assign isolates onto the seventh-pandemic *V. cholerae* O1 El Tor phylogenetic tree (*7*). All isolates mapped to wave 3 of the current pandemic as 2 distinct subclades, PSC-1 and PSC-2, in general agreement with their time of isolation. Both Pakistan subclades are located on the tree close to other isolates of South Asia origin, including Nepal, as well as those from the recent outbreak in Haiti (*23*) (Figure 3). However, the genomic analysis clearly shows that both Pakistan clades represent distinct outbreaks not directly related to *V. cholerae* isolated elsewhere. The geographic distribution of the isolates in PSC-1 and PSC-2 is revealing. Isolates from PSC-1 are largely limited to the non–flood-affected coastal city of Karachi, and only 1 PSC-1 isolate was from the nearby city of Hyderabad, whereas isolates from PSC-2 were from inland flood- and non–flood-affected areas countrywide (Figure 1).

Our root-to-tip distance analyses shows a correlation between the direction of the flow of the Indus River and the spread of *V. cholerae* and suggests that during the floods, 2 or possibly 3 routes of cholera spread in Pakistan. The most parsimonious explanation for the spread of PSC-2 isolates throughout Pakistan was that they followed the river along with the floodwater. In contrast, PSC-1 isolates appear to have originated in the coastal region of Pakistan, and they failed to penetrate far inland. The third possible route is represented by sporadic cholera cases in PSC-2 caused by the flood-associated isolates in areas not affected by the floodwaters but by travel of infected persons.

From our comparative genomics analysis, we identified signature deletions in the VPI-1 and VSP-2 genomic islands. PSC-1 has a unique 3-gene deletion in VPI-1, which includes *aldA* (aldehyde dehydrogenase), *tagA* (a mucinase), and a predicted coding sequence encoding a hypothetical protein. *tagA* plays a role in host cell surface modification (*24*), and its deletion may affect the virulence and transmission of the PSC-1 isolates. To our knowledge, this deletion has not been previously reported; however, the deletion of entire VPI-1 was reported in an isolate from a patient in the United States who had traveled to Pakistan (*25*). A 4-gene deletion

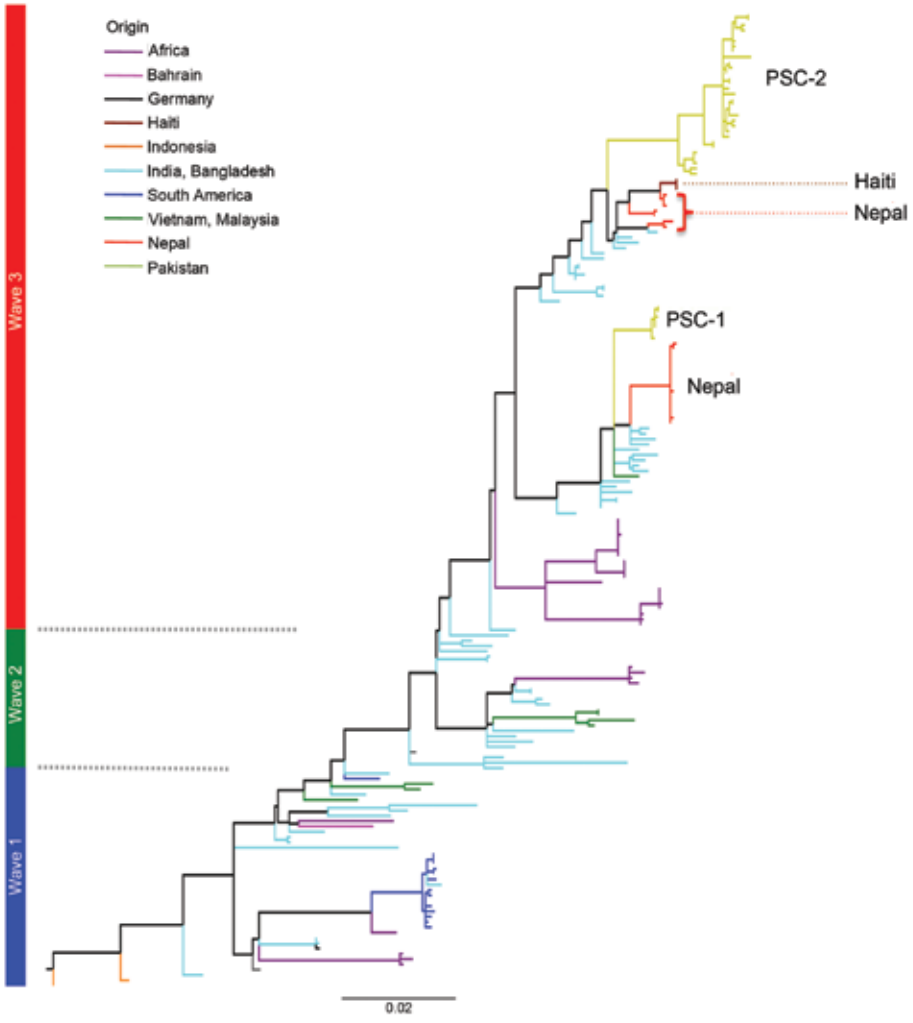

Figure 3. A single-nucleotide polymorphism–based maximum-likelihood phylogeny showing the position of *Vibrio cholerae* O1 El Tor from Pakistan in wave 3 of the seventh-pandemic lineage relative to the Haiti and Nepal strains of Hendriksen et al. (23). Waves 1, 2, and 3 are labeled in blue, green, and red respectively. Scale bar indicates substitutions per variable site.

common to PSC-1 in VSP-2 (VC-0495–498) was similar to those found in isolates from cholera outbreaks in Bangladesh in 2008 (*23*). The 18-gene deletion common to PSC-2 isolates in VSP-2 is similar to deletions found in El Tor isolates from cholera outbreaks in Haiti 2010 and from southeastern China in 2005 (*23*,*26*) because PSC-2 is closer to the Haiti 2010 cholera strains on the phylogenetic map (Figure 3). A gene located in this deletion encodes a putative type IV pilin, which may affect the colonization and virulence potential of these strains. These deletions are consistent with the position of these subclades on the phylogenetic tree of *V. cholerae* O1 El Tor. However, it is perhaps surprising that elements within the 2 pathogenicity islands appear dispensable in both PSC-1 and PSC-2 isolates. The relative impact of these deletions on *V. cholerae* pathogenesis and relative transmissibility remains to be evaluated.

The genetic organization of the SXT locus, which encodes resistance to trimethoprim, sulfamethoxazole, and streptomycin, is similar in both subclades, and as expected, all isolates were resistant to these drugs. In addition, all isolates were resistant to nalidixic acid and had intact VC_1577 (*almG*), VC_1578 (*almF*), and VC_1579 (*almE*) genes, which have recently been shown to explain the genetic basis for resistance to polymixin B/nalidixic acid (*27*). Overall PSC-1 isolates were resistant to more antimicrobial drugs than were SC-2 isolates. For example, all PSC-1 isolates were resistant to tetracycline, whereas all PSC-2 isolates were susceptible. However, *V. cholerae* O1 of both PSCs are sensitive to ciprofloxacin, except 1 from Hyderabad. Therefore, we believe that cholera in Pakistan can effectively be treated by a single dose of ciprofloxacin rather than by a repeatedly higher number of doses of erythromycin (*28*), which may be useful in preventing epidemics during natural disasters.

The position of the PSC-1 and PSC-2 isolates on the global phylogenetic tree of *V. cholerae* O1 places them close to strains from India isolated in 2006 and 2007 and strains from Bangladesh and India isolated in 2004 and 2005, respectively (*8*) (Figure 2). PSC-1 appears to be the most recent emergent subclone, with isolates mainly from

the coastal port city of Karachi. Therefore, we speculate that PSC-1 might have been introduced into Pakistan at this time, either by an unknown route to sites close to the sea or, perhaps more likely, directly from the marine/estuarine ecosystem or might be related to travel into this region.

By contrast, the wider distribution of PSC-2 isolates throughout Pakistan suggests that at the time of sampling this was the major El Tor subclade affecting the country. It is likely that PSC-2 spread through Pakistan predominantly by the flood water because the genetically older isolates were found nearer the source of the river, whereas genetically newer isolates were found further downstream closer to the river outflow into the Arabian Sea. However, a few isolates do not fit the linear regression curve of root-to-tip distance with time. These anomalous isolates might be explained by human travel among different provinces in Pakistan. The phylogeny of the 2 subclades shows unequivocally that PSC-1 and PSC-2 have evolved from 2 different recent ancestors. Thus, during the floods, at least 2 subclades of *V. cholerae* coexisted in Pakistan with different antimicrobial-resistance profiles and patterns of spread: an epidemic within an epidemic.

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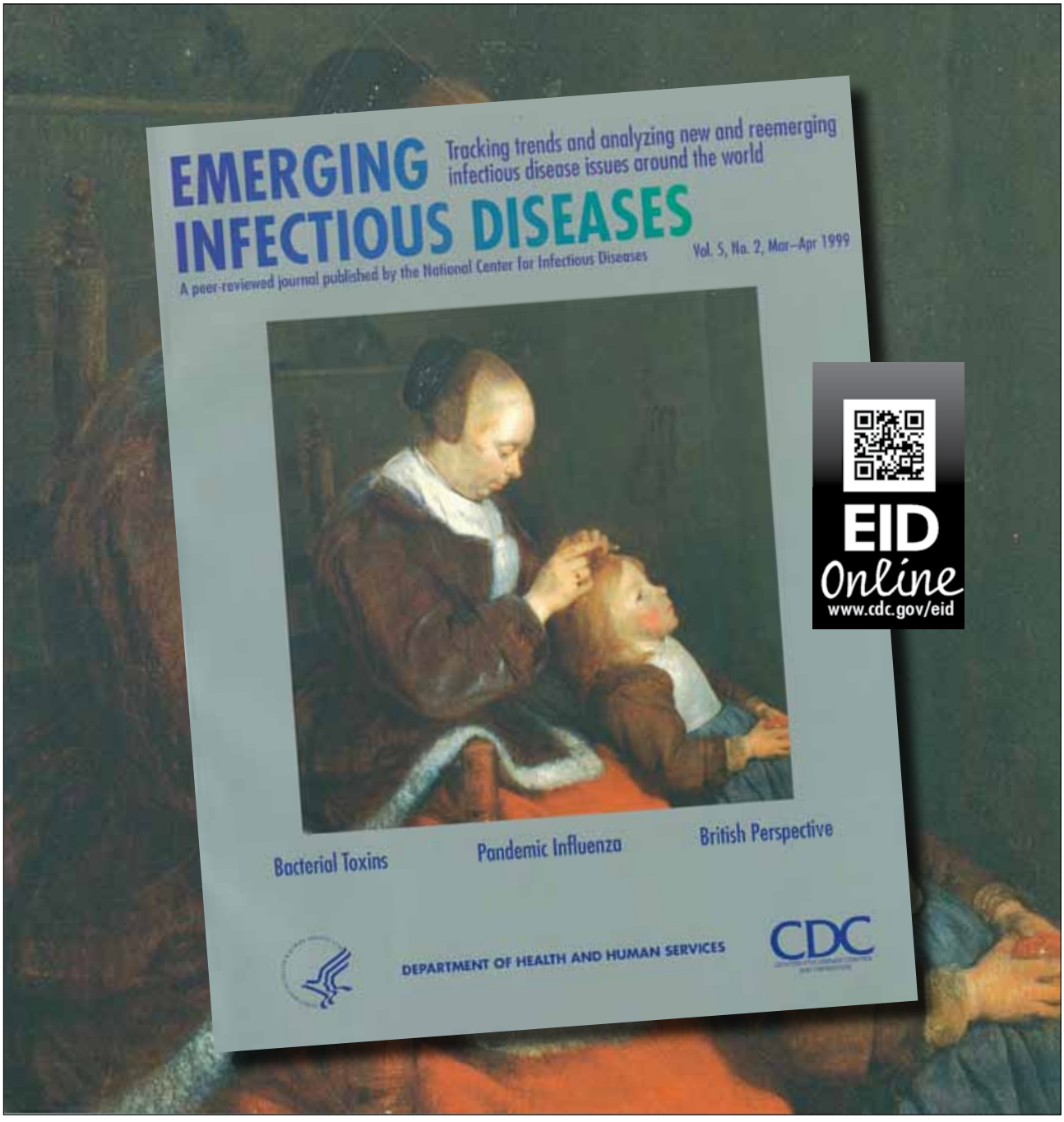

Progenitor "*Mycobacterium canettii*" Clone Responsible for Lymph Node Tuberculosis Epidemic, Djibouti

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"*Mycobacterium canettii*," an opportunistic human pathogen living in an unknown environmental reservoir, is the progenitor species from which *Mycobacterium tuberculosis* emerged. Since its discovery in 1969, most of the ≈70 known *M. canettii* strains were isolated in the Republic of Djibouti, frequently from expatriate children and adults. We show here, by whole-genome sequencing, that most strains collected from February 2010 through March 2013, and associated with 2 outbreaks of lymph node tuberculosis in children, belong to a unique epidemic clone within *M. canettii*. Evolution of this clone, which has been recovered regularly since 1983, may mimic the birth of *M. tuberculosis*. Thus, recognizing this organism and identifying its reservoir are clinically important.

Most "*Mycobacterium canettii*" strains have been isolated in the Republic of Djibouti, where 2 hospitals manage tuberculosis (TB) infections among the Djiboutian population and expatriates (*1*,*2*). A study of clinical and epidemiologic data linked to *M. canettii* infections showed that the proportion of TB cases caused by *M. canettii* was higher among expatriate than among Djiboutian patients and that patients with *M. canettii* infection were significantly younger than those with *M. tuberculosis* infection (*2*). These findings suggested that the Djiboutian population had been immunized against infection by *M. canettii*. No difference was observed in the frequency of the nonpulmonary form of TB caused by *M. tuberculosis* or *M. canettii*.

M. canettii is the progenitor species from which *M. tuberculosis* emerged (*3*–*5*). Genotyping of known *M. canettii* isolates showed that 70% of them belong to a large cluster called A (*1*,*3*). Strains belonging to cluster A were isolated as early as 1983. This observation and the absence of human-to-human transmission support the existence of an environmental reservoir. We report the isolation, since 2010, of 21 new strains of *M. canettii* in Djibouti, of which 7 were associated with 2 lymph node TB outbreaks in children. We show that 17 of the new strains, including the outbreak strains, belong to cluster A. We use draft whole-genome sequencing to demonstrate that this cluster is remarkable among *M. canettii* strains and confirm its epidemic status, which suggests an accelerating emergence of a clone, subsequently called clone A. Within clone A, we identify a single horizontal genetic transfer event, presumably resulting from recombination with closely related mycobacteria. We also investigate CRISPRs (clustered regularly interspaced short palindromic repeats) because these structures, which keep a memory of past infections by bacterial viruses, may provide indirect clues about an environmental reservoir. We take advantage of the clone A sequence data, which is, within *M. canettii*, closest to *M. tuberculosis*, to better describe the emergence of *M. tuberculosis*.

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Materials and Methods

Isolation and Culture

Most samples (sputum, biopsy, or puncture from lymph node; gastric fluid; esophagus; pericardium) came from patients hospitalized from February 2010 to March 2013 in the French Military Hospital Bouffard in Djibouti, Republic of Djibouti (Table 1). One additional sample came from a patient who had been living in Djibouti for 2 years and was hospitalized in the University Hospital in Lyon, France, in August 2011. The samples were collected during the usual care of these patients, and the study was approved by the hospitals' ethics committees.

Of the 22 samples (including 2 samples from the same patient), 10 were processed on site, 1 in Lyon, and the last 11 at the Percy Military Hospital (Clamart, France). After samples were decontaminated by sodium hydroxide (NaOH) in N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH method) (*6*), cultures were done on solid medium (Lowenstein-Jensen) and also in liquid medium for samples sent to France. Susceptibility of the isolates to drugs was measured in liquid medium (BACTEC 960, Becton Dickinson, Le Pont de Claix, France). Identification of the species was made by rapid chromatographic lateral flow assays (SD Bioline TB Ag MPT64 Rapid, Standard Diagnostics, Gyeonggi-Do, South Korea), the DNA strip assay GenoType MTBC (Hain Lifescience, Nehren, Germany), and biochemical analyzes. *M. canettii* strains Percy22, Percy50, and Percy975 were previously described (*1*). Strains were genotyped by using 24 tandem repeat loci (*1*).

Draft Whole-Genome Sequencing and in silico Analysis

The genome of selected strains was sequenced on the HiSeq2000 or MiSeq Illumina platform (BaseClear, Leiden, the Netherlands, or Imagif, Gif-sur-Yvette, France). Raw sequence data files were deposited in the European Nucleotide Archive (ENA project accession no. ERP002514), maintained by the European Bioinformatics Institute.

Single-nucleotide polymorphisms (SNPs) were determined by alignment with reference strains (*M. tuberculosis* H37Rv accession no. NC_000962.3 or *M. canettii* cluster A Percy3 [STB-D CIPT140060008 accession no. NC_019950.1]) as described (*7*; online Technical Appendix 1, wwwnc.cdc.gov/EID/article/20/1/13-0652-Techapp1.pdf). The determination of statistically significant clustering of polymorphic positions was done essentially as described by Croucher et al. (*8*).

A de novo assembly was performed to produce draft genomes. The resulting contigs and additional published *M. canettii* sequence data were compared with *M. tuberculosis* genomes to identify regions that would be shared by all sequenced *M. canettii* strains but absent from *M. tuberculosis* strains.

Table 1. Characteristics of patients from whom *Mycobacterium canettii* isolates were obtained, Djibouti, 2010–2013*

Strain no.	Patient nationality (length of stay, mo)	Hospital	Sex	Isolation date	Age, y†	Sample	TB site	HIV status (CD4/mm^3)	Cluster
Percy975	Djiboutian	Bouffard	M	2010 Feb	28	GF	Pulmonary	Pos (122)	A‡
Percy976	Djiboutian	Bouffard	M	2010 Feb	18	GF	Pulmonary	Neg	A
Percy977	Diboutian	Bouffard	F	2010 Feb	22	GF	Pulmonary	Neg	A
Percy979	Djiboutian	Bouffard	F	2010 Feb	39	GF	Pulmonary	Neg	A
Percy1004	Djiboutian	Bouffard	M	2010 Jun	14	LN puncture	LN	Neg	Singleton
Percy1049	Ethiopian (18)	Bouffard	F	2011 Jan	36	GF	Pulmonary	Pos (9)	A
Percy1060§	Djiboutian	Bouffard	M	2011 Feb	33	Sputum	Diffuse	Pos (235)	A
Percy1062	French (8)	Bouffard	M	2011 Mar	40	GF	Pulmonary	Neg	C
Percy1064	Djiboutian	Bouffard	M	2011 Mar	55	Sputum	Pulmonary	Neg	C
Percy1077	French (12)	Bégin	M	2011 Jul	48	Esophagus biopsy	Esophagus	Pos (UNK)	A
Percy1078	French (13)	Bouffard	F	2011 Sep	3	LN puncture	LN	Neg	A‡
Percy1079	French (13)	Bouffard	M	2011 Sep	1	LN biopsy	LN	Neg	A
Percy1084	French(3)	Bouffard	M	2011 Oct	4	LN puncture	LN	Neg	A‡
Percy1085	French (24)	Lyon	F	2011 Aug	8	LN biopsy	LN	UNK	A
Percy1086	Djiboutian	Bouffard	M	2012 Jan	51	Pericardium biopsy	Diffuse	Pos (52)	A
Percy1101	Djiboutian	Bouffard	F	2011 May	26	GF	Pulmonary	Neg	C‡
Percy1105	French (15)	Bouffard	M	2012 Oct	44	GF	Pulmonary	Neg	A‡
Percy1115	French (4)	Bouffard	M	2012 Dec	3	LN biopsy	LN	Neg	A‡
Percy1116	French (5)	Bouffard	M	2012 Dec	12	LN puncture	LN	Neg	A‡
Percy1129	French (42)	Bouffard	F	2013 Jan	11	LN puncture	LN	Neg	A‡
Percy1130	Djiboutian	Bouffard	M	2013 Mar	35	GF	Pulmonary	Pos (122)	A‡

*TB, tuberculosis; GF, gastric fluid; LN, lymph node; Diffuse, pulmonary and extrapulmonary; Pos, positive; Neg, negative; UNK, unknown.
†Age at isolation date.
‡Ten of the 17 strains selected for draft genome sequencing.
§A second isolate, Percy1050, recovered from a lymph node biopsy specimen, showed the same multiple-locus variable number tandem repeat analysis genotype.

Four different types of CRISPR loci were previously identified in *M. canettii* (*5*) and called III-A, I-C, I-Cvar, and I-E (Table 2; online Technical Appendix 2 Table 1, wwwnc.cdc.gov/EID/article/20/1/13-0652-Techapp2.xlsx). The *M. tuberculosis* CRISPR locus belongs to type III-A. To search for additional CRISPR loci potentially present in the new strains, CRISPRfinder analysis was applied to the draft genome assemblies (*10*). The CRISPRtionary tool was used to compare CRISPR sequence data (*11*).

Results

Epidemiologic Investigation

During February 2010–March 2013, a total of 240 cases of TB were diagnosed in Bouffard Military Hospital (220 Djiboutian and 20 non-Djiboutian patients, including 13 children [patients <15 years]). *M. canettii* was isolated from 21 patients, representing 8.7% of all cases: *M. canettii* was responsible for 4.4% of TB cases in Djiboutians (10 patients) and 55% of TB cases in non-Djiboutians (11 patients). Ten patients had pulmonary TB, 9 had extrapulmonary TB, and 2 showed disseminated infections. The patients were predominantly male (14 male, 7 female) and young (mean age 25.2 years; range 1–55 years). The clinical, biological, and radiologic data did not differ from data from the other patients who had TB diagnosed in this hospital (Table 1). All children had lymph node TB, and conversely, all lymph node TB cases were observed in children.

Nine of the 10 Djiboutian patients with *M. canettii* infection were adults, including 7 case-patients with pulmonary TB (2 persons were HIV positive) and 2 case-patients with disseminated TB (both HIV positive). The last Djiboutian patient was 14 years old and had lymph node TB. The mean age was 31.3 years (range 14–55 years).

The 11 other patients were expatriates (10 from France, 1 from Ethiopia; 2 were HIV positive) with an average duration of stay in Djibouti of 13.8 months (range 3–42 months) (Table 1). Four were adults (3 with pulmonary TB, 1 with extrapulmonary TB [esophagus]; 2 were positive for HIV; age range 36–48 years), and 7 were children (age range 1–12 years). Four cases occurred from August to October 2011 and the last 3 occurred during December 2012–January 2013 (with another 2 suspected cases from which no bacteria could be isolated). All patients had received bacillus Calmette–Guérin vaccine. Inquiries were made concerning each case-patient (within the family, home workers, or at school/work), but no contagious or infected person could be identified. From the beginning of February 2010 through the end of March 2013, a total of 1,661 French children came to Djibouti, according to the French consulate. This provides an estimated probability of declaring a *M. canettii* infection of ≈0.5%.

All isolates were tested and found to be sensitive to rifampin, isoniazid, pyrazinamide, and ethambutol. The expatriate patients were treated at the Bouffard Military Hospital, Bégin Military Hospital, or Lyon Hospital. The

Table 2. Sequence analysis of CRISPR alleles of the *Mycobacterium canettii* isolates, Djibouti, 2010–2013*

Cluster	Strain	No. spacers	Allele code†	Alias‡ (accession no.)
A	Percy3 and all other clone A strains	26	III-A-69@94	STB-D CIPT140060008 (NC_019950)
C	Percy1004	12	III-A-69@74-95-96-89@91-94	
C	Percy32	30	III-A-69@74-95@98-75@91-94	
C	CIPT140010059	29	III-A-69@74-95@98-75@91-94	STB-A CIPT140010059 (NC_015848)
–	Percy79	31	III-A-99@129	
–	Percy301	31	III-A-99@129	
B	Percy214	8	I-C-130@137	STB-H CIPT140070013
B	Percy525	8	I-C-130@137	
C	Percy1101	14	I-C-130@143	
–	Percy25	7	I-C-131-132-144@148	STB-E CIPT140070002
–	Percy65	27 (1 doublet)	I-C-201@205-203-206@226	STB-J CIPT140070017 (NC_019952)
–	Percy327	9	I-C-130-133@137-198@200	STB-L CIPT140070008 (NC_019965)
–	Percy302	50; 53	I-C-149@191-178var-192@197;I-Cvar-337@389	STB-K CIPT140070010 (NC_019951)
–	Percy89	83	I-E-228-230@232-88var-233@240-245@258-278@333	STB-G CIPT140070005
–	Percy99b	58	I-E-227@232-88var-233 @280-334@336	STB-I CIPT140070007
–	Percy157	52	I-E-227@232-88var-233@277	

*CRISPR, clustered regularly interspaced short palindromic repeats.
†The CRISPR type is indicated as prefix (type III-A, I-C, I-Cvar, I-E). Allele codes refer to the spacers dictionary (online Technical Appendix 2 Table 1, wwwnc.cdc.gov/EID/article/20/1/13-0652-Techapp2.xlsx). "69@94" indicates that all spacers from 69 to 94 are present. Spacers 1 to 68 have been previously reported in *M. tuberculosis* (*9*). Spacer numbering in "*M. canettii*" runs from 69 up to 389, i.e., the total number of spacers observed in *M. canettii* is 321.
‡Strains sequenced by (*5*) as draft or completed (European Nucleotide Archive accession no. indicated) genome 88var = TCCAGAGGTCGAAGTGATGTTCGGTGTTCTCCT.

4 adults were successfully treated by rifampin/isoniazid/pyrazinamide/ethambutol for 2 months and then received rifampin/isoniazid for 4 months. The 7 children received the same classic treatment procedure without ethambutol, with 2 exceptions in which ethambutol was added in the second month of treatment because of the enlargement of the first lymph node and appearance of a second lymph node. One of these 2 patients, a 3-year-old child, had surgery 10 months later. For the other patient, the treatment was successful after 6 months.

Genotyping New *M. canettii* Strains and Selecting Strains for Draft Sequencing

The genotypes of the 22 new isolates were compared to published data, which showed that 18 belong to the previously described cluster A (including the 2 isolates derived from the same patient; data not shown) (*1*). Percy1062, Percy1064, and Percy1101, together with Percy32 and 2 historical *M. canettii* strains (CIPT140010060, CIPT140010059), belong to the much smaller and more diverse cluster C (*1*). Percy1004 is more distant.

A total of 17 *M. canettii* strains were selected for draft whole-genome sequencing including 10 cluster A strains (8 strains recovered since 2010 [Table 1] and strains Percy50 and Percy22, collected in 1983 and 2003, respectively) and 7 genetically diverse strains (Percy32, Percy79, Percy157, Percy301, Percy525, Percy1004, Percy1101). Percy302, which was previously fully sequenced under the name STB-K and was shown to be the most remote *M. canettii* strain (*5*), was included for draft re-sequencing as a control. The sequences of these strains were analyzed, together with those of 10 strains previously described (*5*,*11*), representing a total of 27 *M. canettii* strains.

Whole-Genome SNP Analysis

During analysis of all sequenced *M. canettii* and *M. tuberculosis* genomes, 75,412 SNPs were determined, compared with the 13,358 identified within the *M. tuberculosis* complex alone (*7*) (online Technical Appendix 1). The 2 independent sequence datasets for Percy302 (STB-K) clustered closely together (7 differences) as expected. The mean divergence between *M. canettii* isolates was an average of 10 times that inside *M. tuberculosis*, in agreement with previous reports (*4*,*5*). The clustering achieved by single nucleotide polymorphism analysis was in good agreement with the genotyping data. For instance, cluster B and cluster C strains were similarly grouped by both approaches. The clustering of A strains was most remarkable. K116, which was independently investigated (*12*) and for which no genotyping data were available, also belongs to cluster A. This homogeneity is remarkable because cluster A included strains isolated during 1983–2013 (online Technical Appendix 1).

SNP Analysis within Cluster A

A total of 55 SNPs were identified among the 12 cluster A strains by alignment on the fully sequenced genome of cluster A strain Percy3 (STB-D; NC_019950; online Technical Appendix 2 Table 2). A minimum-spanning tree was drawn (Figure 1). There was no homoplasy in this tree, indicating that these single nucleotide polymorphisms did not appear twice independently within this group of strains. The distribution of the polymorphisms along the reference genome was analyzed to detect abnormal densities, potentially resulting from horizontal gene transfer by homologous recombination. Notably, a single instance could be identified. Eighteen polymorphisms fell within a single cluster covering 1,660 bp observed in Percy1129, compared with the other cluster A genomes. This 1% sequence divergent segment covers 2 full genes (online Technical Appendix 2, Table 2). The ratio of nonsynonymous to synonymous SNPs is strikingly different in the 2 groups, consistent with previous observations (*13*–*15*). The ratio is low among the group of clustered SNPs, and remarkably high among the group of unclustered polymorphisms (online Technical Appendix 2 Table 2). Figure 1 shows (blue) the initial position of Percy1129 and its position after removal of this unique genetic transfer event (red). There was no obvious correlation between branch length and strain isolation date. In contrast to the B and C clusters, the A cluster strains clearly belong to an epidemic clone and will subsequently be called clone A.

Rooting the *M. tuberculosis* Phylogenetic Tree

Among *M. canettii* strains, clone A was previously shown to be the closest to *M. tuberculosis* in terms of shared ancestry (*5*). Consequently, clone A sequence data constitute the current best resource to root *M. tuberculosis* (*7*). We merged the list of SNPs reported within *M. tuberculosis* (*7*) with additional polymorphisms deduced from the alignment of the clone A strains on H37Rv to produce a minimum-spanning tree showing precisely the *M. canettii* branching point (red star in Figure 2). The branch containing 4 polymorphisms in Figure 2 demonstrates that the *M. tuberculosis* superlineage containing *M. africanum* and *M. bovis* was the first extant lineage to emerge from the cradle of *M. tuberculosis* in the Horn of Africa (*7*). The blue star indicates the position of the node leading to Percy302 (STB-K), the most genetically diverse *M. canettii* strain. This branching point is significantly closer to clone A than to the red star, indicating a faster mutation rate along the branch leading to the red star, potentially more similar to that observed within *M. tuberculosis*. This might provide indirect evidence for a substantial ecologic change well before this branching point, i.e., a speciation event of *M. tuberculosis* preceding the most recent common ancestor defined by extant lineages.

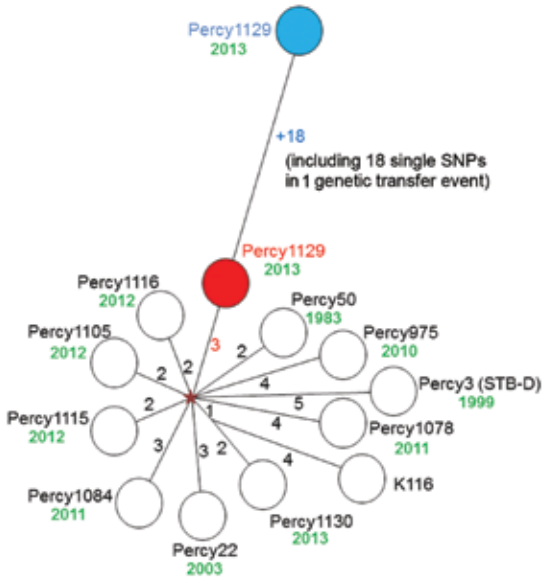

Figure 1. Starburst genealogy within clone A of *Mycobacterium canettii* isolates, Djibouti, 2010–2013. The size of each branch, corresponding to the number of polymorphisms between 2 nodes, is indicated. The tree is based upon 55 polymorphisms, 18 of which are clustered in 1,660 base pairs. The relative position of Percy1129 is shown with (blue) or without (red) these 18 polymorphisms. The isolation year is indicated near each strain. The position of a hypothetical ancestor is indicated by the red star. All cluster A strains are 2 up to a maximum of 5 polymorphisms away from this hypothetical ancestor after removal of the exceptional polymorphism cluster found in strain Percy1129. SNP, single-nucleotide polymorphism.

In silico Study of CRISPR Loci

A single CRISPR type was found in each genome as previously observed, except for Percy302 (STB-K), which contains 2 CRISPR structures, I-C and I-Cvar, comprising 50 and 53 spacers, respectively (*5*). The largest CRISPR allele was found in Percy89 (STB-G), with 83 spacers in its type I-E CRISPR. All clone A strains, including K116, possess an identical type III-A locus composition. Strain Percy1101 belongs to cluster C (online Technical Appendix 1), but its CRISPR structure, type I-C, was different from that of strains of this group (associated with III-A). A total of 321 spacers were detected in the present "*M. canettii*" collection (Table 2; online Technical Appendix 2 Table 1). Locus III-A contributed 61 spacers, locus I-C 97 spacers, locus I-C var 53 spacers, and locus I-E 110 spacers. Three independent events of spacer acquisition from the same source were identified, resulting in only slightly different spacer composition in different CRISPR alleles (Table 2; online Technical Appendix 2 Table 1). Nine spacers matched a prophage in *M. marinum* strain M (within positions 4,821,000 and 4,847,000 of accession no. CP000854.1). Two others matched *Mycobacterium* phages Thibault or Redi. One spacer in the Percy25 (STB-E) type I-C CRISPR allele matched perfectly 36 bp in gene *aftB* (locus tag Rv3805c in H37Rv) (online Technical Appendix 2 Table 1).

Absence of Part of Vitamin B12 Synthesis Pathway in *M. tuberculosis*

One particular region of interest was shown to be specific of the *M. canettii* taxon compared with that of *M. tuberculosis*. This region, which encompassed 3 kb on the Percy3 (STB-D) genome, from position 1,048,604 to position 1,050,991, contains the *cobF* (precorrin) gene and is present in all *M. canettii* strains, although it is absent from all *M. tuberculosis* genomes. This gene is part of a vitamin B12 synthesis pathway, suggesting that this pathway is nonfunctional in *M. tuberculosis*.

Discussion

The prevalence of *M. canettii* in TB patients in the Republic of Djibouti is unique, with >8% of cases reported to Bouffard Hospital during 2010 through early 2013 caused by *M. canettii*. In our experience, *M. canettii* is more frequently the cause of TB among expatriates (particularly children) and severely immunodepressed HIV-positive patients. However, the proportion of *M. canettii* infections is probably biased because the patients consulting at Bouffard Hospital are very likely not representative of the general population. For instance, all French TB patients were treated in Bouffard, and about half were infected by *M. canettii*. Not including the expatriates, the prevalence of *M. canettii* infection is 4%, which is still remarkably high. This raises the possibility that the prevalence of *M. canettii* is underestimated in the population of TB patients in Djibouti, or that additional bias exists in terms of socioeconomic background in the population of TB patients seeking treatment at Bouffard Hospital (*16*). Notably, all infected children had lymph node TB, and all cases of lymph node TB were observed in children. This calls for better surveillance of enlarged lymph nodes in children. *M. canettii* reservoirs likely are not strictly restricted to Djibouti but can be found in neighboring countries and in other large multicultural cities.

Clone A strains constitute an emerging pathogenic clone that appears to be much more successful at infecting humans than are other *M. canettii* representatives, because an almost identical strain has been predominantly isolated over the last 3 decades and represents 70%–80% of all *M. canettii* strains. The 2 outbreaks of lymph node TB reported in 2011 and 2012–2013, mainly in young children, raise again the question of the reservoir for this pathogen and this particular clone, and the reason for its increased virulence. In a mouse model, a clone A strain persisted longer in the lungs than any of the other *M. canettii* strains tested

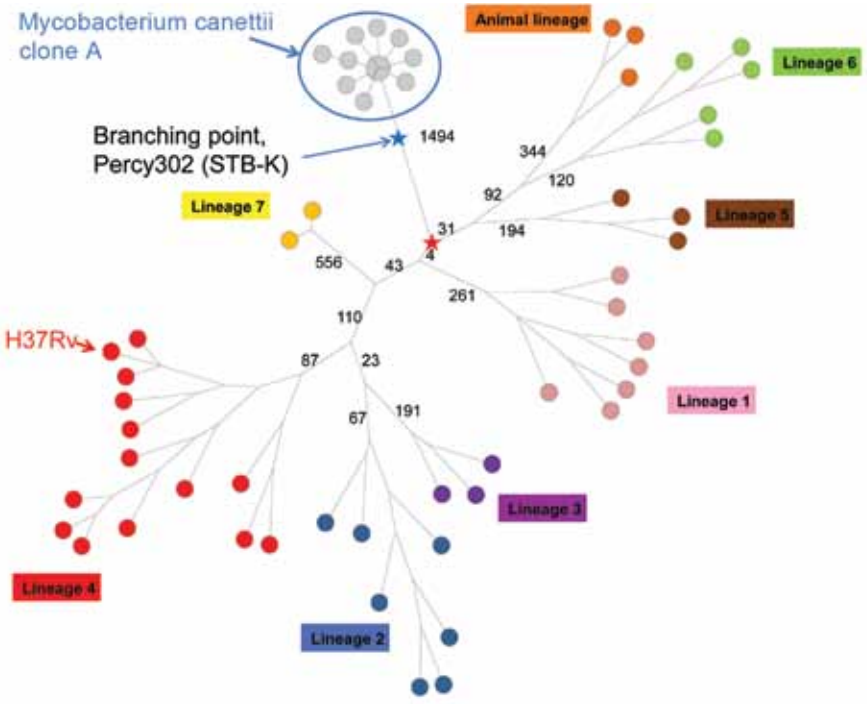

Figure 2. Early evolution of *Mycobacterium tuberculosis* was deciphered using clone A sequence data. A minimum spanning tree was drawn after removal of polymorphisms occurring in clusters, indicative of horizontal gene transfer events. The approximate position of the branching point of Percy302 (STB-K) the most distantly related *M. canettii* strain (*5*) is indicated by the blue star. The red star is the position of the most recent common ancestor of *M. tuberculosis*. The branch lengths of only the most internal branches are indicated. Branch length values inside clone A are <3. The position of the reference strain H37Rv is indicated. Four hundred seventy-one polymorphisms separate the red star from H37Rv. A logarithmic branch length representation was used.

(*5*). This result could explain the difficulties in treating 2 of the young patients. However, for these 2 patients, the extension of lesions might also be the result of a paradoxical upgrading reaction or resistance of the strain to antimicrobial drugs used. Indeed, it was previously reported that *M. canettii* was more resistant in vitro to pyrazinamide and pyrazinoic acid than was *M. tuberculosis* (*2*,*17*,*18*). These points will deserve further investigations. The frequency at which clone A strains infect humans may also reflect a higher success in colonizing a reservoir with which persons in Djibouti are in closer contact. Some of the expatriate patients had been living in Djibouti for short periods of time (4 months for the youngest 3-year-old patient). Although attempts to isolate *M. canettii* from the environment and animals in contact with the infected children have not been successful thus far, efforts in this direction should clearly be reinforced.

Fifty-five SNPs were identified by comparison among the sequenced clone A strains, 18 of which could be linked to a single horizontal gene transfer event with an unknown closely related mycobacterium. It was recently shown in *M. smegmatis* that distributive conjugal transfer could induce multiple genetic transfer events in a single step, and the authors of that study proposed that this mechanism created the genome mosaicism observed among *M. canettii* (*19*). Our observation of a unique transfer event does not support this hypothesis or would suggest that, in *M. canettii*, conjugal transfer is not associated with multiple events.

When only new mutational events are taken into account, the proportion of nonsynonymous mutations and, most notably, the branch lengths within clone A are typical of an *M. tuberculosis* outbreak (*7*,*20*,*21*). The expansion of clone A is thus likely to be very recent. The horizontal gene transfer events can only be explained by the existence of *M. canettii* in a reservoir or inside hosts such as the amoeba (*22*) in which *M. canettii* strains can exchange DNA with other *M. canettii* strains or with closely related mycobacteria that are not infectious for humans.

Links between *M. canettii* Clone A and the *M. tuberculosis* Complex

The finding that only 4 SNPs separate the radiation of *M. tuberculosis* lineages 5–6 and that of lineage 1 suggests that their diversification could correspond to a unique outbreak event, because this distance is consistent with observations of the accumulations of such polymorphisms during an outbreak (*7*,*20*). Along this line, it is tempting to speculate that clone A is reproducing the early steps which led to the speciation of *M. tuberculosis*. This may be favored by a situation in which a relatively naive population, in terms of exposure to *M. tuberculosis* (children and expatriates), is being exposed to the environmental reservoir. Eventually a strain with the appropriate mutation might spread from human to human.

We have been able to identify in clone A 1 horizontal gene transfer event with non–clone A strains or more likely a non–*M. canettii* mycobacterium, presumably occurring in the environment. If the ability of *M. tuberculosis* to spread had been acquired in the environment rather than in the human host, then there would be a possibility

that different *M. tuberculosis* lineages emerged independently from its reservoir. These different lineages might be distinguished by traces of ancestral horizontal gene transfer events, visible in the very internal branches of *M. tuberculosis* evolution, as observed here within clone A. We could not identify any such fossils of early horizontal gene transfer events, which is compatible with a model in which the most recent ancestor of *M. tuberculosis* never lived in the environment. One possibility is that it acquired a key feature leading to speciation during the colonization of its human host, after infection from the environment. Another possibility is that the most recent ancestor of *M. tuberculosis* does not coincide with the speciation of *M. tuberculosis* the obligatory human pathogen (*23*) as suggested here by comparing evolutionary rates toward *M. canettii* clone A and toward *M. tuberculosis*. Clone A may mimic an earlier phase before *M. tuberculosis* speciation. Speciation, associated with the ability to spread from human to human and not only the capacity to cause TB, which is clearly much more ancient, would have resulted from the multiple events of human TB infections caused by *M. canettii*, interspersed with genetic reshuffling of *M. canettii* in the environment. We hope that the list of polymorphisms identified in this investigation will facilitate the analysis of ancient *M. tuberculosis* and allow better positioning of *M. tuberculosis* speciation with respect to its current most recent common ancestor.

CRISPR Diversity

Within the investigated *M. canettii* strains, >300 spacers can be identified. Only a few show significant similarity with sequences in the GenBank nonredundant nucleotides section. One spacer found in a single *M. canettii* strain matches a chromosomal gene, as often seen in *Yersinia pestis* CRISPRs (*24*), suggesting that this chromosomal locus may be the subject of CRISPR interference in this particular strain. Notably, the other matches are with *Mycobacterium* phages, including a *M. marinum* prophage, which may indicate an aquatic reservoir for *M. canettii*.

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Dynamic Modeling of Cost-effectiveness of Rotavirus Vaccination, Kazakhstan

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The government of Kazakhstan, a middle-income country in Central Asia, is considering the introduction of rotavirus vaccination into its national immunization program. We performed a cost-effectiveness analysis of rotavirus vaccination spanning 20 years by using a synthesis of dynamic transmission models accounting for herd protection. We found that a vaccination program with 90% coverage would prevent ≈880 rotavirus deaths and save an average of 54,784 life-years for children <5 years of age. Indirect protection accounted for 40% and 60% reduction in severe and mild rotavirus gastroenteritis, respectively. Cost per life year gained was US $18,044 from a societal perspective and US $23,892 from a health care perspective. Comparing the 2 key parameters of cost-effectiveness, mortality rates and vaccine cost at <US $2.78 per dose, vaccination program costs would be entirely offset. To further evaluate efficacy of a vaccine program, benefits of indirect protection conferred by vaccination warrant further study.

Rotavirus is the leading cause of severe acute gastroenteritis in children worldwide (*1*). Rotavirus vaccines Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium) and Rotateq (Merck & Co., Whitehouse Station, NJ, USA) are in use in the national immunization programs in Australia, the United States, Latin America, and a few European countries. In these high- and middle-income countries, rotavirus effects have decreased markedly after introduction of the vaccine (*2–4*). Universal rotavirus vaccination has not been widely implemented in Asia, and the health effects of rotavirus differ considerably across the continent, with the highest mortality rates concentrated in developing areas. In Central Asia, there are also large variations in the reported rotavirus effects by country (*5*), emphasizing the need for local data to guide the decision on the introduction of the vaccine.

Kazakhstan is the most prosperous country in Central Asia. It has a population of 16 million (*6*) and a land mass equal to approximately half of the continental United States. Kazakhstan has large reservoirs of oil and natural gas and is classified as an upper-middle income economy; its gross national income was US $8,220 per capita in 2011 (*7*), making the country ineligible for international funds to introduce new vaccines. Vaccines included in the national childhood immunization program are fully funded by the government. The health effects of rotavirus in Kazakhstan were estimated at 68 deaths, 4,007 hospitalizations, and 32,500 outpatient visits during 2009 (*5*); another study estimated the total annual cost of rotavirus disease to be US $37.5 million (*8*). No current cost-effectiveness analyses of rotavirus vaccines were available for Kazakhstan.

Recently, 2 economic evaluations of the rotavirus vaccination were conducted in low-income countries in Central Asia (*9*,*10*), but because of differences in rotavirus epidemiology, health care costs, and economy, the results are not generalizable to Kazakhstan. These studies were performed on the basis of static models, which implicitly assume that the probability for disease exposure is constant in time. In contrast, immunization will not only reduce the probability of a vaccinated child to become ill but will also lower the exposure of the virus to others (i.e., herd protection).

Models that account for changes in transmission over time are referred to as dynamic models. Cost-effectiveness studies of rotavirus vaccination performed on the basis of dynamic transmission modeling were recently used in the United States (*11*), England, and Wales (*12*). To the best of our knowledge, this approach has not been applied in middle-income countries or in settings with a transitional economy. These countries face particular challenges because they are not eligible for international financing

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of vaccines, and their resources for new health interventions are limited. Rotavirus vaccine effectiveness has been shown to correlate with income level within a country (*13*). It is possible that rotavirus vaccines may perform worse in middle-income settings than in upper-income countries. Hence, scientifically sound estimates of the effect of rotavirus vaccination are in demand.

We present a cost-effectiveness study of rotavirus vaccination in a middle-income country using dynamic modeling. We incorporated direct effects such as death rates and indirect effects such as herd protection of a nationwide vaccination program. Our purpose for the study is twofold: to inform the impending decision on the introduction of rotavirus vaccination into the national immunization program in Kazakhstan, and to compare the cost-effectiveness of a rotavirus vaccination program in a middle-income country with that reported for high-income settings.

Materials and Methods

We adapted our previously published dynamic model for rotavirus (*14*,*15*) to Kazakhstan. The model is presented in the online Technical Appendix (wwwnc.cdc.gov/EID/article/20/1/13-0019-Techapp1.pdf).

Vaccination Parameters

We modeled the effect of introducing the 2-dose rotavirus vaccine Rotarix in the childhood immunization program in Kazakhstan. We implemented vaccination in the model assuming that the vaccine was effective from the first dose at 2 months, similar to other modeling studies (*15*). We chose a 2-dose rotavirus vaccine versus a 3-dose product because it may be more feasible in practice to achieve high coverage for a vaccine requiring fewer doses. Rotarix demonstrated 96% efficacy against rotavirus gastroenteritis (RVGE) hospitalizations in clinical trials and 90% field effectiveness against hospital admissions in high-income European countries (*26*). A lower field effectiveness range of 76%–79% was reported from the middle-income countries in Latin America (*26*–*28*). Because of lack of clinical trials of rotavirus vaccines in countries like Kazakhstan that are in transitional economies, it is difficult to predict the vaccine performance in these settings. On the basis of the aforementioned findings and our own assumptions, we applied a vaccine efficacy of 80% (range 72%–86%) against severe RVGE and 58% (range 51%–64%) against mild RVGE. The vaccine efficacies were varied by varying the proportions of RVGE infection, and severe RVGE infection in vaccinated children (online Technical Appendix). We did not adjust the vaccine efficacy for specific rotavirus genotypes because the strains circulating in Kazakhstan are globally common (*29*). Pre- and post-licensure data from developing settings indicate that vaccine protection may wane in the second year of life (*30*,*31*). We conservatively assumed vaccine protection to be 1 year, commencing after administration of the last dose at 4 months of age; that is, children were assumed to be fully protected on average until 16 months of age. However, studies from industrialized settings demonstrated high vaccine efficacy through 3 years of life (*32*,*33*). Therefore, we increased duration of vaccine protection to 2 years in a separate scenario analysis.

The vaccination program in our model was hypothetically initiated on January 1, 2012, with a linear buildup of vaccine coverage during the first 6 months. After this period, the vaccination coverage was assumed to be constant at a fixed level. In Kazakhstan, rotavirus vaccine would be administered concomitantly with the diphtheria–tetanus toxoid–pertussis (DTP) vaccine. The reported coverage for 3 doses of DTP in Kazakhstan is 99% (*7*), although a recent study suggests that only 76% of children 12–60 months of age receive all 3 doses of the DTP vaccine without delay (*34*). Considering age restrictions for the administration of rotavirus vaccines, coverage for the rotavirus vaccine may be lower than for other traditional vaccines administered under the World Health Assembly Expanded Programme on Immunization (*35*) because vaccination may not always be on time. We therefore applied 90% coverage in the base case, but varied coverage between 80% and 100% to explore the effect of this parameter on the cost-effectiveness of vaccination. Similarly to other studies, we did not consider an increased risk for intussusception or any other adverse events after rotavirus vaccination (*2*).

Disease Outcomes

We calculated the numbers of rotavirus-associated deaths, hospitalizations and outpatient visits in children <5 years from the modeled incidence of severe RVGE (I_s). We assumed that all children with severe RVGE require outpatient care or hospital care, and on the basis of local data, we modeled that 80% of children who were hospitalized with acute diarrhea sought medical care before admission (*8*). We calculated the numbers of rotavirus homecare episodes (without health care encounters) from the modeled incidence of mild RVGE (I_m), whereas the number of rotavirus-associated deaths and hospitalizations was calibrated to the 2009 estimates of 68 (95% CI 63–74) deaths and 4,007 (95% CI 3,740–4,274) hospitalizations based on a recent study from Kazakhstan (*8*) (online Technical Appendix).

Model Uncertainty and Scenario Analyses

We considered uncertainty related to natural history parameters (Table 1), model calibration, and vaccine efficacy and uptake. The role of adults in rotavirus transmission is a key uncertain factor (*15*). Because no sentinel data in this age group were available, we varied the infectiousness of later rotavirus infections relative to that of the primary infection between 1/5 and 1/10 (*14*,*15*). We also

Table 1. Natural history and vaccine-related parameters used in dynamic modeling of cost-effectiveness of rotavirus vaccination, Kazakhstan

Parameter	Base value [range]	Reference/source
Demographic		
Population during 1980	15,926 million	(*16*)
Birth cohort*	[217,580–367,750]	(*16*,*17*)
Mortality rate in <1 y*	20–54 per 1,000 births	(*16*,*17*)
Mortality rate in 1–4 y*	3.9–6.3 per 1,000 births	(*16*,*17*)
Net yearly migration rate*	−18.6–0.1 per 1,000	(*16*,*17*)
Deaths per year*	[128,570–180,000]	(*16*,*17*)
Natural history		
Duration of maternal protection	70 d	(*18*)
Duration of latency period	0.5 d	(*19*)
Infectious period (days)	8 (first); 6 (second); 4 (later)	(*20*–*22*)
Relative susceptibility	1 (first); 0.62 (second); 0.40 (later)	(*23*)
Relative infectiousness	1 (first); 0.5 (second); [0.1–0.2] (later)	Author assumption
Proportion of infections with RVGE	0.47(first); 0.25 (second); 0.24 (later)	(*23*)
Severe RVGE	0.13 (first); 0.04 (second); 0 (later)	(*23*)
Duration of complete immunity	[6–12 mo]	(*24*)
Vaccination		
Sero-conversion rate	0.96	(*25*)
Relative infectiousness†	0.5	Assumption
Relative susceptibility†	0.62	Author assumption
Prop. of infections with RVGE	0.30 [0.25–0.35]	(*25*–*27*)
Severe RVGE	0.1175 [0.0885–0.139]	(*25*–*27*)
Coverage	0.9 [0.8–1.0]	Author assumption
Duration of complete immunity	12–24 mo]	Author assumption
Fitted‡		
Infectivity parameter, $\beta 0$	1.889–2.605	Author calculation
Seasonal forcing, $\beta 1$	0.025–0.046	Author calculation
Phase angle, θ	0.011–0.251	Author calculation
Mixing (relative susceptibility)§ 0–7m, 8–23 m, 24–35 m	1.077–2.765	Author calculation

*Demographic parameters vary over the time period 1980-2031; only minimum and maximum values are listed in the table. All simulations are performed using the same set of demographic parameters.
†Vaccine efficacy calculated for children with no previous natural infection.
‡Details on the fitted parameters of the five candidate models; see corresponding model fits in online Technical Appendix Table wwwnc.cdc.gov/eid/article/20/1/13-0019-Techapp1.pdf; seasonal forcing: $\beta 0(1 + \beta 1)\sin(2\pi t / 365 + 0)$.
§Relative susceptibility in children <3 years (online Technical Appendix, section 1).

varied the mean duration of complete immunity after rotavirus infection from 6 to 12 months. All models were scored according to how well they fit with the sentinel data, adopting a likelihood-based approach by using the Akaike information criterion. In total, 5 candidate models (online Technical Appendix Table 1) had support and were simulated, both with and without vaccination. For each model, we calculated the yearly numbers of avoided health outcomes resulting from incidence difference with and without vaccination implemented (online Technical Appendix Table 2).

In the economic analysis, we took a weighted average of the incidence differences using Akaike weights (online Technical Appendix). We modeled several different scenarios to account for uncertainty in the calibration process, in vaccine efficacy and vaccine uptake (Table 2). In each scenario, we calculated a weighted model as described above. We analyzed a base case (most likely), best-case, and worst-case scenario to account for uncertainty instead of adopting a probabilistic approach because data on vaccine efficacy and rotavirus-associated health outcomes in Kazakhstan are lacking or sparse. In the base case, we used mean estimates for both vaccine efficacy and calibration values. The best-case scenario was based on the highest vaccine efficacy (86% against severe RVGE and 64% against mild RVGE), in combination with the upper bounds of estimated health outcomes (more events to prevent). The worst-case scenario incorporated the lowest vaccine efficacy (72% against severe RVGE and 51% against mild RVGE) and lower estimates of health outcomes (fewer events to prevent). Vaccine coverage was set to 90% in the base case. Scenarios A and B were constructed as described above, by using coverage of 80% and 100%, respectively (online Technical Appendix Tables 3, 4). Finally, in Scenario C we extended the vaccine protection period to 2 years in line with data from industrialized settings demonstrating high vaccine efficacy through 3 years of life (*32*,*33*). To estimate indirect or herd protection, we compared predictions of the dynamic model with those of a static cohort model, as was previously suggested (*8*) (online Technical Appendix).

Economic Parameters and Cost-effectiveness Analysis

Direct and indirect costs associated with rotavirus disease were recently estimated in a cost-of-illness study

Table 2. Description of scenarios for the economic evaluation of rotavirus vaccination, Kazakhstan

	Vaccine parameters				Children <5 y of age, calibration to 2009 sentinel data			
Scenario	Mean duration of protection, mo	Coverage	Efficacy against severe RVGE	Efficacy against mild RVGE	Deaths	Hospital admissions	Outpatient clinic visits*	Homecare episodes*
Base case	12	0.9	0.80	0.58	68	4,007	I_s-0.2I_h	I_m
Base case, low	12	0.9	0.74	0.51	63	3,740	0.6	0.5I_m
Base case, high	12	0.9	0.86	0.64	74	4,274	1.4	1.5I_m
Scenario A	12	0.89	0.80	0.58	68	4,007	I_s-0.2I_h	I_m
Scenario A, low	12	0.89	0.74	0.51	63	3,740	0.6	0.5I_m
Scenario A, high	12	0.89	0.86	0.64	74	4,274	1.4(	1.5I_m
Scenario B	12	1.0	0.80	0.58	68	4,007	I_s-0.2I_h	I_m
Scenario B, low	12	1.0	0.74	0.51	63	3,740	0.6	0.5I_m
Scenario B, high	12	1.0	0.86	0.64	74	4,274	1.4	1.5I_m
Scenario C	24	0.9	0.80	0.58	68	4,007	I_s-0.2I_h	I_m
Scenario C, low	24	0.9	0.74	0.51	63	3,740	0.6	0.5I_m
Scenario C, high	24	0.9	0.86	0.64	74	4,274	1.4	1.5I_m

*RVGE, rotavirus gastroenteritis; I_s, modeled incidence of severe RVGE; I_h, modeled incidence of hospital admissions; I_m, modeled incidence of mild RVGE.
†The numbers of outpatient clinic and homecare visits were not calibrated.

of RVGE in Kazakhstan (*8*). These costs included direct health care and non–health care costs and indirect costs associated with productivity losses due to the work absenteeism of caregivers and rotavirus-related deaths. For this analysis, cost estimates in 2009 US dollars were inflated to 2012 values by using the consumer price index (Table 3). In the absence of a market price for the rotavirus vaccine in Kazakhstan, we used the 2010 price of pneumococcal vaccine (US $43.00 per dose) purchased by the government. Because the vaccine price is a key determinant of cost-effectiveness, we performed various sensitivity analyses with the price ranging from US $1.00 (assuming program price for traditional Expanded Programme on Immunization vaccines) to US $60.00 per dose (considering a price of pneumococcal vaccine that was the most recent vaccine introduced in the program in Kazakhstan). The program costs included the costs of vaccine doses needed to vaccinate the yearly birth cohorts with 2 doses, a 10% vaccine wastage, and an additional US $267,300 to cover the costs of upgrading the cold chain for rotavirus vaccine in the first year of introduction. A 10% loss from vaccine waste was based on published estimates (*36,37*). The yearly costs of maintaining the cold chain and the costs of training health personnel were estimated in consultation with the Kazakh Ministry of Health. We assumed that rotavirus vaccination does not incur additional costs to parents because it will be administered concomitantly with other vaccines included in the national immunization program.

We assessed the aggregated long-term effect of rotavirus vaccination over a 20-year horizon. All costs and health outcomes were discounted at a rate of 3.0% per year. We conducted cost-effectiveness analyses from the health care system's perspective (including only direct medical costs) and the societal perspective (including indirect costs) using life-years gained as a measure of benefit. We estimated a break-even price for the rotavirus vaccine, in which the total health care costs of the vaccination program were equal to the expected cost savings for the health care system. All results are expressed as mean values with a range to represent realistic vaccination outcomes given the uncertainty in the epidemiologic model. Lacking actual data on uncertainty in the parameter values, we could not express uncertainty in terms of statistical distributions, so we chose to use 1-way sensitivity analyses.

Results

Base Case

Our model projects that the introduction in Kazakhstan of routine rotavirus immunization with 90% coverage and a mean duration of vaccine-induced protection of 1 year would reduce the incidence of severe and mild RVGE in

Table 3. Estimates of projected direct and indirect costs associated with rotavirus disease and rotavirus vaccination, Kazakhstan*

	Cost estimates in 2012 US dollars			
Item (per case)	Direct	Indirect	Total	Reference/source
Rotavirus death	543.33	67,254.13	67,799.46	(*8*)
Severe case (inpatient care)	364.36	181.47	545.83	(*8*)
Moderate case (outpatient care)	32.43	65.57	98.00	(*8*)
Mild case (homecare)	3.49	21.86	25.35	(*8*)
Cost of vaccine per dose, base case	43.00	0	43.00	Authors' assumption
Cold chain upgrade (total first year)	237,300	0	237,300	KMoH
Training costs (first year)	120,096	0	120,096	KMoH
Annual cost of cold chain and training	22,037.74	0	22,037.74	KMoH

*KMoH, Kazakh Ministry of Health.

children <5 years of age within the first year (Figure 1, panels A, B). After ≈4 years of administration of the vaccine program, the dynamics of rotavirus would stabilize, and infection would occur with yearly oscillations. Before the start of the vaccination, the peak incidence of RVGE would be among children <12 months of age. The highest incidence of severe and mild disease postvaccination is found during the second and third years of life, respectively (Figure 1, panels C, D). The age shift is predicted to occur within 3 years of vaccination. The yearly peak is predicted to be delayed by 14–20 weeks compared with the epidemic peak timing without vaccination (online Technical Appendix Figure 4).

During 20 years of vaccination, the predicted incidence of severe RVGE would be reduced by 74% (base case range 64%–80%) of prevaccine levels. The incidence of mild RVGE would be reduced by 56% (range 45%–64%) compared with incidence among unvaccinated children (Figure 1, panels E, F). Our model predicts substantial indirect or herd protection conferred by rotavirus vaccination. The indirect effects account for ≈40% (range 25–33% in relative terms) of the reduction in the projected incidence of severe RVGE, whereas 60% (range 0.28–0.38 in relative terms) of the incidence drop in mild RVGE would be caused by a reduced circulation of rotavirus. Our model projects that over 20 years, a vaccination program with 90% coverage would prevent 881 (range 776–1,004) deaths, 51,891 (range 46,094–57,971) hospital admissions, 370,268 (range 211,825–541,919) outpatient clinic visits, and 1.345 (range 0.641–2.112) million homecare episodes. These values correspond to ≈74% (range 70%–77%) averted deaths, hospitalizations, and outpatient clinic visits and 55% (range 53%–58%) averted homecare episodes compared with the values predicted without vaccination. In that time period, 54,784 (range 48,304–62,442) undiscounted life-years are saved (Table 4).

In the base case, the net undiscounted program costs would be US $530.7 million; the net costs when accounting for cost savings would be US $372.8 (range $325.0–$416.4) million (Table 4). These results would imply a cost of US $23,892 per life-year saved (i.e., incremental cost-effectiveness ratio of US $23,892) in a health care perspective (range $20,557–$27,573) and US $18,044 in a societal perspective ($13,854–$22,779); both estimates were discounted at 3%. Figure 2 shows the cost per life-year gained as a function of the vaccine price per dose in a 20-year perspective. At a cost of US $2.78 (range $2.01–$3.62), the additional cost of the vaccination program would be entirely offset by the cost savings to the health care system corresponding to the medical break-even price.

Scenario Analyses

Varying the vaccination coverage between 80% and 100% (scenarios A and B) did not substantially influence the cost-effectiveness ratio (Table 4). For example,

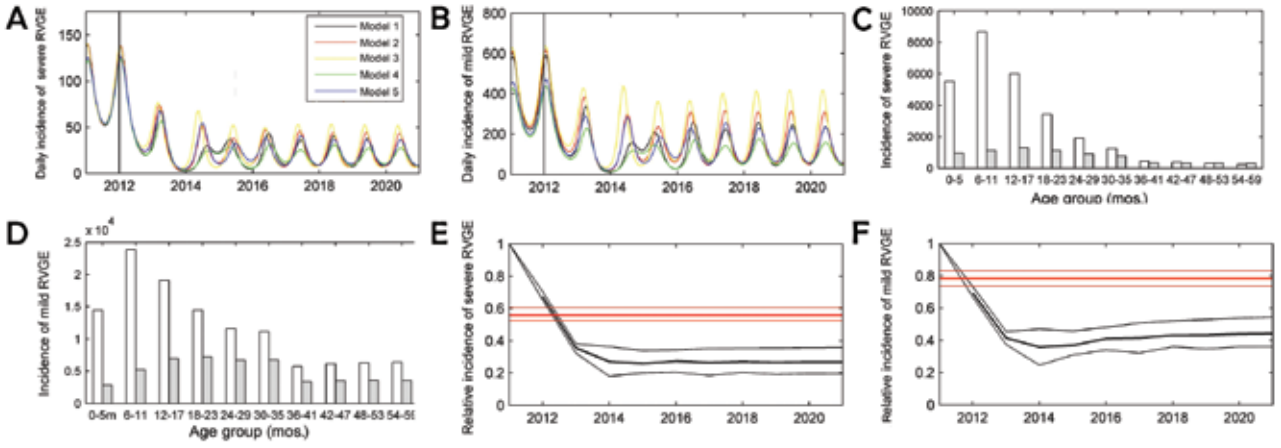

Figure 1. Projected epidemiologic effect of rotavirus vaccination in children <5 years of age in Kazakhstan. A) Estimated daily incidence of severe RVGE (base case scenario) with introduction of rotavirus vaccination in January 2012 in the 5 candidate models. B) Estimated daily incidence of mild RVGE (base case) with introduction of the rotavirus vaccination in January 2012 in the 5 candidate models. C) Yearly age-specific incidence of severe RVGE pre-vaccination (white) and 10 years postvaccination (gray). D) Yearly age-specific incidence of mild RVGE pre-vaccination (white) and 10 years postvaccination (gray). E) Relative incidence of severe RVGE with vaccination compared with the expected incidence without vaccination; the blue curve shows the mean relative incidence with lower and upper bounds predicted by the synthesis of dynamic models, including both direct and indirect effects, while the red curve shows the relative incidence predicted by a static cohort model incorporating only the direct effects (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/1/13-0019-Techapp1.pdf). F) Relative incidence of mild RVGE with vaccination compared with the expected incidence without vaccination; the blue curve shows the mean relative incidence with lower and upper bounds in the synthesis of dynamic models; the red curve shows the relative incidence predicted by a static cohort model.

Table 4. Estimated projected costs in million US dollars and avoided health outcomes of rotavirus vaccination program in Kazakhstan, 2012–2031

Outcome	No vaccination	Base case, 90% vaccine coverage, 1-y vaccination protection			Scenario A, 80% vaccine coverage, 1-y vaccination protection			Scenario B, 100% vaccine coverage, 1-y vaccination protection		
		Mean	Low	High	Mean	Low	High	Mean	Low	High
Avoided outcomes, undiscounted										
Fatal cases	1,310	880	776	1,004	777	681	890	985	876	1 114
In-hospital care	77,205	51,891	46,094	57,971	45,802	40,436	51,447	58,086	52,038	64,396
Out-patient visits	550,896	370,268	211,825	541,919	326,820	185,825	480,935	414,473	239,145	601,983
Home care episodes	2,675,456	1,344,747	640,836	2,112,400	1,163,780	552,160	1,835,487	1,544,096	740,039	2,412,843
Life years gained		54,784	48,304	62,442	48,356	42,375	55,416	61,325	54,534	69,363
Vaccination		530.7	530.7	530.7	471.9	471.9	471.9	589.6	589.6	589.6
In-hospital care	–25.7	–18.9	–16.8	–21.2	–16.7	–14.7	–18.7	–21.2	–18.9	–23.5
Out-hospital care	–16.3	–12.0	–6.9	–17.6	–10.6	–6.0	–15.6	–13.4	–7.8	–19.5
Homecare	–8.5	–4.7	–2.2	–7.4	–4.1	–1.9	–6.4	–5.4	–2.6	–8.4
Indirect costs	–179.4	–122.3	–88.4	–159.7	–107.4	–77.4	–140.9	–137.7	–100.3	–178.9
Total net costs	229.9	372.8	416.4	325.0	333.1	371.8	290.2	411.9	460.1	359.3
Incremental cost-effectiveness ratios, societal perspective										
Discounted 3%	NA	18,044	22,779	13,854	18,280	27,991	13,955	17,775	22,250	13,768
Incremental cost-effectiveness ratios, health care perspective										
Discounted 3%	NA	23,892	27,573	20,557	24,102	23,210	20,620	23,658	27,061	20,526
Threshold prices, 3% discounting										
Medical break-even price†	NA	$2.78	$2.01	$3.62	$2.78	$1.96	$3.60	$2.83	$2.05	$3.65

*Negative values indicate prevented or avoided costs. NA, not applicable.
†The price per vaccine dose at which the vaccinations costs are offset by cost saving generated from lower morbidity and mortality rates.

increasing the coverage to 100% generated only a moderate decrease in the cost per life-year gained (US $23,658 in a health care perspective and US $17,775 in a societal perspective); a marginal change in the cost-effectiveness ratio was also observed when vaccine coverage was decreased to 80%. We included 6 years without vaccination to explore any carryover effects after vaccination is discontinued. The model predicts that such effects are small because infection rates return to prevaccine levels quickly.

Lastly, in scenario C we simulated a 90% vaccine coverage assuming 2-year mean vaccine protection (Table 5; online Technical Appendix Table 5; online Technical Appendix Figure 3). These results suggest a cost of US $22,579 per life-year saved in a health care perspective and US $16,775 in a societal perspective; the medical break-even price was estimated at a cost of US $ 2.95 per dose. Compared with the base case (Table 4), assuming 2 years of vaccination protection reduced the cost per life-year saved by 5.5% in a health care perspective and 7.4% in a societal perspective.

Discussion

Our study evaluated the cost-effectiveness of rotavirus vaccination in a middle-income country by use of a dynamic model. The results indicate that universal rotavirus vaccination in Kazakhstan could prevent 800–1,000 deaths, 46,000–58,000 hospitalizations, 210,000–540,000 outpatient clinic visits, and 0.6–2.1 million homecare episodes during the next 2 decades. Our study suggests that the cost-effectiveness of rotavirus vaccination is determined by 2 key factors: the ability of the vaccines to prevent severe RVGE in children and the market price of the vaccine.

Vaccination also reduces productivity losses because of lower mortality rates and less work absenteeism among parents. However, the small difference between the cost-effectiveness ratios with and without indirect costs is explained by the dominant role of the vaccine costs. In the economic analysis, we calculated the break-even price, representing the price at which the costs of vaccination would be offset by the health care cost savings from avoided cases. With vaccine prices below the break-even price, the vaccination program would become one of

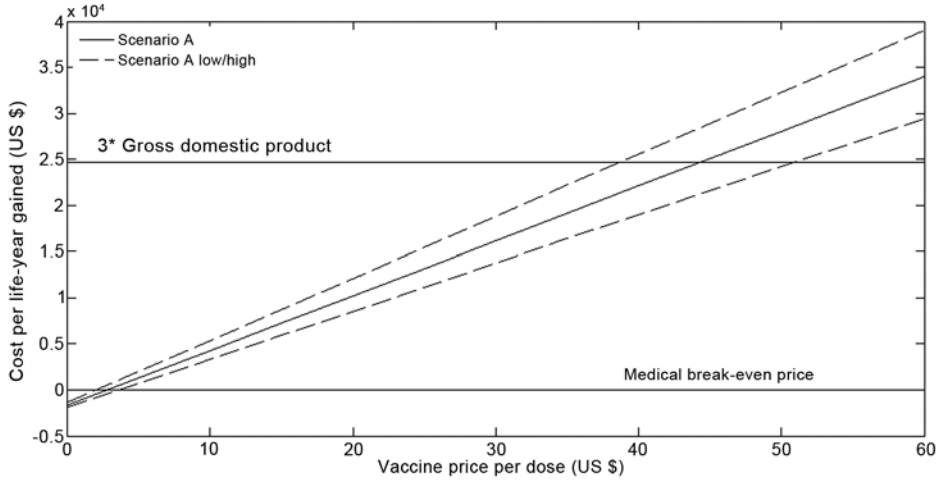

Figure 2. Projected cost (US $) per life-year gained over a 20-year time period (2012–2031) after introduction of rotavirus vaccination in Kazakhstan, according to purchasing price of 1 vaccine dose.

cost saving. We believe that estimating cost per life-year gained and the break-even price of vaccine is informative for decision makers negotiating the price with manufacturers in the absence of an established market price for the product. Whether the cost per life-year represents value for money and is considered cost-effective is a political question for Kazakhstan authorities to decide. WHO has suggested that governments should be willing to pay 3 times gross domestic product per capita per year for a good life-year. For Kazakhstan, this would amount to US $24,660.

A strength of this study is that the results are a synthesis of 5 models and use a likelihood-based approach, in which the models are weighted according to their ability to fit the sentinel data. This approach is common in weather and finance models but not in infectious disease modeling.

Our model demonstrates the role of indirect protection conferred by rotavirus vaccination. In our base case scenario, herd protection accounted for a 40% reduction in the incidence of severe RVGE and a 60% decrease in the incidence of mild RVGE. The contribution of indirect effects to the overall effect of vaccine is an observation also reported by other dynamic modeling studies and supported by empirical data from countries already using rotavirus vaccine in routine immunization programs (*2*,*3*). The incidence reduction in our model is larger than that found by Atkins et al. in a study from England and Wales, where 25% and 40% of the incidence reduction in severe and mild RVGE, respectively, were accounted for by herd protection, assuming a 1-year mean vaccine protection (*38*). This difference may be attributed to differences in assumptions on vaccine-related parameters, the magnitude of the disease burden, population dynamics and other model characteristics. This model has previously been fitted to data from England and Wales with a basic reproductive number of the primary infection of R_0 = 17.6 (*15*), which is smaller than the value estimated for Kazakhstan of R_0 = 19.2 – 2104, thereby suggesting higher transmission pressure in the latter setting.

Data from Finland suggest that vaccine protection may last for >1 year (*39*). We have also tested the model assuming 2 years of vaccine-derived protection. In this case, we found less indirect protection against severe RVGE, roughly representing 20% of the reduction (Table 5; online Technical Appendix Figure 3). The direct effect from vaccination increases because it is calculated from the expected infections in vaccinated children 2–28 months of age, had they not been vaccinated, versus children 2–16 months of age in the base case. We obtained a modest effect from extending the vaccine protection period by 1 year, which may be related to our use of a mean value for the vaccine duration instead of a fixed duration of vaccine protection, implying that some children will experience a shorter duration of protection.

We decided to provide a conservative estimate of the cost-effectiveness of rotavirus vaccination. First, we assumed that direct vaccine-derived protection lasts for 1 year because data on a longer duration of vaccine protection from industrialized countries may not be directly generalizable to Kazakhstan. Second, we assumed that the risk for severe RVGE is age-independent. Vaccination increases the average age of infection, and it is plausible that this risk for severe RVGE is lower for older versus younger children. Third, we applied a lower estimate of vaccine efficacy in our model because Kazakhstan is a developing nation. However, if rotavirus vaccines demonstrate a better efficacy in this country, it may substantially influence the cost-effectiveness of vaccination.

Several limitations in our study warrant care in interpretation of the results. First, our model was fitted to the 2-year sentinel hospital data on rotavirus surveillance on the basis of information from 2 hospitals; hence, changes in annual RVGE incidence and seasonality may have not

Table 5. Estimated projected costs in US dollars and avoided health outcomes from rotavirus vaccination with 2-year protection, Kazakhstan, 2012–2031*

Outcome	No vaccination	Scenario C, 90% vaccine coverage, 2-y vaccination protection		
		Mean	Low	High
		Avoided outcomes, undiscounted		
Deaths	1,310	919	823	1,034
Hospital admissions	77,205	54,163	48,823	59,701
Out-patient visits	550,896	386,479	224,410	558,096
Home care episodes	2,675,456	1,544,202	747,494	2,390,646
Life-years gained	NA	57,183	51,174	64,306
		Avoided costs, undiscounted, US $43 per vaccine dose		
Vaccination	NA	530.7	530.7	530.7
Prevented in-hospital care	25.7	19.7	17.8	21.8
Prevented outpatient care	16.3	12.5	7.3	18.1
Prevented homecare	8.5	5.4	2.6	8.4
Avoided indirect costs	179.4	130.7	95.2	169.2
Total net costs in US$	229.9	362.4	407.8	313.4
		Incremental cost-effectiveness ratios, societal perspective		
Discounted 3%	NA	16,775	21,031	12,952
		Incremental cost-effectiveness ratios, health care perspective		
Discounted 3%	NA	22:759	25:898	19:841
		Threshold prices, 3% discounting		
Medical break-even price†	NA	$2.95	$2.15	$3.79

*NA, not applicable.
†The price per vaccine dose at which the vaccinations costs are offset by cost saving generated from lower morbidity and mortality rates.

been fully captured. Likewise, local data on outpatient visits are sparse. We have attempted to compensate for this by using wide upper and lower confidence bounds on these estimates. Second, the parameters used to characterize natural rotavirus infections were based on those in a study conducted in Mexico and may not properly represent the epidemiology of rotavirus infections in Central Asia. Third, we used continuous aging in the model, which may have introduced a bias arising from persons aging at different rates. Even so, we used a small age band of 1 month, and we tested the model performance without finding severe bias (*14*). Fourth, the choice of simulation period may imply that carryover effects beyond 20 years were disregarded, but the scenario analysis indicates that such effects are small and will not influence the ICERs because of discounting. Fifth, we tested the uncertainty of the vaccine price in the sensitivity analysis, but because of lack of data, we were unable to test the uncertainty of other cost parameters in the model. Finally, because of lack of data, we disregarded improved quality of life in the economic analysis.

In conclusion, rotavirus vaccination in Kazakhstan will provide considerable direct health benefits in terms of reduced illness and deaths. Using the WHO criterion for cost-effectiveness, vaccination would be considered cost-effective under most of the assumptions of our analyses. With a low vaccine price, the avoided disease costs from vaccination will be greater than the vaccination costs. Further study is warranted to measure the benefits of herd immunity conferred by vaccination and to add that information to the current comparison of the costs of illness to those of a national vaccine program.

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Raw Milk Consumption among Patients with Non–Outbreak-related Enteric Infections, Minnesota, USA, 2001–2010

Trisha J. Robinson, Joni M. Scheftel, and Kirk E. Smith

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Learning Objectives

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

- Describe the problem of infections related to the use of raw milk products
- Distinguish the most common infection associated with raw milk consumption in the current study
- Evaluate clinical characteristics of individuals with infections related to raw milk consumption in the current study
- Compare sources of raw milk related to sporadic infection.

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Raw milk has frequently been identified as the source of foodborne illness outbreaks; however, the number of illnesses ascertained as part of documented outbreaks likely represents a small proportion of the actual number of illnesses associated with this food product. Analysis of routine surveillance data involving illnesses caused by enteric pathogens that were reportable in Minnesota during 2001–2010 revealed that 3.7% of patients with sporadic, domestically acquired enteric infections had reported raw milk consumption during their exposure period. Children were disproportionately affected, and 76% of those ≤5 years of age were served raw milk from their own or a relative's farm. Severe illness was noted, including hemolytic uremic syndrome among 21% of *Escherichia coli* O157–infected patients reporting raw milk consumption, and 1 death was reported. Raw milk consumers, potential consumers, and policy makers who might consider relaxing regulations regarding raw milk sales should be educated regarding illnesses associated with raw milk consumption.

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Raw milk is well-established as a vehicle for numerous infectious diseases (*1*–*8*) and has frequently been identified as the source of outbreaks of foodborne illness (*9*,*10*). From 1998 through 2011, a total of 148 outbreaks were documented in the United States associated with the consumption of raw milk products, resulting in 2,384 illnesses, 284 hospitalizations, and 2 deaths (*11*). A recent report concluded that the incidence of reported outbreaks associated with raw dairy products was ≈150 times greater, per unit of product consumed, than the incidence involving pasteurized dairy products (*12*).

Although pasteurization has been available for use in the United States for over a century, a small proportion of the population continues to consume raw milk. In a 2006–2007 population survey comprising 10 states in the Foodborne Diseases Active Surveillance Network, 3.0% of respondents reported consuming raw milk during the previous 7 days (*13*). Farm families have traditionally been the primary consumers of raw milk, but evidence in recent years suggests that the population of raw milk consumers may be changing (*14*). Raw milk advocates tout raw milk for its purported health benefits and better taste, and many persons view raw milk consumption as an opportunity to support local dairies (*15*,*16*). Some raw milk advocates fail to acknowledge the elevated health risk associated with raw milk consumption and minimize the significance of reported outbreaks. In doing so, these advocates convey a false sense of the safety of raw milk to those who are considering consuming this product, and this sense of safety discourages a balanced assessment of the potential risks and benefits involved.

Although outbreaks associated with raw milk occur frequently and receive much media attention, the number of reported cases determined to be outbreak-related likely represents a small proportion of the actual number of illnesses associated with this product. Two lines of evidence support this assumption. First, among reported illnesses caused by enteric pathogens that are laboratory-confirmed, non–outbreak-related (i.e., sporadic) cases far outnumber those associated with recognized outbreaks (*17*). Second, for each reported laboratory-confirmed illness caused by a bacterial or protozoal enteric pathogen, an estimated 26–100 additional illnesses likely occur, depending on the pathogen (*18*). Therefore, any estimates of the number of illnesses associated with raw milk consumption should include an evaluation of sporadic cases, including multipliers to account for underdiagnosis and underreporting. However, little information is available on the number of sporadic cases of illness associated with raw milk consumption.

Minnesota is among the 30 US states that permit raw milk to be sold in some capacity (*19*), allowing for raw milk to be occasionally secured or purchased for personal use at the farm or place where the milk is produced (*20*). To better estimate the true number of human enteric pathogen infections associated with consumption of raw milk, we characterized sporadic enteric illnesses that occurred among patients in Minnesota who reported raw milk consumption during 2001–2010. Our primary objective was to provide better data on the true number of sporadically occurring disease cases associated with raw milk consumption.

Methods

Patients were identified through routine disease surveillance conducted at the Minnesota Department of Health (MDH). Infections caused by *Campylobacter*, *Cryptosporidium*, Shiga toxin–producing *Escherichia coli* (STEC, including O157 and non-O157 serogroups), and *Salmonella* are reportable to MDH by state rule, and active, population-based surveillance is conducted (*21*). All Minnesota residents with a laboratory-confirmed *Campylobacter*, *Cryptosporidium*, STEC O157, non-O157 STEC, or *Salmonella* infection are routinely interviewed by MDH staff using a disease-specific standard questionnaire about symptoms and food, water, animals, and other possible sources of infection during their exposure period. Each questionnaire contains a question about raw milk consumption, including where the raw milk was obtained and when it was consumed. Exposure period is defined as 7 days before illness onset for *Campylobacter*, STEC O157, non-O157 STEC, and *Salmonella* infections and 14 days for *Cryptosporidium* infection.

A case-patient was defined as a Minnesota resident who had a domestically acquired, laboratory-confirmed *Campylobacter*, *Cryptosporidium*, STEC O157, non-O157 STEC, or *Salmonella* infection and a specimen collection date during 2001–2010. Persons were excluded if they refused an interview or were unable to be reached for interview, were part of an outbreak, or traveled internationally during the exposure period. Patients infected with *Campylobacter upsaliensis*, *Cryptosporidium hominis*, and *Salmonella enterica* serotype Typhi were also excluded because these specific species or serotypes have not been documented to be associated with raw milk consumption or other cattle exposures. Patients infected with an unknown species or serotype were eligible for inclusion. The total number of case-patients reporting raw milk consumption was calculated, and we examined the demographic features, severity of illness, and raw milk sources among the case-patients. Estimates of the total number of illnesses that could be attributed to raw milk consumption were calculated by using published pathogen-specific multipliers that account for underdiagnosis (*18*).

Descriptive and univariate analyses were performed by using SAS 9.2 software (SAS Institute, Cary, NC, USA). Case-patients who refused to answer a question or responded don't know or not sure were excluded from relevant analyses. Statistical significance was accepted at $p<0.05$.

Results

During 2001–2010, a total of 20,034 *Campylobacter*, *Cryptosporidium*, STEC O157, non-O157 STEC, and *Salmonella* infections were reported to MDH. Among these cases, 6,695 were excluded for the following reasons: the patient reported international travel (2,648 cases) or refused or was unable to be reached for interview (1,530 cases); the patient was linked to a recognized outbreak (1,244 cases); or the infection was caused by a species or serotype not historically associated with raw milk or other cattle exposures (273 cases).

Of the excluded outbreak cases, 21 occurred during 5 recognized outbreaks associated with raw milk consumption in Minnesota during 2001–2010. These 5 outbreaks resulted in 7 hospitalizations and 1 case of hemolytic uremic syndrome (HUS). One outbreak of *Campylobacter jejuni* infections in 2001 was associated with raw milk consumption at a farm where a ministry group was staying. Two outbreaks of *C. jejuni* infections occurred in 2008: one was associated with raw milk consumption at a family reunion and the other with raw milk purchased from a local dairy farm. In 2010, an outbreak of STEC O157 infections and an outbreak of *C. jejuni* and *Cryptosporidium parvum* infections were associated with consumption of raw milk from the same dairy farm; both outbreaks included several cases associated with milk the consumers had picked up at illegal drop-off sites.

After exclusions, a total of 14,339 cases remained for analysis, including 6,747 *Campylobacter* spp., 1,742 *Cryptosporidium* spp., 1,069 STEC O157, 354 non-O157 STEC, and 4,427 *Salmonella* spp. cases. Among the 14,339 patients, 530 (3.7%) reported consumption of fluid raw milk during their exposure period (Table 1). The median annual number of case-patients reporting raw milk consumption was 53.5 (range 37–64), but this number generally increased over time (Figure 1). Among the 273 persons who were excluded from study because of infection with a species or serotype not historically associated with raw milk or other cattle exposures, only 2 (0.7%) reported raw milk consumption; this was significantly lower than the 3.7% of cases included in the analysis ($p = 0.01$).

Persons with *Campylobacter* infection had the highest percentage of reported raw milk consumption (6.0%), and *Campylobacter* spp. accounted for 407 (77%) of the 530 cases with reported raw milk consumption (Table 1, Figure 1). Among case-patients infected with other pathogens, the percentage, by pathogen, reporting raw milk consumption included: non-O157 STEC, 3.4%; *Cryptosporidium* spp., 3.0%; STEC O157; 1.8%, and *Salmonella* spp., 0.9%. The following data were available regarding the speciation of pathogens from case-patients: 378 *Campylobacter* isolates (*C. jejuni*, 96.8%; *C. coli*, 2.6%; and *C. lari*, 0.5%); 23 *Cryptosporidium parvum* specimens; 36 *Salmonella* isolates (16 serotypes, most frequently *S. enterica* serotype Typhimurium, 10 isolates; *S. enterica* serotype Montevideo, 6 isolates; and *S. enterica* serotype Newport, 5 isolates).

Twelve patients were co-infected with *Campylobacter* spp. and 1 other enteric pathogen: 9 with *Cryptosporidium* spp., 1 with STEC O157, 1 with non-O157 STEC, and 1 with *Salmonella* spp. In addition, 1 patient who was reported to have consumed raw milk was infected with different pathogens at different times during the study period: STEC O157 (including HUS) at 1 year of age and *Salmonella* spp. 1 year later.

Male case-patients comprised 62.6% of study participants reporting raw milk consumption (Table 1). Case-patients reporting raw milk consumption were more likely than the average Minnesotan to be white (96.5% vs. 85.3%; $p<0.001$), and 96.8% were non-Hispanic. Overall, the median age of case-patients reporting raw milk consumption was 17 years (range 9 months to 92 years); 25% were ≤5 years of age, 38% were ≤10 years of age, and 59% were ≤20 years of age (Figure 2). Among patients with STEC O157 infections, those reporting raw milk consumption had a median age of 5 years (range 11 months to 63 years), compared with a median age of 16 years (range 5 months to 92 years) among those who did not report raw milk consumption ($p = 0.02$). Likewise, patients with non-O157 STEC infections who reported raw milk consumption had a median age of 4 (range 1–63) years. Patients with *Cryptosporidium*, *Salmonella*, or *Campylobacter* infection who reported raw milk consumption had median ages of 9, 16, and 18 years, respectively. Illnesses occurring among patients reporting raw milk consumption were disproportionately distributed throughout the year; 35% (186/530) of specimen collection dates occurred during the months of June, July, or August ($p<0.001$).

Table 1. Demographic characteristics of patients with domestically acquired, sporadic enteric infections, Minnesota, 2001–2010

	Patients reporting raw milk consumption			Patients denying raw milk consumption		
Pathogen	No. patients	Median age, y (range)	Male, %	No. patients	Median age, y (range)	Male, %
Campylobacter spp.	407	18 (<1–92)*	63.9*	6,340	33 (<1–96)	56.9
Cryptosporidium spp.	53	8 (1–74)*	52.8	1,689	21 (<1–101)	45.6
Escherichia coli O157	19	5 (<1–63)*	63.2	1,050	16 (<1–92)	46.3
Non-O157 Shiga toxin–producing *E. coli*	12	4 (1–61)	66.7	342	18 (<1–88)	46.3
Salmonella spp.	39	16 (1–78)*	59.0	4,388	28 (<1–98)	46.6
Total	530	17 (<1–92)*	62.6*	13,809	29 (<1–101)	51.3

*Significantly different ($p<0.05$) from persons who denied raw milk consumption.

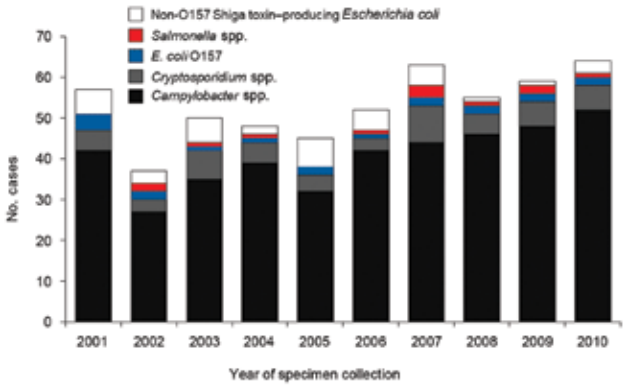

Figure 1. Number of cases of domestically acquired, sporadic enteric infections for which the patient reported raw milk consumption, by year and pathogen (n = 530), Minnesota, 2001–2010.

Seventy (13%) case-patients reporting raw milk consumption required hospitalization for a median of 3 (range 2–27) days for their illness. HUS occurred among 4 (21%) of the 19 patients with STEC O157 infection, including 2 of 4 children <3 years of age. One (8%) of the 12 patients with non-O157 STEC infection developed HUS. An 11-month-old infant with STEC O157 infection died.

Of the 530 case-patients who consumed raw milk, 377 (71%) provided information on the source of their milk, and almost half either obtained it from their own dairy farm (91 consumers, 24%) or from a relative's dairy farm (90 consumers, 24%). Other reported sources included friends (73 consumers, 19%); nonrelative farmers, including direct farm sales (50 consumers, 13%); the workplace or a relative's workplace (39 consumers, 10%); neighbors (16 consumers, 4%); drop-off sites (7 consumers, 2%); or other sources, including at daycare or school (11 consumers, 3%). Those who reported consuming raw milk from their own dairy farm or from a relative's dairy farm were significantly younger than those who reported obtaining raw milk from a nonfamily source (median age 9 years vs. 19 years; $p<0.001$). Among children ≤5 years of age, 76% were reported to have consumed raw milk from their own dairy farm or a relative's dairy farm; this proportion declined with age among pediatric patients, and a steep drop to 9% occurred among those 17–20 years of age, who more frequently reported obtaining raw milk from friends or at work (Figure 3). Among 464 case-patients with known information, 232 (50%) also reported contact with cattle or their environment during the exposure period; 68% of these exposures occurred in persons living or working on a farm or visiting a family member's farm.

Pathogen-specific underdiagnosis multipliers (*18*) were applied to data regarding the 530 cases associated with reported domestic raw milk consumption and resulted in an estimate that 20,502 Minnesotans became ill with sporadic *Campylobacter*, *Cryptosporidium*, STEC O157, non-O157 STEC, or *Salmonella* infection during 2001–2010 after drinking raw milk (Table 2). We applied the percentage of Minnesotans in the Foodborne Diseases Active Surveillance Network population survey who reported consuming raw milk (2.3%) (*13*) to the 2006 state population (5,167,101) and determined that an estimated 118,843 Minnesotans consume raw milk during a given week. If these projected illness data are applied to the projected number of raw milk consumers and the percentage of Minnesotans who consume raw milk was consistently 2.3% during the study period, then an estimated 17.3% of raw milk consumers in Minnesota may have acquired an illness caused by 1 of these enteric pathogens during the 10-year study period.

Discussion

Our study quantifies the number of non–outbreak-related enteric illnesses that could be associated with raw milk consumption. The results indicate that the number of sporadic raw milk–associated illnesses is likely substantial, greatly exceeding the number of cases linked to recognized raw milk–associated outbreaks. Furthermore, the number of cases associated with reported raw milk consumption appears to be increasing, just as the movement to relax regulation of raw milk sales appears to be gaining momentum in many states.

We found that young children were disproportionately affected, and the source of the raw milk they consumed was often their own dairy farm or a relative's farm. This reinforces the risks associated with raw milk consumption for all young children, including those who live on or visit farms. Half of the STEC O157–related and non-O157 STEC–related illnesses occurred among children ≤5 years of age; this finding is of particular concern because of

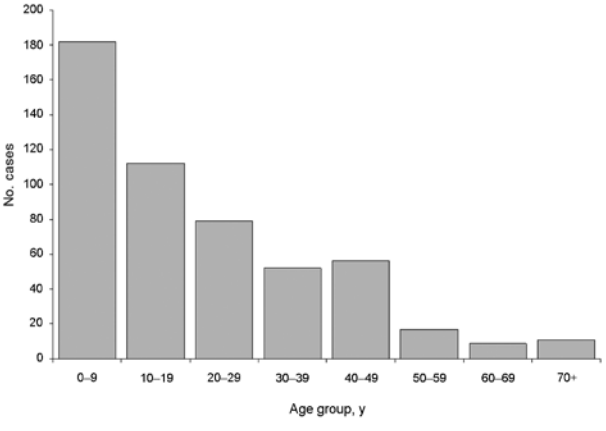

Figure 2. Age distribution among patients with domestically acquired, sporadic enteric infections who reported consumption of raw milk during their exposure periods (n = 518), Minnesota, 2001–2010.

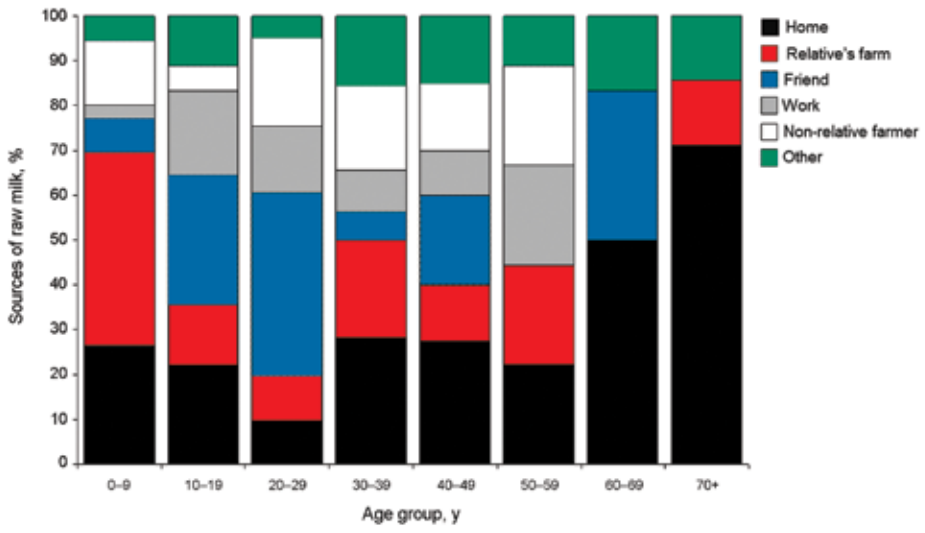

Figure 3. Distribution of reported raw milk sources, by patient age, among patients with domestically acquired, sporadic enteric infections who reported consumption of raw milk during their exposure periods (n = 377), Minnesota, 2001–2010.

the increased risk for HUS among this age group (*22*). In our study, HUS developed in 21% of patients with STEC O157 infections who reported drinking raw milk, and 1 of these patients died. Because of the potential for severe health consequences associated with the consumption of raw milk, the American Academy of Pediatrics and several other professional organizations have adopted a position statement advising that children should only consume milk products that are pasteurized (*23*).

Our study suggests that farm family members, particularly young children, who consume raw milk are susceptible to illness from it. Results of previous studies have shown that 30%–50% of dairy producers surveyed were unaware that their raw bulk tank milk could contain disease-causing microorganisms (*24*,*25*). Therefore, dairy farm families, in particular, should be educated regarding this issue. The differences in disease risk by age group may be a result of acquired immunity among older adolescents and adults who were exposed to pathogens during farm exposures in childhood (*26*).

Most raw milk consumers included in our study were infected with *Campylobacter* spp., and these bacteria have repeatedly been associated with raw milk consumption, both in outbreak investigations and in case–control studies (*27*,*28*). Because of the complexities involved with molecular subtyping for *Campylobacter* spp. (*29*) and because of the high proportion of patients who do not report a source for their raw milk, it is likely that some of the apparently sporadic infections associated with raw milk consumption are actually illnesses related to unrecognized outbreaks.

This study provides evidence that raw milk is also a vehicle for *Cryptosporidium* spp., the second most common pathogen identified among raw milk drinkers. Cryptosporidiosis is a common infection in cattle, and contact with infected animals or their environment is frequently associated with human cryptosporidiosis (*30*,*31*). Although few cryptosporidiosis outbreaks associated with raw milk consumption have been reported (*6*), findings of other studies include an association between raw milk consumption and cryptosporidiosis (*32*,*33*).

We noted an increase in enteric disease cases associated with reported raw milk consumption during the summer months (June–August) compared with other seasons. This trend is in sync with the general seasonality of the enteric pathogens included in this study, and it is also consistent with data from recent studies on the seasonal incidence of *Salmonella* spp. sampled from bulk tank milk, dairy cows, and farm environments (*34*) and the seasonal trend of fecal shedding of STEC O157 by dairy cattle (*35*).

This study had several potential limitations. First, the cases were sporadic, so illness among the raw milk consumers could not be definitively linked to their raw milk

Table 2. Number of sporadic illnesses resulting from raw milk consumption, as estimated by using underdiagnosis multipliers, Minnesota, 2001–2010

Pathogen	No. laboratory-confirmed cases	Multiplier to account for underdiagnosis (*18*)	Estimated no. actual cases
Campylobacter spp.	407	30.3	12,332
Cryptosporidium spp.	53	98.6	5,226
Escherichia coli O157	19	26.1	496
Non-O157 Shiga toxin–producing *E. coli*	12	106.8	1,282
Salmonella spp.	39	29.9	1,166
Total	530	–	20,502

consumption without overestimating the number of illnesses associated with raw milk consumption. For example, illness among some patients could have been associated with direct contact with cattle rather than raw milk consumption. However, 50% of patients did not report cattle contact, and the high-risk nature of raw milk consumption makes it the most likely source for most of the patients who did report concomitant cattle contact. This conclusion is supported by the close demographic and illness similarities among patients with reported raw milk consumption in our study and the outbreak cases reported by Langer et al. (*12*). It is also supported by the very low (0.7%) background level of raw milk consumption among patients with pathogens of a species or serotype not historically associated with raw milk consumption or other cattle exposures.

The second potential limitation is that not all specimens of the pathogens of interest were identified to species or serotype; this was most pertinent for *Cryptosporidium* spp. Therefore, it is possible that some patients were misclassified as eligible, thus underestimating the percentage of raw milk drinkers. The third potential limitation is that refusals to participate in an interview or to answer questions about raw milk could be biased toward raw milk consumers. In contrast to the first limitation, this could potentially have resulted in an underestimation of the number of illnesses associated with raw milk consumption. The fourth potential limitation is that the data available to us for this study did not permit estimation of a true absolute risk for illness associated with raw milk consumption. The fifth and last potential limitation is that the pathogen-specific underdiagnosis multipliers have confidence intervals that were not considered here; therefore, estimates of raw milk–associated illnesses could be substantially higher or lower than we have reported (*18*).

Sporadic cases of illness associated with raw milk consumption far outnumber cases associated with recognized outbreaks. During the study period, the number of patients with sporadic laboratory-confirmed infections who reported raw milk consumption (n = 530) was 25 times greater than the number of raw milk–associated outbreak cases (n = 21) among Minnesota residents. Furthermore, we estimated that up to 20,502 Minnesotans, or 17% of raw milk consumers, may have become ill with enteric pathogens during the study period after consuming raw milk. This finding suggests that outbreaks represent a small number of the illnesses associated with raw milk consumption and that the risk for illness associated with raw milk consumption is far greater than determined based on the occurrence of recognized outbreaks. Findings such as ours should be used to further educate potential raw milk consumers, as well as policy makers who might be asked by constituents to relax regulations regarding raw milk sales.

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Multisite Validation of Cryptococcal Antigen Lateral Flow Assay and Quantification by Laser Thermal Contrast

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Cryptococcal meningitis is common in sub-Saharan Africa. Given the need for data for a rapid, point-of-care cryptococcal antigen (CRAG) lateral flow immunochromatographic assay (LFA), we assessed diagnostic performance of cerebrospinal fluid (CSF) culture, CRAG latex agglutination, India ink microscopy, and CRAG LFA for 832 HIV-infected persons with suspected meningitis during 2006–2009 (n = 299) in Uganda and during 2010–2012 (n = 533) in Uganda and South Africa. CRAG LFA had the best performance (sensitivity 99.3%, specificity 99.1%). Culture sensitivity was dependent on CSF volume (82.4% for 10 μL, 94.2% for 100 μL). CRAG latex agglutination test sensitivity (97.0%–97.8%) and specificity (85.9%–100%) varied between manufacturers. India ink microscopy was 86% sensitive. Laser thermal contrast had 92% accuracy (R = 0.91, p<0.001) in quantifying CRAG titers from 1 LFA strip to within <1.5 dilutions of actual CRAG titers. CRAG LFA is a major advance for meningitis diagnostics in resource-limited settings.

Cryptococcal meningitis is the most frequent form of meningitis among adults in sub-Saharan Africa (*1–4*) and accounts for 20%–25% of AIDS-related deaths in Africa (*5–7*). Although culture is the standard method for definitive diagnosis, detection of cryptococcal antigen (CRAG) in serum or cerebrospinal fluid (CSF) is used for presumptive diagnosis. CRAG screening in peripheral blood is also recommended for HIV-infected persons with CD4 cell counts <100/μL to reduce early deaths while receiving antiretroviral therapy (ART) (*8–10*). CRAG is usually detected by latex agglutination (CRAG latex), which has a sensitivity and specificity >99% (*11,12*). However, CRAG latex testing requires laboratory infrastructure and expertise, electricity, heat inactivation, cold-chain shipping, and refrigeration of reagents. Unfortunately, required infrastructure is usually unavailable in resource-constrained settings in which cryptococcal incidence is greatest (*5*). Thus, India ink microscopy is the primary diagnostic modality, despite having lower sensitivity (*12–14*).

In July 2011, a lateral flow immunochromatographic assay (LFA) (Immy, Inc., Norman, OK, USA) was approved by the US Food and Drug Administration for detection of CRAG in CSF and serum. This assay is a rapid diagnostic test that provides a definitive result in ≤10 min. With its low cost (currently $2/test for resource-limited settings and $5/test for high-income settings), shelf stability at room temperature, and ease of use, this rapid, point-of-care test might expedite diagnosis. Field testing data are needed for this assay.

Quantification of CRAG incidence can be determined by determining CRAG titer. Quantification is clinically useful because higher CSF CRAG titers are predictive of risk for death (*15–17*), and higher serum CRAG titers predict risk for immune reconstitution inflammatory syndrome (IRIS) (*18*). Titers can be determined by using CRAG latex or LFA, but these procedures are rarely used in resource-limited settings because of additional incremental cost per titer dilution. Thus, novel methods of quantification are needed.

In a multisite cohort of HIV-infected persons with suspected meningitis in sub-Saharan Africa, we compared

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CRAG LFA performance with that of traditional diagnostic tests. We demonstrated excellent diagnostic performance by the CRAG LFA in the laboratory and as a point-of-care bedside test. In addition, we demonstrated within this clinical cohort a novel method of CRAG titer quantification for an LFA by using laser thermal contrast measurement (*19*), enabling CRAG titer quantification without traditional serial dilutions.

Materials and Methods

CRAG LFA was performed for 2 cohorts of prospectively enrolled HIV-infected persons with suspected meningitis: a 2006–2009 cohort of 299 hospitalized patients in Kampala, Uganda (*18,20*); and a 2010–2012 cohort of 533 hospitalized patients from G.F. Jooste Hospital, Cape Town, South Africa; Mbarara Hospital, Mbarara, Uganda (*16*); and Mulago Hospital, Kampala, Uganda (NCT01075152, www.clinicaltrials.gov). LFA was performed retrospectively with cryopreserved (–80°C) specimens collected before April 2011 and prospectively thereafter with point-of-care specimens. Persons with suspected meningitis provided written informed consent. Ethical approval was provided by each institutional review board and the Uganda National Council of Science and Technology.

Diagnostic Testing

Quantitative CSF Fungal Culture

The fungal culture procedure in 2006–2009 used 10 μL of CSF cultured on Sabouraud dextrose agar (*12*). This method was insensitive for lower incidence infections. Thus, in 2010, the quantitative culture method was changed to a protocol using a 100-μL input volume of undiluted CSF culture and five 1:10 serial dilutions (*21,22*). Agar plates inoculated with CSF were incubated at 30°C for up to 14 days. The number of discrete colonies found at the highest dilution was multiplied by the dilution to give CFU/mL of CSF. Culture isolates were independently confirmed as *Cryptococcus neoformans* var. *grubii* by multilocus sequence typing (*23*).

CRAG Latex Agglutination

In 2006–2009, a qualitative CRAG latex assay (Meridian, Cincinnati, OH, USA) was performed in Kampala. Reactivity at a ≥1:2 dilution of CRAG was considered a positive result. Semiquantitative CRAG latex titers (*Cryptococcus* Antigen Latex Agglutination Test System; Immy, Inc.) were measured by 2-fold serial dilution on cryopreserved (–80°C) samples in Minnesota. Qualitative concordance between the 2 CRAG assays was imperfect (92%). Thus, results are presented separately.

In 2010–2013, the CRAG latex test kit (Immy, Inc.) was used in Uganda. Reactivity at a 1:2 dilution of CRAG was considered a positive result. CRAG titers were obtained for real-time specimens in Cape Town but for cryopreserved (–80°C) specimens in Mbarara and Kampala. Testing was performed without knowledge of results of alternative assays, except for culture. All laboratories participated quarterly in National Institutes of Health (Bethesda, MD, USA) sponsored external quality assurance testing for fungal culture, India ink microscopy, and CRAG testing.

The 4 cryptococcal tests were not performed for all CSF samples because of insufficient sample volumes (most common), laboratory operating hours, or reagent supply chain difficulties. However, 794 (95%) of 833 samples were tested by ≥3 cryptococcal CSF tests performed prospectively, and 667 CSF samples were tested by LFA.

CRAG LFA

The LFA is a point-of-care dipstick test that uses gold-conjugated, monoclonal antibodies impregnated onto an immunochromatographic test strip to detect cryptococcal capsular polysaccharide glucuronoxylomannan antigen (CRAG) for all 4 *C. neoformans* serotypes (A–D) (*24,25*). If cryptococcal antigen is present in a specimen, suspended, gold-conjugated antibodies bind to the antigen. The gold–antibody–CRAG complex migrates by capillary action up the test strip, interacts with immobilized monoclonal antibodies against CRAG, and forms a red line. The LFA kit contains immunochromatographic test strips, positive controls, and assay diluent that can be stored at room temperature for ≤2 years. To perform the LFA, 1 drop of diluent (≈40 μL) is added to a container of 40 μL of patient specimen. The dipstick is inserted into the container and incubated at room temperature for 10 min (Figure 1).

All LFAs were performed according to manufacturer's instructions by trained operators who had no knowledge of results of all other tests. For 2006–2010, cryopreserved CSF and plasma samples (–80°C) were retrospectively tested by LFA. Beginning in April 2011, the LFA was prospectively performed with CSF as a point-of-care test in the hospital ward and with serum (n = 346) and urine (n = 236) samples in Uganda collected on the day of lumbar puncture. Semiquantitative CRAG LFA titers were determined on cryopreserved samples by using a 2-fold serial dilution starting at 1:25. When samples qualitatively positive but negative at a 1:25 dilution, 2-fold dilutions starting at 1:2 were tested. CSF samples collected during 2010 2012 (n = 414) were qualitatively retested in Minnesota after storage at –80°C to determine inter-reader variability. Readers did not know prior LFA results.

Additional Diagnostic Testing

India ink microscopy was performed on sediment obtained after centrifugation of 1 mL of CSF. Gram staining,

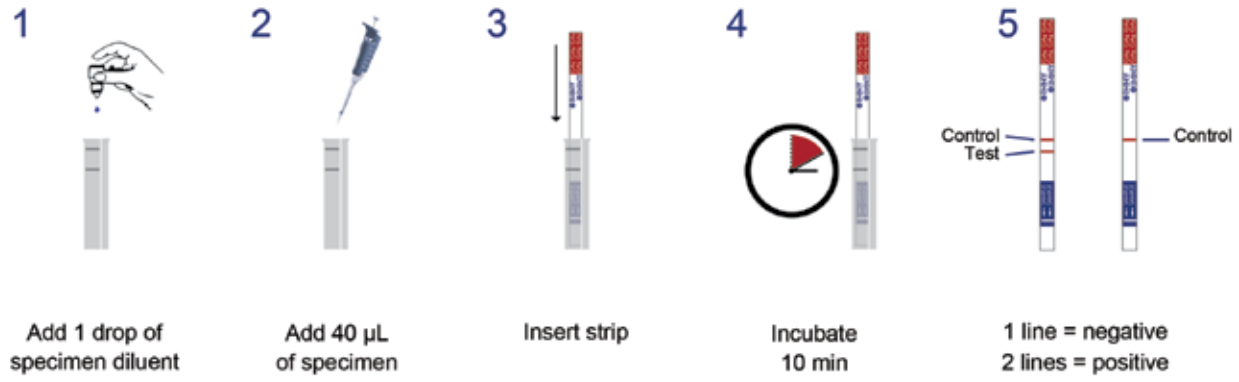

Figure 1. Five steps of the cryptococcal antigen lateral flow assay.

bacterial culture, and staining for acid-fast bacilli were prospectively performed for all specimens.

Thermal Contrast Measurement of CRAG Titer

We used a novel method that provided quantification of LFAs in comparison with semiquantitative CRAG LFA titers by using the heat signature of laser-irradiated gold used in the LFA. To detect gold nanoparticles conjugated to monoclonal antibodies on the LFA line, the line was irradiated with a 0.01 W laser (532 nm, diode pumped; Millenia, Santa Clara, CA, USA) for 30 s, and temperature change (thermal contrast) was recorded with an infrared camera (A20; FLIR ThermoVision, Portland, OR, USA), as described (*19*). Three spots on each horizontal LFA line were irradiated and the average maximum temperature change was calculated. An antigen titer was calculated from the thermal contrast by using a calibration curve established by 2-fold serial dilutions of 3 specimens in triplicate with known CRAG LFA titers ($R^2 = 0.97$). Thermal contrast quantification was performed for 115 positive and 58 visually negative CSF LFA dipsticks sequentially collected in Kampala during 2010–2011. Negative specimens were measured undiluted. Positive specimens were measured by thermal contrast at a 1:250 dilution to demonstrate a wide dynamic range, including specimens visually negative after dilution.

Statistical Analysis

We compared diagnostic performance (sensitivity, specificity, and positive and negative predictive values) for each test versus a composite reference standard. The composite reference standard was defined as a CSF culture-positive (n = 459) or a culture-negative sample with ≥2 positive test results (e.g., India ink microscopy, CRAG latex, or CRAG LFA) and without an alternative etiologic explanation (n = 60). As such, no single positive test result (other than culture) could define the composite reference standard, enabling comparison of performance of the diagnostic tests, yet minimizing bias of the reference standard being defined solely by 1 test (*26*). When only 1 CSF test result was positive, serum cryptococcal antigenemia was used as a second qualifying positive test result (n = 6) to confirm the reference standard (tested during 2010–2012) as discrepant (*26*) on the basis of cryptococcosis pathogenesis in which antigenemia precedes culture-positive meningitis (*7,27,28*).

For thermal contrast measurements, Pearson correlation was used to compare predicted and actual LFA titers. To determine relative benefit from additional resources, we determined the cost of changing from India ink microscopy to CRAG LFA on the basis of the number needed to test (NNT) to detect 1 additional person with infected with *Cryptococcus* spp. We further modeled costs with a probabilistic sensitivity analysis by using TreeAge 2013 (TreeAge Software Inc., Williamstown, MA, USA) on the 95% CI of diagnostic test performance and published cryptococcal prevalence rates (*4*).

Results

Among 832 persons with suspected meningitis during 2006–2012, a total of 525 (63%) had cryptococcal meningitis as defined per the composite reference standard. The 2 cohorts were similar in age, sex, and CD4 cell count (Table 1).

LFA Diagnostic Performance

For 666 CSF samples available for testing, the LFA had a sensitivity of 99.3%, a specificity of 99.1%, a positive predictive value of 99.5%, and a negative predictive value of 98.7% (Table 2). Cryptococcal antigen testing by either latex or LFA was more sensitive than CSF culture. The 2006–2009 culture protocol used only 10 µL of CSF applied by a calibrated loop. This simple quantitative culture method was clinically useful but relatively insensitive (82.4%), and thus probably missed persons with low fungal incidence. The minimum growth on culture was 100 CFU/mL in 2006–2009. During 2010–2012, the culture protocol used 100 µL of CSF as the input volume and showed

Table 1. Demographic characteristics and diagnostic tests performed with specimens from meningitis cohorts, Uganda and South Africa*

Characteristic	2006–2009, retrospective	2010–2012, prospective†		
	Kampala, Uganda	Kampala, Uganda	Mbarara, Uganda	Cape Town, South Africa
Demographic				
No. persons	299	354	142	37
Mean ± SD age, y	36 ± 8	35 ± 9	35 ± 11	37 ± 10
Male sex, no. (%)	168 (56)	174 (49)	87 (61)	22 (59)
CD4 cell count/μL, median (IQR)	19 (7–38)	16 (7–69)	36 (14–74)	65 (43–97)
Diagnostic tests performed, no.				
CSF				
Quantitative culture	282	345	142	37
Latex agglutination	279	345	142	37
India ink microscopy	276	350	142	37
LFA	197	291	142	36
Serum				
Latex agglutination	85	34	NA	NA
LFA	NA	274	49	23
Plasma				
LFA	60	NA	NA	NA
Urine				
LFA	NA	185	51	NA

*IQR, interquartile range; CSF, cerebrospinal fluid; LFA, lateral flow immunochromatographic assay; NA, not available.
†Prospective testing with LFA because point-of-care began in April 2011. Eighty LFAs were conducted on cryopreserved samples.

improved sensitivity (94.2%); the minimum growth detected was 10 CFU/mL. The median quantitative CSF culture grew 150,000 CFU/mL of CSF (interquartile range [IQR] 14,100–455,500 CFU/mL) in 2010–2012. The overlap of specimens with positive results for the 4 cryptococcal diagnostic assays is shown in Figure 2.

Three samples had false-negative results by LFA; all were retested. Of the 1 culture- positive sample with a negative result by LFA, it had a relatively low fungal incidence of 100 CFU/mL (Table 3). The 3 false-negative samples detected by LFA were previously cryopreserved CSF supernatant specimens, and a freeze/thaw artifact or error during storage cannot be excluded. During prospective performance, LFA detection of low fungal incidence in culture was not a systematic problem. Among 10 persons with culture incidence ≤100 CFU/mL in 2011–2012, LFA prospectively detected 10 of 10, CRAG latex detected 9 of 10, and India ink microscopy detected 3 of 10.

Regarding LFA specificity, 8 CSF samples were positive only by CRAG LFA. We initially believed that these samples had LFA false-positive results (Table 4). Culture-positive cyyptococcal meningitis developed 6 weeks later in a person with a possible false-positive LFA result. A second person with a possible false-positive LFA result died of meningitis symptoms several weeks later, and a third person with a possible false-positive LFA result had cryptococcoma masses documented postmortem. Of 8 persons with CSF samples positive by LFA only (negative by culture, India ink microscopy, and CRAG latex), 6 had serum cryptococcal antigenemia. These 6 persons were classified as having true cryptococcal disease. Two persons in the 2006–2009 cohort did not have a peripheral blood specimen collected, and thus were conservatively classified as having false-positive results Their CSF specimens were tested for bacteria, herpes viruses, arboviruses, toxoplasmosis, syphilis, and tuberculosis but no alternative etiology

Table 2. Performance characteristics of cryptococcal diagnostic assays in persons with suspected meningitis, Uganda and South Africa*

Diagnostic test	No.	No. positive/no. tested (%)			
		Sensitivity	Specificity	PPV	NPV
CRAG LFA	666	435/438 (99.3)	226/228 (99.1)	435/437 (99.5)	226/229 (98.7)
CSF culture†	806	459/510 (90.0)	296/296 (100.0)	459/459 (100.0)	296/347 (85.3)
100-μL volume	524	309/328 (94.2)	196/196 (100.0)	309/309 (100.0)	196/215 (91.2)
10-μL volume	282	150/182 (82.4)	100/100 (100.0)	150/150 (100.0)	100/132 (75.8)
India ink microscopy	805	438/509 (86.1)	288/296 (97.3)	438/446 (98.2)	288/359 (80.2)
CRAG latex (Meridian)‡	279	176/180 (97.8)	85/99 (85.9)	176/190 (92.6)	85/89 (95.5)
CRAG latex (Immy)§	749	452/466 (97.0)	283/283 (100.0)	452/452 (100.0)	283/297 (95.3)

*PPV, positive predictive value; NPV, negative predictive value; CRAG, cryptococcal antigen: LFA, lateral flow immunochromatographic assay; CSF, cerebrospinal fluid. All samples tested by CSF CRAG LFA had false-positive results and did not have any other pathogen identified. However, serum or plasma samples were not available for testing for cryptococcal antigenemia to determine if the original result indicated enhanced detection.
†Two quantitative CSF culture procedures were used in 2006–2009 (input volume 10 μL) and 2010–2012 (input volume 100 μL).
‡Meridian, Cincinnati, OH, USA.
§Immy, Inc., Norman, OK, USA.

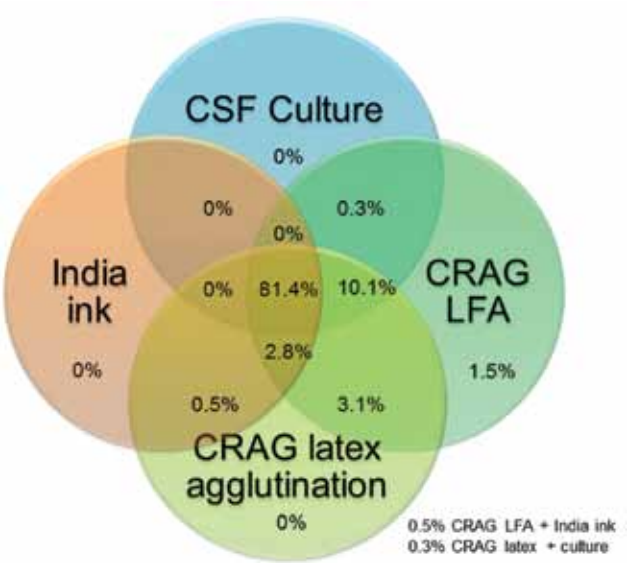

Figure 2. Venn diagram of distribution of 393 cryptococcal meningitis cases tested by 4 diagnostic assays, Uganda and South Africa. CSF, cerebrospinal fluid; CRAG LFA, cryptococcal antigen lateral immunochromatographic flow assay; India ink, India ink microscopy of 1 mL of concentrated CSF specimen. Numbers at the bottom right indicate 2 scenarios in which the Venn diagram does not overlap visually.

was identified. If the 6 samples from persons without documented evidence of cryptococcal meningitis were considered as having false-positive results, the LFA specificity would decrease to 97% (226/234).

CSF CRAG LFA titers correlated with other quantitative measures, such as CSF culture (Pearson r = 0.58, p<0.001) and CRAG latex titers (r = 0.82, p<0.001). When comparing CRAG titers, LFA median titer was 2.5-fold higher (IQR 1.25–5-fold) than CRAG latex titer (e.g., CSF with a CRAG latex titer of 1,024 had an LFA titer of 2,500). This LFA enhancement of higher titers relative to CRAG latex was more prominent at lower titers. For CRAG latex titers ≤256, LFA titers were a median 5-fold (IQR 2.5–10-fold) higher than CRAG latex titers, whereas for CRAG latex titers ≥2,048, LFA titers were a median 2.5-fold (IQR 0–4.4-fold) higher.

LFA readings were concordant for 98.8% (409/414) of prospective samples retested after storage by an independent reader. All discordant results were negative initially on fresh samples read under indirect sunlight in Uganda, but 4 were weakly positive and 1 was strongly positive when retested after cryopreservation in the United States and read under bright fluorescent lighting.

India Ink Microscopy

Sensitivity of India ink microscopy was the lowest (86%) of any test and highly dependent on fungal burden. Sensitivity decreased to 42% (19/45) among persons with CSF cultures having <1,000 CFU/mL. Overall, 1 of 7.2 cryptococcal diagnoses was missed by India ink microscopy (negative predictive value of 80%; (95% CI 76%–84%). If India ink microscopy had been the only diagnostic test used, 8.8% of meningitis cases in Uganda would have been misdiagnosed. Among persons in Uganda who had India ink microscopy–negative results, *Cryptococcus* spp. remained the most common pathogen (20%). The NNT by CRAG LFA was 5.1 (95% CI 4.1–6.5) per additional cryptococcal diagnosis.

By replacing India ink microscopy with CRAG LFA for meningitis diagnostics, we found that overall NNT was 11.4 (95% CI 9.1–15.1) among HIV-infected persons in Uganda with suspected meningitis, and LFA cost per additional diagnosis was US $22.83 (95% CI $18.22–$30.28) for LFA supplies, excluding savings for India ink microscopy costs and inappropriate medications prescribed (e.g., ceftriaxone US $2/day, international wholesale price) (*29*). When we extrapolated cryptococcal prevalence rates in eastern and southern Africa (37% of meningitis cases; 95% CI 35%–39%) (*1*–*4*), the NNT was 20.2 (95% CI 13.3–40.6) at a cost of $40 (95% CI $27–$80) for LFAs per additional diagnosis. In middle- and high-income countries in which laboratory labor is the primary cost component, LFAs would have lower costs than India ink microscopy or CRAG latex.

Peripheral Blood and Urine Samples

Among serum samples tested, sensitivity was 98.3% (114/116) for serum CRAG latex and 99.6% (239/240) for serum LFA among persons with cryptococcal meningitis. Serum LFA specificity was 92% (98/106) for cryptococcal meningitis. The correlation between serum CRAG latex and serum LFA titers was r = 0.87 when tested independently

Table 3. Characteristics of CSF specimens with false-negative results by cryptococcal antigen lateral flow immunochromatographic assay, 2006–2009 cohort, Uganda and South Africa*

CSF culture, CFU/mL	India ink microscopy	CSF latex agglutination test dilution†	CSF CRAG LFA
100	–	1:2 Meridian; 1:1 Immy	–‡
0	+	1:2 Meridian; 1:1 Immy	–‡
0	+	1:2 Meridian; 1:1 Immy	–‡

*CSF, cerebrospinal fluid; CRAG, cryptococcal antigen; LFA, lateral flow immunochromatographic assay; CFU, colony-forming units of Cryptococcus neoformans; –, negative; +, positive.
†Meridian, Cincinnati, OH, USA; Immy, Inc., Norman, OK, USA.
‡Retrospectively performed with cryopreserved specimens.

Table 4. Characteristics of CSF specimens positive only by cryptococcal antigen lateral flow immunochromatographic assay, Uganda and South Africa*

Cohort	CSF LFA titer	Serum CRAG LFA titer	Serum CRAG latex titer	Urine LFA result	Classification	Patient outcome
2006–2009	2	NA	NA	NA	LFA false positive	Unknown
	2	NA	NA	NA	LFA false positive	Unknown
2010–2012	2	512	1:8	–	Cryptococcal meningitis	Began ART; CSF culture-positive result; meningitis developed 6 weeks later
	±	2,048	2,048	–	Cryptococcal meningitis	Died after hospital discharge
	±	32	–	+	Cryptococcal meningitis	Died in hospital
	250	4	–	+	Cryptococcal meningitis	Began ART; minimum CD4 cell count 130 cells/μL
	8	8	128	–	Cryptococcal meningitis	Died after hospital discharge
	±/16†	4,096	2,048	+	Cryptococcal meningitis	Given fluconazole, 800 mg/d; seizure; died; cryptococcoma mass identified postmortem

*CSF, cerebrospinal fluid; CRAG, cryptococcal antigen; LFA, lateral flow immunochromatographic assay; –, negative' NA, not available; ART, antiretroviral therapy; ±, discordant readings by 2 independent readers; +, positive. All test results for CSF culture, India ink microscopy, and CSF CRAG latex were negative.
†Fresh specimen was CRAG latex and LFA negative. CRAG LFA titer of 16 on cryopreserved specimen. Repeat CSF testing results 2 weeks later for neurologic deterioration were positive and remained CRAG latex negative. Thermal contrast showed that all LFA strips had positive results with readings above background.

($p<0.001$). Of 60 plasma specimens retrospectively tested from the 2006–2009 cohort, LFA sensitivity was 100% (specificity unavailable). Plasma LFA titers and serum CRAG latex titers showed a correlation ($r = 0.89$, $p<0.001$), but median LFA titer was 3.3-fold (IQR 2.3–4.3-fold) higher than CRAG latex titer. Strength of the correlation was less between CSF LFA titer and either serum LFA titer ($r = 0.54$, $p<0.001$) or plasma LFA titer ($r = 0.15$, $p = 0.30$). LFA for urine had a sensitivity of 97% (151/156) and a specificity of 85% (68/80) for cryptococcal meningitis. Among urine specimens from persons with false-positive results, 5 of 7 had antigenemia detected by CRAG LFA of serum.

Thermal Contrast

We developed a novel method to quantify CRAG titers from 1 LFA strip by using laser thermal contrast measurement to convert a qualitative LFA into a quantitative assay. The technique measures the change in temperature of gold nanoparticles when laser irradiated, and in comparison with a calibration curve, we predicted CRAG titer (*19*). Among 115 CSF LFA-positive samples and 58 negative samples, the predicted titer by thermal contrast correlated ($r = 0.91$, $p<0.001$) with actual semiquantitative titer by LFA, as performed by traditional 2-fold serial dilutions (Figure 3). Overall, 92% of predicted CRAG titers by thermal contrast were within 1.5 dilutions of the actual semiquantitative titer when measured by serial dilution.

We tested specimens at a 1:250 dilution and detected visually negative specimens (after dilution) by thermal contrast, which demonstrated enhanced sensitivity. All 58 undiluted negative specimens from persons without *Cryptococcus* spp. infection remained negative by thermal contrast, which demonstrated maintained specificity. Thermal contrast removed the subjectivity of borderline, equivocally positive LFA results. Furthermore, because gold is inert, LFA can be subjected to repeated thermal contrast measurements without major changes in measurement. Readings obtained in LFAs were virtually identical ($R^2 = 0.99$) at 2 weeks, 2 months, and 6 months. Thus, archiving of LFA samples for delayed reading is possible (e.g., to centralize laboratory processing for quality control).

Discussion

This multisite study prospectively validated a new point-of-care CRAG LFA in a clinical setting among persons with suspected meningitis in sub-Saharan Africa. These results confirm that the CRAG LFA has ≥99% sensitivity and ≥99% specificity for CSF in the field. We demonstrated that a novel technique, laser thermal contrast, had 92% accuracy in quantifying CRAG titers from 1 LFA strip to within <1.5 dilutions of the actual CRAG titer by serial dilutions ($R = 0.91$, $p<0.001$). LFA performance was more sensitive than that of any other diagnostic test. Conversely, the worst performing test was India ink microscopy, which is the most common cryptococcal diagnostic test in Africa, despite missing 1 in 7 cryptococcal diagnoses and having only an 80% negative predictive value in our cohorts. In facilities with India ink microscopy testing capability, switching to CRAG LFA would result in 5% more meningitis patients with a correct diagnosis in eastern and southern Africa (*4*). In addition, the CRAG LFA now enables diagnostic testing anywhere for the most common cause of meningitis in Africa.

The enhanced LFA analytic sensitivity is reflected by median 2.5-fold higher titers observed for CSF with LFA than with CRAG latex, indicating that LFA is more sensitive to lower antigen levels. Increased sensitivity of the LFA became clinically apparent in CSF specimens

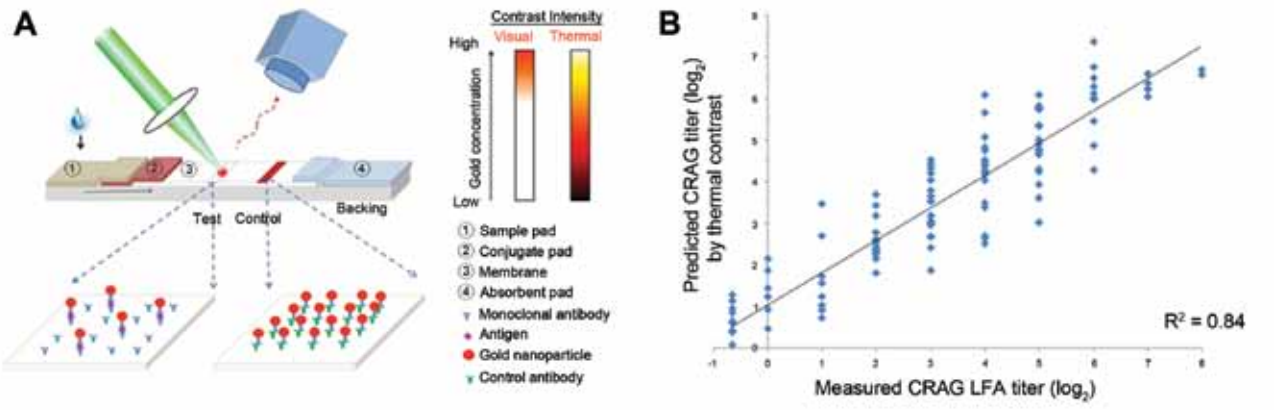

Figure 3. A) Prediction of cryptococcal antigen titer based on laser thermal contrast measurement and concept of lateral flow immunochromatographic assay (LFA) thermal contrast measurement in which a laser irradiates the test line in the LFA (19). The test line is formed by gold–monoclonal antibody–antigen sandwich complex with a monoclonal antibody affixed at the test line. When irradiated by a green laser (532 nm), any gold present absorbs light from the laser and generates heat in direct proportion to the amount of gold (and thereby antigen) present at the test line. This temperature change can be measured by using an infrared camera. B) Association of measured semiquantitative LFA cryptococcal antigen (CRAG) titer starting at a 1:250 dilution by the predicted CRAG titer based on thermal contrast measurement. Measurements on the negative portion of the x-axis are beyond the visual range when specimens were diluted 1:250, yet still detectable by thermal contrast. The Pearson correlation coefficient was r = 0.91 ($p<0.001$, R2 = 0.84) among 115 positive specimens quantified. A total of 58 LFA CRAG–negative specimens established background levels of heat radiation.

from persons positive only by LFA who showed CRAG positivity in serum. We observed 6 such case-patients in the 2010–2012 cohort. Initially, these persons were considered to have false-positive results. However, culture-positive cryptococcal meningitis developed in some persons or these persons died. These findings suggest that early-stage cryptococcal meningitis might be missed by traditional diagnostics. If these cases represent early cryptococcal meningitis, they would argue for expansion of targeted screening for cryptococcal antigenemia in peripheral blood in the setting of unexplained meningitis or repeat testing.

Our study builds upon 5 recent studies validating LFA versus ELISA and latex agglutination in laboratory settings (*24,30–33*). Jarvis et al. validated the LFA in 62 persons with a history of cryptococcal meningitis within the preceding 2 years in South Africa but without any concurrent controls (*24*). At 2 US and 1 Australian reference laboratories, the CRAG LFA had 100% sensitivity and excellent specificity when used to test 18 persons (*31*), 17 persons (*32*), and 25 persons (*33*) with cryptococcal infections, including detection of *C. gattii* (*31–33*). Our study demonstrates excellent performance of LFA as a point-of-care assay.

A point-of-care assay has multiple practical advantages. First, time to diagnosis can be reduced. During prospective implementation, median time between lumbar puncture and reporting CRAG latex results was 4 h and 50 min in Kampala and >24 h in Cape Town. When LFA was performed at bedside, the result was known in ≤10 min. Second, this point-of-care test can be used in rural settings without laboratory infrastructure. Third, cryptococcal guidelines recommend control of intracranial pressure (*8,34–36*). However, manometers are unavailable in Africa. Knowing cryptococcal status beforehand enables intervention during lumbar puncture to therapeutically control intracranial pressure. In contrast, when the cryptococcal diagnosis is not known until after lumbar puncture, the opportunity to intervene to control intracranial pressure has been lost. We suggest CRAG LFA immediately before the lumbar puncture in HIV-infected persons (e.g., with serum, plasma, or fingerstick blood). If results are positive, a manometer could be prioritized for use or, if unavailable, 20 mL CSF could be empirically drained for management of intracranial pressure.

One impracticality of determining CRAG titers in low- and middle-income countries is cost. CRAG titers are useful for predicting survival and risk for paradoxical IRIS, identifying *Cryptococcus* spp. in persons receiving ART who had pre-ART cryptococcal antigenemia, and differentiating cryptococcal-IRIS from cryptococcal relapse (*16,20,28,37,38*). However, each CRAG titer dilution requires the same amount of reagent as the initial test and is labor-intensive. Thermal contrast might be a useful, low-cost technology to increase sensitivity of LFA rapid diagnostic tests and provide quantification of results. Although this possibility is currently a prototype concept, it is probably applicable to any gold nanoparticle–based LFA (e.g., for malaria, *Mycobacterium tuberculosis*–associated lipoarabinomannan, pneumococcus, testing during pregnancy). Future work to construct a handheld, mobile-linked device can leverage this technology to

improve sensitivity and quantification of antigen levels for any gold nanoparticle–based LFA.

In conclusion, the CRAG LFA is a sensitive and specific point-of-care assay for diagnosis of cryptococcal meningitis and has particular applicability in resource-limited settings. Our experience suggests the LFA is more sensitive than current diagnostics and will enable detection of early-stage cryptococcal infection. In addition, the CRAG LFA is practical and may dramatically change the face of meningitis diagnostics worldwide.

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A provisional patent has been filed by J.C.B. for the thermal contrast technique.

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Genetic Variation of *Vibrio cholerae* during Outbreaks, Bangladesh, 2010–2011

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Cholera remains a major public health problem. To compare the relative contribution of strains from the environment with strains isolated from patients during outbreaks, we performed multilocus variable tandem repeat analyses on samples collected during the 2010 and 2011 outbreak seasons in 2 geographically distinct areas of Bangladesh. A total of 222 environmental and clinical isolates of *V. cholerae* O1 were systematically collected from Chhatak and Mathbaria. In Chhatak, 75 of 79 isolates were from the same clonal complex, in which extensive differentiation was found in a temporally consistent pattern of successive mutations at single loci. A total of 59 isolates were collected from 6 persons; most isolates from 1 person differed by sequential single-locus mutations. In Mathbaria, 60 of 84 isolates represented 2 separate clonal complexes. The small number of genetic lineages in isolates from patients, compared with those from the environment, is consistent with accelerated transmission of some strains among humans during an outbreak.

In many areas of the world, cholera remains a major public health problem; it affects millions of persons each year and causes a substantial number of deaths (*1*,*2*). In Bangladesh, cholera transmission is seasonal; 2 annual peaks are initiated by emergence of *Vibrio cholerae* from environmental reservoirs (*3*,*4*). The infectious dose of *V. cholerae* is estimated to be 10^5 to 10^8 CFU; the lower estimates are associated with a buffered stomach (*5*). After the organism enters the body, a physiologic change is induced, which alters the expression of most *V. cholerae* genes (*6*). One outcome of this alteration is a brief hyperinfectious state, during which *V. cholerae* exiting the colon are infectious at a reduced dose (*7*). After returning to the water for 24 hours, *V. cholerae* reverts to a standard infectious state (*7*,*8*). The relative contribution of the recently shed hyperinfectious strains and the strains from the environment to propagation of an outbreak of cholera remains controversial. The terms "slow" and "fast" have been used to distinguish between these 2 modes of transmission; slow refers to the human-to–aquatic environment–to-human pathway (which does not have time constraints), and fast refers to presumed person-to-person or person-to–household environment–to-person transmission (in which transfer is more likely immediately after fecal shedding, when strains are hyperinfectious) (*9*).

To determine the relative contribution of the hypothesized slow and fast routes of transmission during outbreaks, researchers have undertaken microbiological, genetic, and modeling approaches. However, a major problem with the first 2 approaches has been a lack of genetic diversity to track strains. Many methods, including pulsed-field gel electrophoresis (often used for outbreak analysis), detect too few genetic differences between isolates to be useful in tracking the microdynamics of strains. This problem was resolved, in part, by identification of loci containing a variable number of tandem repeats, which provided enough genetic variability to permit tracking of specific strains (*10*–*12*). However, initial studies that used multilocus variable tandem repeat analysis (MLVA) did not sample intensively enough to optimally distinguish between slow and fast transmission. One study, conducted in rural Bangladesh, in which isolates were collected every 2 weeks, showed

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that isolates from different geographic locations collected during different seasons and from clinical and environmental sources had only a few genotypes in common (*12*). Genotypes tended to be similar to one another when isolates were collected during the same season and came from the same geographic location as opposed to coming from different seasons or sources. Although this finding could be interpreted as evidence in support of fast transmission, most environmental isolates were not collected during the same month as most of the clinical isolates, making interpretation of data difficult. In another study, isolates from 100 index case-patients and their household relatives were analyzed (*13*). Remarkably, isolates from persons within a single household were often genetically unrelated, implying either different sources of infection or a single source with multiple genetic lineages. The unexpected variability was reinforced by the observation that unrelated genetic isolates were often isolated from a single fecal sample. However, the study design of sampling 3 households per month did not provide sufficient resolution to address transmission pathways. Mathematical modeling of incidence data from outbreaks has been used to estimate the contribution of fast and slow transmission (*14*,*15*). Although promising, these estimates have been based on clinical surveillance data without more detailed underlying epidemiologic information. Furthermore, it might not be possible to estimate the contribution of 2 transmission mechanisms from incidence data alone when the time scale of slow transmission is similar to that of fast transmission (*16*).

In this study, we used MLVA to characterize 222 clinical and environmental *V. cholerae* O1 isolates from outbreaks in 2 geographically distinct locations. Our objective was to determine genetic relatedness among the isolates, notably clonal relationships between environmental and clinical isolates, and to further explore the relative contribution of different transmission pathways to disease occurrence.

Materials and Methods

Sample Collection

From October 2010 through May 2011, clinical and environmental samples were collected in Chhatak and Mathbaria, Bangladesh. Chhatak is a village located 180 km northeast of Dhaka in the central region, and Mathbaria is a rural area located 150 km south-southeast of Dhaka in the coastal region.

Clinical isolates were obtained from rectal swabs from all patients who visited a health center with signs or symptoms of suspected cholera, representing ≈40% of all suspected cases per week during the peak season of cholera. Clinical samples were collected during 3 consecutive days every week. For characterization of within-host variability of *V. cholerae*, 9–10 isolates were selected from a culture from each of 6 patients at the Chhatak clinic.

Environmental isolates were obtained from water and plankton samples collected at 6 sites (ponds or rivers) in each community, as described (*17*). The same environmental sites were sampled, and the same sampling methods were used each time. Water and plankton samples were collected, concentrated, and analyzed for *V. cholerae* according to standard procedures (*18*,*19*). *V. cholerae* isolation and identification were performed according to standard methods (*19*,*20*). DNA was prepared from broth cultures of *V. cholerae* by using PrepMan Ultra (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. All samples were collected according to protocols approved by institutional review boards at Johns Hopkins University, University of Maryland (both in Baltimore, MD, USA), and icddr,b (Dhaka, Bangladesh).

Genetic Analysis

All PCRs were conducted by the same technician. Fluorescently labeled PCR primers were used to amplify 5 loci with variable-length tandem repeats; a new reverse primer was used for VCA0283 (5′-AGCCTCCTCAGAAGTTGAG-3′) (*13*). The reaction products were separated, detected, and sized by using a 3730xl automatic sequencer, internal lane standards (Liz600), and GeneScan program (all from Applied Biosystems). Genotypes were determined by using published formulas to calculate the number of repeats from the length of each allele except for VC0437, which is calculated by $(x - 252)/7$ (*13*). Five loci were ordered: VC0147, VC0437, VC1650, VCA0171, and VCA0283. A genotype (e.g., 9-4-6-19-11) was interpreted as an isolate having alleles of 9, 4, 6, 19, and 11 repeats at the 5 loci, respectively. Relatedness of isolates was assessed by using eBURST version 3 (http://eburst.mlst.net). Genetically related genotypes are defined as those possessing identical alleles at 4 of the 5 loci and groups of related genotypes (clonal complexes). Additional sensitivity analyses were conducted by using only the 3 more stable loci from the large chromosome (VC0147, VC0437, VC1650); relatedness was determined by identical alleles at 2 of the 3 loci (*13*,*21*).

Statistical Analyses

The null hypothesis was that the genotype isolated from each clinical case has an equal probability of being from each of the genetic lineages (for 5-locus analysis, from 1 of the observed clonal complexes or singletons; for 3-locus analysis, the same genotype) from the environment ($p_1 = p_2 = \ldots = p_n$), which, if true, would be evidence in support of a strong role of the slow transmission route in these outbreaks. A χ^2 test was used to determine whether the null

hypothesis was true. The Simpson index was calculated as a measure of genotype diversity (low values indicating more diversity) within each location as

$$D = \sum_{i=1}^{G} p_i^2$$

where G is the total number of genotypes at a particular location and p_i is the proportion of genotypes of type i.

Results

During the study period, 222 *V. cholerae* O1 Ogawa isolates were obtained from clinical and environmental samples from both locations. In Chhatak, fecal samples from 74 patients yielded 1 isolate from each patient; 6 samples yielded 9–10 additional isolates each. The environmental samples from 6 ponds yielded 5 isolates. In Mathbaria, fecal samples from 56 patients yielded 1 isolate each; the environmental samples from 6 ponds yielded 28 isolates. A total of 51 five-locus genotypes were observed, of which only 3 genotypes were from both Mathbaria and Chhatak, a result consistent with prior observation of geographic differences (*12*). Thus, isolates from the 2 locations were analyzed separately.

Chhatak

Extensive genetic variation was detected among the 138 isolates (Table 1, Simpson index 0.10). The numbers of distinct alleles at loci VC0147, VC0437, VC1650, VCA0171, and VCA0283 were 6, 3, 1, 12, and 8, respectively, with a minimum of 4 and up to 27 repeat units. A total of 30 genotypes (each a unique combination of alleles at the 5 loci) were detected. Only 1 genotype was found in both clinical and environmental isolates. An additional 26 genotypes were found among the clinical isolates, and an additional 3 were found among the environmental isolates.

Analysis of the genotypes by eBURST showed that 26 genotypes clustered into 1 clonal complex (Figure 1, panel A), and 4 genotypes were singletons. The founder of a clonal complex is defined as the genotype with the largest number of single-locus variants (SLVs). In this clonal complex, the founder genotype 9-4-14-21-17 was present in 7 clinical and 2 environmental isolates. The 10 SLV genotypes diverging from the founder genotype were all clinical isolates. Of these 10 SLV genotypes, 3 (9-4-14-23-17; 9-4-14-22-17; 9-4-14-21-18) differentiated further into 9 double-locus variants. Of these, 3 double-locus variant genotypes were differentiated even further into

Table 1. Genotypes of *Vibrio cholerae* isolated from Chhatak, Bangladesh, October 2010–May 2011

Genotype	Source	No. isolates* (1/sample)	No. isolates† (9–10/sample)	Clonal complex
8-4-14-21-18	Human	7		1
8-4-14-22-18	Human	1		1
9-4-14-14-16	Human	8		1
9-4-14-14-17	Human	1		1
9-4-14-9-17	Human	1		1
9-4-14-20-17	Human	2		1
9-4-14-21-12	Human	11		1
9-4-14-21-13	Human	1		1
9-4-14-21-19	Human	1		1
9-4-14-21-18	Human	5	4	1
9-4-14-22-12	Human	2		1
9-4-14-22-16	Human	2		1
9-4-14-23-16	Human	1	1	1
9-4-14-23-17	Human	1	3	1
9-4-14-23-18	Human	5		1
9-4-14-23-19	Human	1		1
9-4-14-25-16	Human	1		1
9-4-14-25-17	Human	15	14	1
9-4-14-21-17‡	Human and environment	5 and 2	18	1
9-4-14-27-18	Environment	1		1
8-4-14-21-11	Environment	1		1
7-4-14-23-17	Human		5	1
9-4-14-13-16	Human		1	1
9-4-14-18-16	Human		9	1
9-4-14-22-18	Human		2	1
9-4-14-22-17	Human		1	1
7-9-14-14-6	Human		1	Singleton, Oct 13
11-9-14-14-17	Human	2		Singleton, Oct 21
12-8-14-15-17	Human	1		Singleton, Oct 11
10-8-14-17-18	Environment	1		Singleton, Nov 1

*Single isolates chosen from the 79 samples
†Multiple isolates from 6 patients
‡Designates the founder genotype defined by eBURST (http://eburst.mlst.net) as the genotype that differs from the largest number of genotypes at a single locus.

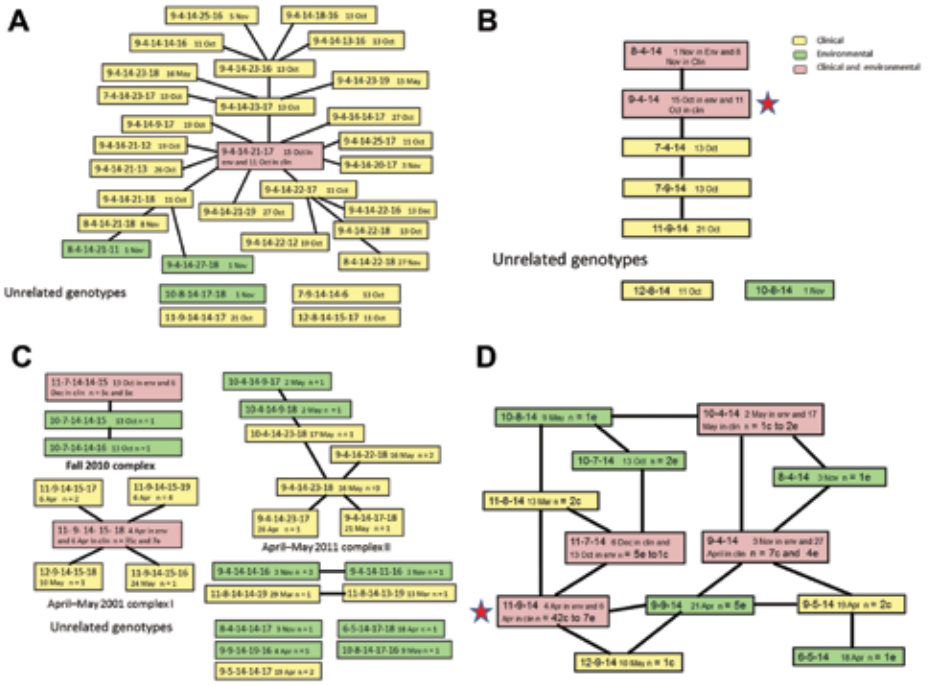

Figure 1. Genetic relatedness between *Vibrio cholerae* genotypes, Bangladesh, 2010–2011. Each genotype is identified by the number of repeats in the allele at the 5 loci VC0147, VC0437, VC1650, VCA0171, and VCA0283. The earliest date of detection is recorded in the box after the fifth allele. The background of the box indicates whether the genotype was detected in clinical isolates only (yellow), environmental isolates only (green), or both (pink). A) Clonal complex of genotypes from Chhatak, Bangladesh, and 4 unrelated genotypes based on a 5-locus genotype. B) Analysis of 3-locus genotypes in Chhatak. C) Five clonal complexes of isolates from Mathbaria, Bangladesh, and 5 unrelated genotypes based on a 5-locus genotype. D) Analysis of 3-locus genotypes from Mathbaria. Env, environmental; clin, clinical.

6 additional variants, often a variant of a locus already noted as a variant.

In any outbreak, time is a critical variable for the temporal sequence of isolations (the earliest date of detection is incorporated into Figure 1 and Figure 2, Appendix [wwwnc.cdc.gov/EID/article/20/1/13-0796-F1.htm]). The founder, and 4 related genotypes, were isolated from multiple cases on the first day, October 11, 2010. Thereafter, on October 13, an additional 6 related genotypes were isolated. The founder was recovered from the environment on October 15. The 10 SLVs were collected from October 11 through November 3. Of the 25 variants, 23 were isolated on the same day or after the genotype next closest to the founder, which is the expected result if the genotypes are derived successively from the founder. If instead of evolving during the outbreak, each genotype existed initially in the environment, then we would expect the probability of finding related genotypes before versus after the founder (or next closest to the founder) to be 0.50. However, we observed 23 of 25 after the founder (or next closest) and, if derived from a random (binomial) process, we would expect to see ≥23 with probability of 10^{-5}. Among the genotypes distant from the founder, only 2 were isolated from the environment, on November 1 and November 8, postdating the first observation of the SLV genotype closer to the founder, which was from a clinical isolate.

Most of the variation occurs in the 2 loci (the fourth and fifth of the genotype) on the small chromosome. If we analyze only the more stable loci, each isolate is described by a 3-locus genotype, and only 7 genotypes are observed. Five genotypes could be related to another genotype by a mutation at 1 locus (Figure 1, panel B), and 2 genotypes were unrelated. The 9-4-14 genotype occurred in 86% (64/74) of the clinical cases sampled.

Variation among isolates from a single fecal sample were also observed (Table 2). Of the 6 fecal samples from which multiple isolates were tested, only 1 yielded 1 genotype for all isolates, 2 yielded 2 genotypes, 2 yielded 3 genotypes, and 1 yielded 4 genotypes. For 5 of 6 fecal samples, all genotypes of the isolates were related variants; variations were seen only in loci of the small chromosome. In 1 sample, 1 isolate was unrelated to any of the other 3 genotypes observed in the other 9 isolates, evidence of multiple isolates involved in disease production. Additional evidence for multiple isolates being involved in disease is provided by the observation that 4 of the 5 genetic lineages in Chhatak were found in clinical samples.

The genetic diversity observed in these environmental and clinical isolates enables crude inference about whether there is >1 mode of transmission. If all genotypes found in the clinical isolates were randomly sampled from the 5 genetic lineages of the 5-locus genotypes, or from 1 of the 7 three-locus genotypes, this would provide evidence for a strong role for slow transmission. However, we observed that 1 lineage predominates ($p<10^{-5}$, χ^2 test, 5-locus set; $p<10^{-5}$, χ^2 test, 3-locus set) consistent with the presence of a nonrandom mode of transmission among patients in Chhatak.

Table 2. Genotypes of the 59 *Vibrio cholerae* isolates from 6 fecal samples from cholera patients, Chhatak, Bangladesh, 2010*

Patient	Collection date	No. isolates	Genotypes	Number	Relation to founder†
1	Oct 11	10	9-4-14-**25**-17	10	SLV
2	Oct 11	9	9-4-14-21-17	5	Founder
			9-4-14-**25**-17	4	SLV
3	Oct 11	10	9-4-14-21-17	7	Founder
			9-4-14-21-**18**	2	SLV
			9-4-14-**22**-17	1	SLV
4	Oct 13	10	**7**-4-14-**23**-17	5	DLV
			9-4-14-**23**-17	3	SLV
			9-4-14-**23-16**	1	DLV
			7-9-14-14-6	1	Unrelated
5	Oct 13	10	9-4-14-**18-16**	9	DLV
			9-4-14-**13-16**	1	DLV
6	Oct 13	10	9-4-14-21-17	6	Founder
			9-4-14-21-**18**	2	SLV
			9-4-14-**22-18**	2	DLV

*9 or 10 isolates/sample. Variant alleles from founder genotype are in **boldface.**
†SLV, single-locus variant; DLV, double-locus variant.

Mathbaria

Significant genetic variation was also observed among the 84 isolates collected in Mathbaria (Table 3, Simpson index = 0.26). The numbers of distinct alleles at loci VC0147, VC0437, VC1650, VCA0171, and VCA0283 were 6, 5, 1, 8, 5, respectively. A total of 24 genotypes were detected, of which 14 were in 56 clinical isolates, 12 in 28 environmental isolates, and 2 in both types of isolate. Almost twice as many genetic lineages detected in Chhatak were detected in Mathbaria: 5 clonal complexes and 5 singletons. Two clonal complexes comprised only 2 genotypes. Among the unrelated (singleton) genotypes, 4 were found only in environmental isolates and 1 was found in a clinical isolate.

In Mathbaria, *V. cholerae* O1 isolates were obtained during the last 3 months of 2010 and during an outbreak during March–May 2011. In 2010, the clonal complex comprised 3 genotypes (Figure 1, panel C). All 3 were in environmental isolates before identification in an isolate from a clinical sample in December. The founder genotype was 1 of the 2 genotypes isolated from the environment on October 13. A second clonal complex of 2 genotypes and an isolate with an unrelated genotype were also collected from the environment at that time.

The annual (seasonal) outbreak of cholera in Mathbaria occurred during March–May 2011; during this outbreak, 3 distinct clonal complexes and 4 unrelated genotypes were detected. The smallest clonal complex was detected in 2 clinical isolates in March. In the largest clonal complex (April–May complex I in Figure 1, panel C), the founder genotype was observed in isolates from the environment on April 4. On April 6, the founder genotype and 2 others were identified in clinical isolates. Over the next 48 days, the founder genotype was detected in 42 isolates (35 from clinical patients and 7 from the environment). In May 2011, another 2 genotypes from this clonal complex were found. The intermediate size clonal complex (April–May complex II in Figure 1, panel C) was first observed in an isolate from a patient on April 26 and subsequently in 2 isolates from the environment on May 2. The founder genotype was observed in 3 clinical isolates on May 16. Subsequently, 3 other SLV genotypes were observed in clinical isolates. A clear pattern of the founder being detected initially and supposedly derived genotypes being detected later was not found for this clonal complex.

When the genotypes were defined only by the 3 large chromosome loci, 12 genotypes were observed, each related to ≥1 of the other genotypes. As shown in Figure 1, panel D, a network best describes the relatedness because no a priori method exists by which to assign which mutation is more likely to have happened. Two genotypes varied by 1 mutation from 4 other genotypes, and 6 genotypes varied by 1 mutation from 3 other genotypes. In the seasonal outbreak, the 11-9-14 genotype was observed in 42 (76%) of 55 of the isolates.

The genetic lineages observed in the clinical isolates were a nonrandom sample of those found in Mathbaria. Although 5 the 10 lineages were from clinical isolates, 1 lineage was found in 43 (77%) of 56 isolates. Assuming that any genetic lineage could cause a clinical case of disease with equal probability, then the observed results are nonrandom ($p<10^{-5}$, χ^2 test, 5-locus set). For the 3-locus analysis, there were 12 genotypes, and 1 occurred in 42 of 56 clinical isolates, a significantly nonrandom distribution ($p<10^{-5}$, χ^2 test, 3-locus set) and, similar to Chhatak, an accelerated mode of transmission (i.e., human-to-human) is likely for certain genotypes.

Discussion

The results of this study show that multiple genetic lineages of *V. cholerae* occur naturally in the environment with geographic and seasonal genetic variation. The genotype patterns of the environmental and clinical isolates in the 2 rural Bangladesh communities indicate 2 things: that identical genotypes can be found in the environment and humans

Table 3. Genotypes of *Vibrio cholerae* isolated from Mathbaria, Bangladesh, October 2010–May 2011

Genotypes	Source	No. isolates	Clonal complex
9-4-14-23-18*	Human	3	1
10-4-14-23-18	Human	1	1
10-4-14-9-18	Environment	1	1
9-4-14-17-18	Human	1	1
9-4-14-22-18	Human	2	1
9-4-14-23-17	Human	1	1
10-4-14-9-17	Environment	1	1
11-9-14-15-18*	Human and environment	35 and 7	2
11-9-14-15-17	Human	2	2
11-9-14-15-16	Human	1	2
11-9-14-15-19	Human	4	2
12-9-14-15-18	Human	1	2
11-7-14-14-15	Human and environment	1 and 5	3
10-7-14-14-15*	Environment	1	3
10-7-14-14-16	Environment	1	3
9-4-14-14-16	Environment	3	4
9-4-14-11-16	Environment	1	4
11-8-14-14-19	Human	1	5
11-8-14-13-19	Human	1	5
9-5-14-14-17	Human	2	Singleton, Apr 19
6-5-14-17-18	Environment	1	Singleton, Apr 18
8-4-14-14-17	Environment	1	Singleton, Nov 3
9-9-14-19-16	Environment	5	Singleton, Apr 4
10-8-14-17-16	Environment	1	Singleton, May 9

*Founder genotype defined as the genotype that has the most single-locus variants.

and that the genotypes in humans are not a random sample of those in the environment. The result of a simple χ^2 test provides evidence that an accelerated human-to-human mode probably contributed to a process that generates genetic uniformity among clinical isolates. Whether the accelerated mode incorporates the hyperinfective state or involves massive numerical increases of a genetic lineage from the earliest cases cannot be distinguished from our analyses.

The rapid expansion of *V. cholerae* in Chhatak fits the pattern of a founder flush event (*22*). The founder flush principle asserts that rapid expansion of population size can be accompanied by relaxation of selection pressure so that genotypes otherwise not detected might be observed. This principle was applied to the presence of novel multilocus sequence genotypes in *V. cholerae* O139 (*23*). In Chhatak, after no clinical isolates were recovered in September and early October, on October 11 and 13, another 7 patients visited the clinic and 11 genotypes were observed among the isolates obtained from rectal swab samples. The expansion continued; over the next 26 days, 50 cases yielded 11 more genotypes. During the spring outbreak in Mathbaria, 2 clonal complexes exhibited additional genetic differentiation but not as dramatic as that in Chhatak. However, the outbreak in Mathbaria was smaller than that in Chhatak.

The appearance of novel MLVA genotypes that differ from the founder over time, as occurred in the Chhatak outbreak, is a microcosm of the evolution seen previously. In Kolkata, India, among *V. cholerae* O139 isolates, the founder genotype appeared shortly after the initial mutation from O1 to O139 and then mutated into multiple novel genotypes; mutations continued over the course of several years (*11*). Similarly, in Dhaka, isolates O1 Ogawa, O1 Inaba, and O139 mutated, and a clear progression of genotypes was documented from year to year (*13*). Previous studies found that differentiation occurred over several years, but in Chhatak, the differentiation occurred within 3 months.

Our analyses of the 3 first chromosome loci demonstrate that the outbreak has a dominant genotype drawn from many in the area. These large (first) chromosome loci are considered to be more stable than the small (second) chromosome loci (*13*,*21*). Although the second chromosome loci might be less useful in the context of evolution across decades (*21*), in this context, the increased resolution of local genetic linages in Chhatak reveals that many genetic variants might occur during rapid expansion, although increased variation is not an obligate part of the expansion, as indicated by the data from Mathbaria.

Multiple genotypes of *V. cholerae* were isolated from single fecal samples, as reported (*13*). However, unlike previous reports, in which only a minority (3 of 9) of samples contained isolates differing only in successive single allelic changes, in the study reported here, such samples accounted for a majority (5 of 6). Thus, whether the different genotypes are part of the same inoculum or differentiated during infection is impossible to determine. In the earlier study, 6 of the 9 fecal samples yielded isolates with unrelated genotypes (i.e., different clonal complexes). The study reported here was conducted in a rural area. Also, the clinical samples analyzed were collected during a seasonal outbreak, as opposed to ongoing infections throughout the year. Most isolates from patients in

the surrounding community were of the same clonal complex; in the previous study, they were not.

Our results provide evidence in support of an accelerated mode of transmission and for multiple strains comprising an infective dose for cholera. As shown by the clinical isolates, a single isolate does not sufficiently describe a single clinical sample; this observation should be included in future clinical studies.

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Population-based Surveillance for Bacterial Meningitis in China, September 2006–December 2009

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During September 2006–December 2009, we conducted active population and sentinel laboratory–based surveillance for bacterial meningitis pathogens, including *Streptococcus pneumoniae*, *Neisseria meningitidis, and Haemophilus influenzae* type b, in 4 China prefectures. We identified 7,876 acute meningitis and encephalitis syndrome cases, including 6,388 among prefecture residents. A total of 833 resident cases from sentinel hospitals met the World Health Organization case definition for probable bacterial meningitis; 339 of these cases were among children <5 years of age. Laboratory testing confirmed bacterial meningitis in 74 of 3,391 tested cases. The estimated annual incidence (per 100,000 population) of probable bacterial meningitis ranged from 1.84 to 2.93 for the entire population and from 6.95 to 22.30 for children <5 years old. Active surveillance with laboratory confirmation has provided a population-based estimate of the number of probable bacterial meningitis cases in China, but more complete laboratory testing is needed to better define the epidemiology of the disease in this country.

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Bacterial meningitis continues to be a major cause of illness and death in neonates and children throughout the world (*1*). The leading vaccine-preventable causes of this disease are *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* type b (Hib). The increasing availability of vaccines has made public health initiatives to control bacterial meningitis disease feasible; however, the incidence of this disease and associated deaths in resource-limited countries continue to exceed those for developed countries (*2*). Accurate pathogen-specific estimates of the number of bacterial meningitis cases are needed to monitor and refine vaccination programs, but such estimates are challenging to obtain in many countries because of limited laboratory-based surveillance capacity.

Thirty-nine infectious diseases are currently routinely reportable in China. However, among the common bacterial meningitis pathogens, only epidemic meningitis caused by *N. meningitidis* is reportable (*3*,*4*). Most reported cases of epidemic meningitis represent clinical diagnoses that lack laboratory confirmation because of the low culture rate for *N. meningitidis.*

In population-based studies, the incidence of acute bacterial meningitis in China ranges from 12.4 to 19.2 cases/100,000 children <5 years of age (*5*,*6*). Available studies suggest that the main causal pathogens of bacterial meningitis in China are *N. meningitidis*, Hib, and *S. pneumoniae* (*7*–*9*). In population-based studies in Hefei and Nanning, China, the incidence of meningitis caused by Hib was 10.66 and 0.98 cases/100, 000 children <5 years of age, respectively (*6*,*10*), and the incidence of

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S. pneumoniae meningitis was 1.5 and 1.3 cases/100,000 children <5 years of age, respectively (*6*,*11*).

In 2006, the Acute Meningitis and Encephalitis Syndrome Project was initiated in China (*12*,*13*) to determine the incidence and epidemiology of vaccine-preventable causes of meningitis and encephalitis and to improve the laboratory capacity for diagnosis of these diseases. Using data from the project's active surveillance system, we report the etiology, epidemiology, and estimated incidence of bacterial meningitis in 4 Chinese prefectures during September 2006–December 2009.

Materials and Methods

Disease Surveillance

The Acute Meningitis and Encephalitis Syndrome Project was launched in September 2006 in Jinan Prefecture, the capital of Shandong Province, and Yichang Prefecture, which is in western Hubei Province (Figure 1). In April 2007, the project areas were expanded to Shijiazhuang Prefecture, the capital of Hebei Province, and Guigang Prefecture, which is in southeast Guangxi Province. Detailed methods are reported elsewhere (*12*). In brief, 6 hospitals were selected as sentinel hospitals in each prefecture, including the largest general infectious disease and children's hospitals. Efforts were made to collect diagnostic specimens (serum and CSF) for laboratory testing from all clinical cases of meningitis and encephalitis syndrome. All other hospitals in each prefecture were designated as nonsentinel hospitals, at which epidemiologic data, but not specimens, were collected on reported acute meningitis and encephalitis syndrome (AMES) cases (*13*).

An AMES case-patient was defined as a person of any age who experienced acute onset of fever with change in mental status and/or meningeal signs (e.g., neck stiffness, headache). AMES case-patients were considered to have probable bacterial meningitis (PBM) if laboratory test results demonstrated at least 1 of the following: a turbid appearance of CSF; leukocytosis (>100 cells/mm^3; reference 0–8 cells/mm^3); or leukocytosis (10–100 cells/mm^3) and elevated level of protein (>100 mg/dL; reference 20–40 mg/dL) or decreased glucose (<40 mg/dL; reference 50–80 mg/dL). These signs are consistent with the World Health Organization (WHO) PBM case definition (*14*). Case-patients with any blood or CSF laboratory test results positive for specific bacterial pathogens were defined as confirmed bacterial meningitis case-patients.

Clinic doctors in sentinel and nonsentinel hospitals reported cases of illness meeting the AMES case definition to the county Center for Disease Control and Prevention (CDC) through passive surveillance. Public health doctors performed case investigations within 48 hours after receiving case reports from sentinel hospitals, and they collected case information, including date of birth, date of symptom onset, and initial clinical diagnosis, for cases in nonsentinel hospitals.

Active surveillance was performed every 10 days in both sentinel and nonsentinel hospitals. Public health doctors

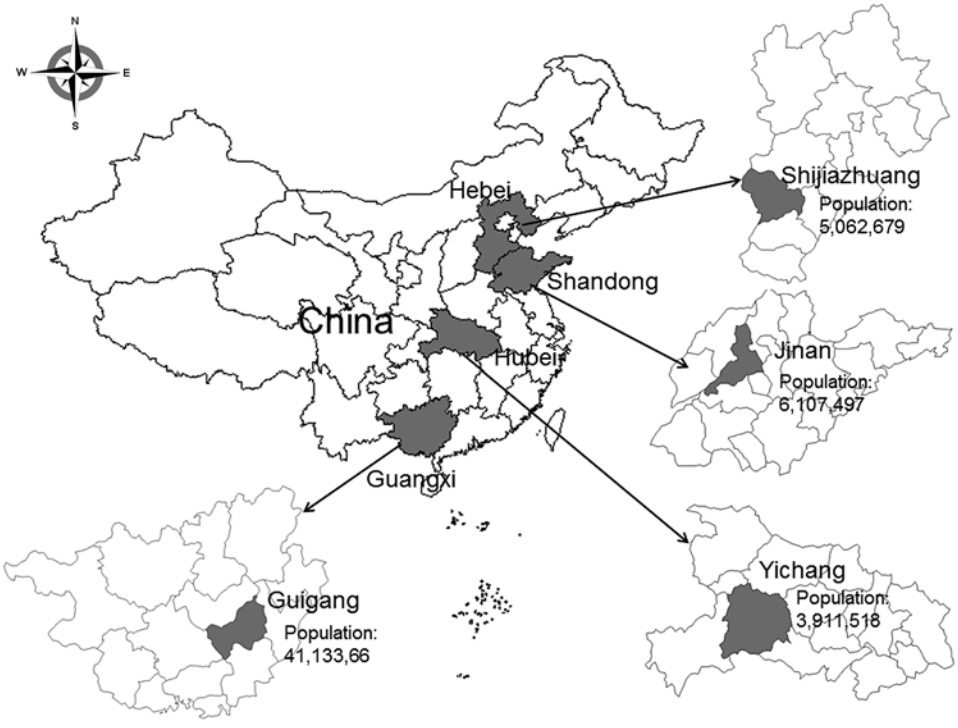

Figure 1. Location and population of 4 prefectures in China in which active population and sentinel laboratory–based surveillance for bacterial meningitis pathogens was conducted through the Acute Meningitis and Encephalitis Syndrome Surveillance System during September 2006–December 2009. The province in which each prefecture is located is indicated on the main map.

reviewed admission and discharge records in the pediatric, neurology, and infectious disease inpatient and outpatient departments and queried clinic doctors to identify and report any missed AMES cases. Computer files in sentinel hospitals were also reviewed to identify all cases with 12 specific initial diagnostic codes: Japanese encephalitis (JE), viral encephalitis, viral meningitis, viral meningoencephalitis, other encephalitis, meningococcal meningitis, purulent meningitis, cerebrospinal meningitis, TB meningitis, TB meningoencephalitis, other meningitis, and other diagnosis. The key indicators for surveillance quality were that blood and CSF specimens were obtained from >90% and 70%, respectively, of AMES case-patients.

Specimen Collection and Laboratory Testing

For this study, serum and cerebrospinal fluid (CSF) specimens were collected from patients at the sentinel hospitals as part of routine diagnostic testing. Testing performed at reference laboratories was done on de-identified specimens. CSF specimens were collected in hospitals with the patient's or parent's signed informed consent. The ethical review committee of the Chinese CDC determined that additional informed consent was not required because this study was for surveillance and used standard clinical specimens.

Physicians in sentinel hospitals collected blood (≥3 mL) and/or CSF specimens from the AMES case-patients. For CSF specimens, routine examination, including the leukocyte count and glucose and protein levels, was conducted by sentinel hospital laboratories according to the usual physicians' requests; latex agglutination, bacterial culture of CSF, and blood cultures were performed at the physician's discretion. Two kinds of latex agglutination reagents (bioMérieux, Marcy-l'Etoile, France, and Bio-Rad, Marnes-la-Coquette, France) were used according to the manufacturers' guidelines. All specimens (serum or CSF) were stored at or below –20°C and transported to prefecture CDC laboratories and, subsequently, to provincial CDC laboratories. Prefecture CDC laboratories performed JE virus IgM ELISA testing on all CSF and serum specimens (*12*).

Bacterial isolates from hospital laboratories were transported to the provincial CDC laboratories for confirmatory testing and testing for antimicrobial drug susceptibility. These bacteriology laboratories tested CSF specimens for *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* by using real-time PCR. Staff in all laboratories had received training from US Centers for Disease Control and Prevention and Chinese CDC bacteriologists. The primers have been described (*15*,*16*). Shandong and Hubei provincial CDC laboratories tested all CSF specimens that were negative for JE; other virus testing for Jinan and Yichang Prefectures was done at the Shandong and Hubei laboratories, respectively. Hebei and Guangxi provincial CDC laboratories tested all PBM case specimens according to the WHO definition noted above (*14*). A subset of specimens and isolates was forwarded to the Chinese CDC bacterial laboratory (Beijing) to confirm PCR results and to analyze the strains by using multilocus sequence typing.

Data Collection and Analysis

Demographic, clinical, and laboratory data from the sentinel hospitals were entered in an EpiData database (http://epidata.dk/). Data from nonsentinel hospitals were compiled by using standardized Excel spreadsheet software (Microsoft, Redmond, WA, USA).

To estimate the PBM incidence in the 4 prefectures, we first calculated the proportions of PBM cases among resident AMES case-patients who had CSF specimens collected in sentinel hospitals, stratified by age and initial diagnosis. We then multiplied these proportions by the number of resident cases (tested and untested in sentinel and nonsentinel hospitals), again stratified by age and initial diagnosis, and divided by the prefecture population under surveillance. The χ^2 test was used to determine significance of differences in rates and percentages.

Results

Overall Surveillance by Province

A total of 7,876 AMES cases were reported, 4,712 from sentinel hospitals and 3,164 from nonsentinel hospitals (Table 1). The percentage of residents among AMES case-patients varied by location of the prefectures and between sentinel and nonsentinel hospitals; >50% of cases in sentinel hospitals in 2 prefectures were in persons from outside the prefecture (*12*). Overall, the incidences of AMES cases for prefecture residents were similar; in general, there were ≈10 cases/100,000 residents (range 9.1 13.5 cases/100,000). However, the rate was higher among children <5 years of age, among whom AMES incidence among prefecture residents ranged from 30.8 to 96.9 cases/100,000 children (Table 1).

Demographic Features of the AMES Cases and Bacterial Testing

Among the AMES case-patients identified in sentinel hospitals, 42.2%–81.7% were children <15 years of age, but age distribution differed between the 4 prefectures (Table 2). CSF specimens were obtained from 60.0%–83.9% of case-patients in the 4 prefectures. Bacterial testing rates for cases in sentinel hospitals varied by testing method and prefecture; CSF culture, CSF PCR, and blood culture were the most frequently used methods. Use of CSF culture ranged from 20.8% in Jinan Prefecture to 53.7% in Yichang Prefecture, and use of CSF real-time PCR ranged from 13.7% in Shijiazhuang Prefecture to 60.1% in Jinan.

Table 1. Surveillance for AMES cases in 4 China prefectures, September 2006–December 2009*

Variable	Province/prefecture				Total
	Shandong/Jinan	Hubei/Yichang	Hebei/Shijiazhuang†	Guangxi/Guigang	
Total population (no. <5 y of age)	6,107,497 (353,672)	3,911,518 (236,036)	5,062,679 (314,180)	4,113,366 (261,302)	
Project duration, mo	40	40	32	32	
Total no. cases	2,647	1,631	2,093	1,505	7,876
Cases in sentinel hospitals, no. (%)	1,200	1,055	959	1,498	4,712
Residents	607 (50.6)	979 (92.8)	439 (45.8)	1,474 (98.4)	3,499 (74.3)
Nonresidents	593 (49.4)	76 (7.2)	520 (54.2)	24 (1.6)	1,213 (25.7)
Residents <5 y of age	170 (44.2)	161 (92.5)	149 (37.2)	671 (98.4)	1,151 (70.1)
Nonresidents <5 y of age	215 (55.8)	13 (7.5)	252 (62.8)	11 (1.6)	491 (29.9)
Cases in nonsentinel hospitals, no. (%)	1,447	576	1,134	7	3,164
Residents	1,241 (85.8)	574 (99.7)	1,067 (94.1)	7 (100.0)	2,889 (91.3)
Nonresidents	206 (14.2)	2 (0.3)	67 (5.9)	0 (0)	275 (8.7)
Residents <5 y of age	193 (87.7)	105 (100.0)	360 (96.0)	4 (100.0)	662 (94.0)
Nonresidents <5 y of age	27 (12.3)	0 (0)	15 (4.0)	0 (0)	42 (6.0)
Cases among prefecture residents per year/100,000 persons					
Residents	9.08	11.91	11.16	13.50	
Residents <5 y of age	30.79	33.81	60.75	96.87	

*AMES, acute meningitis and encephalitis syndrome.
†Only 13 counties in Shijiazhuang Prefecture were included in the project.

Use of any testing method for bacterial pathogens ranged from 58.7% to 79.1% in the 4 prefectures (Table 2).

Frequency of PBM and Results of Bacterial Testing

Overall in sentinel hospitals, 833/3,464 (24.0%; percentages ranged from 15.8% for Yichang Prefecture to 31.0% for Shijiazhuang Prefecture) of AMES cases with CSF specimens met the WHO definition of PBM (*14*) (Table 3); 339 (40.7%) occurred in children <5 years of age. Among the AMES case-patients, children <5 years of age were more likely than older patients to have PBM (29.1% vs. 21.5%).

The proportion of AMES cases attributed to PBM in each age group also varied by prefecture. In Yichang, only 19 (16.0%) of 119 PBM cases were in children <5 years of age. However, in other prefectures, a large proportion of cases occurred in this age group. In Jinan, 101 (35.1%) of 288 PBM cases were in children <5 years of age, and in Shijiazhuang, 118 (66.3%) of 178 PBM cases were in children <5 years of age.

PBM cases were detected each month during the study period (Figure 2), but there was a slight peak in cases during June–August. In the 2 prefectures with the most marked seasonality (Jinan and Guigang), the peaks occurred during

Table 2. Demographic features and bacterial laboratory testing for AMES case-patients in sentinel hospitals in 4 China prefectures, September 2006–December 2009*

Age, sex, and laboratory testing for case-patients	No. (%) patients by province/prefecture				Total no (%), n = 4,712
	Shandong/Jinan, n = 1,200	Hubei/Yichang n = 1,055	Hebei/Shijiazhuang, n = 959	Guangxi/Guigang, n = 1,498	
Case-patient age, y					
<2	152 (12.7)	85 (8.1)	232 (24.2)	387 (25.8)	856 (18.2)
2–4	233 (19.4)	89 (8.4)	169 (17.6)	295 (19.7)	786 (16.7)
5–14	393 (32.8)	271 (25.7)	383 (39.9)	380 (25.4)	1,427 (30.3)
15–29	170 (14.2)	189 (17.9)	86 (9.0)	110 (7.3)	555 (11.8)
30–44	135 (11.3)	197 (18.7)	33 (3.4)	88 (5.9)	453 (9.6)
≥45	117 (9.8)	224 (21.2)	56 (5.8)	238 (15.9)	635 (13.5)
Case-patient sex					
M	741 (61.8)	642 (60.9)	586 (61.1)	936 (62.5)	2,905 (61.7)
F	459 (38.3)	413 (39.1)	373 (38.9)	562 (37.5)	1,807 (38.3)
Case-patient specimen testing					
CSF examination	1,007 (83.9)	751 (71.2)	575 (60.0)	1,131 (75.5)	3,464 (73.5)
Blood, first culture	26 (2.2)	507 (48.1)	311 (32.4)	662 (44.2)	1,506 (32.0)
CSF culture	250 (20.8)	567 (53.7)	345 (36.0)	754 (50.3)	1,916 (40.7)
CSF latex agglutination	80 (6.7)	282 (26.7)	95 (9.9)	286 (19.1)	743 (15.8)
CSF real-time PCR	721 (60.1)	540 (51.2)	131 (13.7)	407 (27.2)	1,799 (38.2)
Any testing method	851 (70.9)	834 (79.1)	563 (58.7)	1,143 (76.3)	3,391 (72.0)
No. case-patients positive for Japanese encephalitis virus	136	29	20	111	296

*AMES, acute meningitis and encephalitis syndrome; CSF, cerebrospinal fluid.

Table 3. Demographic information for AMES case-patients meeting the World Health Organization definition for probable bacterial meningitis, China, September 2006–December 2009*

Demographic variable	% PMB case-patients (no. with CSF specimen/no. total), by province/prefecture				% total PBM case-patients (no. with CSF specimen/no. total), n = 3,464
	Shandong/Jinan, n = 1,007	Hubei/Yichang, n = 751	Hebei/Shijiazhuang, n = 575	Guangxi/Guigang, n = 1,131	
Total PBM patients	28.6 (288/1,007)	15.8 (119/751)	31.0 (178/575)	21.9 (248/1,131)	24.0 (833/3,464)
Case-patient age, y					
<2	28.9 (39/135)	26.8 (11/41)	47.0 (95/202)	22.0 (54/246)	31.9 (199/624)
2–4	29.5 (62/210)	16.7 (8/48)	25.0 (23/92)	24.7 (47/190)	25.9 (140/540)
5–14	30.6 (102/333)	16.1 (25/155)	20.9 (37/177)	20.8 (57/274)	23.5 (221/939)
15–29	23.3 (31/133)	14.1 (22/156)	28.1 (16/57)	20.4 (21/103)	20.0 (90/449)
30–44	28.4 (29/102)	17.1 (28/164)	19.0 (4/21)	24.7 (21/85)	22.0 (82/372)
≥45	26.6 (25/94)	13.4 (25/187)	11.5 (3/26)	20.6 (48/233)	18.7 (101/540)
Case-patient sex					
M	30.3 (191/630)	17.1 (79/463)	30.7 (107/349)	20.9 (147/705)	24.4 (524/2,147)
F	25.7 (97/377)	13.9 (40/288)	31.4 (71/226)	23.7 (101/426)	23.5 (309/1,317)
Case-patient place of residence					
Residents	31.9 (166/521)	15.4 (106/687)	25.7 (45/175)	21.6 (241/1,116)	22.3 (558/2,499)
Non-residents	25.1 (122/486)	20.3 (13/64)	33.3 (133/400)	46.7 (7/15)	28.5 (275/965)
Yearly PBM incidence/100,000 population					
All residents					
Crude rate	0.82	0.81	0.33	2.20	
Adjusted rate*	2.61	1.84	2.26	2.93	
Residents <5 y old					
Crude rate	4.16	2.16	3.22	14.21	
Adjusted rate†	8.41	6.95	14.47	22.30	

*AMES, acute meningitis and encephalitis syndrome; PBM, probable bacterial meningitis.
†Adjusted rate was calculated based on PBM positivity among resident case-patients by age and initial clinical diagnosis applied to all cases without cerebrospinal fluid specimen and profile. Two assumptions were made when adjusting the rate: (1) the subset tested is a representative specimen of AMES cases, and (2) there was a homogenous rate of infection within a given prefecture, age group, and initial clinical diagnosis stratum.

July–September and June–July, respectively, consistent with peak periods for JE cases (*12*).

The crude incidence rates of PBM ranged from 0.33 cases/100,000 persons in Shijiazhuang to 2.20 cases/100,000 persons in Guigang. After adjustment based on PBM case positivity rates by age and initial diagnosis, the estimated PBM incidence rate in the 4 prefectures was ≈2 cases/100,000 persons (range 1.84 cases/100,000 persons for Yichang to 2.93 cases/100,000 persons for Guigang). Among children <5 years of age, the estimated PBM incidence in the prefectures varied >3-fold, from 6.95 to 22.30 cases/100,000 children for Yichang and Guigang, respectively (Table 3).

Among 3,391 case-patients to have clinical specimens tested by any bacterial detection method, 74 (2.2%) were laboratory confirmed to have bacterial meningitis: 26 cases caused by *N. meningitidis*, 9 cases caused by Hib, and 39 cases caused by *S. pneumoniae*. Among confirmed cases, 18 (24.3%) were confirmed by CSF culture and 62 (83.8%) by real-time PCR. Confirmation rates by real-time PCR were higher than those by any other method (3.4% vs. 0.2%–1.3%; p<0.01) (Table 4).

AMES cases with an initial clinical diagnosis of meningococcal meningitis or purulent meningitis were more likely than cases with other initial diagnoses to meet the WHO case definition for PBM (41.7%) and to have a higher

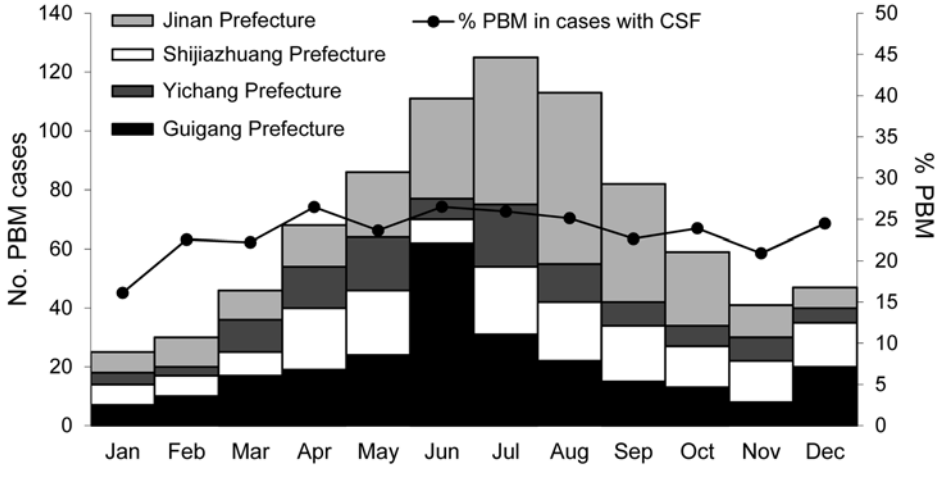

Figure 2. Number and percent of probable bacterial meningitis (PBM) cases by month among acute meningitis and encephalitis syndrome case-patients in 4 China prefectures, September 2006–December 2009.

Table 4. Bacterial testing results for specimens from AMES case-patients, China, September 2006–December 2009*

Test	No. cases positive/no. tested	% Positive	No. cases positive, by pathogen		
			Neisseria meningitidis†	*Haemophilus influenzae* type b	*Streptococcus pneumoniae*
Blood culture		0.2	3	0	0
CSF culture	3/1,506	0.9	1	5	12
Latex agglutination	18/1,916	1.3	6	1	3
Real-time PCR	10/743	3.4	25	7	30
Any method	62/1,799	2.2	26	9	39

*AMES, acute meningitis and encephalitis syndrome; CSF, cerebrospinal fluid.
†One case was serogroup A, 3 were serogroup B, and 18 were serogroup C; serogroup could not be determined for 4 cases

rate of laboratory confirmation for any 1 of the 3 bacterial pathogens (11.9%) (Table 5). Of the 74 laboratory-confirmed cases, 34 (45.9%) had an initial clinical diagnosis of meningococcal meningitis or purulent meningitis, and the remaining 40 (54.1%) cases had an initial diagnosis of some other clinical syndrome or etiology (Table 5). The proportion of laboratory-confirmed bacterial cases among the PBM cases was higher than among the other tested AMES cases (5.5% vs. 0.8%, respectively; p<0.01).

The epidemiologic features of laboratory confirmed bacterial meningitis varied by pathogen. Confirmed *N. meningitidis* cases were predominantly serogroup C (18 [69%] cases) (Table 4). Half of the confirmed *N. meningitidis* case-patients were <15 years of age. Confirmed *S. pneumoniae* cases were present in each age group, but predominately among children <2 years of age (12 [30.8%] cases) and in persons >45 years of age (8 [20.5%] cases). The confirmed Hib cases were all in children <2 years of age. Among children in this age group, *S. pneumoniae* was the predominant pathogen (12 cases) followed by Hib (9 cases) and *N. meningitidis* (3 cases).

The 74 confirmed bacterial meningitis cases occurred across all months; however, 22 (85%) *N. meningitidis* cases occurred in the first half of the year. Hib and *S. pneumoniae* cases occurred in all seasons.

Discussion

During September 2006–December 2009, we identified >7,000 AMES cases in 4 China prefectures. The overall incidence was ≈10 cases/100,000 residents, and the estimated PBM incidence was ≈2 cases/100,000 persons for the entire population and 7 22 cases/100,000 children for residents <5 years of age. Using a variety of laboratory testing methods, we confirmed 74 bacterial meningitis cases among the AMES cases; real-time PCR had the highest positivity rate among the methods used for pathogen testing.

N. meningitidis, *S. pneumoniae*, Hib, and JE virus are serious but vaccine-preventable causes of meningitis and encephalitis in China. Surveillance for bacterial meningitis and viral encephalitis has previously been conducted separately, and only JE and *N. meningitidis* (epidemic meningitis) are reportable diseases (*12*). In our study, CSF specimens were obtained from 73.5% of AMES case-patients in sentinel hospitals in the 4 prefectures. This percentage exceeds the surveillance indicator for CSF specimen collection (i.e., 70% among all AMES cases) and shows the feasibility of using active and passive surveillance with sentinel laboratory testing, as used in our study population, to detect bacterial meningitis.

Table 5. Initial clinical diagnoses of AMES and probable bacterial meningitis cases in sentinel hospitals in 4 China prefectures, September 2006–December 2009*

Initial diagnosis	No. (%) AMES cases	No. AMES cases with CSF specimen/no. (%) meeting WHO definition of PBM	No. AMES specimens tested/no. (%) confirmed positive for any bacteria
Meningococcal meningitis	19 (0.4)	13/6 (46.2)	12/5 (41.7)
Purulent meningitis	280 (5.9)	255/156 (61.2)	244/29 (11.9)
TB meningitis	149 (3.2)	132/55 (41.7)	114/1 (0.9)
TB meningoencephalitis	48 (1.0)	33/17 (51.5)	31/0
Japanese encephalitis	142 (3.0)	102/29 (28.4)	110/0
Viral encephalitis	1,631 (34.6)	1,173/221 (18.8)	1,146/12 (1.0)
Viral meningitis	231 (4.9)	179/38 (21.2)	170/3 (1.8)
Viral meningoencephalitis	181 (3.8)	137/31 (22.6)	135/2 (1.5)
Other encephalitis	1,201 (25.5)	842/155 (18.4)	814/12 (1.5)
Other meningitis	70 (1.5)	57/14 (24.6)	55/0
Cerebrospinal meningitis	10 (0.2)	8/2 (25.0)	8/1 (12.5)
Other diagnosis†	731 (15.5)	526/109 (20.7)	539/9 (1.70)
Data missing	19 (0.4)	7/0	13/0
Total	4,712 (100)	3,464/833 (24.0)	3,391/74 (2.2)

*AMES, acute meningitis and encephalitis syndrome; CSF, cerebrospinal fluid; WHO, World Health Organization; PBM, probable bacterial meningitis; TB, tuberculosis.
†Other diagnosis were cases that met the AMES case definition (e.g., suspected CNS infection and high fever and convulsion with unknown reason) but that were not included among the 11 specific initial diagnosis listed above.

PBM cases were reported during all months in each of the 4 prefectures, but there was a slight peak during June–August in Jinan and Guigang Prefectures, locations that had most of the confirmed cases of JE during the JE epidemic season (*12*). The percentage of PBM cases among the AMES case-patients with CSF specimens varied substantially by age group among the 4 prefectures. These differences may be due to several factors, including variations in ecologies, population characteristics, and the use of vaccines. In 1986, Shandong Province integrated meningococcal polysaccharide A vaccine into its provincial Expanded Program on Immunization and since then has achieved systematic vaccination of children. The Hib conjugate vaccine was available for children in the 4 prefectures studied, but its use was generally limited to urban areas, and parents had to pay for the vaccine. *S. pneumoniae* vaccine was introduced in China at the end of 2008, but its use is limited because the vaccine price is extremely high (*17*).

We estimated that, during September 2006–December 2009, the annual incidence of PBM among the population of the 4 prefectures studied was ≈2 cases/100,000 persons. For children <5 years of age, the estimated incidence (6.95 22.3 cases/100,000 children) was lower than that for Poland and Bulgaria before Hib vaccine was introduced (29 and 35 cases/100,000 children, respectively) (*18*,*19*). Our estimated incidence among children <5 years of age was also lower than that for Mongolia (68 cases/100,000 children 2–5 years of age) and Korea (91 cases/100,000 children <5 years of age) (*20*,*21*), but the incidence in our study was similar to that found in other studies in China (*5*,*6*) and other Asian countries, such as Thailand (27 cases/100,000 children <5 years of age) (*22*). The lower incidence among children <5 year of age in our study may be due to regional factors, wide use of *N. meningitidis* vaccine, and moderate use of the Hib vaccine in the 4 prefectures.

N. meningitidis, Hib, and *S. pneumoniae* are the most common causes of acute bacterial meningitis; most studies indicate that these pathogens are responsible for >75% of all cases of bacterial meningitis overall and for 90% of cases in children (*23*). In our study, the relative proportion of cases caused by *N. meningitidis* (35.1%) was lower than that in a previous study in China (48.4% in Beijing) (*7*), and the proportion of cases caused by *S. pneumoniae* (52.7%) was relatively higher. For children <2 years of age, the predominant pathogens causing bacterial meningitis were *S. pneumoniae* and Hib. The lower proportion of *N. meningitidis*–related meningitis cases among young children may be the result of the wide use of the meningococcal polysaccharide vaccine in this age group.

By comparing the initial clinical diagnoses in our study with the bacterial meningitis laboratory results, we found that some clinical diagnoses (e.g., meningococcal meningitis and purulent meningitis) were most likely to represent cases with a confirmed bacterial cause. However, more than half of laboratory-confirmed bacterial meningitis cases had other initial clinical diagnoses. These findings point to a need to conduct sensitive laboratory testing for all suspected AMES case-patients, particularly for those meeting the WHO PBM case definition (*14*). Moreover, 12.2% of the laboratory-confirmed bacterial meningitis cases initially had another diagnosis, indicating the challenges posed trying to encompass all clinical diagnoses relevant for syndromic surveillance of bacterial meningitis.

Overall, the percentage of AMES cases confirmed as caused by *N. meningitidis*, *S. pneumoniae*, or Hib (2.2% of all tested cases and 9.0% of PBM cases) was substantially lower than the percentage in other studies (25% in Bangladesh for bacterial meningitis, 44.6% in Yemen for the acute meningitis) (*24*,*25*). The factors leading to low positive rates for bacterial meningitis were complex. One key factor is the indiscriminate use of antimicrobial drugs in clinical practice and by patients in China (*11*): 62% of all AMES case-patients and 66% of PBM case-patients in our study had used antimicrobial drugs before clinical specimens were collected. In addition, some case-patients were referred from smaller hospitals where conditions for specimen collection, storage, and transport may have also affected testing results. Other contributing factors were the enrollment of all clinically diagnosed meningitis and encephalitis case-patients, which likely included a wider range of viral pathogens, particularly JE, among cases that met the WHO PBM case definition, as well as some illnesses that were ultimately determined to not be of infectious etiology.

Among the laboratory methods we used for bacterial testing, real-time PCR had a substantially higher detection rate than other assays. This finding suggests the value of using real-time PCR to confirm suspected bacterial meningitis cases. Increased use of PCR will require making such tests more widely available in hospitals and public health laboratories in China and other countries attempting to evaluate bacterial causes of meningitis.

This study has several limitations. First, not all CSF specimens were tested by commonly used diagnostic methods (blood and CSF culture) in hospitals or by real-time PCR in the 4 provincial CDC laboratories. Testing in hospital clinical laboratories in China is done at the discretion of treating physicians and is often not done because of the limited ability of patients to pay for testing. In 2 provinces, only CSF specimens from patients with PBM were tested by PCR. Hence, our study likely missed many bacterial meningitis cases, particularly among case-patients whose illnesses did not meet the PBM case definition. Second, because of limited laboratory testing in nonsentinel hospitals, we relied on adjustment by clinical diagnosis to calculate estimated rates of PBM; inconsistent clinical diagnosis of nontested cases may have caused inaccuracies in these estimates.

Last, because laboratory testing had low positivity rates for confirmed bacterial meningitis, we did not find enough confirmed cases to derive accurate incidence rates for each pathogen, thus limiting the generalizability of these data.

Despite the limitations, this project has provided useful insights into the incidence and epidemiology of bacterial meningitis in China. It is clear that active surveillance linked to laboratory confirmation is critical to estimate the number of bacterial meningitis cases. On the basis of our findings, we suggest that bacterial meningitis surveillance in China should be enhanced in several ways. First, wider use of appropriate specimen collection and handling is needed, along with laboratory testing to confirm the causative pathogens, especially for cases in young children. In the study setting, this enhanced surveillance should include ensuring appropriate specimen collection in all cases and not charging hospital patients for testing. These enhancements will require better training of physicians and hospital staff and more rigorous quality control regarding specimen collection. Second, real-time PCR is the most sensitive bacterial testing method and should be used in the sentinel sites. These efforts will help to improve active surveillance so that disease incidence and pathogen-specific etiologies for bacterial meningitis can be more accurately determined by age group and used to develop recommendations for the use of meningococcal conjugate vaccines and to determine the need for routine Hib and *S. pneumoniae* vaccinations for young children in China.

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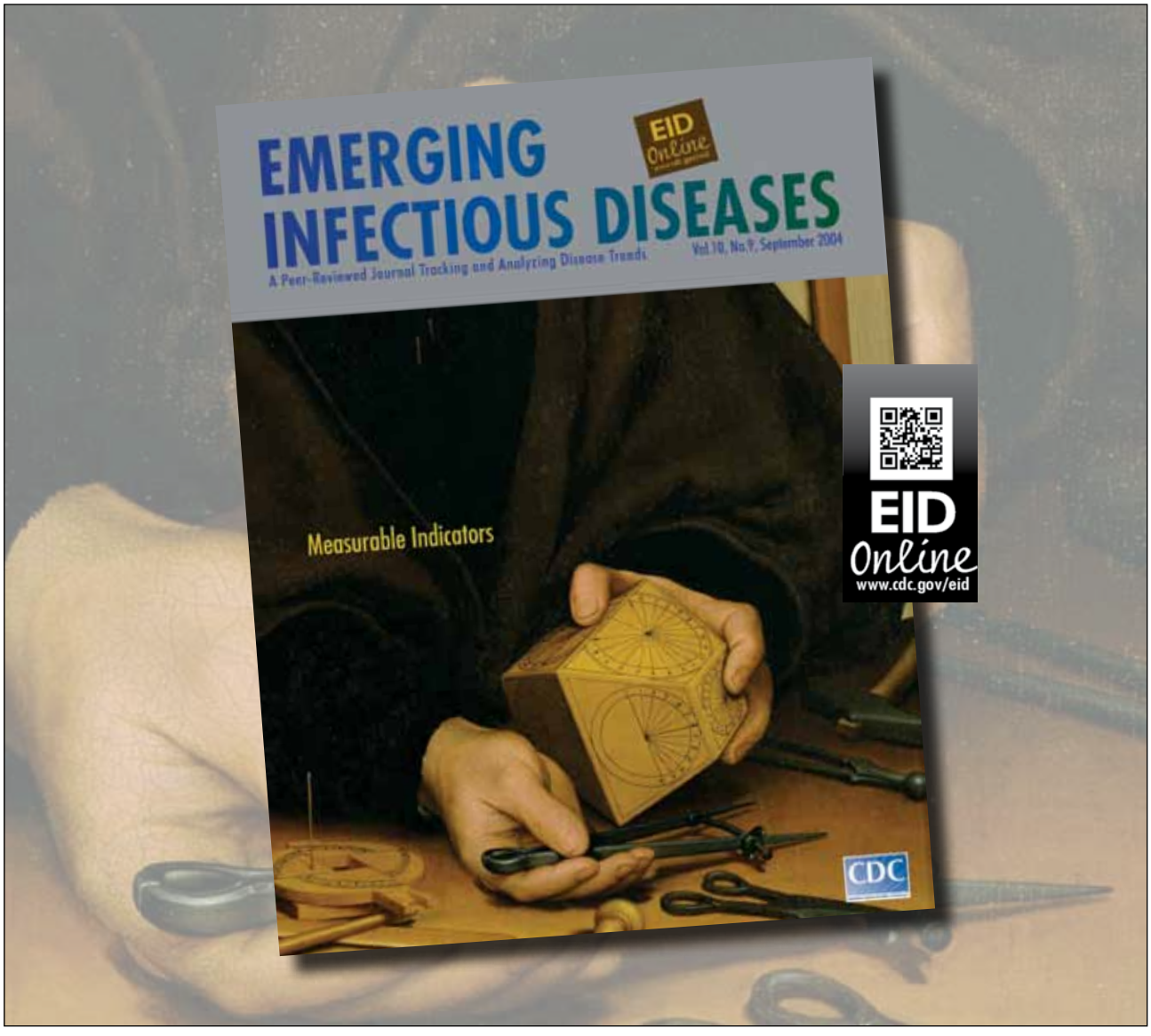

Effects of Drinking-Water Filtration on *Cryptosporidium* Seroepidemiology, Scotland

Colin N. Ramsay, Adam P. Wagner, Chris Robertson, Huw V. Smith, and Kevin G.J. Pollock

Continuous exposure to low levels of *Cryptosporidium* oocysts is associated with production of protective antibodies. We investigated prevalence of antibodies against the 27-kDa *Cryptosporidium* oocyst antigen among blood donors in 2 areas of Scotland supplied by drinking water from different sources with different filtration standards: Glasgow (not filtered) and Dundee (filtered). During 2006–2009, seroprevalence and risk factor data were collected; this period includes 2007, when enhanced filtration was introduced to the Glasgow supply. A serologic response to the 27-kDa antigen was found for ≈75% of donors in the 2 cohorts combined. Mixed regression modeling indicated a 32% step-change reduction in seroprevalence of antibodies against *Cryptosporidium* among persons in the Glasgow area, which was associated with introduction of enhanced filtration treatment. Removal of *Cryptosporidium* oocysts from water reduces the risk for waterborne exposure, sporadic infections, and outbreaks. Paradoxically, however, oocyst removal might lower immunity and increase the risk for infection from other sources.

Each year since 2005, Health Protection Scotland has received reports of 500–700 laboratory-confirmed cases of cryptosporidiosis (10–14 cases/100,000 population/year); seasonality is usually markedly biphasic, peaking in spring and early autumn. Cryptosporidiosis is caused by ≥1 species/genotypes of the protozoan parasite in the genus *Cryptosporidium*, which infects a wide variety of animals including humans. The most common human pathogens are *Cryptosporidium hominis* and *C. parvum*. Characteristic signs of infection are profuse, watery diarrhea, often accompanied by bloating, abdominal pain, and nausea or vomiting. Illness is typically self-limiting but can last for 2–3 weeks; studies suggest an association with long-term health sequelae, such as reactive arthritis and postinfection irritable bowel syndrome (*1*,*2*). Moreover, severe, chronic diarrhea or even life-threatening wasting and malabsorption can develop in persons with severely compromised immune systems, particularly those with reduced T-lymphocyte counts, in the absence of immunotherapy (*3*).

Drinking water contaminated with *Cryptosporidium* oocysts is a recognized risk factor for human illness (*4*–*6*). Before or after treatment, water can be contaminated by a variety of sources, including livestock, feral animals, or humans (*7*). Oocysts can remain infectious in the environment for prolonged periods and are resistant to regular drinking-water disinfection treatments. For preventing human exposure, oocysts must be physically removed from water supplies; however, inadequate water filtration can expose persons to risk for infection from viable oocysts (*8*–*11*).

Where drinking-water filtration has been enhanced to reduce oocysts counts, the incidence of reported clinical *Cryptosporidium* infection has been reduced (*6*,*11*). However, reported rates of infection are subject to variation, depending on factors such as local laboratory testing criteria, and exposure source attribution depends on the quality of risk-factor exposure data. Therefore, assessing trends in clinical infection rates might not be sufficiently sensitive for detecting changes in single-exposure risks. Variations in other risk factors (e.g., foreign travel, direct animal contact) can also obscure an effect associated with reduced exposure to oocysts in drinking water. Assessment of the effects of changes in environmental oocyst exposure would ideally be based on measuring population-level indicators, rather than relying on reported (self-selected) cases of laboratory-confirmed cryptosporidiosis. Alternatively, longitudinal variation in levels of antibody

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to *Cryptosporidium* oocyst proteins could be used to detect associations with variations in oocyst exposure.

The association between seropositivity and exposure to *Cryptosporidium* oocysts in drinking water has been investigated. Low levels of oocysts have been detected in 65%–97% of surface-water supplies, suggesting that many populations may be at risk (*12*). Elevated serologic responses have been detected in those whose drinking-water source is surface water rather than groundwater. The risk for oocyst exposure might therefore be higher for surface water than for groundwater sources (*13*–*15*), even after conventional filtration (*13*). However, chronic low-level exposure to oocysts in environmental sources, including drinking water, can stimulate protective immunity. Strong serologic responses to oocyst antigens have been associated with such environmental exposures (*16*,*17*).

To decrease the risk for waterborne *Cryptosporidium* infection from drinking-water supplies, the water industry established several barrier water treatment systems. In Scotland, water treatment has significantly reduced the concentration of *Cryptosporidium* oocysts in final (posttreatment) tap water. Before September 2007, however, the Loch Katrine system, which supplies the towns of Glasgow and Clyde, did not have such a filtration treatment. The risk from drinking unfiltered water was demonstrated in 2000, when an outbreak of cryptosporidiosis occurred among Glasgow residents living within the Loch Katrine supply area (*18*). To decrease this risk, in September 2007, enhanced treatment (rapid gravity filtration and coagulation) was introduced to the Loch Katrine supply system. This new system provided an opportunity to assess the public health effects of improving the standard of water filtration.

We investigated the prevalence of antibodies to the 27-kDa *Cryptosporidium* oocyst antigen among residents living in the Loch Katrine supply area (Glasgow) before and after the introduction of filtration and compared these with levels in a control population (Dundee) where no such change to drinking-water treatment occurred. Our main objective was to determine whether an association exists between prevalence of antibody response to the 27-kDa antigen and the standard of drinking-water treatment (filtered vs. unfiltered).

Methods

Study Sites and Populations

The study received approval from the Multi-Research Ethics Committee for Scotland and was conducted from April 2006 through October 2008. Volunteer blood donors were recruited in Glasgow (population 580,000; western Scotland) and Dundee (population 142,000; eastern Scotland). Each area receives drinking water from a separate surface-sourced system. Before September 2007, the Glasgow water supply received only rough screening treatment, but after that date, it was upgraded to match the specifications at Dundee (Clatto reservoir), the control system, which received rapid gravity filtration and coagulation. During the study period, no significant changes were made to the Dundee water treatment system, and no waterborne or other outbreaks of cryptosporidiosis were reported for either location. Blood samples for this study were collected during 4 periods (matched for seasonality): April–July 2006 (period 1), August–October 2006 (period 2), April–July 2008 (period 3), and August–October 2008 (period 4).

Waterborne Oocyst Data

Potential exposure to waterborne oocysts was assessed by examining data on oocyst counts, which was routinely collected from the respective supplies for regulatory purposes. To reduce sampling bias, large sample volumes (≈1,000 L), collected before and after filtration, were used for oocyst detection. Filtamax filters (Genera Technologies, Newmarket, UK) were used for posttreatment water sampling; Cuno (3M, Bracknell, UK) filters were used for pretreatment water sampling because of their higher turbidity.

Blood Sample Collection and Donor Questionnaires

Blood donors from Glasgow and Dundee were informed of the aims of the study and asked to consent to participate for the duration of the project. At each blood donation session attended during the study period, volunteers were asked to confirm their agreement to allow a sample to be used for the study and to provide information about recent known exposure to potential risk factors for *Cryptosporidium* infection. Blood samples (1 mL) were sent in heparinized containers to the Scottish Parasite Diagnostic Laboratory for analysis.

Western Blot

Serum samples were analyzed by using immunoblot (mini-format) to measure IgG response to the 27-kDa antigen. The analytical methods are described elsewhere (*14*,*16*,*17*). However, because locally available human serum for use as positive control was insufficient, the positive control was derived from serum from a rabbit that had been immunized with a soluble lysate of *C. parvum* in Freund's complete adjuvant.

Before each blood collection period, fresh rabbit control serum was prepared, aliquoted, and frozen. Serum samples (Dundee and Glasgow) from each collection period were tested in the same set of test runs, which enabled comparison of potential differences between the 2 geographic locations. Oocysts (Iowa isolate) were imported from the University of Arizona (Tucson, AZ, USA). The intensity of the serologic response to the antigen was digitally analyzed by using a Gel Doc 2000 Imaging System

(Bio-Rad, Hercules, CA, USA). The intensity of each band was standardized by comparing the response intensity of each serum sample against a positive control (expressed as percentage positive response [PPR]). PPR standardization was performed by comparing the intensity of the study serum band with that of the positive control band from the same blot.

Statistical Methods

To achieve a power of 95% for detecting a difference of at least 10% in seropositivity between prefiltration and postfiltration blood samples in the Glasgow cohort, 700 donors were required from Glasgow and 290 from Dundee. These numbers were based on results of the McNemar test and an assumption that ≈30% of the prefiltration samples would be seropositive. According to results of a standard normal test for differences in proportions, the power to detect a seropositivity difference between the cohorts of at least 10% would be >90%.

Several statistical methods, including univariate (χ^2 or Fisher exact test) and multivariate techniques, simultaneously considered several explanatory variables and serologic values. The type of multivariate regression model used was dictated by the distribution of the response variables: linear regression for continuous (PPR) and logistic regression for binary (positive/negative) responses.

Each participant should have had serologic results from 4 periods, and results were expected to correlate with each other. To enable repeated observations from the same participants over time, we used mixed-effect regression models. These methods maximized the data at each period and accounted for new participants recruited to replace those lost to attrition.

Measurement of Seropositivity

Antibody levels in immunized rabbits are probably higher than those in humans exposed to low levels of oocysts in the environment. Seropositivity was measured relative to the positive (rabbit serum) control (0 to ≥100% PPR). Some of the analyses used the actual PPR measurements; others required a binary measure (positive/negative response). For the latter analyses, a PPR level had to be selected to designate what constituted a positive response. Seropositivity can therefore vary, depending on the cutoff threshold value used to compare with the positive control.

Other studies of *Cryptosporidium* antibody seroprevalence that used serum from human patients rather than immunized rabbits have used a 20% cutoff threshold to designate positivity (*13*–*15*,*19*). In such studies, *Cryptosporidium* antibody seroprevalence has been 48%–76% of the study populations. Because our study used 20% PPR as the cutoff threshold, overall prevalence of antibodies against *Cryptosporidium* was similar for the entire study cohort (75%). Had the cutoff been increased to 30% PPR, then 64% of the entire cohort would have been designated as having a positive serologic response.

The choice of PPR cutoff threshold affected our ability to detect significant changes in the proportion of each cohort who were antibody positive. To determine what effect different PPR cutoffs would have on the analyses, we conducted a sensitivity analysis; as the PPR cutoff value increased, the chance of detecting a significant difference also increased.

Multivariate Analysis of Risk Factors and Serologic Responses

Logistic models were fitted to the logistic response by dichotomizing the response at different PPR cutoff values; those >20% were designated as positive. Within the logistic models, we fitted a series of contrasts, which enabled us to test differences in serologic responses linked to differences in risk factor exposures between the cohorts and between collection periods. From the 8 main observation categories (2 cities and 4 periods), 7 comparisons (contrasts) were generated and used to assess variations in the proportions of persons with a positive serologic response. The main study hypothesis was that the introduction of filtration to the Glasgow supply would be associated with a change in seroprevalence levels in the Glasgow cohort. The prefiltration/postfiltration effect in Glasgow, compared with this effect in Dundee, was the major comparison used in the sensitivity analysis for choosing the optimal PPR cutoff for the main statistical analyses. Analyses of logarithmically transformed PPRs were used to identify relative differences across collection periods and city cohorts. We also compared the exposure risk profiles of those who had no (0) detectable responses with those who had some (>0) serologic responses by using χ^2 tests.

Unless otherwise stated, we used R software version 2.80 (www.R-project.org) for the statistical analysis and Minitab statistical software version 14 (www.minitab.com) for information collation. In general, a significance level of 5% ($p<0.05$) was used for all analyses. However, because repeated tests were conducted for the same variable (the serologic response), the level used to assess statistical significance was corrected by using the Bonferroni correction. Because there were 31 demographic risk factor questions, a corrected significance level of 0.0016 (0.05/31), rather than the standard 0.05, was used.

Results

Study Participants

The original cohort consisted of 791 blood donors from Glasgow and 260 from Dundee. However, not all the original participants remained in the study, and some were

replaced by new recruits (Table 1). The total number of study participants was therefore 1,437. Some participants did not donate blood during all 4 collection periods; others donated ≥1 time during some collection periods.

Oocyst Counts

Before September 2007, the oocyst detection rate in Clatto (final water) averaged 4.4×10^{-4} oocysts per 10 L (Table 2). During this period, consumers of Loch Katrine water were exposed to 13.2×10^{-4} oocysts per 10 L, which is 3 times higher. Therefore, the risk for waterborne infection was potentially greater for Glasgow residents. After filtration was introduced at Loch Katrine, the oocyst count decreased to zero in final water, representing complete removal of waterborne oocysts. During this period, consumers of Clatto water were also exposed to fewer oocytes (average 1.15×10^{-4} oocysts/10 L, reduced from the original 4.4×10^{-4} oocysts/10 L). Thus, both cohorts were exposed to reduced oocyst contamination in the drinking water, but the magnitude of reduction was greater for the Glasgow cohort (100% reduction) than for the Dundee cohort (75%). Speciated oocysts were *C. parvum, C. bovis, C. ubiquitum,* and other environmental genotypes. However, *C. hominis* was never isolated from either drinking water supply during the study period.

Questionnaire Responses

Few statistically significant differences in demographic or exposure risk factor variables were found between the 2 cohorts (Table 3). The relative similarity in demographic and risk factor profiles between the Glasgow and Dundee cohorts indicated that the populations were comparable.

During the first period, more Glasgow participants consumed bottled water and fewer consumed unboiled drinking water than did their Dundee counterparts ($p<0.001$). Fewer participants in each cohort reported swimming after September 2007 than before ($p = 0.011$ and $p = 0.015$, respectively).

Serologic Responses

According to univariate analyses, the only significant difference was that participants who had no (0) antibody response were significantly younger ($p<0.001$) than those with a detectable (>0) serologic response. The importance of age to participant serologic response was also assessed by using a linear model fitted to the square root of the serologic response (to normalize the distribution). In this model, age was associated with a statistically significant difference in the serologic response ($p<0.0001$); for each additional year of age, the serologic response increased by ≈0.35%.

In this study, serologic response to *Cryptosporidium* oocysts was positive for 75% of participants in both cohorts. The mean serologic response showed an overall increase over the 3-year period, as measured by the proportion with a serologic response (positive/negative, logistic model) and the average PPR (linear model) in both cohorts (Figure). Both cohorts showed an overall increase over the whole 3-year period, as measured by the proportion with a serologic response (positive/negative, logistic model) and by the average PPR (linear model) (Figure). For periods 1 and 2, the responses for the Glasgow cohort were higher than those for Dundee. However, for the Glasgow cohort, the average serologic response (PPR) for period 3 dropped below that for period 2. This finding coincided with the introduction of enhanced filtration treatment at Loch Katrine in September 2007. The Glasgow response during period 3 was also lower than the Dundee response during period 3. Although for period 4, the average serologic response among participants in both cohorts again increased, the mean response for Glasgow remained lower than that for Dundee.

Further analysis of the data, with the second linear mixed effects model fitted to the log of the PPR, indicated that mean serologic responses for Glasgow participants decreased by 32%, compared with those for Dundee participants, after enhanced filtration began at Loch Katrine (Table 4). This step-change reduction was statistically associated with introduction of the new treatment.

The mixed regression analysis of other risk factors for exposure to *Cryptosporidium* oocysts identified 4 positive findings, although none was statistically significant (by Bonferroni correction). Average serologic response to *Cryptosporidium* oocyst antigen was lower among participants who owned pets than among those who did not ($p = 0.034$), higher among those who had been swimming in the United Kingdom ($p = 0.091$), higher among those who consumed water from private supplies ($p = 0.087$), and lower among those who drank bottled water ($p = 0.051$).

Table 1. Participants in study of antibodies against *Cryptosporidium* in drinking water, Scotland, UK, 2006–2008

	Study period			
Participants	1 (2006 Apr–Jul)	2 (2006 Aug–Oct)	3 (2008 Apr–Jul)	4 (2008 Aug–Oct)
Previous participants*	1,051	750	608	911
New recruits	0	133	253	0
Total	1,051	883	861	911
By city				
Glasgow	791	671	638	698
Dundee	260	212	223	213

*Donated blood at least 1 time since period 1.

Table 2. Concentration of oocysts in drinking water before and after September 2007 installation of filtration system in Glasgow, Scotland, UK

Location, period	Concentration of oocysts/10 L		Reduction, %
	Raw	Final	
Dundee			
Before	0.000850	0.000439	48
After	0.001979	0.000115	94
Glasgow			
Before	0.004219	0.001320	69
After	0.000855	0.000000	100

Using the interactions model, we investigated the effect of introducing filtration in Glasgow and how this was associated with the sources of water consumed. The coefficients of all of these potential interactions were negative, providing evidence that enhanced filtration at Loch Katrine reduced serologic responses among all participants in Glasgow, regardless whether they drank only unboiled tap water, only bottled water, or both. Serologic response reduction was largest among participants who consumed any bottled water (either solely or in combination with unboiled tap water). However linear, quadratic, and cubic analyses indicated no evidence of a direct statistically significant association between the reported quantity of bottled water consumed and the reduced serologic response (i.e., no dose–response effect).

Discussion

Opportunities to study the public health effects of major infrastructure changes are uncommon. Blood donors provide a convenient sample; they are relatively healthy, accessible, and generally cooperative. However, because they are predominantly younger to middle-aged adults, they are not completely representative of the population. Hence, the results of this study might be less applicable to children or elderly persons.

In this survey, serologic response to the 27-kDa *Cryptosporidium* oocyst protein was detected in 64%–75% of the total cohort. These findings are consistent with those from other populations served by surface-water sources (*14*,*19*). However, the aspects of this study that advance previous knowledge are as follows: the study involved a large number of participants in distinct cohorts followed over a considerable period, there was a defined intervention in one population but not the control population, and we sought to collect data on demographics and changes to biologically plausible risk factor exposures each time a participant donated a blood sample. The prospective cohort study design is a relatively robust epidemiologic method; high participant numbers enabled robust statistical analyses.

We were primarily interested in evidence of serologic response to oocyst exposure spanning a long period, as opposed to recent, acute exposure. Other serologic studies have assessed IgG serologic responses to the 15/17-kDa complex and the 27-kDa protein (*13–15*,*17*,*19*). After exposure to *Cryptosporidium* oocysts, a serologic response to both of these antigen groups usually peaks 4–6 weeks later (*20*). The 15/17-kDa marker declines to baseline levels in 4–6 months, but the 27-kDa marker remains elevated for at least 6–12 months. Because the 27-kDa response is considered a reliable marker for exposure to *Cryptosporidium* oocysts, we do not believe that our not investigating serologic responses to the 15/17-kDa complex devalues our findings.

The Western blot method compared each serum sample with a positive control. Ideally, the positive control serum would have been derived from clinically ill persons, but because we did not have access to enough such serum to compare with >3,700 participant samples, we used rabbit-derived positive control serum. Although this form of calibration is recognized as acceptable for serologic studies, it is a possible limitation to this study despite the fact that our primary focus was detecting evidence of low level oocyst exposure, not confirming a clinical diagnosis of infection.

By using several statistical models, we detected a marked and dramatic step-change reduction in the seroprevalence of antibodies against *Cryptosporidium* among blood donors from the Loch Katrine water supply area after introduction of enhanced water filtration. We detected no corresponding step-change in seroprevalence among the control (Dundee) participants. The collated evidence suggests that

Table 3. Demographic characteristics of participants in study of antibodies against *Cryptosporidium* in drinking water, Scotland, UK, 2006–2008*

Characteristic	Location, water filtration status			
	Dundee		Glasgow	
	Before	After	Before	After
Mean age, y	42.3 (40.8–43.9)	45.5 (43.8–47.2)	38.8 (37.9–39.6)	42.7 (41.7–43.6)
Female, %	44.2 (38.3–50.3)	45.2 (38.7–52)	47.1 (43.9–50.3)	43.1 (39.6–46.8)
Swam in past 12 mo, %	64.6 (58.6–70.2)†	48.3 (41.6–55.1)‡	58.4 (55.1–61.5)†	50.1 (46.4–53.7)‡
Have with <5 children, %	8.5 (5.7–12.5)†	9.5 (6.2–14.3)‡	7.4 (5.9–9.2)†	7.5 (5.8–9.7)‡
Have pets, %	43.8 (37.9–49.9)†	38.8 (32.4–45.5)‡	32.3 (29.3–35.4)†	30.8 (27.5–34.3)‡
Drink unboiled water, %	96.2 (93.1–97.9)†	95.2 (91.4–97.4)‡	89.2 (87.1–91.1)†	88.4 (85.8–90.5)‡
Drink bottled water, %	69.2 (63.4–74.5)[1]	63.2 (56.4–69.4) ‡	77.4 (74.6–80)†	73.7 (70.3–76.8)‡

*Before, before filtration in Glasgow (periods 1 and 2); after, after filtration in Glasgow (periods 3 and 4).
†Percentage of persons who answered "yes" to the question at period 1, period 2, or both.
‡Percentage of persons who answered "yes" to the question at period 3, period 4, or both.

this effect was mainly, if not solely, attributable to the introduction of filtration to the Loch Katrine water supply.

Given the fact that the serologic response levels (PPR) continued to increase after the oocysts were eliminated from the Loch Katrine water source, this study supports evidence that oocysts from other environmental sources stimulate background immunity levels. Exposure to oocysts through other sources (e.g., animal contact and contaminated food) might be at least as common as exposure through contaminated drinking water and might be more likely to transmit a higher dose of oocysts.

The study demonstrated that serologic responses to *Cryptosporidium* oocyst exposure were more likely (although not statistically significantly) to be higher among participants who ingested water while swimming in an indoor swimming pool or drank tap water from a private supply than among those who did not. The protective effect of antibody levels induced by such exposures is unknown; clinical cases of cryptosporidiosis have also been associated with swimming pools and private water supplies (*21*,*22*). More frequent use of water for recreational and other purposes might therefore increase the overall level of oocyst exposure, but it might also confer some resistance to infection on a per-event basis (*23*).

The observation in this study that age correlates with increased serologic response to *Cryptosporidium* antigen has been observed among persons with gastrointestinal and other infections (*24*–*26*). Children are more susceptible to gastrointestinal infection (including cryptosporidiosis) than adults, partly because adults have higher serum/mucosal antibody levels induced by the number of pathogen exposures during their lifetime (*15*,*26*).

Because partial (probably protective) immunity develops among persons with previous or ongoing exposure to *Cryptosporidium* oocysts, contamination of drinking-water sources might not necessarily manifest itself as detectable cases or outbreaks among local residents, but it might affect casual consumers more. Increased rates of clinical disease might not be the inevitable result of ongoing chronic low-level contamination of the water supply (*4*). The apparently complete removal of *Cryptosporidium* oocysts from drinking water supplied by Loch Katrine might have decreased the risk for waterborne illness. However, this reduction of low-level immune system stimulation might have paradoxically increased risk for infection from other sources of exposure.

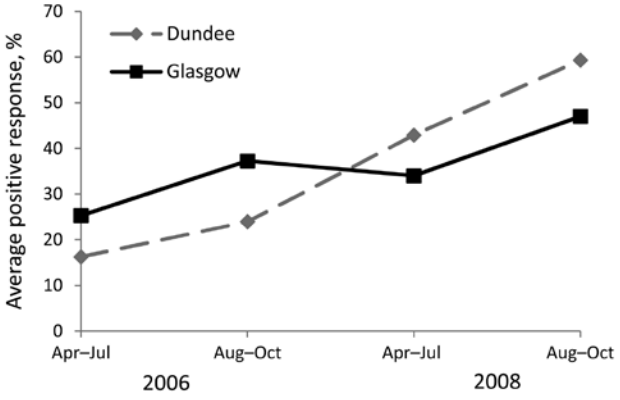

Figure. Mean percentage positive response (PPR) (IgG) to the 27-kDa antigen of *Cryptosporidium* oocysts among blood donors in Glasgow and Dundee, Scotland, 2006–2008. This graph represents the (model estimate) geometric mean PPR for an average participant followed up for the 4 time periods. The plot does not represent the proportion of participants for whom serologic response to the 27-kDa antigen was positive.

Table 4. The coefficients and significance of the terms used in the second linear mixed effects model fitted to the log of the percentage positive response*

Model element	Coefficient		Coefficient exponentiated	% Difference
	Value (95% CI)	p alue	(95% CI)	(95% CI)
Intercept	2.79 (2.61 to 2.98)	0.0000	16.32 (13.57 to 19.63)	NA
City†	0.09 (0.02 to 0.15)	0.0077	1.09 (1.02 to 1.17)	19 (5 to 33)
Pre/post filtration periods‡	0.29 (0.24 to 0.33)	0.0000	1.33 (1.27 to 1.4)	66 (54 to 79)
Period 2 vs. period 1§	0.16 (0.1 to 0.22)	0.0000	1.17 (1.1 to 1.25)	34 (20 to 49)
Period 4 vs. period 3¶	0.12 (0.06 to 0.19)	0.0004	1.13 (1.06 to 1.21)	26 (11 to 42)
Pre/postfiltration, Glasgow vs. Dundee#	–0.17 (–0.22 to –0.12)	0.0000	0.84 (0.8 to 0.88)	–32 (–40 to –23)
Period 2 vs. period 1, Glasgow vs. Dundee**	0.06 (0 to 0.12)	0.0690	1.06 (1 to 1.13)	12 (–1 to –26)
Period 4 vs. period 3, Glasgow vs. Dundee††	0.07 (0 to 0.14)	0.0475	1.07 (1 to 1.15)	14 (0 to 29)
Minimum donor age‡‡	0.02 (0.01 to 0.02)	0.0000	1.02 (1.01 to 1.02)	NA

*Calculated for study of antibodies against *Cryptosporidium* spp. in drinking water, Scotland, 2006–2009. NA, not applicable.
†Negative value implies fewer/lower serologic responses in Glasgow than in Dundee, averaged over the 4 periods.
‡Positive value implies more/higher responses in periods 3 and 4 compared with periods 1 and 2, averaged over the periods and cities. Periods 3 and 4 are post filtration in Glasgow.
§Positive value implies more/higher responses during period 2 compared with period 1, averaged over the cities.
¶Positive value implies more/higher responses during period 4 compared with period 3 averaged over the cities.
#Negative value implies fewer/lower responses after filtration (period 3 and 4) at Glasgow than at Dundee. This is a comparison of the change in response from periods 1 and 2 with periods 3 and 4 in Glasgow with the same change in Dundee.
**Positive value implies more/higher change in responses at Glasgow than at Dundee in period 2 compared with period 1.
††Positive value implies more/higher change in responses at Glasgow than at Dundee in period 4 compared with period 3.
‡‡Earliest age at which a sample was collected from a donor.

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Use of Electronic Death Certificates for Influenza Death Surveillance[1]

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Surveillance for influenza deaths has been used to gauge the severity of influenza seasons. Traditional surveillance, which relies on medical records review and laboratory testing, might not be sustainable during a pandemic. We examined whether electronic death certificates might provide a surveillance alternative. We compared information retrieved from electronic death certificates that listed influenza (or a synonym) with information retrieved from medical charts on which influenza deaths were reported by traditional means in Los Angeles County, California, USA, during the 2009 influenza A(H1N1) pandemic and 2 subsequent influenza seasons. Electronic death certificate surveillance provided timely information, matched the demographics and epidemiologic curve of that obtained from traditional influenza-related death surveillance, and had a moderately positive predictive value. However, risk factors were underreported on death certificates. Because surveillance by electronic death certificates does not require obtaining and reviewing medical records, it requires fewer resources and is less burdensome on public health staff.

Each year in the United States, more deaths are estimated to be caused by influenza than by AIDS (*1*,*2*). Influenza viruses commonly mutate, and concern that a new influenza pandemic will arise is always present. Hence, extensive clinical, syndromic, and virologic surveillance for influenza is conducted in the United States and worldwide. For determining the severity of each influenza season, recording the number of deaths from influenza has long been part of the national system. Although in the United States, most estimates of influenza deaths use a complex algorithm involving data from death certificates and virologic surveillance, in select situations, case reports of individual deaths are used (*3*,*4*). For example, since 2004, influenza-related deaths among children have been nationally reportable, and during the 2009 influenza A(H1N1) pandemic, laboratory-confirmed influenza-related deaths among persons of all ages were reported by state health departments to the Centers for Disease Control and Prevention (*5*,*6*).

Individual case reports (ICRs) of laboratory-confirmed influenza-related deaths provide useful information about the strain of influenza that caused the death, the demographic characteristics of the persons who died, and traditional and novel risk factors for death (*7*–*9*). Deaths are initially reported to health departments by hospitals, physicians, and medical examiners. Health departments collect medical records, laboratory results, specimens for confirmation at public health laboratories, and occasionally interviews of health care providers to determine whether the initial case report meets the definition of a laboratory-confirmed influenza-related death (*5*).

However, collecting and reviewing detailed medical records and laboratory confirmation reports can be time-consuming and labor-intensive. During pandemics, the infrastructure and resources needed to perform public health surveillance of individual influenza deaths can become limited right when the demand for knowledge about disease trends increases. Resources for performing a full, or even limited, investigation of individual influenza-associated deaths might not be available (*10*). Therefore, during pandemics, automated surveillance systems might prove useful for influenza death surveillance.

To evaluate usefulness of an automated influenza death reporting system during and after the 2009 influenza A(H1N1) pandemic, we investigated all death certificates in Los Angeles County, California, USA, on which influenza was listed as a direct or indirect cause of death from August 2009 through April 2012. We compared the sensitivity, positive predictive value, and timeliness of an electronic death reporting system (EDRS) with that of traditional influenza death surveillance based on ICRs.

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Methods

Case Finding and Case Definitions

ICRs

Since April 2009, all influenza deaths have been reportable to the Los Angeles County Department of Public Health. For this study, initial ICRs were received by fax and by electronic web reporting from infection prevention personnel at hospitals, private medical practices, the coroner's office, and other jurisdictions. For each initial ICR, Department of Public Health staff obtained medical records and laboratory results; from these original medical records, trained staff at the Los Angeles County Department of Public Health extracted demographic and risk factor information by using a standard form. Information extracted included the presence of congenital and/or neurologic conditions, immunosuppressive conditions, diabetes, cardiovascular or pulmonary conditions, renal dysfunction, patient's height and weight, and other information. Obesity was defined as body mass index ≥30 for adults or ≥95th percentile for those <19 years of age. A death reported on an ICR was defined as death of a person 1) who resided in Los Angeles County (except for the cities of Long Beach and Pasadena, which maintain their own health departments); 2) whose influenza infection was considered in whole, or in part, to have contributed to the death; and 3) who had a documented positive influenza test result. Laboratory tests included virus culture, PCR, direct or indirect fluorescent antibody test, or a rapid test.

EDRS

Since October 2007, all deaths that occur in Los Angeles County (except those that occur in Long Beach and Pasadena) are recorded by the electronic filing of death certificates into the EDRS at Los Angeles County Department of Public Health. As part of the 122 Cities Mortality Reporting System, each week an algorithm is used to search the EDRS at the Los Angeles County Department of Public Health for death certificates on which influenza was listed as the underlying or contributing cause of death (*11*). Because death certificates are searched at the time of initial filing at Department of Public Health, at that time they have not yet had been coded by the International Classification of Diseases, Tenth Revision, for causes of death. Therefore, specific text strings representing influenza or its synonyms (e.g., flu, swine, H1N1, H1, HINI, N1H1) are used to identify influenza in the cause of death or other significant conditions sections by using SAS software version 9.2 (SAS Institute, Cary, NC, USA). As indicated in the 122 Cities Mortality Reporting System guidelines for reporting influenza, any certificates that mention death from *Haemophilus influenzae* and/or parainfluenza virus are excluded.

For this study, trained staff at the Los Angeles County Department of Public Health used a standard form to extract information about demographics and risk factors from the death certificates. Information extracted included the presence of congenital and/or neurologic conditions, immunosuppressive conditions, diabetes, cardiovascular or pulmonary conditions, renal dysfunction, and other conditions. A case-patient was recorded as obese if the word "obese" or "obesity" was listed on the death certificate. The EDRS defined an influenza death as death of a person who 1) died in Los Angeles Country, regardless of his/her residence, and 2) had influenza (or a synonym listed above) recorded on the death certificate in the week during which the death certificate was originally filed. Medical records and laboratory results were also obtained for each influenza death reported by the EDRS, data were abstracted by using the same form used for deaths reported on ICRs, and cases were reviewed by trained staff at the Los Angeles County Department of Public Health to determine whether the influenza death reported in the EDRS met the case definition for an influenza death reported on ICRs.

Analysis

The pandemic period was defined as August 30, 2009, through April 30, 2010. To determine whether the 2 surveillance systems provided similar descriptions of the demographics, underlying conditions, and trends of influenza deaths, we used *t*-tests, χ^2 tests, and Fisher exact tests, as appropriate, to compare their results.

We calculated the timeliness of each surveillance system for deaths that occurred during the pandemic period. For cases reported by ICR, lag time was defined as the number of days that elapsed from the date of death until the date the death was confirmed by medical and laboratory record review as being influenza related. Lag time was calculated only if the case had been reported at the time of or after death (cases that had initially been reported before death, as part of surveillance for influenza-related intensive care unit admissions, were removed from this analysis). For cases reported by the EDRS, lag time was defined as the number of days that elapsed between the date of death and the date that the death was identified.

For the pandemic period and for the subsequent 2 influenza seasons combined (August 2010–July 2012), we calculated the sensitivity and positive predictive value for the EDRS and used the ICR system as the standard. Data were stored in Microsoft Access (Redmond, WA, USA) databases and analyzed by using SAS software version 9.3 (SAS Institute).

Cases were identified as part of routine public health surveillance for influenza-related deaths. As such, no explicit ethical approval was necessary or sought for this study. A high standard of patient confidentiality was maintained.

Table 1. Demographic variables associated with influenza-related deaths obtained by 2 surveillance systems, Los Angeles County, California, USA, 2009–2010*

Demographic	Surveillance system		p value
	EDRS, n = 85	ICR, n = 105	
Age, y			>0.8
Mean	45.4	45.1	
Median	49	47	
Range	0–90	0–94	
Sex, no. (%)			>0.5
M	37 (44)	50 (48)	
F	48 (56)	55 (52)	
Race, no. (%)†			>0.3
Asian	6 (7)	6 (6)	
Black	5 (6)	9 (9)	
Latino	48 (56)	54 (51)	
White	25 (29)	28 (27)	
Other	1 (1)	2 (2)	
Unknown	0	6 (6)	

*EDRS, electronic death reporting system; ICR, individual case report.
†Percentages may add to >100% because of rounding.

Table 2. Comparison of underlying variables associated with influenza-related deaths obtained by 2 surveillance systems, Los Angeles County, California, USA, 2009–2010*

Condition†	Surveillance method, no. (%)	
	EDRS	ICR
Any	53 (62)	102 (97)
Developmental disability	3 (4)	13 (12)
Pregnancy	1 (1)	3 (3)
Obesity	2 (2)	52 (50)

*EDRS, electronic death reporting system; ICR, individual case report.
†p<0.002.

Results

During 2009–2010, a total of 105 influenza-related deaths were reported by ICRs and 85 by the EDRS; reported patient demographic characteristics were similar (Table 1), specifically, percentages of female patients (53% and 54%), percentages of Hispanic patients (51% and 56%), and median patient ages (47 and 49 years), respectively. However, risk factors for influenza death were recorded on only 53 (62%) of the 85 electronic death certificates but on 102 (97%) of the 105 ICRs (Table 2).

The dates of death reported by each of the 2 surveillance systems were similar (Figure 1). Each system showed peak activity during surveillance weeks 43–48 in 2009 (corresponding to October 18, 2009–December 5, 2009) and low activity during 2010.

The lag times for confirming (ICR) or identifying (EDRS) influenza-related deaths were also similar for the 2 systems (Table 3). After 56 of the 105 ICR deaths with negative lag times were excluded, the mean and median reporting lag times for deaths reported by ICR (n = 49) were 24 and 4 days, respectively (range 0–325 days, interquartile range 1–16 days), and the mean and median reporting lag time for deaths reported by the EDRS (n = 85) were 16 and 11 days, respectively (range 3–86 days, interquartile range 9–16 days). Of the deaths reported by ICR, 5 were reported ≥50 days after death because of extensive testing at the coroner's office, which increased the mean lag time for deaths reported by ICR, but the difference between the reported mean lag times was not statistically significant.

Of the 85 deaths identified by the EDRS during the pandemic period, 60 met the ICR definition for influenza-related death; positive predictive value was 71%. Of the 25 deaths identified by the EDRS that did not meet the case definition, 14 did not have a positive influenza laboratory test result, 10 patients did not live in Los Angeles County although they died in Los Angeles County, and 1 patient was mistakenly selected by the automated algorithm as having influenza although the causative organism listed was *H. influenzae*. A total of 43 of the 105 of the deaths identified by ICR were reported by the EDRS; sensitivity was 41% (Figure 2). Of note, an additional 17 deaths identified by the EDRS met the ICR case definition after medical record review, but these had not been reported to the Los Angeles County Department of Public Health through normal reporting mechanisms.

During the postpandemic period (August 2010–July 2012), totals of 53 and 35 influenza-related deaths were reported by ICRs and the EDRS, respectively. Of the 35 deaths reported by the EDRS, 30 were verified as laboratory-confirmed influenza-related deaths of Los Angeles County residents. During the postpandemic period, the sensitivity of the EDRS compared with that of ICRs was 45% and the positive predictive value was 86% (Figure 3).

Discussion

This study demonstrated that surveillance that used automated text searches of electronic death certificates was timely, matched the demographics and the epidemiologic curve of traditional influenza-related death surveillance in Los Angeles County, and had a moderate positive predictive value. Because no medical records needed to be obtained or reviewed, surveillance by the EDRS required fewer resources and was less burdensome on public health staff and hospitals.

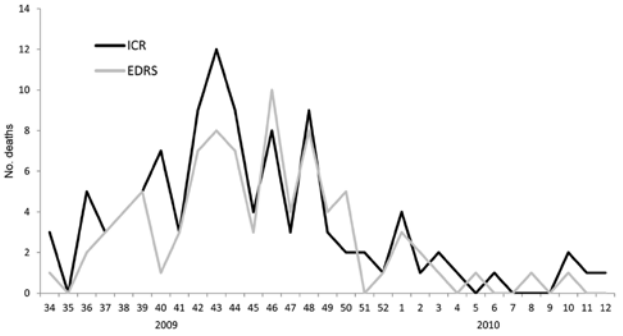

Figure 1. Influenza-related deaths by MMWR (Morbidity and Mortality Weekly Report) week of death reported by 2 surveillance systems, Los Angeles County, California, USA, 2009–2010.

Table 3. Comparison of lag times associated with reporting influenza-related deaths obtained by 2 surveillance systems, Los Angeles County, California, USA, 2009–2010*

Reporting delay, d†	Surveillance method	
	EDRS, n = 85	ICR, n = 49
Mean	16	24
Median	11	4
Range	3–86	0–325

*EDRS, electronic death reporting system; ICR, individual case report.
†p>0.20.

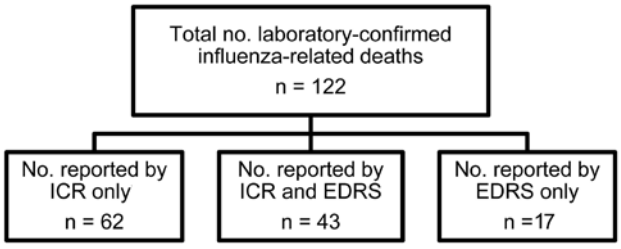

Figure 2. Report source of laboratory-confirmed influenza-related deaths in Los Angeles County, California, USA, April 2009–April 2010. ICR, individual case report; EDRS, electronic death reporting system.

The major limitation of using death certificates as a primary surveillance tool is that relatively fewer risk factors (including obesity or neurodevelopmental diagnoses, which were newly identified risk factors for severe influenza during the 2009–2010 influenza A[H1N1] pandemic) were listed on the death certificates than on ICRs (*8*,*12*). For example, although 50% of the confirmed deaths reported by ICRs were actually in obese persons, the death certificates rarely listed obesity as being a cause of death or as another significant condition. For identifying obesity as a risk factor for severe complications of influenza A(H1N1) virus infection, medical record review and additional efforts to contact hospitals were needed to obtain the height and weight of the patients. Furthermore, the sensitivity of the EDRS was <50% compared with that of traditional reporting, suggesting that many doctors do not consider influenza as a significant condition to list on death certificates or are not aware of a positive laboratory result when the death certificate is signed.

The number of influenza deaths reported to a health department by any means (death certificates or ICRs) almost certainly underestimates the true number of influenza deaths in a population (*13*). It reflects only those patients who were tested, had adequate specimens for testing, had sensitive tests performed, and were reported to the health department. A recent study estimated that the total number of deaths from pandemic influenza in the Americas was 2–4 times that of reported laboratory-confirmed cases (*14*). Consequently, estimates of the total number of influenza-related deaths in the United States are routinely made through a statistical algorithm based, in part, on standardized International Classification of Disease, Tenth Revision, codes indicated on death certificates, including codes for influenza, pneumonia, respiratory disease, or cardiac disease (*3*,*4*). Because death certificates must be standardized and coded, years can pass between the end of an influenza season and the final estimate of influenza-related deaths that occurred during that season. Therefore, contemporary data, even if underestimated, from ICRs or from death certificates that have not yet been standardized can be useful for local influenza control policies by estimating relative virulence of a given influenza season and identifying risk factors and demographic groups at highest risk for death.

This study has several limitations: it was performed in only 1 jurisdiction, death certificate coding practices might differ in other jurisdictions, and the overall sample size was small. However, Los Angeles County has a diverse population of 9.8 million and multiple reporting sources (≈100 hospitals and ≈25,000 physicians), so the base of reporting was broad and diverse. Because the 122 Cities Mortality Reporting System/EDRS algorithm for identifying influenza and pneumonia on death certificates is processed only 1 time each week, there is a built-in time lag between when the death certificate is filed and when a death is identified in EDRS. Furthermore, because the system looks only at the first time a death certificate is filed (often before cause of death has been determined), it might miss death certificates that are later amended to cite influenza as a cause of death. In fact, of the 105 laboratory-confirmed ICR deaths, 16 were reported by the Office of the Coroner in Los Angeles County. For all 16 patients, death certificates were initially filed with cause of death deferred. Thus, these deaths were not detected by our weekly EDRS search algorithm, although for 13 (81%) of the 16, influenza was ultimately listed on the amended death certificates. To enhance the timeliness and sensitivity of EDRS surveillance, the database should be analyzed more often than 1 time each week and should include amended death certificates. Furthermore, text strings in death certificates should be manually evaluated at regular intervals to ensure that all appropriate terms are included when searching for possible influenza-related deaths.

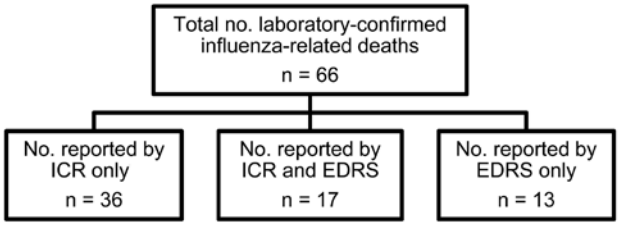

Figure 3. Report source of laboratory-confirmed influenza-related deaths in Los Angeles County, California, USA, August 2010–April 2012. ICR, individual case report; EDRS, electronic death reporting system.

In summary, as an increasing number of jurisdictions have or plan to use EDRSs (*15*), public health officials might find an EDRS useful for the surveillance of influenza or other emerging diseases. Surveillance that used electronic death certificates and a text-based search algorithm for influenza was able to accurately describe the population affected by influenza during the influenza A(H1N1) 2009 pandemic in a timely and efficient manner. During a pandemic, when surveillance resources can be overwhelmed, use of electronic death certificates to identify and analyze influenza deaths might be a reasonable option. At a minimum, investigation of influenza deaths reported by death certificates might identify additional confirmed influenza-related deaths not reported through traditional mechanisms.

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At the time of the study, Dr Bancroft was a medical epidemiologist at the Los Angeles County Department of Public Health. Her research interests include improving surveillance for respiratory viruses and hepatitis and investigation of health care–associated infections.

Ms Lee is an epidemiologist at the Los Angeles County Department of Public Health. Her research interest is analyzing statistical reports and health status indicators by using death data at various geographic levels for studies of conditions such as ovarian cancer and echinococcosis.

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Human Parechovirus Infection, Denmark

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Human parechoviruses (HPeVs) often cause severe illness among young children. National surveillance with routine testing of all cerebrospinal fluid, fecal, and tissue samples was conducted during January 2009–December 2012 in all counties in Denmark (6,817 samples from 4,804 children were screened for HPeV). We detected HPeV RNA in 202 (3.0%) specimens from 149 persons. Young infants were at highest risk for HPeV, and 9 (6%) of the HPeV-infected children died, probably of their HPeV illness. HPeV3 was the most common genotype identified, and 5 closely related clades of HPeV3 circulated in Denmark throughout the study period. Our study adds perspective on the prevalence and clinical and molecular virologic characteristics of HPeV infection.

Human parechoviruses (HPeVs) have recently been recognized to cause a variety of symptoms ranging from mild diarrhea to sepsis and meningitis, particularly among young children. HPeVs belong to a large family of nonenveloped, positive-sense, single-stranded RNA viruses, the *Picornaviridae*, which comprises 12 genera (and 5 proposed genera). Six genera are associated with human infections: cardiovirus (saffold virus), cosavirus, enterovirus (EV), hepatovirus (hepatitis A), kobuvirus (Aichi virus) and HPeV. HPeV1 and HPeV2, originally known as echovirus 22 and 23 of the EV genus, respectively, were reclassified in 1999 as a separate genus (*Parechovirus*) on the basis of genetic and biologic differences (*1*). Since the reclassification, the number of known HPeVs has increased and now totals 16 genotypes (www.picornaviridae.com/parechovirus/hpev/hpev.htm).

HPeV1 is known to be associated with asymptomatic infection. HPeV3 seems to be more or less well established (*2*–*4*). The remaining known HPeVs are clinically unexplored.

Whereas HPeV3 has been reported to be associated with sepsis-like syndrome, meningitis, encephalitis, and hepatitis in neonates and young infants (*2*), most HPeV infections are asymptomatic or associated with mild respiratory and/or gastrointestinal symptoms (*4*). HPeV incidence has been reported to show a seasonal pattern in temperate climates, with different types cocirculating simultaneously (*5*). Although HPeV infections are relatively common in most settings, experience from long-term population surveillance is somewhat sparse. We used 4 years of national laboratory surveillance to describe the molecular epidemiology of HPeV, including phylogenetic characteristics of the emerging HPeV epidemic, in Denmark.

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Materials and Methods

Study Design

During January 2009–December 2012, a total of 6,817 specimens were collected from 4,808 children from all regions of Denmark: 2,006 cerebrospinal fluid (CSF) samples from 1,952 children; 1,963 fecal samples from 1,608 children; 1,057 blood samples from 1,025 children; 682 respiratory samples from 610 children; 571 autopsy samples from 228 children; 302 swab specimens from 291 children; and 236 other samples. In general, samples were collected from patients with symptoms compatible with EV infection, particularly meningitis, sepsis-like illness, respiratory symptoms, and/or diarrhea. The CSF samples were collected from patients who typically had fever and/or clinical signs compatible with meningitis or encephalitis. The fecal samples were collected from a mixture of patients with community-acquired infection and hospitalized patients who typically had fever, diarrhea, and/or meningitis/sepsis-like illness. Autopsy material was analyzed for EV and HPeV as part of standard procedure irrespective of symptoms. Samples were sent by mail to the Department of Microbiological Diagnostics and Virology at the Statens Serum Institut (Copenhagen, Denmark) to undergo viral diagnostic and isolation and real-time PCR for EVs and HPeVs. HPeV infections detected in the same person within a 3-week period were considered to be the same infection. During the study period, the Department of Microbiological Diagnostics and Virology was the only laboratory conducting HPeV diagnostics in Denmark; therefore, the data reflect all HPeV infections detected in Denmark.

Laboratory Analyses

Sample Preparation

Only fecal samples and biopsy samples needed special preparation before use of the general nucleic acid extraction protocol. Fecal samples were prepared as a 10% wt/vol suspension in minimal essential medium, followed by centrifugation at 3,500 × *g* for 30 min to remove inhibitors. Biopsy samples were suspended in Lysis/Binding Buffer from the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany), followed by homogenization.

Nucleic Acid Isolation

Nucleic acids were extracted from 200 mL sample material. All sample types, except CSF, were processed by using the MagNa Pure 96 DNA and Viral NA Small Volume Kit on the MagNa Pure 96 instrument (Roche Diagnostics) according to the manufacturer's specifications. Nucleic acids from CSF were isolated by using the QIAamp DNA Blood Mini Kit on the QIAcube instrument (QIAGEN, Hilden, Germany) following the manufacturer's specifications.

Amplification and Detection

For amplification, 5 mL of extracted nucleic acids were used per reverse transcription PCR (RT-PCR) reaction (total volume 25 mL) by using the OneStep RT-PCR Kit (QIAGEN). The reaction mixtures contained 1 μmol/L of each primer and 0.2 μmol/L probe. The primers and probe used have been published (*6*), and the reaction mixture also contained an assay for EV. The HPeV-specific probe was labeled with a Hex dye. The Mx3005P real-time thermocycler (Agilent Technologies A/S, Hoersholm, Denmark) was used for amplification and detection with the following settings: 50°C for 20 min, 95°C for 15 min, followed by 45 cycles of 95°C for 15 s and 55°C for 1 min.

Genotyping

We amplified 256–259 bp of the viral protein (VP) 3/VP1 region (from position 2159–2458 in relation to L02971) in a nested PCR. The first-round RT-PCR was conducted by using primers Harv1-F and Harv1-R (*7*) with a OneStep RT-PCR kit (QIAGEN) and the following thermocycler conditions: 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 95°C for 30 s, 42°C for 30 s, and 60°C for 45 s. The second-round PCR was conducted by using primers Harv2-F and Harv2-R (*8*) and the following thermocycler conditions: 95°C for 6 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s. This protocol was followed by a final extension at 72°C for 10 min. PCR amplification was followed by sequencing.

Before sequencing, PCR products were treated with exo-SAP IT (GE Healthcare, Buckinghamshire, UK). PCR products were sequenced by using the dideoxynucleotide chain termination method with the ABI Prism BigDye Terminator Cycle Sequencing Reaction kit on an ABI Prism 3100 automated sequencer (Applied Biosystems, Naerum, Denmark). Sequencing was performed with the forward and reverse primers from the second-round PCR. Sequences were assembled in BioNumerics 6.5 (Applied Maths, Kortrijk, Belgium). The sequences have been submitted to GenBank under accession nos. KF300772–KF300885.

Phylogenetic Analysis

Assembled sequences were aligned with reference sequences by using the Simmonic sequence editor (www.virus-evolution.org). Phylogenetic and molecular evolutionary analyses were conducted by using MEGA 5.0 software (www.megasoftware.net). Genetic distances were calculated by using the Jukes-Cantor parameter at the nucleotide level, and the phylogenetic trees were constructed by using the maximum-likelihood method with 500 bootstrap replications (*8*).

Results

Sample Material

Study Population

During January 2009–December 2012, from 6,817 samples from 4,804 children, we detected HPeV RNA in 202 (3%) specimens from 149 (3%) children from all counties of Denmark. Of the 149 individual HPeV cases, 25 (17%) were detected in CSF samples, 105 (70%) in fecal samples, and 19 (13%) in a variety of clinical specimens (8 pharynx/tonsil swabs, 2 bronchio-lavage fluids, and 9 biopsy specimens from children who died unexpectedly [5 from abdominal lymph nodes, 1 from the small intestine, 2 from the large intestine, and 1 from pulmonary tissue]). Of the 149 individual HPeV cases, 125 had sufficient material or RNA for further VP3/VP1 regional subtyping.

Frequency of HPeV-infected Patients

HPeV was detected in 52 (3%) of 1,744 patients in 2009, in 31 (2%) of 1,729 patients in 2010, in 33 (2%) of 1,939 patients in 2011, and in 33 (2%) of 1,405 patients in 2012. Of the 149 HPeV-positive patients, 52 (35%) tested positive in 2009, 31 (21%) in 2010, 33 (22%) in 2011, and 33 (22%) in 2012.

HPeV3 infections occurred in all months of the year, albeit with a clear seasonal pattern; only few cases occurred during the winter and spring months of November–May, with a marked increase during June and July, peaking during the fall months of September and October (Figure 1).

HPeV1 infections show a seasonality pattern very similar to that of the HPeV3 infections, although no cases were observed during March–June.

Epidemiology of HPeV-infected Persons

Age was inversely associated with risk for HPeV infection among children <5 years of age: the overall median age of HPeV-infected children was 39 days (interquartile range [IQR] 22–71 days), and the median age of HPeV-negative children was 16.3 years (IQR 318 days–41.8 years). Children with HPeV3 infection (median age 37 days [IQR 19–59 days) were significantly younger than children with HPeV1 (median age 199 days [IQR 84–303 days]).

Clinical Features of HPeV Infection

Information about symptoms and the source of the sample was available for 89 (60%) of children with HPeV. A wide range of symptoms were reported: slightly more cases were associated with meningitis (36 cases) than diarrhea (29) and fever (19), but sepsis-like symptoms, convulsions, apathy, and general discomfort also were reported (Table).

Virologic and Molecular Findings

Our sequencing of 1 of the least conservative regions of the capsid (VP3/VP1) resulted in the following: 90 HPeV3, 21 HPeV1, 8 HPeV6, 4 HPeV5, and 2 HPeV4. The genotype was assigned by BLAST analysis of the sequence against all published sequences in GenBank (http://blast.ncbi.nlm.nih.gov/).

HPeV3 was more frequently associated with disease in neonates than was any other HPeV genotype. The mean age of HPeV3-infected children was 1.6 months, compared with 8.9 months for HPeV1-infected children (n = 20), and 8.1 months for HPeV6-infected children (n = 6) (*t* test for mean age difference, p = 0.01). Sequences were available from 8 of the 9 children who died unexpectedly; of these, 4 sequences were HPeV1, 2 were HPeV3, 1 was HPeV5, and 1 was HPeV6. Of the 36 patients with reported meningitis, HPeV types were available for 32; of these, 30 (94%) types were HPeV3, and 2 (6%) were HPeV1. Of the 4 patients with sepsis-like syndrome, sample material for typing was available for 3; all 3 were identified as HPeV3. HPeV types were available for 25 of the 29 patients with reported diarrhea; of these, 19 (76%) types were HPeV3, 4 (16%) were HPeV1, and 2 (8%) were HPeV6. All 25 CSF samples were HPeV3. HPeV1 and HPeV3 were detected throughout the study period; HPeV6 was detected in all years except 2010; and HPeV5 emerged only in 2012.

Sequence data from 124 of these 125 samples were of sufficient quality and were used to create phylogenetic trees (Figure 2, Appendix, wwwnc.cdc.gov/EID/article/19/12/13-0569-F2.htm), which also included reference sequences obtained from GenBank for each genotype identified in this study. The phylogenetic analyses revealed the existence of 5 closely related clades of HPeV3 circulating in Denmark throughout the study period, with most clades found all years of the study, implying cocirculation of these clades without genetic selection of either clade in the sequenced area. In addition, there was no particular geographic distribution of the individual clades. The other genotypes appear to follow the same pattern of circulation and of little evolutionary change within each genotype over time. However, data for the genotypes other than HPeV3 were insufficient to substantiate a division into individual clades.

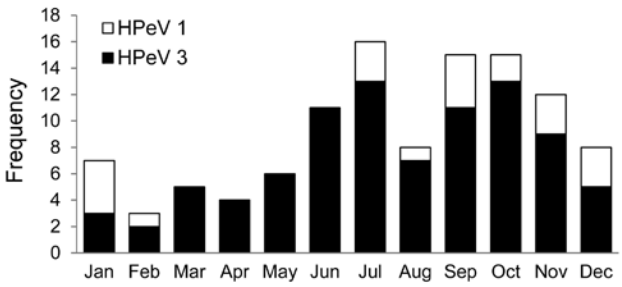

Figure 1. Genotype-specific seasonal distribution of laboratory-confirmed human parechovirus (HPeV) type 1 (n = 21) and HPeV3 (n = 90) in children <5 years of age, Denmark, 2009–2012.

We created a phylogenetic tree (Figure 3) including a representative strain from each of the HPeV3 clades detected in our study in Denmark, combined with matching HPeV sequence from Europe available in GenBank. This tree showed that clades 1–4 were most closely related to strains from Spain (*9*) and Italy (*10*) during 2006–2009, whereas clade 5 was identical (in the sequenced area) to a simultaneous strain from Germany (*11*). We found an intragenotype variation of 3.9%, within HPeV1 of 5.6%, within HPeV4 of 11.6%, within HPeV5 of 1.6%, and within HPeV6 of 3.5%.

Discussion

Our study establishes HPeV as important differential diagnosis in young and often severely ill children in

Table. Clinical characteristics of human parechovirus–infected children, Denmark, 2009–2012

Clinical characteristic	Patients, no. (%), N = 89
Fever >38.5°C	19 (21.3)*
Diarrhea and fever	29 (32.6)
Meningitis	36 (40.5)†
Sepsis-like syndrome	4 (4.5)
Sudden infant death syndrome	1 (1.1)‡

*Of the 19 patients for whom fever was the main symptom reported, 4 infants also were reported to be "irritable."
†Three of the 36 patients with meningitis also were reported to have convulsions.
‡The child who was found dead in bed (sudden unexpected infant dead), was reported to have had liquid diarrhea for 2 d before death.

Figure 3. Phylogenetic analysis of strains of human parechovirus type 3 from Europe compared with a representative strain of each of the 5 clades found in Denmark (squares). Maximum-likelihood analysis with bootstrap values >70% are shown. Scale bar indicates nucleotide substitutions per site.

Denmark with fever and/or central nervous system symptoms. The sample material received for diagnostic testing, where a significant portion of the HPeV-positive samples are CSF, illustrates the severity of these infections. Without routine testing for HPeV of infants suspected to have EV infection (EV and HPeV3 infections are indistinguishable in infants [*12*]), these children's illnesses would not have been diagnosed, which would have resulted in unfocused treatment, such as unnecessary antimicrobial drug therapy and unnecessary long hospital stays, as suggested by Wolthers et al. (*13*). In our selected material (selected for suspected enteroviral disease), HPeV3 was by far the most prevalent HPeV type and was the only genotype detected in CSF. The high prevalence of HPeV3 contrasts with findings from other studies (*5*,*14*), which have established HPeV1 as the most common virus circulating in the population. However, these studies did not select the patients on the basis of symptoms because the children were part of healthy birth cohorts from prospective diabetes studies. In Denmark, although HPeV infections were detected throughout the year, the illness showed a marked seasonality with peaks during July and September, coinciding with the EV season. These findings underscore the need for HPeV and EV differential diagnostics in young children who have nonbacterial meningitis and severe disease in general caused by the similar clinical characteristics of HPeV and EV (*12*). The observed seasonality (i.e., occurrence of HPeV3 every year) indicates an endemic pattern of HPeV infection in Denmark. This finding contrasts with other HPeV studies from the United Kingdom and the Netherlands, where a biannual cycle of HPeV3 infections has been observed; the reason for this difference is unknown (*15*,*16*).

In our study, the children infected with HPeV (and in particular HPeV3) were very young (median age 39 days), which is consistent with prior studies (*17*,*18*). However, a recent study from Japan (*5*) has established that adults also can be infected with HPeV3, possibly resulting in epidemic myalgia.

We also showed that HPeV3 in Denmark did not undergo significant genetic diversification during 2009–2012, at least not in the sequenced part of the capsid (VP3/VP1) (Figure 2, Appendix). This observation was in line with comparisons of the strains from Denmark with strains from Europe in which only limited variation periodically (1994–2012) and geographically was observed (Figure 3). The genetic distances in the trees are small (which further substantiates the results of limited evolutionary change). These indications of limited genetic evolution correspond well with results from a study in the Netherlands that described HPeV during 2000–2007 (*19*). The reason for the lack of intratypic diversification of the otherwise intertypically heterogenic capsid area is unknown. Perhaps most infections are subclinical and do not elicit an immunologic response, which might otherwise cause the virus to evolve to escape the immune response. Structural constraints might also exist that limit the genetic variation to ensure proper capsid formation.

Our data supported previous findings that HPeV3 is commonly associated with meningitis/sepsis symptoms among young infants, and we found that convulsions and apathy were reported among children with such severe cases. Because HPeV3 has been reported to cause white matter injury in 9 of 10 infected neonates, resulting in a range of complications from cerebral palsy in 1 child to epilepsy in another child to learning disabilities in a third child (*20*), focused longitudinal follow-up and cognitive evaluation of these children during childhood might be indicated to better understand the long-term consequences of this emerging viral disease.

In conclusion, HPeV was a clinically significant virus rivaling EVs in young children in Denmark. In particular, HPeV3 was circulating in our clinically selected material, with 5 different clades cocirculating over most of the years of the study. The patients were from all around Denmark demonstrating the need for central or disseminated HPeV diagnostics in Denmark so that HPeV is tested for in all young children suspected of infection with HPeV or EV. Testing might possibly reduce the length of hospitalization and limit the inappropriate use of antimicrobial drugs. Furthermore, doctors and especially pediatricians should be aware of the symptoms of HPeV-associated diseases and should be encouraged to collect a sample (primarily CSF and preferably

combined with a fecal sample) for diagnostics. Centralized surveillance of this virus could provide deeper insight into the behavior of HPeV and might shed light on the clinical significance of the HPeVs other than type 3.

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Molecular Barriers to Zoonotic Transmission of Prions

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The risks posed to human health by individual animal prion diseases cannot be determined a priori and are difficult to address empirically. The fundamental event in prion disease pathogenesis is thought to be the seeded conversion of normal prion protein to its pathologic isoform. We used a rapid molecular conversion assay (protein misfolding cyclic amplification) to test whether brain homogenates from specimens of classical bovine spongiform encephalopathy (BSE), atypical BSE (H-type BSE and L-type BSE), classical scrapie, atypical scrapie, and chronic wasting disease can convert normal human prion protein to the abnormal disease-associated form. None of the tested prion isolates from diseased animals were as efficient as classical BSE in converting human prion protein. However, in the case of chronic wasting disease, there was no absolute barrier to conversion of the human prion protein.

Prion diseases are rare fatal neurodegenerative conditions that affect humans and animals. The human diseases include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease, and fatal familial insomnia. Most cases of human prion disease are apparently spontaneously occurring (sporadic CJD [sCJD]) or are associated with mutations in the human prion protein gene, designated *PRNP* (genetic CJD, Gerstmann-Sträussler-Scheinker disease, or fatal familial insomnia). A small minority of cases are acquired by inadvertent human-to-human transmission during medical or surgical treatments (iatrogenic CJD).

In contrast, animal prion diseases are generally acquired. This applies to scrapie in sheep, transmissible mink encephalopathy, and chronic wasting disease (CWD) in deer and elk. No credible evidence exists of a link between scrapie and any human prion disease, despite the endemicity of scrapie in many parts of the world and the consequent likely human exposure to the scrapie agent, which has been attributed partly to a species barrier between sheep and humans. However, strong epidemiologic, pathologic, and molecular evidence does indicate that the epidemic of bovine spongiform encephalopathy (BSE), primarily in the United Kingdom during the 1980s, resulted in a zoonotic form of CJD termed variant CJD (vCJD). BSE/vCJD is the only known zoonotic prion disease strain.

After identification of BSE and vCJD, active surveillance for animal prion diseases in Europe and elsewhere has identified rare atypical prion diseases in sheep and cattle. These include Nor98 or atypical scrapie in sheep (*1*) and 2 prion diseases of cattle, bovine amyloidotic spongiform encephalopathy or L-type BSE (*2*) and H-type BSE (*3*), both of which have a pathology and epidemiology distinct from classical or C-type BSE (*4*). In addition to these new (or newly described) diseases of farmed sheep and cattle, CWD in cervids is an acquired, probably contagious disease that affects captive and free-ranging deer and elk populations primarily in North America (*5*).

Their distinctive epidemiology, clinical features, neuropathology, PrP biochemistry, and transmission characteristics suggest that scrapie, atypical scrapie, C-type BSE, H-type BSE, L-type BSE, and CWD represent distinct prion strains in their respective species (*6*,*7*). Within scrapie and CWD, natural strain variation also occurs. The prion hypothesis posits that the posttranslational conformational conversion of a host's normal cellular prion protein (PrP^{C}) by the abnormal form of the prion protein (PrP^{Sc}) is the fundamental event in prion disease pathogenesis and that PrP^{Sc} itself constitutes the infectious agent. It follows that an aspect of prion host range may be a species barrier operating at the molecular level that depends on compatibility between the PrP^{Sc} from 1 species and the PrP^{C} from another. Similarities in the species-specific primary *PRNP* sequences may account for part of this effect, but prion strain and host *PRNP* polymorphic genotype, both of which

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probably find expression in the conformation of PrP, affect susceptibility in ways not yet fully understood.

A relatively simple empirical approach to assessing this molecular barrier is to use cell-free PrP conversion assay techniques (*8*,*9*) to determine the relative efficiency of PrP conversion using natural "seeds" from an infectious prion source from the brain of 1 species and a normal brain "substrate" from another species. We have previously reported the use of protein misfolding cyclic amplification (PMCA) as a model of cross-species prion transmission of C-type BSE in cattle and sheep to humans (*10*). Here we report a comparative study of the ability of sheep, cattle, and deer prions to convert normal human PrP in this same cell-free system.

Methods

Ovine, bovine, and cervine frozen brain tissue from prion disease–affected and –unaffected animals were obtained by request from the Animal Health Veterinary Laboratory Agency TSE Archive (AHVLA, Weybridge, UK). The cases and brain regions supplied were selected on the basis of proven disease status and of brain region with an expected high PrP^{Sc} load, characteristic of the particular prion disease. The prion disease status of the animals involved was determined at AHVLA and/or the Canadian Food Inspection Agency's Ottawa laboratory (Ottawa, ON, Canada) by neuropathology and PrP immunohistochemistry. The classical scrapie specimen was of brain stem from a field suspect of the animal prion protein gene *Prnp* ARQ/ARQ genotype, and a brain stem specimen from an unaffected scrapie suspect of the same genotype was also supplied. The atypical scrapie specimen was of parietal cortex, also from a field suspect, but of the ARQ/AHQ genotype, and a corresponding negative control animal specimen was also supplied. The C-type BSE samples were of brain stem from confirmed positive C-type BSE suspects obtained through passive surveillance, and the corresponding negative control specimen was similarly obtained. Both the H- and L-type BSE specimens were of frontal cortex from successful experimental bovine transmissions conducted at AHVLA. Mid-brain tissue from a confirmed CWD-positive and control-negative (unaffected) elk (both with *Prnp* codon 132MM genotype) was also supplied through the AHVLA.

Frozen half brains from inbred transgenic mouse lines expressing human PrP^{C} of the *PRNP* codon 129 methionine (129MM) and valine (129VV) genotypes (*11*–*13*) were used for PMCA substrate preparation. The production of PMCA substrates from stably transfected human 293F cells overexpressing human PrP^{C} (exogenous *PRNP* codon 129M and endogenous *PRNP* codon 129MM) has been described (*14*). A *PRNP* codon 129 valine expressing counterpart was engineered by suppressing expression of endogenous *PRNP* codon 129MM expression with RNAi and transient transfection with a *PRNP* codon 129 valine expression vector (designated 129V).

Human brain tissues (frontal cortex) were sampled from frozen half brains collected at autopsy with the appropriate consent for tissue retention and research use. The vCJD specimen was from a patient (*PRNP* codon 129MM) with definite vCJD as defined by established criteria. The non-CJD human brain specimens used for PMCA substrate preparation were frontal cortex from patients with Guillain-Barré syndrome (129MM) and dementia with Lewy bodies (129VV). sCJD specimens from patients with the MM1 and VV2 subtypes of the disease were used as reference standards in certain Western blotting experiments. Ethical approval for the use of these tissues in this study is covered by Local Research Ethics Committee 2000/4/157.

Brain homogenates were prepared by using a manual homogenizer and chilled conversion buffer (150 mM NaCl, 1% Triton X-100, 1X protease inhibitor cocktail in 1× phosphate-buffered saline) to obtain a final 10% wt/vol solution. The homogenized tissue was cleared by centrifugation at 2,000 rpm for 40 s in a refrigerated centrifuge (4°C), and the supernatant was aliquoted and stored at -80°C (*15*).

We prepared homogenates (10% wt/vol) of C-type BSE, scrapie, CWD, L-type BSE, H-type BSE atypical scrapie, and vCJD brain. We followed the same method used for the substrate brain homogenate.

PMCA experiments were carried out in PCR tubes. Aliquots of 10% substrate brain homogenate (or 20% cell extracts) were mixed with 10% prion disease brain seeds in a final volume of 120 μL. Low molecular weight heparin was included at 100 μg/mL in all PMCA reactions (*15*). Before sonication, 19 μL of the PMCA reaction mixture (termed "frozen" sample) was taken for comparison with the amplified sample (termed "sonicated" sample). The reactions were incubated into the microplate horn of a programmable sonicator (Misonix 4000, Misonix, Farmingdale, NY, USA) at 37°C. A total of 96 PMCA cycles were performed comprising 20 s of sonication (at an amplitude of 90%) followed by 29 min 40 s of incubation for every cycle (*16*).

Tissue homogenates and PMCA reaction products were digested with proteinase K (50 μg/mL for 1 h at 37°C) and detected by Western blotting (*10*). Detection was with 3F4 or 6H4 antibodies diluted in 1× phosphate-buffered saline, 0.05% Tween 20 (*10*). The monoclonal antibody 9A2 was obtained from Central Veterinary Institute Wageningen UR (Lelystad, the Netherlands) (*7*). Membranes were developed by using peroxidase conjugated secondary antibody and a luminescent peroxidase substrate ECL-Plus (*10*). Finally, blots were exposed to a photographic film and the image acquired using the XRS digital CCD camera system (Bio-Rad Laboratories, Hercules, CA, USA). The antibody 6H4 recognizes an epitope in the protease-resistant

core of human PrP spanning amino acids 145–153, and it cross-reacts with bovine, ovine, and cervine PrP. 3F4 also reacts with a sequence in the protease resistant core of the human PrP spanning positions 106–112, but 3F4 does not recognize PrP from species other than humans and hamsters. The combination of protease-digestion and detection by 3F4 in these experiments therefore provides a very sensitive method for detecting newly formed human protease-resistant prion protein (PrP^{res}) (*10*,*17*). The antibody 9A2 reacts with human PrP amino acids 99–101 and cross-reacts with ovine, bovine, and cervine PrP.

Results

PrP was confirmed in all animal brain samples in the form of 2 major bands in the 20–40-kDa molecular mass range, probably corresponding to full-length diglycosylated PrP (upper band) and N terminally truncated diglycosylated or full-length monoglycosylated PrP (lower band). The levels of PrP and the electrophoretic pattern were broadly similar between prion disease and corresponding negative control (unaffected) animal brain samples (Figure 1, panel A). However, proteinase K digestion showed differences in the amount of PrP^{res} contained in these samples. PrP^{res} was most abundant in classical scrapie and CWD samples; lower levels were seen in C-, H- and L-type BSE samples and were barely detectable at this level of sensitivity in atypical scrapie (Figure 1, panel B). Normalizing the Western blot PrP^{res} signal by adjusting sample loading volumes demonstrated the expected PrP^{res} relative mobilities and glycosylation types (data not shown). When larger volumes of brain homogenate were analyzed, a characteristic <10-kDa band was present in the atypical scrapie specimen (Figure 1, panel C).

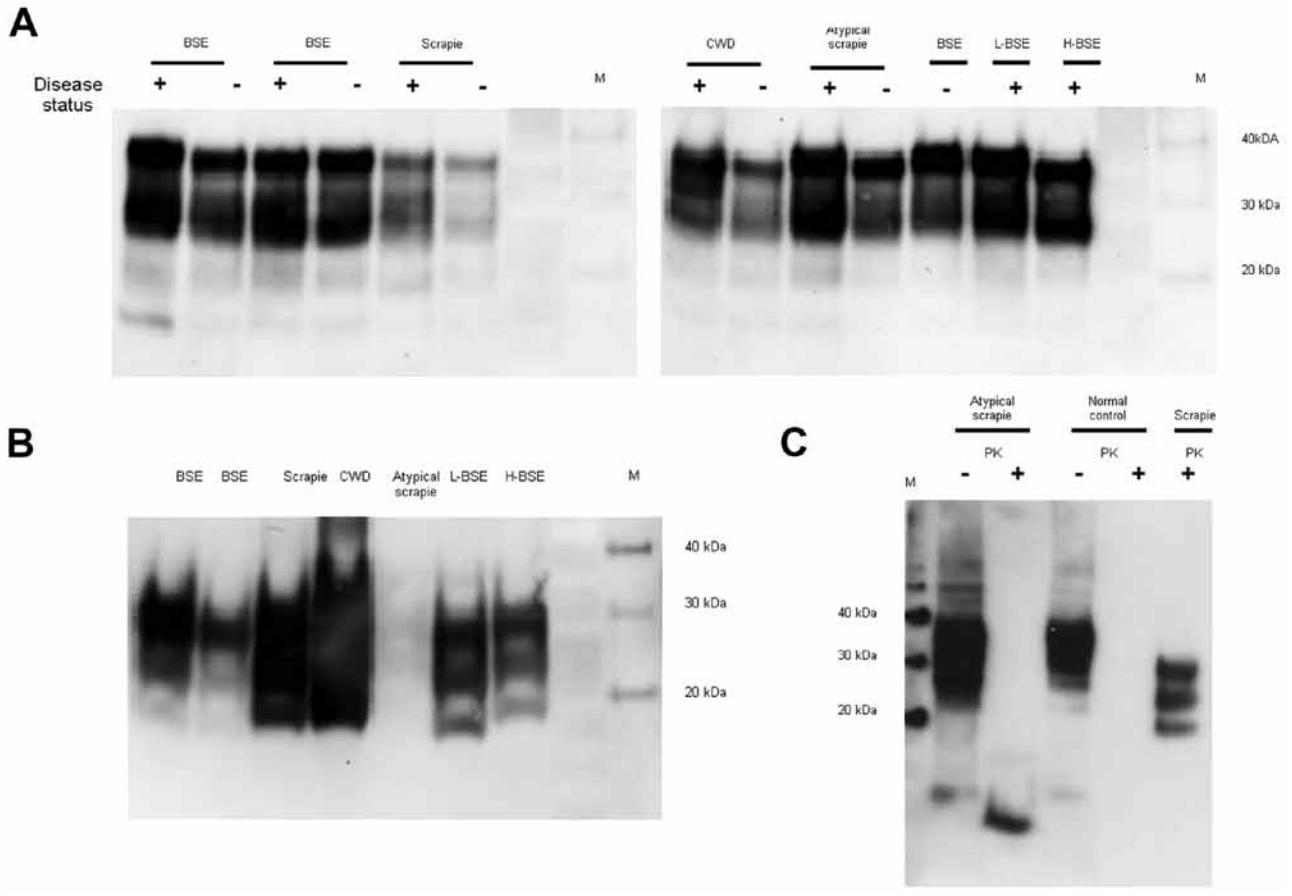

Figure 1. Determination of total PrP and PrP^{res} level in animal tissues. To characterize the PrP expression levels (total PrP), brain homogenates were analyzed by Western blot without digestion with PK. Nineteen microliters of each 10% wt/vol homogenate was loaded in each lane (A). To detect the PrP^{res} in the samples, PK digestion (50 μg/mL) was performed to remove PrP^{C}, and the samples were then reanalyzed (B). The atypical scrapie and matched normal control animal samples were further analyzed by Western blot both with (+) and without (–) prior PK digestion (C) by comparing 3 μL of undigested homogenates with 100 μL of the PK-digested sample concentrated by centrifugation. Five microliters of a PK-digested classical scrapie brain homogenate was analyzed in parallel for comparison. The detection antibody was 6H4 in (A) and (B) and 9A2 in (C). PrP, prion protein; PrP^{res}, protease-resistant PrP; PK, proteinase K; PrP^{c}, normal cellular PrP; M, molecular marker; BSE, bovine spongiform encephalopathy; +, animal prion disease sample; –, matched normal animal control sample; CWD, chronic wasting disease; L-BSE, L-type BSE; H-BSE, H-type BSE.

We evaluated the susceptibility of the human PrP (129MM) to in vitro conversion first using the human brain homogenate substrate. Western blotting with antibodies 3F4 and 6H4 both showed readily detectable amplification in the samples seeded with C-type BSE and vCJD. (Figure 2, panel A, lanes 2, 4, and 14, compared with lanes 1, 3, and 13). Scrapie, L-type BSE, H-type BSE, and atypical scrapie reactions did not show detectable human PrP^{res} formation with the 3F4 antibody (Figure 2, panel A, lanes 6, 10, 12, and 16). However, 3F4 detected human PrP^{res} in the reaction seeded with the CWD brain homogenate (Figure 2, panel A, lane 8). Humanized transgenic mouse (129MM) brain substrate similarly showed efficient amplification of vCJD and C-type BSE and readily detectable amplification of CWD PrP^{res} using 3F4 (Figure 2, panel B). Faint bands were seen in L-type BSE and H-type BSE PMCA reactions when the 6H4 antibody was used. These bands most likely represent conversion of endogenous bovine PrP^{C} from the inoculum converted to PrP^{res}, rather than conversion of human PrP^{C} from the substrate (compare lanes 11 and 12 for 6H4 and 3F4 in Figure 2, panels A, B).

Dilutions of CWD brain homogenate in substrates prepared from human brain, transgenic mouse brain, and 239F cells expressing human PrP (129M or 129V) were compared for their ability to support amplification. Irrespective of origin, all 3 *PRNP* 129M-containing substrates supported amplification, albeit with slightly different efficiencies (Figure 3, panels A, B, C). All three *PRNP*

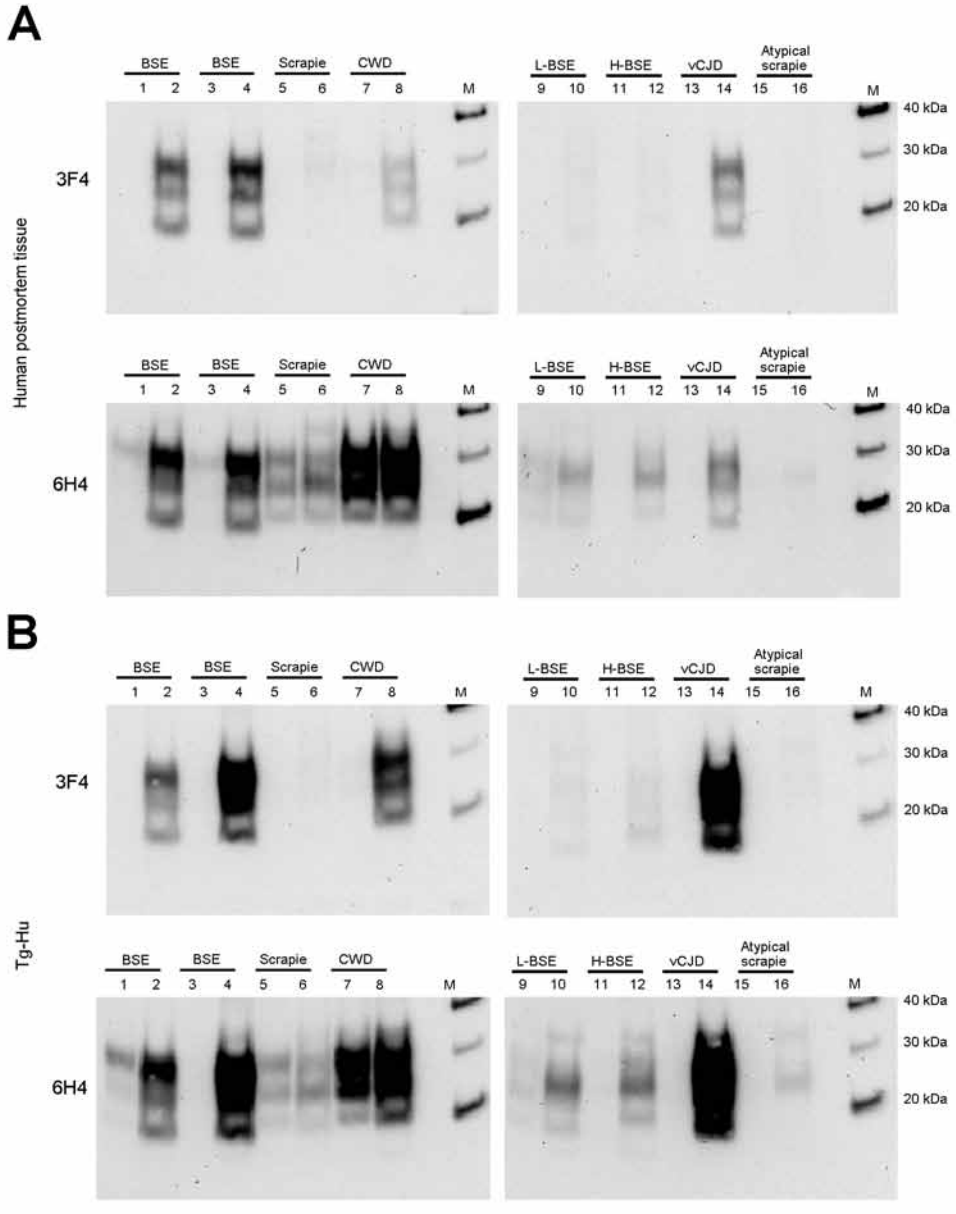

Figure 2. PMCA of *PRNP* codon 129MM human brain homogenate and humanized transgenic mice brain homogenate seeded with C-BSE, scrapie, CWD, L-BSE, H-BSE, vCJD, and atypical scrapie. PMCA reactions using *PRNP* 129MM human brain homogenate (human postmortem tissue) (A) and *PRNP* 129MM humanized transgenic mouse brain homogenate (Tg-Hu) (B) were seeded (1:3) with animal prion disease brain as indicated. Lanes 1, 3, 5, 7, 9, 11, 13, and 15 show the samples without PMCA. Samples in lanes 2, 4, 6, 8, 10, 12, and 14 were subjected to PMCA. Western blotting used the antibody 3F4 that enables the specific detection of human PrP. To compare the PrP^{res} levels into the seeds (before the PMCA), antibody 6H4 was also used. PMCA, protein misfolding cyclic amplification; BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; vCJD, variant Creutzfeldt-Jakob disease; PrP, prion protein; PrP^{res}, protease-resistant PrP; M, molecular marker.

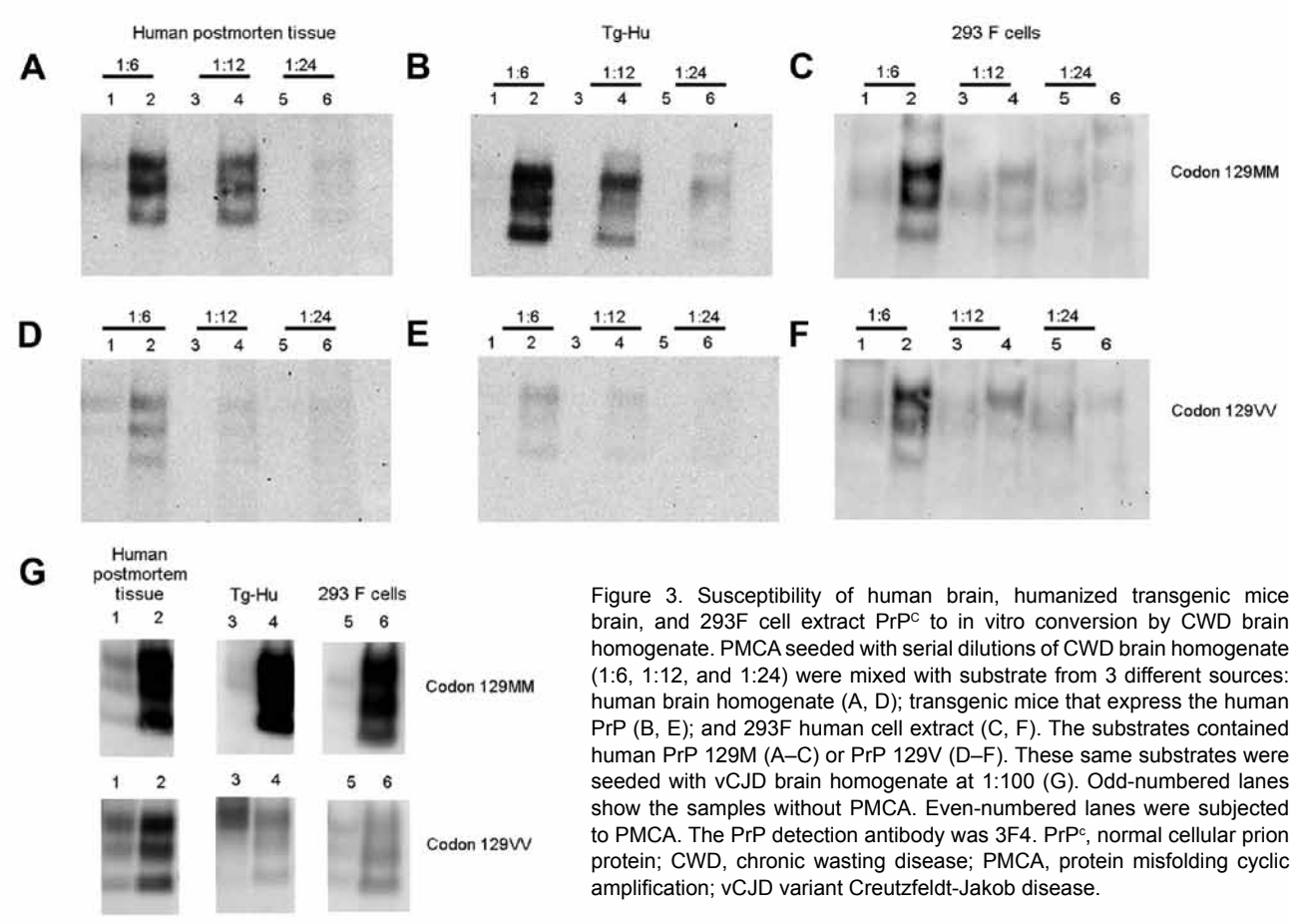

Figure 3. Susceptibility of human brain, humanized transgenic mice brain, and 293F cell extract PrP^{C} to in vitro conversion by CWD brain homogenate. PMCA seeded with serial dilutions of CWD brain homogenate (1:6, 1:12, and 1:24) were mixed with substrate from 3 different sources: human brain homogenate (A, D); transgenic mice that express the human PrP (B, E); and 293F human cell extract (C, F). The substrates contained human PrP 129M (A–C) or PrP 129V (D–F). These same substrates were seeded with vCJD brain homogenate at 1:100 (G). Odd-numbered lanes show the samples without PMCA. Even-numbered lanes were subjected to PMCA. The PrP detection antibody was 3F4. PrP^{c}, normal cellular prion protein; CWD, chronic wasting disease; PMCA, protein misfolding cyclic amplification; vCJD variant Creutzfeldt-Jakob disease.

129V-containing substrates also supported amplification (Figure 3, panels D, E, F), although the level of amplification was lower than for the 129M equivalent. PMCA reactions using vCJD brain homogenate as a seed were conducted by using these same substrates and are shown for comparison (Figure 3, panel G).

Next, we estimated the relative PrP^{res} amount in each sample by densitometry and adjusted the volume of 10% brain homogenate from the different animal prion diseases used in the PMCA reaction to give roughly equivalent amounts of PrP^{res} seed in each reaction (Figure 4). The amount of PrP^{res} in the atypical scrapie specimen was so low that a maximum volume of homogenate was used. The results of the Western blot analysis of these seed PrP^{res} normalized PMCA reactions, using antibody 3F4, confirmed that amplification efficiency was a function of seed/substrate compatibility and not simply PrP^{res} abundance.

C-type BSE, vCJD, and CWD amplification products were normalized and diluted (1:3, 1:6, 1:12, 1:24) in fresh human brain tissue homogenate (129MM) and subjected to a second round of PMCA. The CWD and C-type BSE PMCA reaction products retained their ability to convert further human 129M PrP, albeit at a lower efficiency than vCJD (Figure 5).

Western blot analysis of PrP^{res} produced by a PMCA reaction using human brain homogenate (129MM) seeded with CWD brain homogenate (Figure 6, lane 2) showed that this PrP^{res} shared the mobility and general glycosylation profile of type 1 PrP^{res} from sCJD brain (MM1 subtype) (Figure 6, lane 1). It was distinct from that of type 2 PrP^{res} characteristic of sCJD (VV2 subtype) and vCJD (Figure 6, lanes 3 and 4, respectively).

Discussion

Multiple factors govern the transmission of prions in experimental settings. In addition to infectious dose and route, a species or transmission barrier phenomenon is well recognized. Within the theoretical confines of the prion hypothesis, the most obvious basis of a species barrier effect would be dissimilarity in *PRNP* sequence between the infectious source and the exposed individual. However, *PRNP* sequence similarity alone does not seem to accurately predict whether prions are transmissible between species, perhaps because interactions between PrP^{C} and PrP^{Sc} occur as native PrP^{C} and misfolded and aggregated PrP^{Sc}

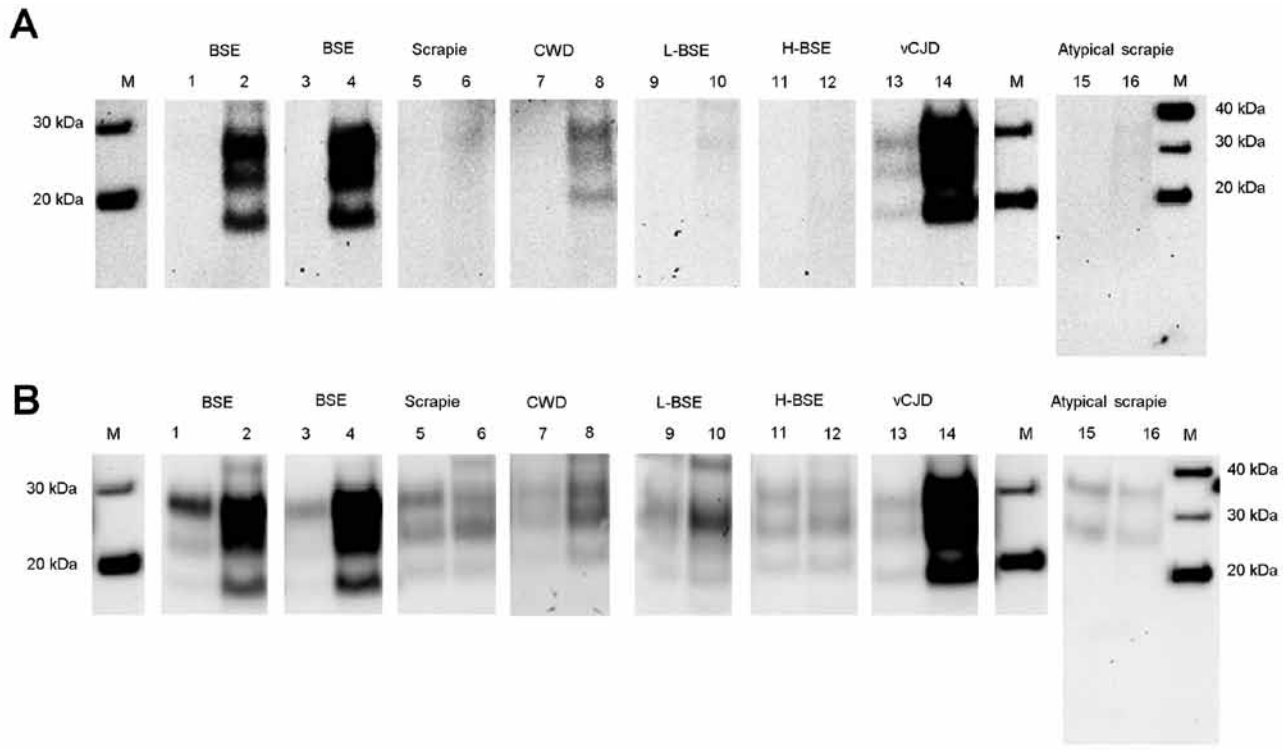

Figure 4. Relative conversion efficiency of human PrP (129M) by different animal prion disease samples. Brain homogenates from animal prion diseases were seeded at different volumes adjusted to give roughly equivalent amounts of seeding PrP^{res} and amplified by using PrP 129M-containing human brain substrate. Human PrP^{res} formation was detected by the 3F4 antibody (A) and seed and newly formed PrP^{res} detected using the 6H4 antibody (B). PrP, protein prion; PrP^{res}, protease-resistant PrP; M, molecular marker; BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; L-BSE, L-type BSE; H-BSE, H-type BSE; vCJD variant Creutzfeldt-Jakob disease; M, molecular marker.

conformers. The possible effects of species-specific sequence difference on PrP^{C} folding are not well understood. Neither is the secondary and higher order structure of PrP^{Sc} except for clear evidence that different prion strains are associated with different PrP^{Sc} conformers and glycotypes (reviewed in *4,18*) and that these might exist as a quasispecies or molecular cloud (*19*). Under such a scenario molecular compatibility might be difficult to predict.

To isolate and study molecular effects, we have previously conducted cell-free PrP conversion experiments by PMCA using homogenates of bovine and ovine prion disease brain samples to seed brain homogenates containing human PrP, assessing the extent of conversion by detection of human PrP^{res}. These studies showed that samples of C-type BSE (which is a known human pathogen and the cause of vCJD) efficiently converted human PrP, with a codon 129 preference similar to that of vCJD (MM>MV>VV), whereas samples of classical scrapie (which is not thought to be a human pathogen) failed to convert human PrP to a measurable extent. Equally importantly, a sheep BSE isolate resembled C-type BSE and vCJD in its ability to convert human PrP, thus underscoring influence of strain over sequence similarity in determining what might be termed a molecular transmission barrier (*10*).

Here we applied the same approach to a series of animal prion diseases whose risk to human health is poorly characterized. Our results show that under the PMCA conditions used, L-type BSE, H-type BSE, and atypical scrapie isolates fail to produce detectable human PrP^{res}. The CWD isolate used converted human PrP^{C}, albeit less efficiently than C-type BSE. This observation remained true whether the input animal prion disease brain homogenate was normalized by tissue weight or by PrP^{res} abundance and whether the PMCA substrate was from human brain, *PRNP* humanized murine brain, or a human-derived and human PrP^{C} overexpressing cell line. The conversion of human PrP^{C} by CWD brain homogenate in PMCA reactions was less efficient when the amino acid at position 129 was valine rather than methionine. Furthermore, the form of human PrP^{res} produced in this in vitro assay when seeded with CWD, resembles that found in the most common human prion disease, namely sCJD of the MM1 subtype.

Previous attempts to determine the transmissibility of these prion diseases to humans and thus assess their zoonotic potential have used experimental challenge of nonhuman primates, humanized PrP transgenic mice, and cell-free assays with sometimes conflicting results. Successful

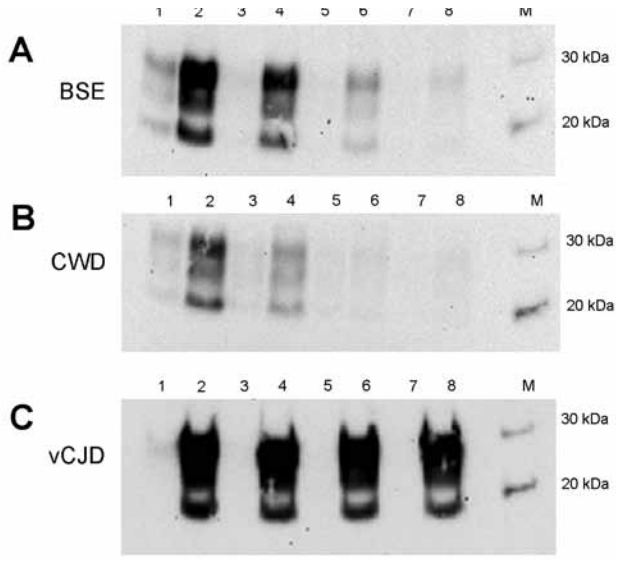

Figure 5. Properties of C-BSE, CWD, and vCJD amplification products in a second round of PMCA. Hu-C-BSE, hu-vCJD, and hu-CWD (from a previous round of PMCA) were supplemented with fresh human brain homogenate and subjected to a second round of PMCA. The reactions were normalized by PrP^{res} level and the product diluted (1:3, 1:6, 1:12, 1:24) in fresh human brain homogenate (*PRNP* codon 129MM) before PMCA. Odd numbers correspond to samples without PMCA; even numbers correspond to the reactions after PMCA (A, B, and C). The PrP detection antibody was 3F4. C-BSE, C-type bovine spongiform encephalopathy; CWD, chronic wasting disease; vCJD variant Creutzfeldt-Jakob disease; PMCA, protein misfolding cyclic amplification; hu, human; PrP^{res}, protease-resistant prion protein; M, molecular marker.

transmission of CWD and L-BSE to certain nonhuman primates has been reported: L-type BSE showing a different pathologic profile and a shorter incubation period than C-type BSE (*20–23*). However, Kong et al. (*24*) reported that CWD failed to transmit to humanized PrP 129M overexpressing mice inoculated with an elk brain homogenate. In contrast, Beringue et al. (*25*) reported that humanized PrP 129M overexpressing mice were susceptible to L-type BSE and suggested that L-type BSE was more virulent than C-type BSE and presented a zoonotic risk. H-type BSE reportedly failed to transmit to these same mice. Sandberg et al. (*26*) and Tamgüney et al. (*27*) confirmed the previous report of Kong et al. that CWD fails to transmit to transgenic mice, irrespective of whether 1) the mice expressed bovine, ovine, or human PrP; 2) the mice expressed the human 129M or 129V PrP allelic variants; or 3) the CWD isolates were from mule deer, elk, or white-tailed deer.

Cell-free approaches to modeling human susceptibility to animal prion prion diseases also have been published (*8*,*10*,*28–31*). Raymond et al. (*28*) compared the ability of CWD, C-type BSE, sheep scrapie, and CJD brain homogenates to convert human PrP^{C} metabolically labeled and purified from transfected cells. These experiments obtained limited conversion of human PrP^{C} by CWD, C-type BSE, and scrapie. In contrast to our study, this early cell-free system failed to distinguish between scrapie and C-type BSE in their ability to convert human PrP^{C}; however, it indicated a substantial molecular barrier to conversion of human PrP^{C} by CWD PrP^{Sc} (*28*,*29*), which agrees with this report. Kurt et al. (*31*) reported that PMCA using human PrP^{C} overexpressing transgenic mice brain (both 129M and 129V lines) as substrate failed to support amplification when seeded with CWD cervine brain homogenate. Cervidized *Prnp* transgenic mouse brain homogenate can support CWD prion replication (*32*), and extensive in vitro conditioning of a CWD isolate by PMCA in a cervidized substrate (or passage in cervidized mice) was sufficient to overcome the barrier and enable efficient in vitro amplification in a humanized transgenic mouse substrate (*33*). Direct comparison of these studies is made difficult by the differences in approach (in vivo vs. in vitro), the different transgenic constructs used, and the technical details of the cell-free conversion assays undertaken (Table). An additional possibly significant difference between these studies is the nature of the CWD isolate used. CWD affects different deer species (some of which show allelic variation in their *Prnp* sequence), but CWD also occurs as different biologic strains of agent (*34–36*). Different strains of CWD may have a role in determining transmissibility and conversion efficiency. Recently, Meyerett et al. (*37*) reported the in vitro strain adaptation of a CWD isolate by serial PMCA, similar to that produced by in vivo subpassage.

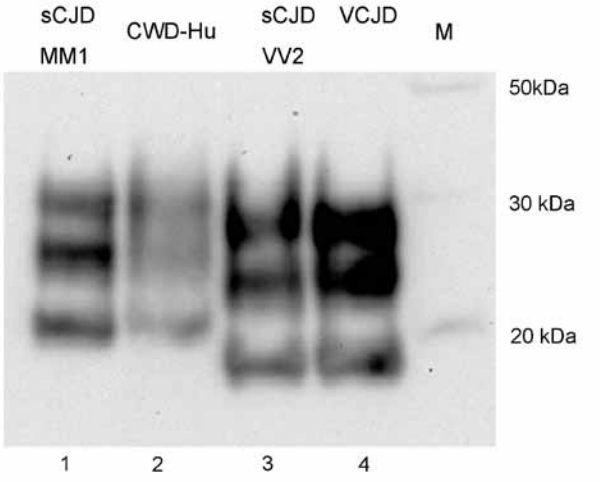

Figure 6. PrP^{res} typing of the CWD amplification product. The CWD PMCA product derived from amplification in a human brain homogenate substrate (*PRNP* codon 129MM) was compared by Western blotting with PrP^{res} from human brain samples from cases of sCJD of the MM1 subtype, sCJD of the VV2 subtype, and variant CJD. The PrP detection antibody was 3F4. PrP^{res}, protease-resistant prion protein; CWD, chronic wasting disease; PMCA, protein misfolding cyclic amplification; sCJD sporadic Creutzfeldt-Jakob disease; vCJD, variant CJD; hu, human; M, molecular marker.

Table. Comparison of the outcomes of experimental transmission and in vitro conversion studies of chronic wasting disease in human, humanized, and nonhuman primate model systems*

Donor animal inoculum	In vivo			In vitro			Ref
	Species inoculated	Animal (expression levels)/codon 129	Transmission	PrP source (expression levels)/codon 129	Method	Conversion	
Mule deer, white-tailed deer, and elk				Cells 129M and 129V	C-FA	Pos	(28,29)
Elk	Humanized PrP transgenic mice	Tg-40 (1×)/129MM Tg-1 (2×)/129MM	Neg Neg				(*24*)
Mule deer	Squirrel monkeys	*Saimiri sciureus* (1×)	Pos				(*20*)
Mule deer, white-tailed deer, and elk	Humanized PrP transgenic mice	Tg(HuPrP)440 (2×)	Neg				(*27*)
Mule deer, white-tailed deer, and elk	Squirrel monkeys Cynomolgus macaques	*Saimiri sciureus* (1×) *Macaca fascicularis* (1×)	Pos Neg				(*22*)
Mule deer and white-tailed deer				Tg-6816 (16×)/129M Tg-7823 (5×)/129V	PMCA PMCA	Neg Neg	(*31*)
Mule deer	Humanized PrP transgenic mice	Tg-45 (4×)/129MM Tg-35 (2×)/129M Tg-152 (6×)/129VV	Neg Neg Neg				(*26*)
White-tailed deer	Humanized PrP transgenic mice	HuMM (1×)/129MM HuVV (1×)/129VV	Neg Neg				(*11*)
Mule deer				Tg-440 (2×)/129MM	PMCA	Pos (after in vitro conditioning)	(*33*)
Elk				Human brain (1×)/129MM	PMCA	Pos	This article
				Human brain (1×)/129VV	PMCA	Pos	
				HuMM (1×)/129MM	PMCA	Pos	
				HuVV(1×)/129VV	PMCA	Pos	
				293F cell line(4×)/129M	PMCA	Pos	
				293F cell line(2×)/129V	PMCA	Pos	

*Numbers in parentheses denote the stated expression levels of PrP^c into the animal species and cell lines used. PrP, prion protein; C-FA, cell-free assay; pos, positive; neg, negative; PrP^c, host's normal cellular PrP. Blank cells indicate no reported data.

The most directly comparable in vivo study to that reported here is Wilson et al. (*11*), in which a similar series of atypical animal prion diseases were used to challenge transgenic mice expressing physiologic levels of human PrP^C. Atypical scrapie; C-, H-, and L-type BSE; and CWD all failed to produce disease (or signs of infection) on first passage in these mice (*11*). The use of different animal prion disease isolates (and possibly differing species and strains of CWD) might explain this discrepancy; however, a more fundamental difference might be that the in vivo and in vitro model systems assess different aspects of the agent and its replication. The in vivo model is undoubtedly more complex and arguably more physiologically relevant, and the readout is disease; however, it remains disease in a mouse, in which the *PRNP* sequence alone is human. The in vitro cell-free model does not assess disease as such, only the compatibility of particular combinations of seed and substrate homogenates (some of which, in these examples, were entirely of human origin) to produce PrP^{res}. Differences between the in vivo and in vitro models are exemplified by the comparison of C-type BSE, and vCJD. Both amplify well in PMCA using humanized (129MM) brain homogenate as a substrate (*10*), whereas intracranial inoculation of C-type BSE into humanized (129MM) mice fails to produce disease (*12*), unless first experimentally transmitted to sheep or goats (*13*,*38*,*39*).

The interpretation of different amplification efficiencies as a semiquantitative measure of relative risk is tempting but is probably premature and almost certainly an oversimplification. The testing of more isolates, especially of CWD in deer and elk, is advisable before any firm conclusions can be drawn. Additionally, possible strain-specific effects on amplification efficiency by the precise PMCA experimental conditions are difficult to discount and might complicate interpretation. The relative amplification efficiencies of C-, H-, and L-type BSE might differ intrinsically because certain strains of sheep scrapie appear to, even when amplified in homologous sheep substrates (*40*). However, we can say

with confidence that under the conditions used here, none of the animal isolates tested were as efficient as C-type BSE in converting human PrP^C, which is reassuring. Less reassuring is the finding that there is no absolute barrier to the conversion of human PrP^C by CWD prions in a protocol using a single round of PMCA and an entirely human substrate prepared from the target organ of prion diseases, the brain.

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Rapidly Fatal Hemorrhagic Pneumonia and Group A *Streptococcus* Serotype M1

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We report 3 cases of fulminant hemorrhagic pneumonia in previously health patients. Sudden-onset hemoptysis and dyspnea developed; all 3 patients and died <12 h later of massive pulmonary bleeding, despite aggressive supportive care. Postmortem analysis showed that the illnesses were caused by group A *Streptococcus emm1*/sequence type 28 strains.

Streptococcus pyogenes or group A *Streptococcus* (GAS) is a versatile pathogen that can cause serious diseases, including bacteremia, cellulitis, puerperal sepsis, meningitis, pneumonia, and necrotizing fasciitis (*1,2*). This pathogen also causes streptococcal toxic shock syndrome, a severe response to streptococcal pyrogenic exotoxins (Spe proteins), which trigger overproduction of inflammatory cytokines, leading to tissue damage, organ failure, and shock (*3,4*). Despite improved awareness and treatment, GAS infections remain among the top 10 infectious causes of fatal disease in humans (*1*).

Periodic resurgences of invasive GAS infections in industrialized countries have been linked to emergence of dominant GAS clones resulting from horizontal gene transfer (*5–8*). The ability of these clones to cause life-threatening disease in healthy persons probably depends on expression of virulence factors that facilitate penetration of host cell barriers and evasion of immune defenses (*3,4,8*).

More than 250 GAS types have been identified in sequencing studies of the hypervariable region of the *emm* gene, which encodes the M surface protein (*1,2*). The *emm* types seem to correlate with tissue tropism of the organism, and M protein augments GAS virulence by interfering with antibody and complement deposition, facilitating formation of microcolonies and neutralizing antimicrobial peptides, and stimulating proinflammatory and procoagulatory activities (*3,4*). M1 protein and M1 fragments released by neutrophil proteases can also provoke pulmonary hemorrhage, inflammation, and tissue destruction by their interaction with fibrinogen, which causes neutrophils to release heparin-binding protein, a mediator of vascular leakage (*9*). The hypervirulent *emm*1 GAS clone also harbors prophages encoding SpeA proteins and extracellular streptodornase D (Sda1), which can also enhance virulence and dissemination (*8,10*). We report 3 cases of rapidly fatal, hemorrhagic pneumonia in previously healthy patients that were determined to be caused by infection with hypervirulent GAS.

The Study

Patient 1 was a 74-year-old Italian man who came to the emergency department (ED) of Vittorio Emanuele Hospital in Catania, Italy, in July 2012. Patient 2 was 65-year-old German woman who arrived in Rome from Frankfurt in March 2011 and came directly from the airport to the ED of the University of Rome Medical Center. Patient 3 was a 40-year-old Japanese man who came to the ED of the Catholic University Medical Center in Rome in February 2012, 3 days after arriving in the city. All 3 patients had sudden-onset hemoptysis, dyspnea, and fever (temperature 37.5–38.5°C), which had rapidly worsened over 2–3 h. Their medical histories were unremarkable.

All 3 patients had acute respiratory failure requiring ventilator support and hemoconcentration; hyponatremia; increased levels of serum creatinine, lactate dehydrogenase, lactic acid, and brain natriuretic peptides; leukocytosis (7,000–8,000 cells/mm^3, 3,200–4,000 neutrophils); and increased levels of C-reactive protein (>400 mg/mL) and D-dimer (>2,000 μg/mL). Computed tomography of the chest showed patchy opacification throughout the lungs and multifocal confluent parenchymal opacities (Figure, panel A). Bronchoalveolar lavages were bright red and contained numerous erythrocytes, gram-positive cocci resembling streptococci, and no polymorphonuclear leukocytes. In spite of aggressive supportive care and empirical therapy with ceftriaxone (2 g intravenously) and levofloxacin (500 mg intravenously), the conditions of the patients deteriorated rapidly, and all 3 died of massive pulmonary hemorrhage ≤12 h after symptom onset.

Autopsy specimens showed bilateral hemorrhagic pleural effusions (1,500–2,000 cells/mL), heavy, blood-engorged lungs (1,600–1,700 g), and patent hilar structures, but no

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thoracic or abdominal lymphadenopathy. Lungs showed hemorrhage and edema (Figure, panel B). Microscopic analysis of the lungs showed necrosis and bacteria (Figure, panel C). Admission blood cultures (3 sets/patient) were positive at the 12-h reading. *S. pyogenes* was identified by using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (Bruker Daltonik, Breman, Germany) and isolated in all culture bottles, bronchoalveolar lavage cultures (pure colonies, 10^6 CFU/mL), and postmortem lung tissue cultures.

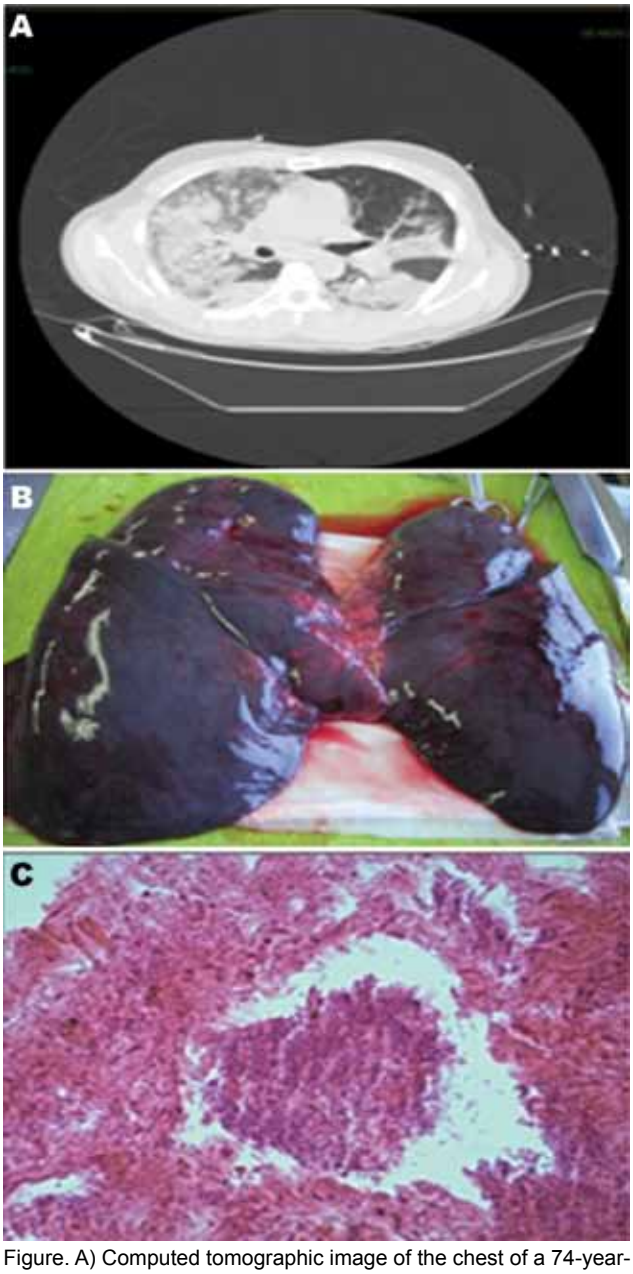

Figure. A) Computed tomographic image of the chest of a 74-year-old patient (patient 1) with fatal hemorrhagic pneumonia, Catania, Italy, showing multifocal confluent parenchymal opacities. B) Postmortem view of the lungs showing hemorrhage and edema. C) Microscopic evidence of necrosis and bacteria in the lungs (original magnification ×40).

All isolates were susceptible to erythromycin, tetracycline, amoxicillin, penicillin, and clindamycin by Etest (bioMérieux, Marcy l'Etoile, France). Results were interpreted according to European Committee on Antimicrobial Susceptibility Testing breakpoints (www.eucast.org/clinical_breakpoints). Results of testing for urinary *Legionella pneumophila* and *S. pneumoniae* antigens, β-glucan, galactomannan, and HIV and toxicology panels were negative for all 3 patients. Commercially available PCRs for respiratory tract samples showed negative results for major respiratory viruses and bacterial pathogens. Antibody titers against streptolysin O were <200 IU.

The *emm* typing, which was performed by PCR using protocols and the database of the Centers for Disease Control and Prevention (Atlanta, GA, USA; www.cdc.gov/ncidod/biotech/strep/M-ProteinGene_typing.htm), showed that all 3 GAS strains were *emm*-type 1 and had identical sequences at the 5′ end of the *emm* gene, indicative of the *emm* 1.0 allele. Strains were tested for multiple virulence genes (Table) by using PCR and primers described elsewhere (*11*) or designed with the VectorNTI program (Invitrogen, Carlsbad, CA, USA). They were identical in terms of the *spe* genotype (*speA*+, *speB*+, *speC*–, *speG*+, *speI*–, *speJ*+, *smeZ*+, *ssa–)*; the presence of *SLO*, *sag*A, *sag*BC, and *sda*1 genes; and the absence of *PAM*, *prt*F, and *sof* genes. Multilocus sequence typing was performed as described (http://spyogenes.mlst.net/) and showed an identical sequence type (ST) (ST28/CC28) for the isolates from patients 1 and 3 and a new single-locus variant of ST28 (designated ST648) for the isolate from patient 2. All cases were caused by GAS strains harboring the *emm1.0* allele and *Sda1*, *smeZ, speA, speB, speG*, and *speJ* genes.

Conclusions

A total of 1.3%–23.8% invasive GAS infections involve pneumonia, but these infections are more common in developing countries (*1,6,7,12*). Successful management depends on early diagnosis and initiation of effective treatment (*2,3*). However, despite early aggressive supportive care and empirical antimicrobial drug therapy, which was later confirmed to be appropriate by antibiogram results, all 3 patients we describe died within 8–12 h of symptom onset from massive pulmonary bleeding and acute respiratory failure. Initial presentations were fairly nonspecific, and diagnoses were made postmortem. The roles of GAS in the rapid progression of disease were highlighted by the abundance of GAS found the lungs, extensive and severe pulmonary damage, and virulence factor profiles of the isolates, all of which included *sda1*, *smeZ*, *speA*, *speB*, *speG*, *speJ*, and *emm-1* genes (*4,5*).

Table. Primers used for identification of virulence genes by PCR for group C *Streptococcus* isolates

Gene*	Primer name†	Primer sequence, 5′→3′	Amplicon size, bp
slo	**MS442**	GGTAACCTTGTTACTGCTAATGCTGA	400
	MS443	TAATGGAAATATCGACTGGTGTAGTG‡	400
speA	**speA-fw**	CTCAACAAGACCCCGATCCAAG	500
	speA-rew	ATTTAGAAGGTCCATTAGTATATAGTTGC‡	500
speB	MS384	GGCATGTCCGCCTACTTTACCGA	800
	MS385	CAGGTGCACGAAGCGCAGAAG‡	800
speC	MS410	TACTGATTTCTACTATTTCACCTATCATC	447
	MS411	TCTGATTTTAAAGTCAATTTCCTGG‡	447
speG	MS412	GCTATGGAAGTCAATTAGCTTATGCAG	448
	MS413	CCGATGTATAACGCGATTCCGA‡	448
speI	speI-up	GGTCCGCCATTTTCAGGTAGTTT	516
	speI-rew	ACGCATACGAAATCATACCAGTAG‡	516
speJ	MS414	CACTCCTTGTACTAGATGAGGTTGC	508
	MS415	ACGCATACGAAATCATACCAGTAG‡	508
sagBC	**sagBC-fw**	GCAGCTAGTTGCTCAACATTTAATG	600
	sagBC-rew	CATAGGCAGTCGCCTGATTCC‡	600
prtF	MS400	CGGAGTATCAGTAGGACATGCGGA	882
	MS401	CTCCCACCAACATTGCTTAATCCA‡	882
PAM	**PAM-fw**	GCAGACGACGCTAGAAATGAAGTA	900
	PAM-rew	CCTGCTTGTGGTGCTTGACCTTTAC‡	900
sof	MS402	ATGCCTGGTTGGGTATCTTCGGT	406
	MS403	AGAGAACAAAACGTTCTGCGCCTA‡	406
ssa	**ssa-fw**	GTAGTCAGCCTGACCCTACTCCAGAAC	621
	ssa-rew	ACTGATCAAATATTGCTGCAGGTGC‡	621
sda1	**MS431**	GGGTCTATAAGAAAAGTGGGCAAAG	439
	MS432	TGATCGTAAAGGTGGGATGCAGTA‡	439

**slo,* streptolysin O; *speA*, streptococcal pyrogenic toxin A; *speB*, streptococcal cysteine protease; *speC*, streptococcal pyrogenic toxin C; *speG,* streptococcal pyrogenic toxin G; *speI*, streptococcal pyrogenic toxin I; *speJ;* streptococcal pyrogenic toxin J; *sagBC*, streptolysin S-associated gene B/C protein; *prtF,* fibronectin-binding protein; *PAM*, plasminogen-binding protein; *sof,* serum opacity factor; *ssa,* streptococcal superantigen; *sda*1, streptodornase D.
†Primers indicated in **boldface** were created with the VectorNT program (Invitrogen, Carlsbad, CA, USA). Other primers were described by Santagati et al. (*11*).
‡Reverse primers.

Invasive bacterial disease requires virulence factors that facilitate interactions of the microbe with host tissues and subvert defenses of the immune system. In the *emm1* GAS clone, progression to systemic infection is also favored by mutations in the 2-component control of virulence regulatory system, which enhances resistance to subepithelial immune defenses and facilitates deep-tissue penetration. These mutations markedly alter transcription profiles of invasive GAS isolates than those of pharyngeal mucosal isolates (*3–5,8*), strongly upregulating *sda1* transcription and markedly downregulating expression of the gene encoding cysteine protease SpeB (*6–12*). The *sda1* gene facilitates avoidance of neutrophil extracellular traps by the pathogen and serves as a selective force for a control of virulence regulatory system mutation (*7,13*). SpeB protease enables accumulation and activation of broad-spectrum host protease plasmin on the microbial cell surface, thereby promoting infection spread to normally sterile sites (*2,3,14*). Invasive GAS strains also produce increased levels of toxins, including some that destroy immune cells, and superantigens (e.g., SpeA, SpeJ) that dysregulate the immune response of the host (*3*).

Host factors also affect clinical presentation and disease progression, which explains why diseases of different severity can be caused by genetically indistinguishable *emm1* strains with no evidence of regulatory gene mutation (*2,4,7*). Elucidation of these host and bacterial factors involved in the pathogenesis of these rare but life-threatening infections may be useful for improving disease prognosis (*6*).

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Pathogenic Pseudorabies Virus, China, 2012

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In 2012, an unprecedented large-scale outbreak of disease in pigs in China caused great economic losses to the swine industry. Isolates from pseudorabies virus epidemics in swine herds were characterized. Evidence confirmed that the pathogenic pseudorabies virus was the etiologic agent of this epidemic.

Pseudorabies virus (PRV), also called Aujeszky disease virus or suid herpesvirus type 1, is a member of the *Alphaherpesvirinae* subfamily within the family *Herpesviridae*. This pathogen has major economic consequences in pig husbandry (*1*–*3*). The PRV genome is a double-stranded linear DNA molecule ≈143 kb long and contains at least 72 genes (*1*,*4*). PRV can infect many kinds of mammals, including ruminants, carnivores, and rodents (*2*,*3*,*5*). However, pigs have been confirmed to be the primary hosts and reservoir of this virus (*6*–*8*). PRV infection is characterized by nervous system disorders and death in newborn piglets, respiratory disorders in older pigs, and reproductive failure in sows (*7*,*8*). Like other α herpesviruses, PRV infection can be a lifelong latent infection in the peripheral nervous systems of infected pigs, and these latently infected pigs can infect others under certain conditions (*7*–*9*). In this way, PRV causes devastating disease in pigs and economic losses worldwide.

Vaccination of pigs with attenuated live or inactivated vaccines is widely performed to reduce the huge economic losses caused by PRV infection (*10*–*12*). Although vaccination confers protection against disease, it does not prevent infection from a wild-type strain. Thus, both the virus in the vaccine and the super-virulent wild-type strain can establish latency within the same animal (*13*–*15*).

We report an outbreak of PRV infection that devastated the swine-producing regions of China in 2012. We systematically investigated the outbreak to identify the causative agent.

The Study

In January 2012, a previously unknown severe disease was observed in pigs on several farms in northern and eastern China. In Shandong Province, >80,000 pigs were infected. The affected pigs had high fever (≥40.5°C), anorexia, coughing, respiratory distress, conjunctival serous and mucinous secretion, and posterior paralysis. The disease was first observed in older pigs and spread within 2–3 days to younger pigs. Duration of disease was 5–7 days. Rate of illness reached 50%, and mortality was 3%–5%. Most pig deaths were recorded on the third day after monitoring began. Abortion was observed in ≈35% of sows that were 70–90 days pregnant. Viscera (e.g., lung, kidney, heart, liver, and spleen) and serum samples were collected from dead pigs from different provinces. Pathologic examination showed the most striking gross lesions were consolidated in the lungs (Figure 1, panel A), with edema and hemorrhage (Figure 1, panel B). In addition, foci of yellow-white necrosis were observed in the kidneys of some dead pigs (Figure 1, panel C).

To gain insight into the etiologic agent of the disease, we conducted extensive and systematic diagnostic testing, including PCR, ELISA, viral isolation, immunohistochemical staining, and standard bacteriologic culture, to evaluate the specimens. Marc-145 cells, inoculated with various tissue homogenates, showed cytopathic effects. A specific PRV monoclonal antibody was used, and immunopositive cells were observed in infected tissue (online Technical Appendix Figure 1, panels A, B, wwwnc.cdc.gov/EID/article/20/1/13-0531-Techapp1.pdf). The PCR for inocula samples showed that many glycoprotein (g) genes of PRV could be amplified by using the primers specific to the unique gene fragments (online Technical Appendix Figure 2). The PRV gE-ELISA assays (IDEXX Laboratories, Westbrook, ME, USA) indicated that serum samples from the sick pigs contained antibodies against wild-type, virulent PRV glycoprotein E but not against the vaccine strain (Table). All these results indicated that PRV was the causative agent of this disease. Our results also ruled out other suspected agents, such as classical swine fever, African swine fever, porcine reproductive and respiratory syndrome virus, and some bacterial infections. The 3 isolates found here are referred to as NVDC-PRV-BJ, NVDC-PRV-HEB, and NVDC-PRV-SD, according to the provinces from which they were isolated.

The pigs vaccinated with attenuated live PRV vaccines still showed clinical signs of PRV during the outbreak. To confirm the presence of PRV in these herds, 15 pigs were vaccinated with the current vaccine strain and then challenged

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Figure 1. Necropsy specimens from pigs infected with pseudorabies virus. A) Pulmonary consolidation in the lung. B) Edema and hemorrhage of lung. C) Kidney with many yellow-white necrotic spots (arrows).

with the NVDC-PRV-SD strain 21 days after vaccination. The results indicated that the vaccinated pigs had not been given completely effective protection against infection and exhibited obvious clinical signs of disease similar to the typical symptoms observed in the field, suggesting that the virulence of the newly isolated PRV strains had changed. This virulence may have caused the deaths of infected pigs.

To better understand the genetic relationship of the 3 PRV isolates found here to other PRV isolates, we amplified and sequenced the 15 major genes of the 3 isolates (online Technical Appendix Table 1). Compared with other PRV isolates (online Technical Appendix Table 2), there was a 21-nt insertion from nucleotide positions 185–205 in the gC gene, similar to SA215, BJ, DG, Ea, Fa, and P-PrV strains (Figure 2, panel A), which shared 100% nt identity with each other. The gD gene, like isolates Ea, Fa, SA215, and Yangsan, had 6 nt at positions 801–806 and shared 99.4%–99.8% identity with each other (Figure 2, panel B). There were 2 insertions of 6 discontinuous nucleotides each at positions 138–140 and 1472–1474 in the gE gene (Figure 2, panel C). These had 99.9%–100% identity with each other. The rest of the genes from the 3 isolates had no nucleotide insertions or deletion in common with the other PRV isolates. We further analyzed the relationship of these 3 isolates with other PRV isolates using a phylogenetic tree based on the gE gene; the 3 isolates formed a tightly clustered branch and were very closely related to other isolates from Asia (online Technical Appendix Figure 3).

Conclusions

We describe and analyzed a major outbreak of PRV in pigs in China. In these herds, all pigs had been vaccinated against PRV 3 times a year, at approximately the same time as each other. The disease spread to >6 provinces

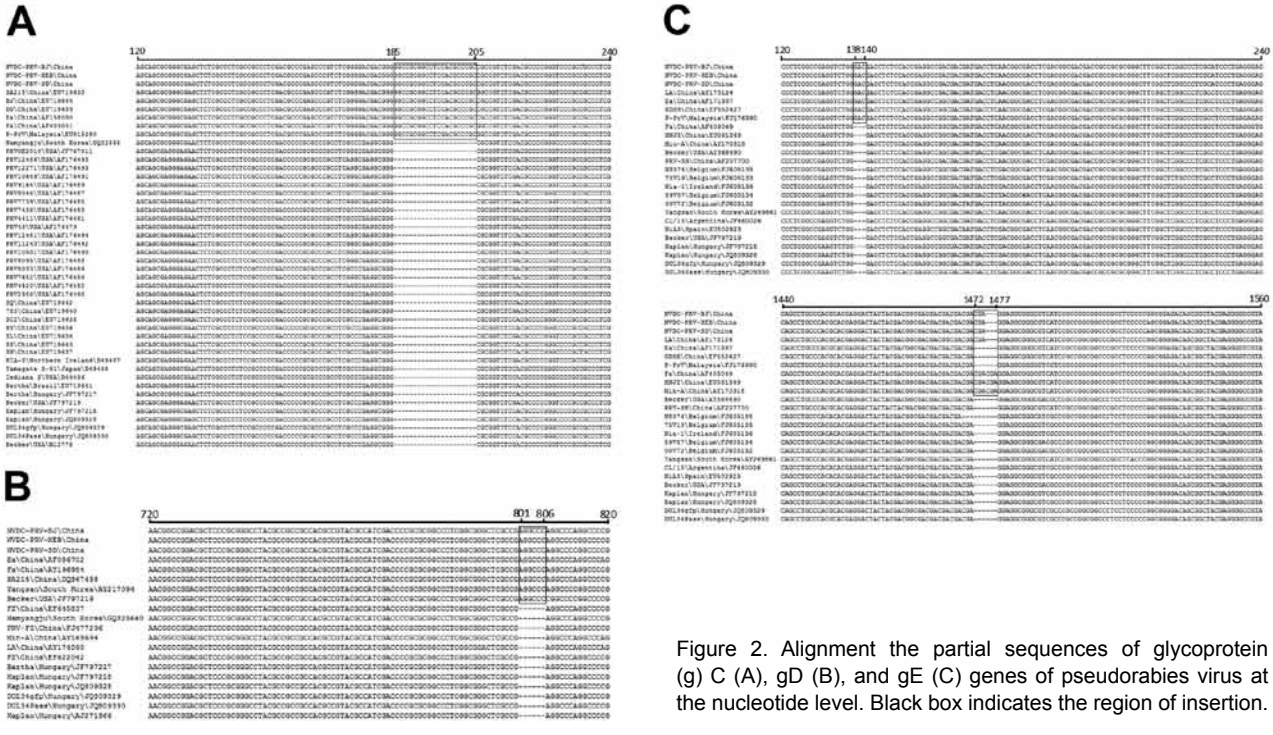

Figure 2. Alignment the partial sequences of glycoprotein (g) C (A), gD (B), and gE (C) genes of pseudorabies virus at the nucleotide level. Black box indicates the region of insertion.

Table. Antibodies to PRV gE in serum from PRV-infected pigs from different provinces, China, 2012

Sample origin province	Collection date	gE ELISA S/N ratio*
Shandong	2012 Jan	0.115
Beijing	2012 Feb	0.240
Hebei	2012 Feb	0.169
Tianjin	2012 Feb	0.171
Liaoning	2012 Mar	0.168
		0.274
		0.168
		0.173
		0.177
		0.179
		0.168
		0.158
		0.143

*S/N ratio was calculated as a ratio of the absorbance of a well with serum to the absorbance of a control well without serum. Serum with an S/N ratio of ≤0.60 was considered positive. PRV, pseudorabies virus; g, glycoprotein.

(including autonomous cities and regions) and caused considerable economic losses among local pig farms.

PRV has been recognized as a source of infection for pigs and continues to circulate globally among herds (*9*). In particular, pigs receiving attenuated live PRV vaccines showed typical clinical symptoms, which suggest that these isolates might have evolved new types of pathogenicity.

Even though the PRV isolates showed nucleotide insertions in the gC, gD, and gE genes, the molecular mechanisms underlying their high pathogenesis have yet to be elucidated. The origin of these lethal isolates within China is still obscure, although phylogenetic trees based on the gE gene here indicated that the 3 isolates are more closely related to the Asia PRV isolates, especially the China isolates, than to isolates from other countries. Because the virulence and origin of PRV is thought to be associated with multiple factors, whether such insertions are related to the virulence of PRV remains an issue and requires further investigation.

In summary, our study indicates that an outbreak of disease in pigs in China, which was of unprecedented scale, was caused by PRV infection. Other pathogens were ruled out. Our findings highlight the need to prevent and control the spread of this virus.

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Novel Avian Coronavirus and Fulminating Disease in Guinea Fowl, France

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For decades, French guinea fowl have been affected by fulminating enteritis of unclear origin. By using metagenomics, we identified a novel avian gammacoronavirus associated with this disease that is distantly related to turkey coronaviruses. Fatal respiratory diseases in humans have recently been caused by coronaviruses of animal origin.

Fulminating disease (also referred to as X disease) of guinea fowl (*Numida meleagris*) is an acute enteritis characterized by intense prostration and a very high death rate, leading to the almost complete destruction of affected flocks. Lesions are generally limited to severe enteritis and, in some birds, pancreatic degeneration. This disease is uncommon, but its fulminating evolution raises concerns of differential diagnoses with highly pathogenic avian influenza.

Fulminating disease has been reported for decades in the French guinea fowl industry, and although its viral origin was previously suspected, the virus remained unknown. During the 1980s, French groups investigated the etiology of the disease. Because propagation on cells or embryos remained unsuccessful and molecular tools were unavailable, etiologic hypotheses were based mostly on electron microscopy findings. The groups reached different conclusions, proposing candidates such as toga-like virus (*1*), reovirus, or herpesvirus (*2*). More recently, astroviruses have been associated with catarrhal enteritis in guinea poults (i.e., young guinea fowl) (*3*), but these viruses were not detected in birds affected by fulminating disease (data not shown).

We investigated field cases and performed an experimental reproduction of fulminating disease and identified its agent by using unbiased high-throughput sequencing. We propose a gammacoronavirus of a novel genotype as the most likely causal agent of fulminating disease. Coronaviruses (CoVs) are zoonotic. The novel human Middle East respiratory syndrome CoV, a betacoronavirus that was first isolated in 2012 in Saudi Arabia, is most closely related to *Tylonycteris* bat CoV HKU4 and *Pipistrellus* bat CoV HKU5 (*4*); severe acute respiratory syndrome–CoV originated from a betacoronavirus that spread from bats to civets and humans (*5*).

The Study

We investigated field cases of fulminating disease among 5 flocks of guinea fowl in France during 2010 and 2011; in all cases, the birds' clinical signs were limited to severe prostration, a dramatic decrease in water and feed consumption, and a daily death rate of up to 20%. From each affected flock, 5–10 sick birds were euthanized, and necropsy was performed. Most birds showed marked congestive enteritis and a whitish, enlarged pancreas. Livers, spleens, pancreas, kidneys, and intestinal tracts from the birds were placed into 10% buffered formalin for histopathologic examination or stored at -80°C for virologic analyses.

Fifteen 6-week-old guinea fowl poults were housed in 2 poultry isolators. One group of 5 birds that had been orally inoculated with the clarified and filtered (0.45-μm mesh) intestinal contents of diseased birds from 1 field case flock shared an isolator with another group of 5 uninoculated guinea poults, placed as contact birds. A third group of 5 uninoculated birds were placed in the other isolator. Inoculated and contact birds showed severe prostration as early as 2 days after infection and died or had to be euthanized within 6 days. Uninoculated birds showed no clinical signs of illness throughout the experiment. Necropsy was performed on birds that died or were euthanized, and comprehensive samples of tissues and fluids were obtained for subsequent investigations. At necropsy, the most notable lesion was an acute and marked enteritis; in some birds, a mildly enlarged and whitish pancreas was observed.

The intestinal contents of experimentally infected poults were pooled, clarified by centrifugation, and pelleted by ultracentrifugation (100,000 × *g*, 2 h). The pellets were treated with RNase (Ambion, 20 μg/mL) and DNase (Invitrogen, 10 U/μL) (both from Life Technologies, Grand

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Table 1. Distribution of reads generated by sequencing of pooled intestinal contents from guinea fowl poults with fulminating disease France, 2010–2011*

Read type	No. (%) reads
Total reads generated	476,430
Cellular reads	142,739 (30)
Bacterial reads	246,787 (51.8)
Archaea reads	35,271 (7.4)
Phage, plant, and insect virus reads	32,477 (6.8)
Eukaryote virus reads	19,155 (4.0)
Coronavirus reads	1,441 (7.5)†

*Sequencing was performed by using MiSeq (Illumina, San Diego, CA, USA) and analyzed by using the GAAS (http://gaas.sourceforge.net/) algorithm with an expected value of 10^{-3} (*7*).
†Percent within eukaryote virus reads.

Island, NY, USA) to concentrate the viral material. RNA and DNA were extracted separately, and a random reverse transcription PCR was performed, as described (*6*), to generate unbiased PCR products of ≈300 bp. High-throughput sequencing was performed by using a MiSeq platform (Illumina, San Diego, CA, USA). A total of 476,430 sequences were generated (Table 1), 10.8% of which matched with known viral sequences, as determined by using GAAS software (http://gaas.sourceforge.net/) (*7*) with an expected value of 10^{-3}; 7.5% of the eukaryotic viral reads were similar to avian CoVs, such as turkey CoV (TCoV) and infectious bronchitis virus (IBV). A total of 1,444 reads related to CoVs were aligned against the most similar TCoV genome available in GenBank (TCoV/CA/MG10; accession no. EU095850). The reads fairly aligned to almost the whole TCoV genome; the overall coverage was 78.86% (data not shown). A CoV-specific reverse transcription PCR was performed and the result was positive, specifically in intestinal tissues of experimentally infected birds (Table 2) (*8*). Furthermore, immunochemistry was performed by using a monoclonal antibody specific for TCoVs (*9*). We observed an intense, cytoplasmic, and granular labeling in enterocyte villi of inoculated birds only (Figure 1), suggesting a substantial intestinal replication of a TCoV-related virus. A 3,680-nt consensus full sequence of the spike (S) gene was completed by classical PCR and Sanger sequencing (GenBank/EMBL accession no. HF544506). A BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search followed by a phylogenetic analysis performed on the complete S gene showed that guinea fowl fulminating enteritis virus corresponds to a distinct genotype of CoV, clustering within *Gammacoronavirus* genus, which also includes TCoV and

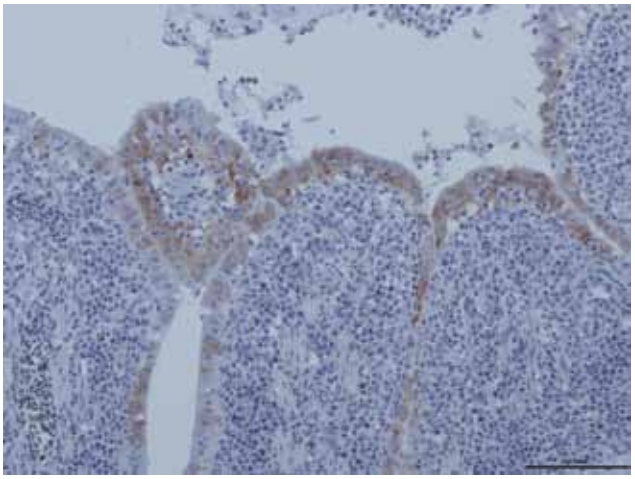

Figure 1. Replication of guinea fowl coronavirus in intestinal epithelium cells of experimentally infected birds as evidenced by immunohistochemical testing with a turkey coronavirus–specific monoclonal antibody, France, 2010–2011 (*9*). Scale bar indicates 0.1 mm.

IBV (*10*). The complete S gene sequence of guinea fowl CoV (GFCoV) was most similar to that of the TCoV S gene (minimum Kimura distance 22.6% at the nucleotide level between GFCoV/FR/2011 and TCoV/VA/74/2004, TCoV/CA/MG10, TCoV/IN/517/1994, or TCoV/ATCC). The GFCoV S gene was more similar to those of North American TCoV than to those of European (French) TCoV strains (Figure 2) (*11*).

CoVs are positive-sense RNA viruses that are subject to frequent mutations and recombination events, resulting in the emergence of novel viruses, such as severe acute respiratory syndrome (in 2003) and Middle East respiratory syndrome CoV in humans (*4*,*5*). Avian CoVs associated with nonclinical carriage or with respiratory, genital, renal, or enteric diseases have been identified in many avian species (*12*). CoV infection causes mild enteritis in different avian species, mainly turkeys, partridges, and quails. Avian CoVs are usually classified as gammacoronaviruses, although a few bird CoVs have also recently been described as deltacoronaviruses (*13*).

In the past, a recombination event led to the emergence of TCoV: the S gene of IBV recombined with an unknown virus (likely of avian origin), which resulted in a host change (chicken to turkey) and a tropism switch (respiratory to enteric). IBV and TCoV share <36% similarity for the S gene,

Table 2. Tissue tropism of coronavirus in experimentally and naturally infected guinea fowl poults, as detected by reverse transcription PCR, France, 2010–2011*

Case	Duodenum	Ileum/colon	Pancreas	Spleen	Bursa of Fabricius
Inoculated	5/5	5/5	0/5	0/5	1/5
Contact	3/5	5/5	0/5	0/5	1/5
Uninfected control	0/5	0/5	0/5	0/5	0/5
Field†	5/5	5/5	NT	NT	NT

*Data are no. of coronavirus PCR–positive birds/total no. of birds. NT, not tested.
†Intestines (pooled duodenum, ileum, colon samples) from 5 field cases were tested (5–10 birds tested per clinical case). The 5 cases were selected on the basis of clinical signs (severe prostration and death rate >50%).

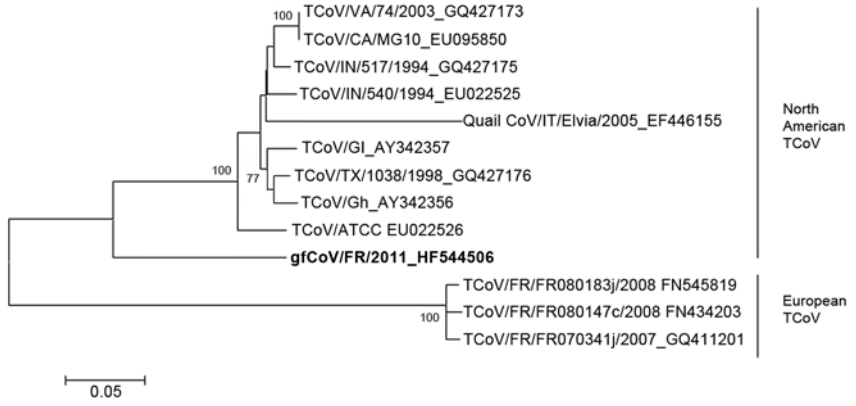

Figure 2. Phylogenetic analysis of the complete spike (S) gene of the fulminating disease of guinea fowl coronavirus (gfCoV, in **boldface**) in relation to turkey coronaviruses (TCoVs) at the nucleotide level. The tree was generated by using MEGA 5.05 (http://megasoftware.net/mega.php) by the neighbor-joining method, Kimura 2-parameter model, and pairwise deletion. Bootstrap values (1,000 replicates) >75 are indicated on the nodes. Only a partial S gene sequence (1,657 nt) was available for quail CoV/Italy/Elvia/2005. Scale bar indicates Kimura distance.

but their full genomes are >86% similar (*14*). Although the origin of GFCoV is still unknown, the distance of its S gene to TCoV and IBV S genes suggests not only a common ancestor but also a current separate evolutionary path. A quail CoV similar to TCoV has been described (*15*); its available S gene sequence also clusters with North American TCoV (Figure 2). However, the comparable partial S gene sequences of quail CoV/Italy/Elvia/2005 and GFCoV differ greatly (genetic distance 30%). A few cases of guinea fowl fulminating disease are diagnosed each year in France but have no apparent epidemiologic link to each other. The severity of the disease in the field may suggest a poor adaptation of the pathogen to guinea fowl. This pathologic pattern differs greatly from TCoV enteritis in turkeys and makes GFCoV of potential interest for comparative studies of CoV pathobiology. Virus reemergence may indicate that ≥1 other species may also be asymptomatic carriers.

Conclusion

We identified an avian gammacoronavirus related to TCoVs in fulminating disease of guinea fowl. The epidemiologic reservoir of this virus still needs to be clarified, and the sequencing of the full genome of the pathogen is warranted to fully assess its phylogenetic relationship with other gammacoronaviruses, its epidemiology, and its origin.

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Fatal Metacestode Infection in Bornean Orangutan Caused by Unknown *Versteria* Species

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A captive juvenile Bornean orangutan (*Pongo pygmaeus*) died from an unknown disseminated parasitic infection. Deep sequencing of DNA from infected tissues, followed by gene-specific PCR and sequencing, revealed a divergent species within the newly proposed genus *Versteria* (Cestoda: Taeniidae). *Versteria* may represent a previously unrecognized risk to primate health.

We describe the identification of a previously genetically uncharacterized species within the newly proposed Taeniid (Cestoda) genus *Versteria* (*1*), which caused fatal metacestode infection in a captive juvenile Bornean orangutan (*Pongo pygmaeus*). The orangutan was born in Colorado, USA, on April 4, 2007, and, after maternal rejection, was transported to the Milwaukee County Zoo in Milwaukee, Wisconsin, USA, for adoption by a surrogate mother on February 7, 2008. On December 27, 2012, keepers noted that the orangutan was exhibiting loss of appetite and an intermittent, moist cough. The animal became increasingly lethargic and was found dead 2 days later.

The Study

Postmortem examination revealed diffuse hemorrhages in the lungs (which did not collapse), splenomegaly, a pale mottled liver, and thoracic and pericardial effusions. Diagnostic microbiologic examination of tracheal washes and lung tissue identified only common environmental bacteria, and tests for viruses and fecal examination for parasites were all negative. Histopathologic examination of the liver revealed cystic structures containing eukaryotic parasite cells between ≈4 and 5 μm in diameter (Figure 1). Similar cells were observed in the parenchyma and blood vessels of lung and spleen (not shown). On the basis of these results and clinical observations, the cause of death was determined to be acute respiratory distress due to disseminated infection with an unknown parasite.

Because attempts to identify the parasite by morphologic features were inconclusive, total DNA extracted from infected organs was subjected to deep sequencing to detect molecular sequences of pathogens. DNA was isolated from liver, lung, and spleen by using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN Inc., Valencia, CA, USA), followed by treatment with RNase (Epicenter Biotechnologies, Madison, WI, USA) to remove RNA. DNA libraries were then generated by using the Nextera DNA Sample Prep Kit (Illumina, San Diego, CA, USA) and sequenced on an Illumina MiSeq instrument as described (*2*). Resulting sequence data were analyzed by using CLC Genomics Workbench 5.5 (CLC bio, Aarhus, Denmark). Briefly, low quality (<q30) and short (<100-bp) sequences were removed, sequences were aligned against an orangutan (*P. abelii*) genome (*3*), and nonmapped sequences were subjected to de novo assembly.

Deep sequencing of total DNA from infected tissues resulted in ≈2,400,000 sequences after quality trimming. Subtractive mapping against the orangutan genome removed ≈97% of these sequences. De novo assembly of the remaining ≈50,000 sequences resulted in 293 contiguous sequences, 7 of which had high similarity to GenBank sequences corresponding to *Taenia* spp. (expected values <1 × 10^{-18}). Subsequent mapping of nonhost sequences against the *T. solium* genome (*4*) resulted in 8,494 matches.

On the basis of deep-sequencing results, PCR primers were used to amplify 3 mitochondrial genes informative for resolving relationships within the Taeniidae (Table). For 12s ribosomal RNA (12s rRNA), primers CES12sF (5′-AGGGGATAGGACACAGTGCCAGC-3′) and CES12sR (5′-CGGTGTGTACMTGAGYTAAAC-3′) were modified from GenBank accession nos. KC344674–KC344701. For cytochrome c oxidase subunit I (*cox1*), published primers JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (5′-TAAAGAAAGAACATAATGAAAATG-3′) were used, and for NADH dehydrogenase subunit 1 (*nad1*), published primers JB11 (5′-AGATTCGTAAGGGGCCTAATA-3′) and JB12 (5′-ACCACTAACTAATTCACTTTC-3′) were used (*5*). PCRs were conducted in 20-μL volumes with 1-μL DNA template by using the Phusion kit (New England Biolabs Inc., Ipswich, MA, USA), cycled as follows: 98°C, 30 s; 35 cycles of 94°C,

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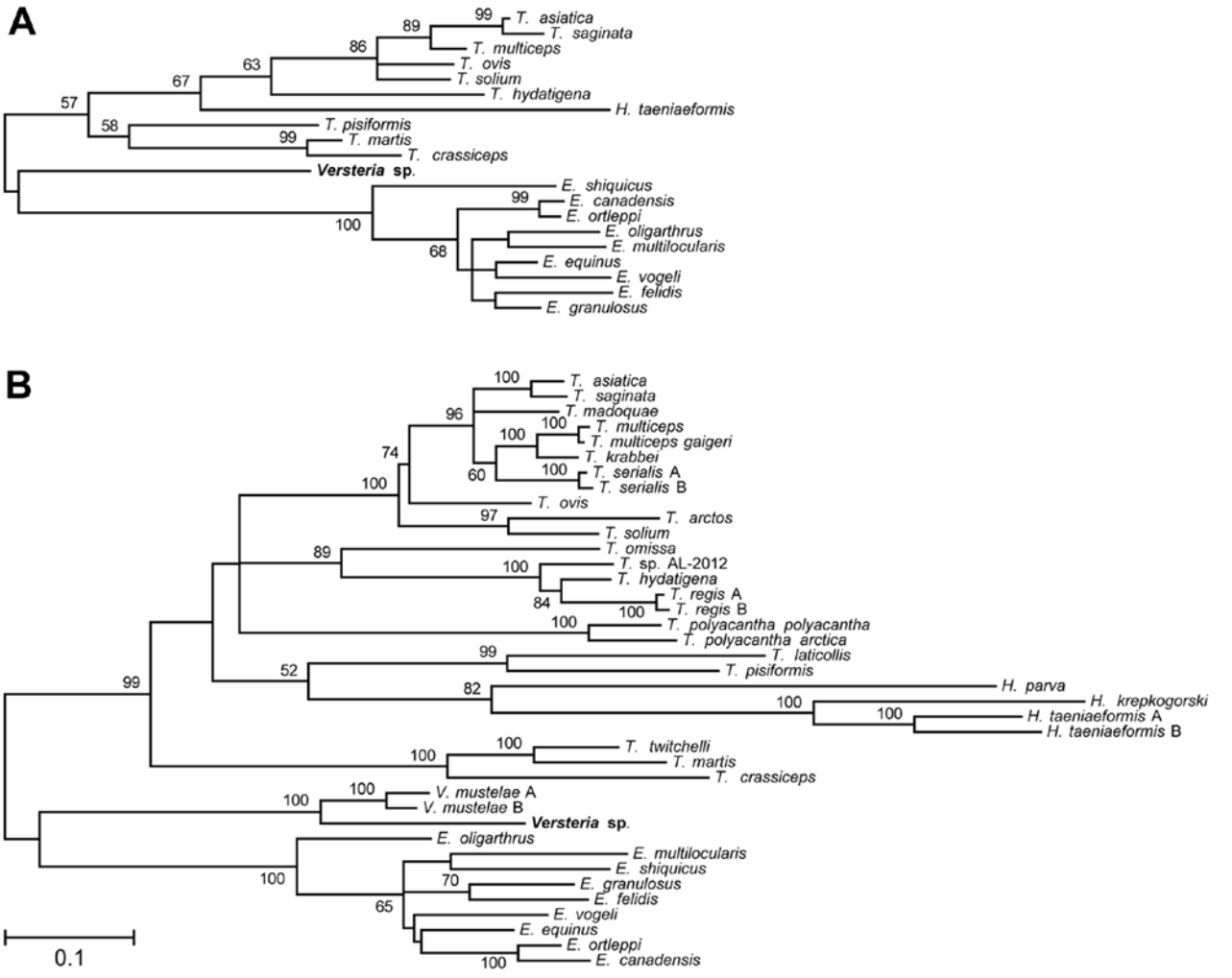

Figure 2. Phylogenetic trees of the Taeniidae, including newly generated sequences derived from tissues of a fatally infected Bornean orangutan. Trees were constructed from DNA sequence alignments of 12s rRNA (A) and concatenated *cox1*/*nad1* (B) sequences from the orangutan (*Versteria* sp.; bold; accession nos. KF303339–303341) and representative *Echinococcus*, *Hydatigera*, *Taenia,* and *Versteria* sequences from GenBank (see Table 1). The maximum likelihood method was used, with the likeliest model of molecular evolution chosen for both datasets by using MEGA5.2 software (*6*). Models of molecular evolution and tree likelihood values are HKY+G, -lnL = 2279.42 for 12s rRNA, and GTR+G+I, -lnL = 11582.71 for *cox1*/nad1. Numbers next to branches indicate bootstrap values (%), estimated from 1,000 resamplings of the data (only bootstrap values ≥50% are shown). Scale bar indicates nucleotide substitutions per site.

10 s, annealing, 30 s, 72°C, 30 s; and final extension at 72°C for 10 min (annealing temperatures for 12s rRNA, *cox1*, and *nad1* were 60°C, 50°C, and 55°C, respectively).

Amplicons underwent electrophoresis on 1% agarose gels stained with ethidium bromide, were purified with the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA), and were Sanger-sequenced in both directions by using PCR primers on ABI 3730xl DNA Analyzers (Applied Biosystems, Carlsbad, CA, USA) at the University of Wisconsin Biotechnology Center. Sequence chromatograms were edited and assembled by using Sequencher version 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were aligned with homologous sequences from all taeniid species in GenBank as of April 7, 2013 (Table). To construct phylogenetic trees, we used the maximum-likelihood method in MEGA5.2 software (*6*).

Figure 2 shows phylogenetic trees of newly generated 12s rRNA (panel A) and concatenated *cox1*/*nad1* (panel B) sequences and representative taeniid sequences. The trees closely agree with recently published taeniid phylogenies (*1*). Concatenated *cox1*/*nad1* sequences from the orangutan cluster with *V. mustelae* (formerly, *T. mustelae*) with 100% bootstrap support, placing the organism within the newly proposed genus *Versteria* (*1*) with confidence. However, the new *cox1* and *nad1* sequences

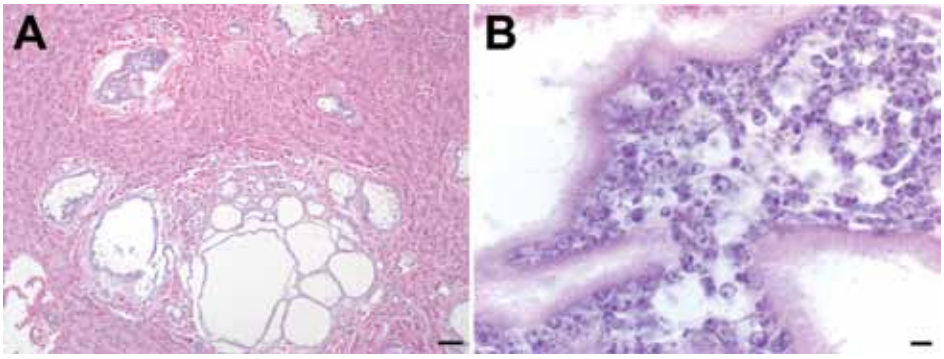

Figure 1. Microscopic images of liver sections from a Bornean orangutan fatally infected with *Versteria* metacestodes. Images of liver sections stained with hematoxylin and eosin (H&E) stain were captured at 10× magnification (A; scale bar = 30 μm) and 100× magnification; B; scale bar = 5 μm). Large numbers of parasite cells can be seen within well-defined cystic structures separated from the surrounding host tissue by clearly visible membranes.

are 12% different from those of published *V. mustelae* sequences. This degree of divergence is equal to or greater than that separating established *Echinococcus* and *Taenia* spp. (*7*) (Figure 2).

Members of the newly proposed genus *Versteria* have morphologic features that distinguish them from members of the other taeniid genera, such as miniature rostellar hooks, small scolex, rostellum, and suckers; a short strobili; and a small number of testes (*1*). However, no such distinguishing morphologic features could be identified by microscopy in the case described here. *V. mustelae* tapeworms infect multiple small animal intermediate host species and have been found in the upper midwestern United States in a hunter-killed fox squirrel (*Sciurus niger rufeventer*) with hepatic cysts (*8*). The definitive hosts of *V. mustelae* tapeworms are small carnivores of the family Mustelidae, such as weasels and martens (*9*). The genus *Versteria* also contains *V. brachyacantha* (*10*) tapeworms, which infect the African striped weasel (*Poecilogale albinucha*), but sequences of this species are not represented in GenBank. North American *V. mustelae* tapeworms are capable of asexual multiplication in the intermediate host (*11*); however, sequence data are only available for Eurasian specimens (*7*). The parasite described herein could thus represent a novel species or a previously genetically uncharacterized North American *V. mustelae* variant.

Conclusions

This study illustrates the utility of deep sequencing for diagnosing and characterizing enigmatic parasites. Similar methods have aided in the discovery of RNA viruses (*12*), but their application to eukaryotic pathogens has lagged, presumably because of technical challenges associated with distinguishing host from parasite DNA. In this light, it is noteworthy that our efforts were greatly facilitated by the availability of an orangutan genome against which to perform in silico subtractive mapping (*3*). As more host genomes become available, and as costs of equipment, reagents, and bioinformatics software decline, such methods promise to enter the diagnostic mainstream, as a complement to traditional morphologic and molecular approaches.

Encysted taeniid metacestodes can remain dormant for years before asexual multiplication (*13*); thus, this animal could have become infected at virtually any point in its life. Rapid progression to fatal disease could indicate an underlying condition, such as immune deficiency. Alternatively, this particular *Versteria* species may be inherently virulent.

Regarding source of infection, orangutans engage in geophagy (*14*), a behavior that this animal frequently practiced, suggesting that the infectious agent could have been obtained from contaminated soil. However, other sources (e.g., food, water, fomites) cannot be excluded. Infectious eggs could have entered the orangutan's environment through direct deposition by a definitive host or through complex pathways of environmental transport. To date, no other animals in the zoologic collections in Colorado or Wisconsin, where the orangutan was housed, have experienced similar disease, nor have similar infections been reported in persons, to our knowledge.

In any case, this animal's rapid and severe disease progression raises concerns about the health of captive apes in similar settings. Moreover, the close evolutionary relationship between orangutans and humans (*3*) raises concerns about the parasite's zoonotic potential.

Acknowledgments

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Table. GenBank accession numbers of taeniid DNA sequences used in phylogenetic analyses

Taxon	Origin	GenBank accession nos.*		
		12s rRNA	*cox1*	*nad1*
Echinococcus				
E. canadensis	Kazakhstan	NC_011121	NC_011121	NC_011121
E. equinus	United Kingdom	AF346403	AF346403	AF346403
E. felidis	Uganda	AB732958	AB732958	AB732958
E. granulosus	United Kingdom	NC_008075	NC_008075	NC_008075
E. multilocularis	Japan	NC_000928	NC_000928	NC_000928
E. oligarthrus	Panama	NC_009461	NC_009461	NC_009461
E. ortleppi	Argentina	NC_011122	NC_011122	NC_011122
E. shiquicus	Tibet	NC_009460	NC_009460	NC_009460
E. vogeli	Colombia	NC_009462	NC_009462	NC_009462
Hydatigera				
H. krepkogorski	China		AB731762	AB731762
H. parva	Spain		AB731760	AB731760
H. taeniaeformis (A)	China	NC_014768	NC_014768	NC_014768
H. taeniaeformis (B)	Finland		AB731761	AB731761
Taenia				
T. arctos	Finland		GU252130	GU252132
T. asiatica	Korea	NC_004826	NC_004826	NC_004826
T. crassiceps	Canada	NC_002547	NC_002547	NC_002547
T. hydatigena	China	NC_012896	NC_012896	NC_012896
T. krabbei	Norway		EU544578	EU544631
T. laticollis	Finland		AB731727	AB731727
T. madoquae	Kenya		AB731726	AB731726
T. martis	Croatia	NC_020153	NC_020153	NC_020153
T. multiceps	China	NC_012894	NC_012894	NC_012894
T. multiceps gaigeri	Iran		HM101469	HM101470
T. omissa	Canada		JX860631	JX860632
T. ovis	New Zealand	AB731675	AB731675	AB731675
T. pisiformis	China	NC_013844	NC_013844	NC_013844
T. polyacantha arctica	Greenland		EU544594	EU544646
T. polyacantha polyacantha	Denmark		EU544583	EU544636
T. regis (A)	Kenya		AM503328	AM503346
T. regis (B)	Kenya		AM503329	AM503347
T. saginata	Africa	NC_009938	NC_009938	NC_009938
T. serialis (A)	Kenya		AM503319	AM503336,
T. serialis (B)	Kenya		AM503322	AM503339
T. solium	China	NC_004022	NC_004022	NC_004022
T Taenia. sp. AL-2012	Finland		JX860629	JX860630
T. twitchelli	Russia		AB731759	AB731759
Versteria				
V. mustelae (A)	Finland		EU544567	EU544620
V. mustelae (B)	Russia		EU544571	EU544624
Versteria sp.†	United States	KF303339	KF303340	KF303341

*Multiple sequences were chosen to capture the maximum extent of intraspecific genetic divergence within highly diverse taxa (variants arbitrarily labeled A or B).
†Sequences generated in this study.

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Detection of Infectivity in Blood of Persons with Variant and Sporadic Creutzfeldt-Jakob Disease

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We report the presence of infectivity in erythrocytes, leukocytes, and plasma of 1 person with variant Creutzfeldt-Jakob disease and in the plasma of 2 in 4 persons whose tests were positive for sporadic Creutzfeldt-Jakob disease. The measured infectivity levels were comparable to those reported in various animals with transmissible spongiform encephalopathies.

Among humans, Creutzfeldt-Jakob disease (CJD) is a low incidence disease (≈1 case per million per year) that occurs as either a sporadic (sCJD) or a familial/genetic (fCJD) form. Whereas familial disease forms are linked to a mutation in the prion protein gene (*Prnp*), no clear epidemiologic risk factors have been identified for sporadic disease forms. sCJD is not a uniform disorder in terms of clinical and neuropathological phenotype. sCJD cases are classified as type 1 or 2 according to the polymorphism at codon 129 of the protease-resistant prion protein (PrP) sequence (methionine/valine) and to the electromobility of the proteinase K–resistant core of the abnormal PrP (PrP^{res}) (*1*). Type 1 and type 2 isoforms in sCJD are believed to correspond to different transmissible spongiform encephalopathy (TSE) agents

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Despite their relative rarity, several hundred iatrogenically transmitted CJD cases were identified during the past 60 years (*2*). Some data supporting the presence of infectivity in the blood of sCJD-affected patients were reported following the intracerebral inoculation of blood fractions from affected patients into rodents. These observations remain ambiguous because other studies did not confirm them (*3*,*4*).

In 1996, a new form of CJD, named variant CJD (vCJD), was identified in humans. Variant CJD was demonstrated to be caused by the agent that causes bovine spongiform encephalopathy in cattle (*5*). In the United Kingdom, 4 vCJD transmissions (3 clinical cases and 1 asymptomatic infection) were probably caused by the transfusion of non–leuco-depleted erythrocyte concentrates prepared from donors who later had positive test results for vCJD (*6*). More recently, a presumed additional case of vCJD infection was reported in the United Kingdom in a hemophilic patient who had received fractionated plasma products, including some units linked to a donor who had vCJD diagnosed (*7*). Despite the epidemiologic evidence of bloodborne transmission in vCJD, bioassays performed on conventional rodent models failed to demonstrate the presence of infectivity in the blood (*8*). The lack of TSE transmission in conventional rodent models could be a consequence of a low infectivity level in blood from vCJD- and sCJD-affected patients (as described in sheep and rodent TSE models) (*9*) or of the existence of the species barrier phenomenon that limits the transmission of human prions to these animal models. The development during the last decade of transgenic mice models expressing PrP from others species that abrogate the species barrier now offers the potential to detect low level of infectivity (*10*).

In this study, we used 2 transgenic mouse models that displayed a high sensitivity to the vCJD or sCJD TSE agents to estimate the infectious titer in certain blood fractions from vCJD- and sCJD-affected patients. According to legislation of the United Kingdom, Germany, and France, the experimental protocol, including the use of human samples, was approved by UK National CJD Research & Surveillance Unit tissue bank: REC reference number 2000/4/157-German TSE reference center: Ref Nr 11/11/93, PHRC ref 2004-D50-353 for patient from France.

The Study

Previous studies reported a high sensitivity in transgenic mice overexpressing bovine PrP (tgBov) for the detection of the bovine spongiform encephalopathy agent. To demonstrate that tgBov also displays a high sensitivity to vCJD infection, we titrated to endpoint a vCJD isolate (10% brain homogenate) by intracerebral inoculation in this model (Tg110) (*11*). Considering the potential diversity

Table 1. Titration of sCJD and vCJD isolates in transgenic mice expressing the human or bovine prion protein*†

	sCJD MM1 in tgHu		vCJD in tgBov	
Dilution	Positive transmission in mice	Incubation period, d	Positive transmission in mice	Incubation period, d
Not diluted	6/6	186 ± 10	6/6	249 ± 2
10^{-1}	6/6	213 ± 15	6/6	283 ± 15
10^{-2}	6/6	240 ± 13	6/6	316 ± 21
10^{-3}	6/6	263 ± 24	6/6	342 ± 10
10^{-4-}	6/6	296 ± 26	6/6	453 ± 66
10^{-5}	6/6	323 ± 29	4/6	499 ± 17
10^{-6}	1/6	316	1/6	502
10^{-7}	0/6	>650	0/6	>700
Infectious titer, ID_{50}/g of brain (95% CI)	$10^{6.67}$ ($10^{6.33}$–$10^{6.97}$)		$10^{6.33}$ ($10^{5,84}$ –$10^{6.82}$)	

*sCJD, sporadic Creutzfeld-Jakob Disease; tgHu, human PrP gene ;PrP, protease-resistant prion protein; vCJD, variant CJD; tgBov transgenic mice overexpressing bovine PrP, ID, infectious dose.
†Successive 1/10 dilutions of 10% brain homogenate (frontal cortex) from patients affected by vCJD and sCJD were injected intracerebrally to tgHu (n = 6) and tgBov (n = 6) mice, respectively. Those 2 patients were different from the 1 whose blood was tested in bioassay (Table 2). Mice were euthanized when they showed clinical signs of infection or after 650 days postinfection. Mice were considered infected when abnormal prion protein deposition was detected in the brain by western blot by using Sha31 monoclonal antibody, which recognizes amino acids 145–152 (YEDRYYRE) of the sheep prion protein. Infectious titers were estimated by the Spearman-Karber method (*14*).

of TSE agents that may cause sCJD, we decided to focus only on type 1 homozygous for methionine at codon 129 of the PRP gene (MM1) sCJD cases. An endpoint titration of a MM1 sCJD 10% brain homogenate was performed in a mouse model that express the methionine 129 variant of the human PrP gene (tgHu:Tg340) (*12*). This enabled confirmation of the capacity of the tgBov and tgHu models to detect the vCJD and sCJD MM1 agent, respectively, up to a 10^{-6} dilution of the reference brain homogenates (Table 1; *13*). This value was within the range of the brain/blood relative infectivity reported in various TSE animal models (*9*,*14*).

In the next step of our experiment, blood fractions (erythrocytes, plasma, and leukocytes) from 1 vCJD-confirmed patient were injected intracerebrally in tgBov mice. Similarly, plasma samples from 4 sCJD MM1 patients were inoculated with tgHu (Table 2). The blood fraction

Table 2. Intracerebral inoculation of blood components collected from 1 vCJD and 4 sCJD cases (MM1) in transgenic mice expressing the bovine or human prion protein gene*†

Mouse model	Donor	Specimen	Inoculated mice	Positive mice	Incubation period, d	ID/mL (95%CI)‡
tgBov	vCJD	Leukocyte	24	3	476, 567, 576	2.23 (0–4.87)
		Plasma	24	1	453	2.12 (0–6.52)
		Erythrocyte	24	1	433	2.12 (0–6.52)
tgHu	sCJD case 1	Plasma	14§	1	338	3.70 (0–11.65)
		Brain	6	6	216 ± 2	NA
	sCJD case 2	Plasma	24	0	>700	0 (0- 6.24)
		brain	6	6	217 ± 5	NA
	sCJD case 3	Plasma	24	1	233	2.12 (0–6.52)
		Brain	6	6	205 ± 5	NA
	sCJD case 4	Plasma	24	0	>700	0 (0–6.24)
		Brain	6	6	207 ± 3	NA
tgHu	Control human	Plasma	12	0	>650	NA
tgBov	Control human	Plasma	12	0	>650	NA
tgHu	Control human	PBS	12	0	>700	NA
tgBov	Control human	PBS	12	0	>700	NA
tgHu	Control human	Brain	24	0	>700	NA
tgBov	Control human	Brain	24	0	>700	NA
tgHu	Control human	None	24	0	>750	NA
tgBov	Control human	None	24	0	>750	NA

*vCJD, variant Creutzfeld-Jakob disease; sCJD, sporadic Creutzfeld-Jakob disease; dpi, days postinfection; ID, infectious dose; tgBov, bovine prion protein; tgHu, human prion protein;; PBS, phosphate-buffered saline.
†The leukocyte(s) from a single vCJD case corresponding to a starting volume of 3 mL of blood were suspended in 1 mL of 5% glucose solution. The leukocyte suspension and the crude erythrocytes were homogenized by using a high speed cell disrupter. The leukocyte and erythrocyte homogenates (vCJD case) and crude plasma (vCJD and sCJD cases) were intracerebrally injected into mice (20 µL per mouse). For the 4 sCJD MM1 cases, brain homogenate (10%, temporal cortex) were also inoculated in tgHu. Mice were euthanized when they showed clinical signs of infection or after 650 or 750 dpi. Mice were considered infected when abnormal protease-resistant prion protein; deposition was detected in brain tissue by using Western blot analysis with Sha31 monoclonal antibody: epitope amino acids 145–152 (YEDRYYRE) of the sheep PrP sequence. For samples showing 100% attack rate, incubation periods are reported as mean (± SD). For other samples, individual incubation period of CJD-positive mice are presented; their infectious titers were estimated by using limiting dilution titration method (application of Poisson model) described by Brown et al (*13*).
‡Leukocyte titer is expressed as ID/mL of the starting whole blood. Plasma and erythrocyte titers are expressed as ID/mL of inoculum.
§24 mice were inoculated; 10 died because of the acute toxicity of the sample.

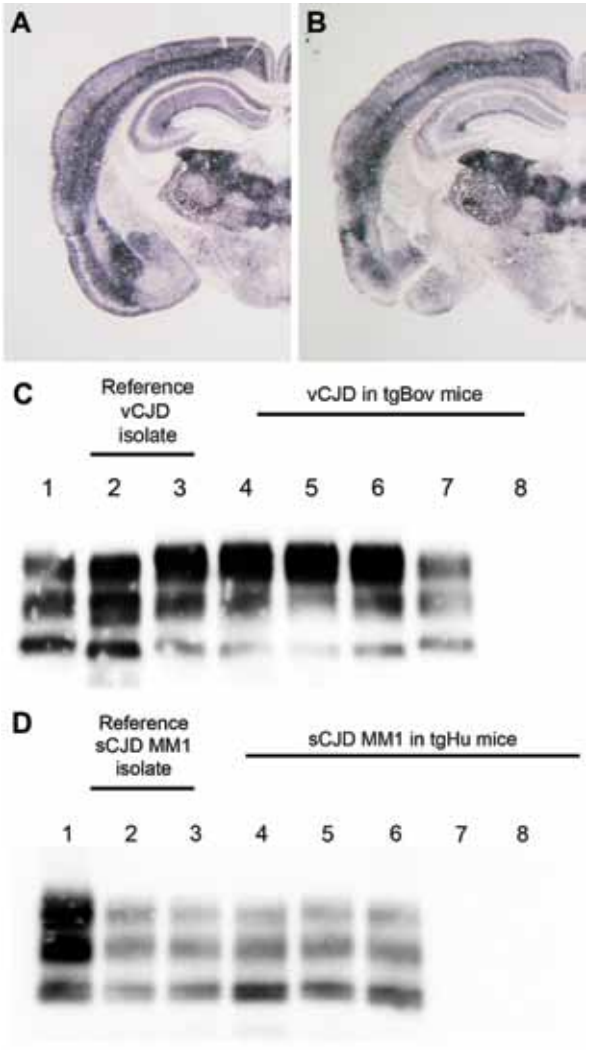

Figure. Abnormal prion protein (PrP^{res}) detection by using Western blot (WB) and paraffin-embedded tissue (PET) blot in the brain of transgenic mice expressing the methionine 129 variant of the human prion protein (PrP) (tgHu) or bovine PrP (tgBov). A, B) PET blot PrP^{res} distribution in coronal section (thalamus level) of tgHu mice inoculated with sporadic Creutzfeldt-Jakob disease (sCJD) MM1 isolates (10% brain homogenate): A) reference isolate used for the endpoint titration in Table 1; B) sCJD case 1 (Table 2). C) PrP^{res} WB of variant Creutzfeldt-Jakob disease (vCJD) reference isolate (used for endpoint titration in Table 1) and tgBov mice inoculated with the same vCJD reference isolate or vCJD blood fractions. Lane 1, WB-positive control; lanes 2 and 3, reference vCJD isolate; lane 4, leukocytes; lane 5, erythrocytes; lane 6, plasma; lane 7, WB-positive control; lane 8, healthy human plasma in tgBov. D) (PrPres Western blot of the sCJD reference isolate (used for endpoint titration in Table 1) and tgHu mice inoculated with the same sCJD reference isolate and plasma from sCJD cases. A proteinase K–digested classical scrapie isolate in sheep was used as positive control for the blots in panels C and D. (PrPres immunodetection in PET and Western blots was performed by using Sha31 monoclonal antibody (epitope: 145YEDRYYRE152 of the human PrP). Lane 1, WB-positive control; lanes 2 and 3, reference sCJD MM1 isolate; lane 4, brain tissue from case 1; lane 5, plasma from case 1; lane 6, plasma from case 3; lane 7, plasma from case 2; lane 8, plasma from case 4.

preparation was performed by using laboratory scale hematologic protocols (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/1/13-0353-Techapp1.pdf), not by following the procedure applied by blood banking services. This method implies that the leucodepletion that is applied to blood labile products in most countries to reduce the vCJD bloodborne transmission risk was not performed. Brain tissue samples from each of the 4 sCJD cases were also inoculated with tgHu. On the basis of the incubation period (Table 2) and PrP^{res} distribution pattern in the brain as assessed by using paraffin-embedded tissue blot, the TSE agents in those isolates were indistinguishable from those in the MM1 sCJD case that was used for endpoint titration (Figure, panel A).

No TSE clinical signs or PrP^{res} accumulation were observed in the tgBov or tgHu mice inoculated with phosphate-buffered saline or brain and plasma from healthy human controls. The 3 blood fractions from the vCJD-affected patient caused a positive result but low attack rate among tgBov mice (Table 2). On the basis of these results, infectivity in erythrocytes and plasma was estimated to be 2.12 infectious dose (ID)/mL of inoculum. In leukocytes, the infectious titer was estimated to be 2.23 ID/mL of whole blood. According to these values and the hematocrit of the sample (online Technical Appendix), the global infectious titer whole blood in the tested patient would be ≈4.45 ID/mL. Such infectious level is approximately equivalent to 1.4 μg of the reference vCJD brain sample that was endpoint-titrated (Table 1).

In tgHu mice, positive transmission was observed among mice inoculated with 2 of 4 plasma samples (Table 2). The infectious titers in both positive plasma samples were estimated to be 2.12 and 3.7 ID/mL of plasma, which is equivalent to 0.3–0.5 μg of the reference sCJD MM1 brain sample that was endpoint titrated (Table 1). However, because of the limited number of mice inoculated (n = 24) and the overall sensitivity of the assay (upper CI limit 6.24 ID/mL), the absence of transmission in mice inoculated with the 2 other plasma samples cannot be interpreted conclusively

In tgBov inoculated with vCJD and tgHu inoculated with sCJD, the PrP^{res} banding patterns observed by Western blot in animals challenged with brain homogenate and blood components were identical (Figure, panels C, D). These results support the contention that the TSE agent propagated in tgBov mice and tgHu were vCJD and sCJD agents, respectively.

Conclusions

The data reported here confirm the presence of infectivity in erythrocytes, leukocytes, and plasma from vCJD-affected patients and demonstrate unambiguously the presence of infectivity in the plasma of some, but not all, sCJD-affected patients. The infectivity levels that we

measured in the tested vCJD and sCJD blood components were comparable to those reported in various TSE animal models. The number of cases included in our study was limited; a new experiment that would include a larger number of cases and different blood fractions from sCJD cases will be necessary to refine the data. However, these results represent a substantial input for assessing the risk for interindividual bloodborne transmission of sCJD and vCJD.

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Contact Tracing for Influenza A(H1N1)pdm09 Virus–infected Passenger on International Flight

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In April 2009, influenza A(H1N1)pdm09 virus infection was confirmed in a person who had been symptomatic while traveling on a commercial flight from Mexico to the United Kingdom. Retrospective public health investigation and contact tracing led to the identification of 8 additional confirmed cases among passengers and community contacts of passengers.

On April 27, 2009, influenza A(H1N1)pdm09 virus infection was confirmed in a passenger who had traveled on a commercial flight from Mexico to the United Kingdom (UK) (*1*). This was the first identified imported case of A(H1N1)pdm09 infection in the UK. The person departed Mexico on April 20, 2009, and arrived in Birmingham, UK, 9.5 hours later on April 21, 2009. We describe the contact-tracing investigation of passengers on the flight and estimate the risk for transmission of A(H1N1)pdm09 virus to the passengers.

The Study

During the flight aboard a Boeing 767-300 airplane from Mexico to the UK, the index A(H1N1)pdm09 patient (case-patient 1) was seated in the rear cabin. Bulkheads and toilets divided the airplane cabin into 3 sections (front, middle, and rear). On the implicated flight, 282 passenger seats were available. Case-patient 1 is believed to have become symptomatic on April 18, 2009 (2 days before departing Mexico) and continued to be symptomatic during the flight. Reported symptoms were fever, cough, headache, myalgia, and chills. A nasopharyngeal swab sample obtained on April 24 was PCR positive for A(H1N1)pdm09 virus. A traveling companion of case-patient 1 (case-patient 2) was asymptomatic during the flight but symptomatic on April 23, 2009; a nasopharyngeal swab sample obtained on April 25th was PCR positive for A(H1N1)pdm09 virus.

By using information from the airline's passenger manifest, we identified close contacts of case-patient 1. Close contacts were defined as passengers seated in the same row as or in the 2 rows in front of or behind the row in which case-patient 1 sat. This definition is consistent with World Health Organization guidance for post-flight influenza contact tracing (*2*).

Beginning April 29, 2009, close contacts of case-patient 1 were interviewed by telephone; a structured questionnaire was used. Because case-patients 1 and 2 had the first identified cases of A(H1N1)pdm09 infection in the UK, they had already been extensively interviewed. Thus, we extracted relevant information from those interviews and did not re-interview the patients. Data for these 2 persons are not included in the calculation of post-flight attack rates.

Passengers on the flight were considered to be post-flight case-patients if they had influenza-like illness ≤7 days after arrival in the UK and had positive test results for A(H1N1)pdm09 virus. Influenza-like illness was defined as fever, measured or subjective, plus ≥2 of the following signs or symptoms: cough, sore throat, rhinorrhea, myalgia, headache, vomiting, and diarrhea. Using case-patient 1 as the initiator of the chain of transmission, we categorized post-flight case-patients as first-generation case-patients. Persons with cases arising from first-generation cases were categorized as second-generation case-patients. Passengers identified as close contacts but who did not meet these criteria were not regarded as case-patients. To identify other A(H1N1)pdm09 cases in the UK associated with this flight, we reviewed the Health Protection Agency's First Few Hundred national database (*3*).

Thirty-nine passengers on the flight (all of whom lived in the UK) were identified as close contacts of case-patient 1, and 37/39 were asymptomatic during the flight. The 2 passengers who were symptomatic (cough and subjective fever) during the flight sat within 1 row of case-patient 1; both had test results negative for A(H1N1)pdm09 infection. All close contacts were interviewed within 3 weeks (median 13 days, range 8–20 days) of disembarkation.

Two of the 37 case-patients who were asymptomatic during the flight later tested positive for A(H1N1)pdm09 infection. One of these persons (case-patient 2, the traveling companion of case-patient 1) was seated next to case-patient

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1; the other person (case-patient 3) was seated 2 rows behind case-patient 1. Therefore, after excluding case-patient 2, the attack rate for persons identified as first-generation cases and close contacts of case-patient 1 was 1/38 (2.6%, 95% CI 0.5%–13.5%).

Details of 6 additional confirmed cases that were identified after a review of a national database are shown in the Table (cases 4–9). Four of these cases were first-generation cases (cases 4–7). The others (cases 8–9) were classified as second-generation cases and occurred in persons who had not been on the flight and had no travel history but were known to have had direct contact with persons who had been on the flight.

Case-patients 4 and 5 had been seated next to each other in the middle section of the cabin, 4 rows in front of case-patient 1. They were situated directly in front of the bulkhead separating the middle and rear sections of the cabin. Case-patients 6 and 7 were seated within 3 rows of each other and 5 and 8 rows, respectively, behind case-patient 1 in the rear section of the cabin. The attack rate for passengers sitting elsewhere in the plane and not regarded as close contacts of case-patient 1 was 4/238 (1.7%, 95% CI 0.5%–4.3%), whereas the attack rate for all passengers was 5/276 (1.8%, 95% CI 0.8%– 4.2%). Altogether, 4 of the confirmed cases were identified among the 96 passengers seated in the rear section of the cabin, where the attack rate was 4.2% (95% CI 1.2%–10.3%).

Conclusions

The investigation of passengers on this flight and their contacts identified 9 cases of PCR-confirmed A(H1N1)pdm09 infection: the index case-patient, who had been symptomatic while traveling; 6 other passengers on the same flight; and 2 members of the public who had exposure to persons who had been asymptomatic passengers on the flight. Of the 6 confirmed case-patients on the flight, only 2 (including case-patient 2, the traveling companion of case-patient 1) had been seated within 2 rows of case-patient 1.

It cannot be definitively stated that A(H1N1)pdm09 virus was transmitted from case-patient 1 to other passengers during this flight; however, several reasons support our assumption that such transmission did occur. At the time of disembarkation, there were no known cases of A(H1N1)pdm09 virus infection in the UK, and no other plausible sources of infection were identified. These facts increase the likelihood that the 2 second-generation cases identified are directly attributable to passengers on the flight who were identified as first-generation case-patients. The distribution of possible first-generation cases within the aircraft reflects previous reports describing the in-flight transmission of influenza viruses and A(H1N1)pdm09 virus (*4*–*7*). Symptom onset for the 6 first-generation cases occurred 1–5 days after disembarkation, and although it is possible that infection could have occurred in Mexico at any time before embarkation, this timeline is also within the range for in-flight transmission and consistent with the known epidemiology of A(H1N1)pdm09 virus (*8*).

It is also plausible that this flight was multiply seeded with asymptomatic infected persons. In particular, it is possible that the first-generation case-patients seated >2 rows away from case-patient 1 may have been exposed to A(H1N1)pdm09 virus by other unidentified, infected persons.

This study had limitations. First, contact tracing was limited to close contacts of case-patient 1. Second, not all rear-cabin passengers were tested or interviewed. Last, confirmation was based on PCR testing of a single nasopharyngeal swab sample, so it is possible that some infections were missed, leading to an underestimate of transmission risk.

The results of this study suggest that where in-flight transmission of a novel virus is suspected, restricting contact tracing to passengers within a 2-row zone may result in a failure to identify other cases (*5*,*6*,*9*). Such an outcome may have implications with regard to the global spread of a new disease.

Table. Selected characteristics of persons with confirmed cases of influenza A(H1N1)pdm09 virus infection, United Kingdom, April 2009*

Case-patient no.	Symptomatic during flight	No. rows from index case-patient	Day of symptom onset, April 2009, no. days before/after flight
1†	Yes		18th, 3 before
2‡	No	Same	23rd, 2 after
3	No	2 behind	24th, 3 after
4	No	4 in front	26th, 5 after
5	No	4 in front	24th, 3 after
6	No	5 behind	22nd, 1 after
7	No	8 behind	24th, 3 after
8§	NA	NA	25th, 4 after
9¶	NA	NA	26th, 5 after

*Characteristics were determined during flight-related contact tracing. NA, not applicable.
†Case-patient 1, the index patient, had the first laboratory-confirmed case of A(H1N1)pdm09 infection in the United Kingdom.
‡Case-patient 2 was the traveling companion of case-patient 1.
§Case-patient 8 was not on the flight and is a secondary case-patient who is believed to have been exposed to the virus by case-patient 2.
¶Case-patient 9 was not on the flight and is believed to have been exposed to the virus by a passenger who was on the flight but who had test results negative for A(H1N1)pdm09 virus.

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Dr Shankar was a member of the regional response team for influenza A(H1N1)pdm09 virus infection in the West Midlands. His areas of interest include control of communicable diseases, surveillance, vaccine preventable diseases, and the epidemiology of health care–associated infections.

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Dobrava-Belgrade Virus in *Apodemus flavicollis* and *A. uralensis* Mice, Turkey

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In 2009, human Dobrava-Belgrade virus (DOBV) infections were reported on the Black Sea coast of Turkey. Serologic and molecular studies of potential rodent reservoirs demonstrated DOBV infections in *Apodemus flavicollis* and *A. uralensis* mice. Phylogenetic analysis of DOBV strains showed their similarity to *A. flavicollis* mice–borne DOBV in Greece, Slovenia, and Slovakia.

The genus *Hantavirus,* family *Bunyaviridae,* contains human pathogenic viruses that cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (*1*). HFRS in Europe is caused mainly by Puumala virus and different genotypes of Dobrava-Belgrade virus (DOBV) (*2*). In Asia, Hantaan virus and Seoul virus cause most HFRS cases. Hantaviruses are enveloped viruses with a single-stranded 3-segmented RNA genome of negative polarity. The small (S), medium, and large genome segments encode the nucleocapsid protein, the glycoproteins Gn and Gc, and an RNA-dependent RNA polymerase, respectively.

Hantaviruses have been detected in various rodent, shrew, mole, and bat species (*3*). They are transmitted to humans by inhalation of aerosols that are contaminated with urine, feces, and saliva of infected reservoir hosts. The human pathogenic DOBV was first isolated from a yellow-necked field mouse (*Apodemus flavicollis*) and, subsequently, from a striped field mouse (*A. agrarius*) and a Caucasian wood mouse (*A. ponticus*) (*1*). The association of DOBV with these different *Apodemus* species seems to determine its human pathogenicity, with the *A. flavicollis*–associated genotype Dobrava being the most life threatening (*1*).

Few reports about hantavirus seroprevalence in human and rodent populations in Turkey occurred before 2009 (*4*,*5*). In February 2009, the first hantavirus outbreak among humans in this country was described in 2 provinces in the western Black Sea region (*6*; Figure 1). DOBV-reactive antibodies were reported for 7 of 200 patients who had renal symptoms in a region near the Aegean Sea (*7*). In 2010, DOBV RNA was detected by a nucleic acid test in urine from a person in Istanbul Province who was experiencing fatigue, diffuse pain, nausea, and vomiting (*8*). The reservoir host(s) and virus strain(s) causing human infections on the Black Sea coast of Turkey remained unknown.

The Study

After the hantavirus outbreak in 2009, a National Hantavirus Study Group was founded in Turkey. Coordinated by this group, in June 2009, a total of 173 rodents and 2 shrews were collected from 7 sites in the rural area of Bartin Province in the western Black Sea Region, where suspected human hantavirus cases had been reported (Figure 1).

To determine the presence of DOBV in rodent tissue samples, we developed a single tube reverse transcription quantitative PCR (RT-qPCR) using the QuantiTect Probe RT-PCR kit (QIAGEN, Hilden, Germany) and novel primers and probe (online Technical Appendix Table, wwwnc.cdc.gov/EID/article/20/1/12-1024-Techapp1.pdf). For that purpose, spleen, liver, and lung samples from each animal were pooled and homogenized in sterile phosphate-buffered saline (pH 7.0). RNA extraction was performed by using a commercial viral nucleic acid isolation kit (Vivantis, Selangor Darul Ehsan, Malaysia). The reaction mixture (total 25 μL) of the RT-qPCR assay consisted of 1.5 μmol/L of each primer, 0.8 μmol/L of the probe, and 5 μL of RNA. Positive (100 copies of DOBV genotype Dobrava isolate small (s) segment containing control plasmid) and negative control (ultrapure water) reactions were also included in each test. Thermal cycling was carried out in a RotorGene 6000 (QIAGEN) at 50°C for 30 min, 95°C for 15 min, then 45 cycles of 15 s at 94°C, 30 s at 60°C, and 1 min at 72°C.

The RT-qPCR revealed 23 DOBV RNA-positive rodents, including 10 *A. flavicollis mice*, 12 *A. uralensis* mice, and 1 *Rattus rattus* rat (Tables 1, 2). The viral RNA load varied from 7.2×10^2 to 4.6×10^6 copies/mL. Most DOBV-positive rodents were from neighboring trapping sites in Akbas, Bogaz, and Kumluca Districts of Bartin

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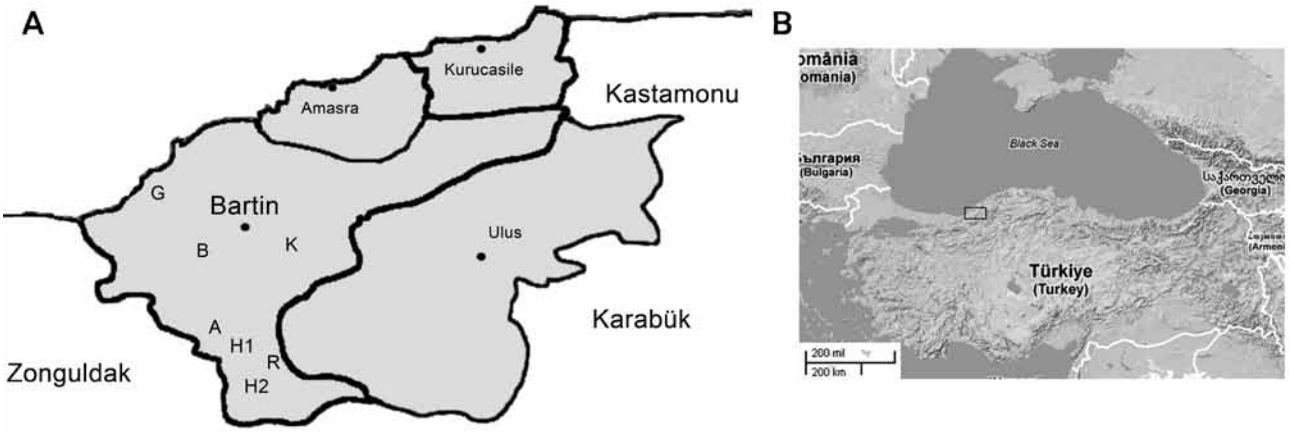

Figure 1. Regional map of Turkey showing the Bartin province (41°38′9′N, 32°20′15′′E), where trapping of small mammals was conducted. Abbreviations indicate locations at which captures were performed: A, Akbas; B, Bogaz; H1, Hanyeri; H2, Hasankadi; G, Guzelcehisar; K, Kumluca; R, Region 49. Panel A corresponds to the region (Bartin Province) indicated by box in panel B.

Province (Figure 1). The reason hantavirus RNA could not be detected in bank voles (Table 1) might be because a DOBV-specific RT-qPCR was used that is unable to detect bank vole–associated PUUV.

Serum samples from all 173 rodents were screened by a commercial enzyme immunoassay (Hantavirus DxSelect; Focus Diagnostic, Cypress, CA, USA) that used anti-mouse IgG and IgM conjugates (Sigma-Aldrich Chemie, Taufkirchen, Germany). The assay found that 11 (6.4%) and 36 (20.8%) animals contained hantavirus-reactive IgM and IgG, respectively (Table 1). Eight (4.6%) serum specimens were positive for both IgM and IgG, whereas 3 (1.7%) and 28 (16.2%) samples were positive for either IgM or IgG, respectively. The hantavirus seroprevalence varied between the different trapping sites and rodent species investigated (Table 1). Serologic status of the 23 RT-qPCR positive animals was proven by indirect immunofluorescence assay (IFA) as described by manufacturer (Euroimmune, Lübeck, Germany) except using fluorescein isothiocyanate (FITC)–labeled anti-mouse IgG conjugate (Sigma-Aldrich Chemie). The IgG ELISA results were confirmed for 5 samples, whereas 5 additional samples demonstrated a different reactivity in ELISA and IFA (Table 2).

All DOBV RT-qPCR positive samples were subjected to conventional S-segment RT-PCR, which produced an amplification product of 790 bp for sequence analysis. After denaturation of RNA at 70°C for 5 min, cDNA was synthesized by using Moloney murine leukemia virus RT and random hexamers (MBI Fermentas, Vilnius, Lithuania), by incubating at 25°C for 10 min and thereafter at 37°C for 1 h. PCR amplification was carried out by adding 3 μL of cDNA into a reaction mix containing 75 mmol/L Tris-HCl (pH 8.8), 20 mmol/L $NH_4(SO_4)_2$, 2.4 mmol/L $MgCl_2$, 10 pmol of each primer, 0.2 mmol/L dNTP, and 5U Taq DNA polymerase (MBI Fermentas). The initial denaturation for 6 min at 94°C, was followed by 40 cycles each at 94°C for 1 min, 52°C for 1 min, 72°C for 2 min, and a final extension at 50°C for 1 min and 72°C for 10 min. Analysis by agarose gel electrophoresis revealed amplification products of the expected size for 21 samples. The PCR products were purified by using PCR/Gel purification kit (GeneMark

Table 1. Detection rate of hantavirus-reactive IgM and IgG by ELISA, and hantavirus RNA by RT-qPCR in small mammals from different trapping sites, Turkey, 2009*

Location (map code/no. rodents)	No. positive animals/total no. animals											
	Apodemus spp.			*Myodes* spp.			*Rattus rattus*			Other†		
	IgM	IgG	RNA	IgM	IgG	RNA	IgM	IgG	RNA	IgM	IgG	RNA
Region 49 (R/11)	0/3	0/3	0/3	1/8	3/8	0/8	–	–	–	–	–	–
Akbaş (A/64)	0/39	1/39	8/39	2/18	14/18	0/18	–	–	–	0/5	0/5	1‡/5
Bogaz (B/20)	0/13	0/13	4/13	–	–	–	0/8	0/8	0/8	0/2	0/2	0/2
Guzelcehisar (G/17)	0/17	0/17	2/17	–	–	–	–	–	–	–	–	–
Hanyeri (H1/13)	0/8	0/8	2/8	0/5	3/5	0/5	–	–	–	–	–	–
Hasankadi (H2/33)	1/12	0/12	1/12	6/18	10/18	0/18	0/3	1/3§	1/3§	–	–	–
Kumluca (K/14)	1/14	4/14	5/14	–	–	–	–	–	–	–	–	–
Total no. positive/no. tested (%); N = 173	2/106 (1.9)	5/106 (4.7)	22/106 (20.7)	9/49 (18.3)	30/49 (61.2)	0/49	0/11	1/11 (9.1)	1/11(9.1)	0/7	0/7	1/7(14.2)

*RT-qPCR, reverse transcription quantitative PCR; –, not found.
†*Pitymys* spp., *Microtus* spp., *Mus musculus, Crocidura suaveolens.*
‡*Cytochrome b*–based genetic species determination was not performed.
§Results from different animals.

Table 2. Serologic reactivity of 23 DOBV RT-qPCR–positive rodents by ELISA and IFA, Turkey, 2009*

Animal no.	Species (*cyt b*)†	Location/district‡	Hantavirus serology		
			ELISA		IFA
			IgM	IgG	IgG
94-09	*Apodemus uralensis*	Akbas	Neg	Neg	Neg
95-09	*A. uralensis*	Akbas	Neg	Neg	NS
121-09	*A. flavicollis*	Akbas	Neg	Neg	Neg
123-09	*A. flavicollis*	Akbas	Neg	Neg	Pos
124-09	*A. uralensis*	Akbas	Neg	Neg	BL
130-09	*A. uralensis*	Akbas	Neg	Neg	Neg
139-09	*A. flavicollis*	Akbas	Neg	Pos	Pos
142-09	*A. flavicollis*	Akbas	Neg	Pos	Neg
151-09	*A. uralensis*	Bogaz	Neg	Neg	Neg
162-09	*A. uralensis*	Bogaz	Neg	Neg	Neg
169-09	*A. flavicollis*	Bogaz	Neg	Pos	Neg
177-09	*A. uralensis*	Bogaz	Neg	Neg	Neg
178-09	*A. uralensis*	Guzelcehisar	Neg	Neg	NS
180-09	*A. uralensis*	Guzelcehisar	NS§	NS	NS
100-09	*A. uralensis*	Hanyeri	Neg	Neg	Neg
102-09	*A. uralensis*	Hanyeri	Neg	Neg	Neg
106-09	*Rattus rattus*	Hasankadi	Neg	Pos	Neg
25-09	*A. flavicollis*	Hasankadi	Pos	Neg	Neg
78-09	*A. flavicollis*	Kumluca	Neg	Pos	Pos
80-09	*A. uralensis*	Kumluca	Neg	Pos	Pos
81-09	*A. flavicollis*	Kumluca	Neg	Pos	Pos
134-09	*A. flavicollis*	Kumluca	Neg	Pos	Pos
137-09	*A. flavicollis*	Kumluca	Neg	Neg	Pos

*DOVB, Dobrava-Belgrade virus; RT-qPCR, reverse transcription quantitative PCR; IFA, immunofluorescence assay; NS, no serum sample available; BL, borderline.
†*Cytochrome b*–based genetic species determination following a recently published protocol (*9*).
‡In alphabetical order.

Technology Co., Taichung City, Taiwan) and cloned into pJet1.2 blunt (MBI Fermentas). Plasmid DNA was directly subjected to sequencing in CEQ 8000 Genetic Analyzer (Beckmann Coulter, Brea, CA, USA) using the Dye Termination Cycle Sequencing Kit (Beckmann Coulter).

The nucleotide sequence identity among the obtained 21 S-segment sequences (GenBank accession nos. KF615886–KF615906) was very high, reaching 99.2 %. For phylogenetic analysis 2 novel DOBV strains found in *A. flavicollis* (Table 1, number 81/09; accession no. HQ406826) and *A. uralensis* (Table 1, number 80/09; accession no. HQ406825) were selected that showed a nucleotide sequence identity of 99.1%, whereas the amino acid sequence identity of the corresponding part of the nucleocapsid protein was 98.7%. A phylogenetic analysis of these 2 *Apodemus*–derived DOBV sequences from Turkey confirmed their strong similarity to *A. flavicollis*–associated DOBV genotype Dobrava sequences from Greece, Slovenia, and Slovakia (Figure 2). The sequence divergence of the 2 sequences from Turkey to other genotype Dobrava sequences was found to be 3.8% at nucleotide level (0.7% at amino acid level). The sequence divergence to DOBV sequences from *A. ponticus* and *A. agrarius* mice was found to be much higher, up to 15.2% and 6%, respectively.

Conclusions

Human infections with members of the *A. flavicollis*–associated DOBV lineage have been described in Slovenia, Greece, Serbia, Montenegro, the Czech Republic, and Hungary (*2*). The current study of rodents in Turkey identified DOBV genotype Dobrava (DOBV-Af) as a potential causative agent of the hantavirus outbreak on the Black Sea coast of Turkey. This conclusion is in agreement with previous findings in patients from this region (*6*,*8*,*10*).

Notably, this study demonstrated closely related DOBV sequences in *A. flavicollis* and *A. uralensis* mice and a high DOBV prevalence in both species. This observation might indicate multiple DOBV spillover to *A. uralensis* mice. In addition, we found a single spillover infection here in rats (*R. rattus*). Similarly, spillover infections of DOBV genotype Dobrava (DOBV-Af) in *Mus musculus* and *A. sylvaticus* mice and of DOBV genotype Kurkino (DOBV-Aa) in *A. flavicollis* mice have been reported (*11*,*12*). Alternatively, the frequent detection of DOBV-specific nucleic acid in *A. uralensis* mice may indicate that this rodent species functions as a reservoir. Previously, in a study in Czech Republic *A. uralensis* mice were found to contain hantavirus antigen (*13*). These findings underline the current problem of identifying hantavirus reservoirs (*14*) as was recently also observed for European Tula virus (*15*). The detection of hantavirus-reactive antibodies and the absence of hantavirus RNA in *Myodes* species might be explained by DOBV spillover infections or, alternatively, by the presence of another hantavirus not detected by the RT-qPCR assay used here. Therefore, future studies

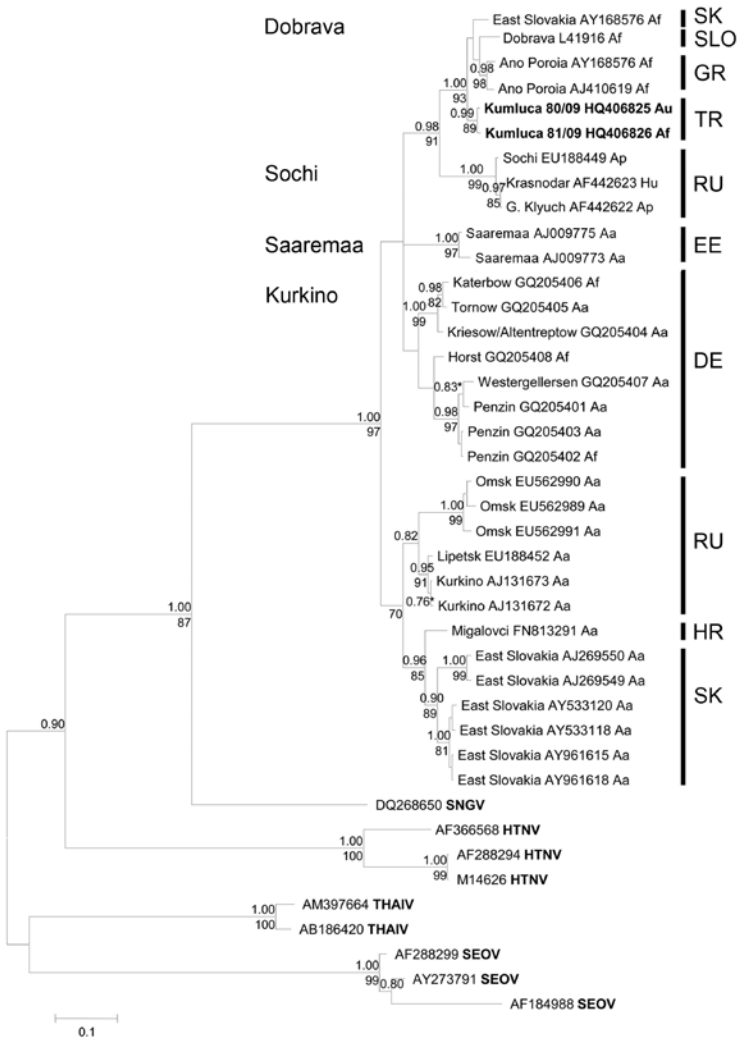

Figure 2. Bayesian phylogenetic tree, based on an alignment of 450-nt long region of the small segment from various Dobrava-Belgrade virus lineages and other Murinae-associated hantaviruses. Posterior probabilities for Bayesian analysis are given under the branches and bootstrap values above the branches. Values lower than <0.7% and <70% are not shown. The sequences were aligned with ClustalW included in the BioEdit software package version 2.1 (http://www.mbio.ncsu.edu/bioedit/page2.html). The phylogenetic analyses were performed by using MrBayes 3.1.2 with Bayesian Metropolis-Hastings Markov Chain Monte Carlo (MCMC) tree-sampling methods based on 2 MCMC runs consisting of 4 chains of 2,000,000 with a burn-in of 25% and second by maximum-likelihood bootstrap analysis with 1,000 pseudoreplicates using MEGA5 (www.megasoftware.net). The Hasegawa-Kishino-Yano model with a discrete gamma distribution, to model evolutionary rate differences among sites (2 categories [+G, parameter = 0.8874]) according to jModeltest (http://code.google.com/p/jmodeltest2/) was used. Af, *Apodemus flavicollis*; Au, *A. uralensis*; Ap, *A. ponticus*; Hu, human; Aa, *A. agrarius*; SNGV, Sangassou virus; HTNV, Hantaan virus; THAIV, Thailand virus; SEOV, Seoul virus; SK, Slovakia; SLO, Slovenia; GR, Greece; TR, Turkey; RU, Russia; EE, Estonia (island Saaremaa); DE, Germany; HR, Croatia. Scale bar indicates number of nucleotide substitutions per site.

on sympatrically occurring *Apodemus* species at multiple sites in Turkey along the distribution range of *A. uralensis* mice need to confirm whether these rodents have a role as a potential reservoir host of DOBV. In addition, a comprehensive study in different rodent and other small mammal species should determine whether hantaviruses other than DOBV are present.

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Increasing Threat of Brucellosis to Low-Risk Persons in Urban Settings, China

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Cases of brucellosis were diagnosed in 3-month-old twins and their mother. An epidemiologic survey suggested that raw sheep or goat meat might be the source of *Brucella melitensis* infection. This finding implies that the increasing threat of brucellosis might affect low-risk persons in urban settings in China.

Brucellosis, a zoonotic disease, causes severe pain and impairment in humans. In 2012, the Chinese Center for Disease Control and Prevention (China CDC) reported 39,515 new cases of human brucellosis, and this number is increasing by 10% each year. Generally, brucellosis is associated with persons who are occupationally in contact with *Brucella* spp.–infected animals or products (*1*,*2*). However, in this report, we present a cluster of cases of brucellosis in a family living in Guangzhou, China. These data illustrate a trend of human brucellosis threatening theoretically low-risk persons in an urban setting and suggest a need for eradicating or controlling Brucella spp.–infected animals and products in China.

Case Reports

Congenital brucellosis was diagnosed in patients 1 and 2, who were 3-month-old twins (online Technical Appendix Table, wwwnc.cdc.gov/EID/article/20/1/13-0324-Techapp1.pdf). They were prematurely delivered by cesarean section on July 6, 2012, at the Provincial Maternity and Child Care Center (Guangzhou, China). The boy (patient 1, Apgar score 9–10/1–10 min) had a birthweight of 2.3 kg, and the girl (patient 2, Apgar score 9–10/1–10 min) had a birthweight of 1.8 kg. They received standard care for preterm neonates at the hospital. They were discharged once their weight reached 2.5 kg; this happened for patient 1 at 3 weeks of age and for patient 2 at 4 weeks of age (July 29 and August 3, 2012, respectively).

On October 2, 2012, the boy was examined at the hospital for irregular fever up to 39°C. On October 9, he was readmitted to the hospital with a fever of 38°C and weight of 5.0 kg. Chest radiograph showed signs of increased bronchovascular shadows. Mezlocillin sodium and sulbactam sodium (4:1) and ribavirin were administered, but the patient did not improve. On the same day, the girl had a cough and low-level fever (37–37.5°C) but was not hospitalized. On October 16, *B. melitensis* was isolated from a blood culture from patient 1, in whom brucellosis with alveobronchiolitis, abnormal hepatic function, and moderate anemia were initially diagnosed when he was 3 months and 10 days of age.

On October 17, the twins were transferred to an infectious disease hospital, where they had extensive physical and laboratory examinations (Table 1). During 57 days of hospitalization, the boy received general and specific therapies for brucellosis. Brucellosis and glucose-6-phosphate dehydrogenase deficiency were diagnosed in the girl, and she received appropriate treatment. At the time of discharge (December 12), the twins were well and without fever. They left the hospital for home care, which was supervised by a local general practitioner who provided rifampin and sulfamethoxazole for up to 6 weeks.

Patient 3 (the mother of patients 1 and 2), a 31-year-old woman who was admitted to a hospital on July 4, 2012 for threatened premature labor at 34 weeks and 2 days' gestation. Chorioamnionitis phase I was diagnosed that day. On July 6, the patient gave birth to twins through a uterine lower segment cesarean section due to early rupture of the amniotic membrane. Postnatally, the mother was in generally good clinical condition without specific complaint and was discharged for home care on July 11. When *Brucella* infection was diagnosed in her son (patient 1), she was hospitalized for suspected *Brucella* infection (online Technical Appendix Table). Brucellosis was diagnosed, and she was treated as an outpatient with a 4-week course of rifampin and doxycycline and 1-week course of streptomycin. Her symptoms of brucellosis rapidly improved.

Serologic and bacteriologic tests were conducted for diagnosis of *Brucella* infection. On October 17, 2012, blood samples were taken from all 6 members of the patients' family. By standard tube agglutination test, the twins and their mother tested positive for *Brucella* antibodies with titers of 400 (twins) and 800 (mother), whereas results for the twins' father and grandparents were negative. *Brucella* antibodies from the twins' blood samples were detected 3 times with titers of 400, 200, and

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Table 1. Clinical and laboratory data on twin patients on admission to the infectious disease hospital

Variables*	Patient 1, twin boy	Patient 2, twin girl	Reference range (children)†
Temperature, °C	38	37.0	36–37
Pulse, beats/min	128	128b	120
Respiratory rate, breaths/min	34	36	30–35
Blood pressure, mmHg	76/42	76/45	80/48
Weight, kg	5.7	5.6	
Erythrocyte count, cells/L	4.99×10^{12}	4.69×10^{12}	$3.5–5.5 \times 10^{12}$
Hemoglobin, g/L	85	89	120–160
Hematocrit, %	26.3	27.5	40–50
Erythrocyte sedimentation rate, mm/h	5	No record	<10
Leukocyte count, cells/L	7×10^{9}	8.51×10^{9}	$4.0–10.0 \times 10^{9}$
Differential count, %			
Neutrophils	18.8	10.7	50–75
Eosnophils	3	1.1	0.5–5
Basophiles	0.2	0.3	0–1.0
Lymphocytes	74.3	83.9	20–40
Monocytes	3.7	4	3.0–10.0
Platelet count, per L	462×10^{9}	456×10^{9}	$100–300 \times 10^{9}$
Sodium, mmol/L	103	135.4	135–145
Potassium, mmol/L	33	No record	3.4–4.8
Glucose 6-dehydrogenase, U/L	4,254	1,363	≥2,500
Lactate dehydrogenase, U/L	356	322	100–380
Alanine aminotransferase, U/L	31	48	5–40
Aspartate aminotransferase, U/L	54	64	5–40
Alkaline phosphatase, U/L	508	642	30–390
Total bilirubin, μmol/L	8.49	5.17	5.10–22.2
Total protein, g/L	51	49	60–68
Albumin, g/L	37	40	35–55
Globulin, g/L	14	9	20–35
C-reactive protein, mg/L	0.2	0.21	0.03–5
Creatine kinase, U/L	49	203	24–194
Creatine kinase-MB, U/L	32	25	0–25
Brucella antibody titer, SAT	400	800	<100
Blood culture	*B. melitensis*	*B. melitensis*	
Hepatitis B surface antigen	Negative	Negative	
Antibody to hepatitis B surface antigen, IU/L	6.77	Negative	
Hepatitis B e antigen	Negative	Negative	
EBV IgA	Negative	Negative	
Cytomegalovirus IgM	Negative	Negative	
Herpes simplex virus 1, 2 IgM	Negative	Negative	
Influenza virus A + B antigens	Negative	Negative	
Mycoplasma IgM	Negative	Negative	
Chlamydia pneumoniae IgM	Negative	Negative	
Toxoplasma IgM	Negative	Negative	

*Major items are presented from clinical testing. EBV, Epstein-Barr virus.
†The ranges used at this hospital are not all for children, and may not be appropriate for the twin patients. The values may be affected by the laboratory methods in different hospitals.

200 on November 10, 18, and 29, respectively. Plasma from the twins' cord blood tested positive by a rose bengal plate test, but results were indeterminate or negative by standard tube agglutination test (titer <50). Samples from patients 1–3 were collected on October 17, and on October 25, after 8 days of blood cultures on commercial agar plates 3 *Brucella* strains were isolated. A *Brucella* sp. was repeatedly isolated in blood samples collected on November 10 but not in samples collected on November 18 and 29, after the patients were treated with rifampin. The mother's breast milk was collected before and after she was treated for brucellosis, and *Brucella* sp. was not isolated from these samples.

Brucella DNA was tested by quantitative PCR of blood cultures from 3 patients, from patients' cord blood, and from a positive control (Figure 1, panel A). Additionally, the specific DNA bands for *B. melitensis* were identified from each patient's blood culture by an abbreviated *B. abortus*, *melitensis*, *ovis*, and *suis* (AMOS) PCR (*3*) but were not observed from the twins' cord blood, possibly due to low levels of *Brucella* DNA (Figure 1, panel B).

Bacterial isolates were characterized as *B. melitensis* biotype 3 (Table 2) (*4*). By multilocus variable-number tandem repeat analysis of 16 samples (*5*), these *Brucella* isolates from the twins and their mother were genetically identical. They were all genotyped as 16 loci, with variable number of tandem repeats of 1 5 3 13 2 3 3 2 6 22 9 6 9 11 4 5, which was phylogenetically closer to #20081716 and #9900139 strains prevalent in Spain (Figure 2) but differed from strains prevalent in Kyrgyzstan (*6*).

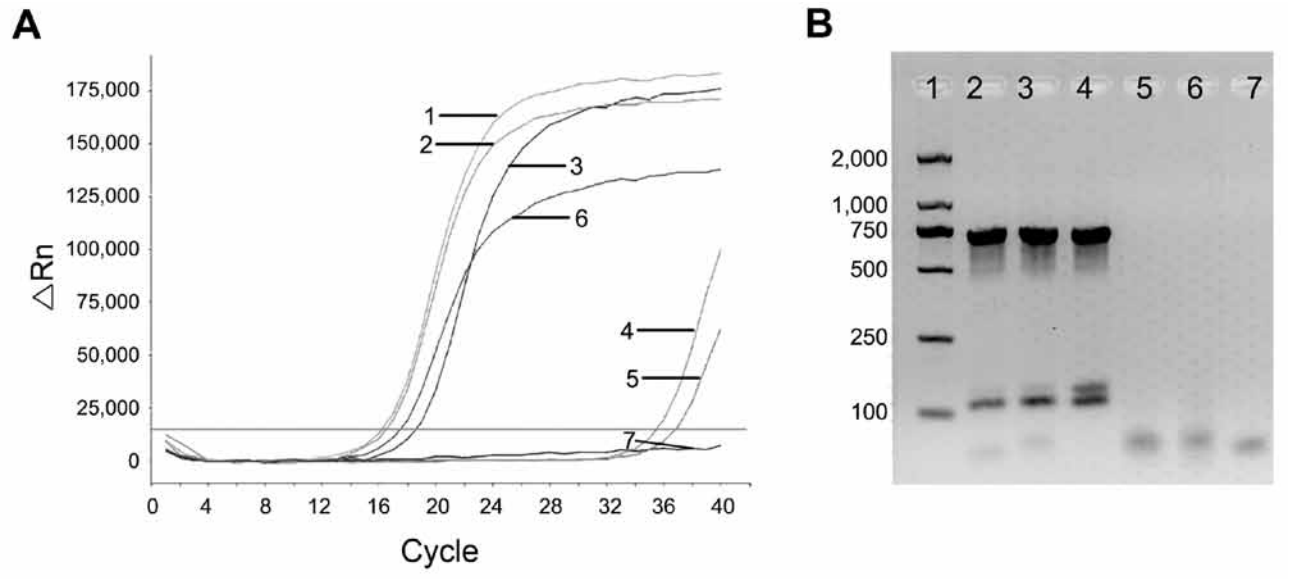

Figure 1. Detection and identification of *Brucella* DNA. A) Detection of *Brucella* DNA by quantitative PCR. Numbers indicate amplification curves with cycle threshold (C_t) values representative of samples. Curve 1, sample from patient 1 with 16 C_t value; curve 2, sample from patient 2 with 16 C_t; curve 3, sample from patient 3 with 17 C_t; curve 4, stem cells of cord blood from patient 1 with 34 C_t; curve 5, stem cells of cord blood from patient 2 with 34.5 C_t; curve 6, positive control with 18 C_t, curve 7, negative control with no C_t. B) Amplification of *Brucella* DNA by AMOS-PCR. Numbers indicate the amplified DNA bands representative of samples. Lane 1, DNA molecular weight marker, values along the left side are base pairs; lane 2, sample from patient 1; lane 3, sample from patient 2; lane 4, sample from patient 3; lane 5, stem cells from cord blood of patient 1; lane 6, stem cells from cord blood of patient 2; lane 7, negative control.

Conclusions

Patients with brucellosis usually have occupations that involve interaction with animals or clinical or laboratory veterinary work. There are reports of human brucellosis related to blood transfusion (*7*), bone marrow transplantation (*8*), transplacental transmission (*9*), breast feeding (*10*), or sexual activity (*11*). In this study, a cluster of brucellosis was identified in 3 patients from a 6-member family. However, the mother and other family members denied having risk factors associated with brucellosis. During the mother's pregnancy, she had fever and aching bones, while the grandmother occasionally prepared steamed stuffed buns containing raw sheep or goat meat, which reportedly were bought at the supermarket or local butcher's shop. Raw meat might therefore constitute the source of the *Brucella* infection.

Table 2. Bacteriological and biochemical features of *Brucella* strains

Strain	TZ (twin boy)	TS (twin girl)	ML (mother)
CO_2 requirement	–	–	–
H_2S production	–	–	–
Dye inhibition*			
Thionin	+	+	+
Basic fuchsin	+	+	+
Mono-specific anti-serum agglutination†			
A	+	+	+
M	+	+	+
R	–	–	–
Lysis test by *Brucella* spp. phage‡			
$Tb10^4$	–	–	–
Tb	–	–	–
Wb	±	±	±
BK_2	+	+	+
Identification			
Species	*B. melitensis*	*B. melitensis*	*B. melitensis*
Biovar	3	3	3

*A final concentration of 20 μg/mL dyes was used in the testing (*4*).
†The bacterial isolate was tested for agglutination by mono-specific anti-serum samples to *Brucella* antigens A, M, and R (rough), respectively.
‡Bacterial isolate was tested for lysis by specific *Brucella* phages of Tb, Wb, and BK2.

In recent years human brucellosis cases have spread quickly from rural to urban areas and increased sharply in persons in China who do not fit into standard risk categories. Guangzhou, a major city in southern China, is located far away from the *Brucella*-endemic areas of northern China but has recorded increasing numbers of human brucellosis: >60 cases in the past 5 years (China CDC, unpub. data). Live animals and raw meat products are frequently transported across the whole country, and cases of brucellosis have been recorded in all regions of the country (*12*). About 85% of brucellosis cases have been attributed to *B. melitensis* from infected sheep or goats (*12*,*13*), which put ordinarily low-risk persons at much higher risk when they consumed or handled infected animal meat and milk (*14*). The increasing numbers of cases of brucellosis indicates that the strategy of vaccination and quarantine for infected

Key	Strain	Host	Isolated in	Species-biovar	Group
2010Valdezate#048	#20081716	Human	Spain	*B. melitensis*	East Mediterranean
2010Valdezate#014	#9900139	Human	Spain	*B. melitensis*	East Mediterranean
2011Kilic#068	BRU-S068	Human	Turkey	*B. melitensis* 3	East Mediterranean
2011Kilic#074	BRU-S074	Human	Turkey	*B. melitensis* 3	East Mediterranean
2007AlDahouk#063	BfR 29	Human	?	*B. melitensis* 2	East Mediterranean
2012Ferreira#008	LNIV-479Bm3-05	Sheep	Portugal	*B. melitensis* 3	East Mediterranean
2007AlDahouk#030	BfR 5	Human	Turkey	*B. melitensis* 2	East Mediterranean
2012Ferreira#063	LNIV-128Bm3-03	Sheep	Portugal	*B. melitensis* 3	East Mediterranean
2007AlDahouk#014	BCCN 96-142	Human	France	*B. melitensis* 3	East Mediterranean
2012Ferreira#004	LNIV-913Bm3-10	Sheep	Portugal	*B. melitensis* 3	East Mediterranean
2012Ferreira#005	LNIV-912Bm3-09	Sheep	Portugal	*B. melitensis* 3	East Mediterranean
2012Ferreira#019	LNIV-563Bm3-06	Sheep	Portugal	*B. melitensis* 3	East Mediterranean
2012Ferreira#028	AFSSA-03/4805/7430	Sheep	Portugal	*B. melitensis* 3	East Mediterranean
2012Ferreira#067	LNIV-718Bm3-07	Sheep	Portugal	*B. melitensis* 3	East Mediterranean
2011Kilic#073	BRU-S073	Human	Turkey	*B. melitensis* 3	East Mediterranean
2011Kilic#066	BRU-S066	Human	Turkey	*B. melitensis* 3	East Mediterranean
2012Ferreira#006	LNIV-66Bm3-01	Sheep	Portugal	*B. melitensis* 3	East Mediterranean
2012Ferreira#022	AFSSA-02/6425	Sheep	Portugal	*B. melitensis* R	East Mediterranean
2007AlDahouk#072	BfR 39	Human	Portugal	*B. melitensis* 2	East Mediterranean
2007AlDahouk#071	BfR 38	Human	Germany	*B. melitensis* 3	East Mediterranean
Queried-strain	BRU-TZ	Human	China	*B. melitensis* 3	East Mediterranean

Figure 2. Genetic relationship between the strain isolated in this study (BRU-TZ) and other *Brucella melitensis* strains. The variable number of tandem repeats were obtained for phylogenetic analysis at multiple-locus variable-number tandem repeat analysis bank version-4 (http://mlva.u-psud.fr) (*5*,*6*). The phylogenetic tree was plotted on the differences in variable number of tandem repeats at 16 loci obtained by multiple-locus variable-number tandem repeat analysis.

animals has failed in China. One possible reason is the limited efficacy of the current vaccines (*2*,*15*), but a primary reason is that the policies for eradication and control of *Brucella*-infected animals and their products have not been adequately implemented.

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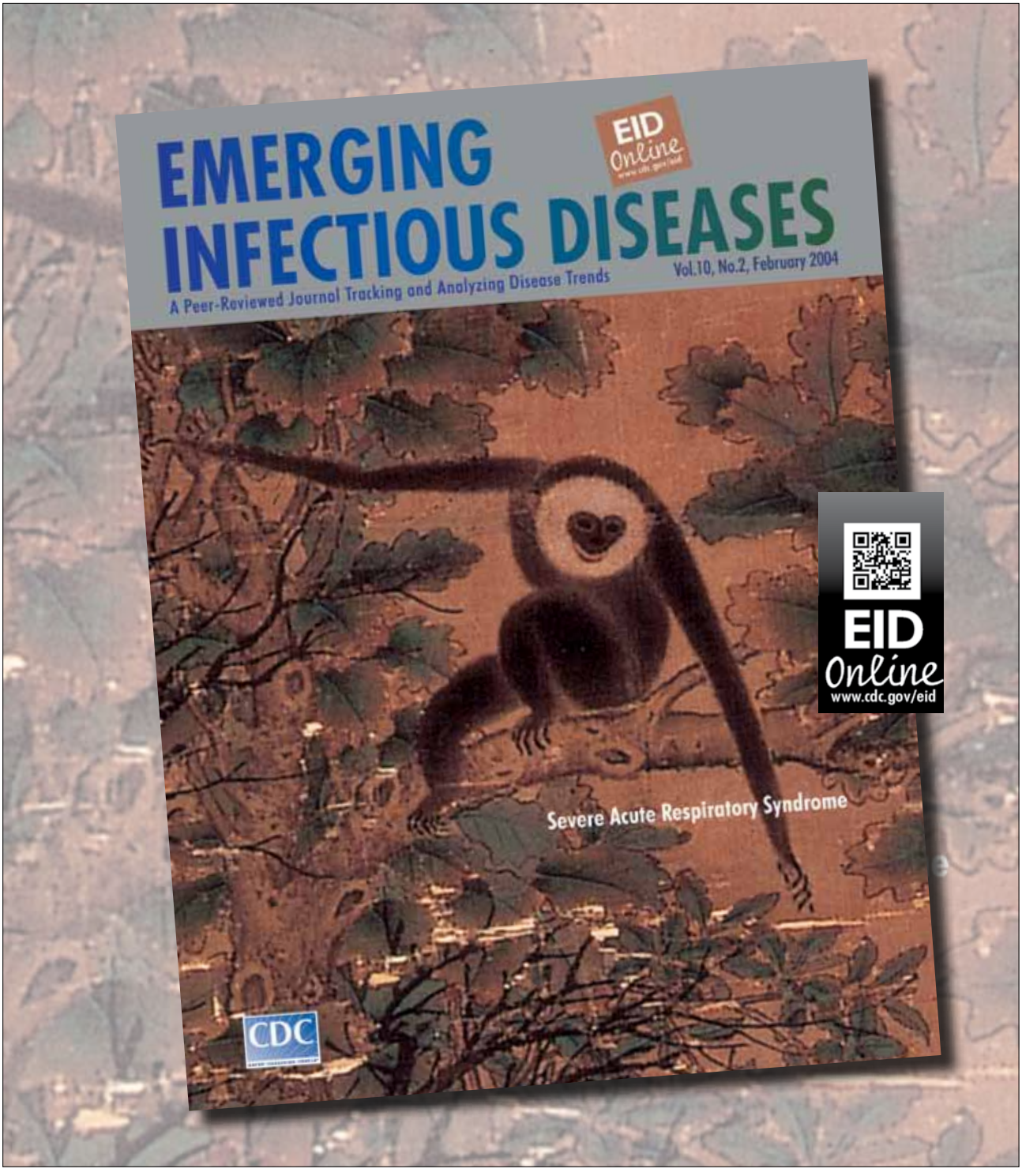

Schmallenberg Virus Infection among Red Deer, France, 2010–2012

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Schmallenberg virus infection is emerging in European domestic and wild ruminants. We investigated the serologic status of 9 red deer populations to describe virus spread from September 2010 through March 2012 among wildlife in France. Deer in 7 populations exhibited seropositivity, with an average seroprevalence of 20%.

In summer and fall 2011, an unidentified disease was reported in dairy cattle in Germany and the Netherlands, causing decreased milk production, fever, and diarrhea (*1*,*2*). The virus associated with these clinical signs was identified as a new member of the genus *Orthobunyavirus* of the Simbu serogroup and named Schmallenberg virus (SBV) (*2*). This virus was later associated with abortions and congenital malformations in calves, lambs, and kids in several European countries (*3*). Serologic testing among wild cervids in Belgium revealed antibodies against Schmallenberg virus in roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) (*4*). Seroprevalence was already high (27% on average) in wild cervids in October 2011 in Belgium, suggesting that the virus began circulating months earlier (before August 2011). It has recently been shown that SBV had already circulated in *Culicoides* vectors in Belgium during August and September 2011 (*5*). Although SBV has been closely monitored among domestic ruminants in France, suggesting that clinical cases and antibodies appeared almost at the same time during 2011–2012 (*6*), little is known about the geographic spread of SBV in wildlife. To correct this lack of data, we conducted a serologic study using serum specimens collected from red deer in different regions in France.

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The Study

Blood samples from 502 red deer, which had been either killed by gunshot or captured, were collected within 9 French departments (administrative units) during 1 or 2 sampling seasons (i.e., during September 2010–January 2011 and September 2011–March 2012). The serum specimens were first screened by using an SBV indirect ELISA (i-ELISA) that was previously validated for the serum specimens from cattle, sheep, and goats (ELISA ID Screen Schmallenberg Virus Indirect, Bicupule; ID Vet, Montpellier, France) (*7*). The results were expressed as S/P values using the cutoff recommended for domestic species (S/P = [optical density sample (S)/optical density positive control (P)] × 100); S/P<60%, negative; S/P>70%, positive; and S/P 60–70%, doubtful result). Serum specimens were also tested with a new competitive ELISA (c-ELISA; ELISA ID Screen Schmallenberg Virus Competitive; ID Vet). Positive results by c-ELISA corresponded to a percentage of inhibition (PI) <50, doubtful result if 40>PI≤50, and negative when PI >50. The antigen used in both c-ELISA and i-ELISA is the same N recombinant protein. A subset of samples were also subjected to a seroneutralization test (SNT) as described (*7*).

Of 502 serum specimens, 492 could be tested by using i-ELISA and 486 by using c-ELISA. The 2 ELISA methods exhibited a 92% match (449/486). Because our samples (taken from dead animals in nonsterile conditions) generated bacterial contamination or cytotoxicity, conclusive SNT results were available from 114 animals only: 64 samples with positive or doubtful i-ELISA results and 50 samples (S/P>20) with negative i-ELISA results. A large part of the serum specimens that were positive or doubtful by ELISA methods were also positive for SBV by SNT, suggesting a good specificity of both methods, though slightly better for c-ELISA than for i-ELISA (Table 1). Many serum specimens that tested negative by i-ELISA or c-ELISA (all collected during 2011–2012) were positive by SNT (Table 1). Even though the c-ELISA kit appeared slightly more sensitive than the i-ELISA kit, these results suggest that SNT is the most sensitive technique for detecting antibodies against SBV in a recently infected population of red deer. These results are consistent with the fact that SNT and c-ELISA are able to detect IgG and IgM, whereas i-ELISA detects only IgG that appears after the IgM adaptive response (E. Breard, pers. comm.). Considering the performance of serologic methods in that study, seroprevalence was finally estimated as the proportion of positive or doubtful serum specimens by using the c-ELISA kit.

The number of samples collected in each department, the proportion of positive specimens, and the date of first

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Table 1. Serologic results for red deer serum samples tested for Schmallenberg virus with SNT, i-ELISA, and c-ELISA*

	SNT method			
	SNT positive, n = 97		SNT negative, n = 17	
ELISA method	Positive or doubtful	Negative	Positive or doubtful	Negative
i-ELISA	57	40	7	10
c-ELISA	67	30	6	11
i-ELISA and c-ELISA	49	22	6	9

*SNT, seroneutralization test; i-ELISA, indirect ELISA; c-ELISA, competitive ELISA.

observation of seropositive result are indicated in Table 2. The 56 serum specimens collected during September 2010–February 2011 in northeastern and southwestern France (Bas-Rhin and Pyrenees-Atlantiques departments) were negative by both ELISAs. From September 2011 through March 2012, a total of 7 of 9 departments exhibited at least 1 seropositive specimen by c-ELISA. Among these 7 departments, the average seroprevalence was 20% (95% CI 16%–24%), with significant variations between the 7 departments exhibiting seropositive results (8%–49%) (χ^2 = 67.4, df = 6, p<0.001). Seroprevalence was not influenced by the animal's age, suggesting an equal exposure of fawns born in 2011 and older animals (χ^2 = 0.16, df = 2, p = 0.92). It is thus likely that SBV had not spread to France before red deer in France gave birth to young in spring 2011 (mid-May to early June) (*8*). Seroprevalence varied significantly with the period (χ^2 = 25.0, df = 2, p<0.001). On average, seroprevalence was higher in December 2011–January 2012 (31%; 95% CI 25%–37%]) compared with September–November 2011 (7%; 95% CI 3%–12%) or February–March 2011 (14%; 95% CI 7%–22%).

These results suggest that SBV was actively circulating during fall 2011 until mid-November or early December. In agreement with the findings of Linden et al. for Belgium (*4*), we consider that the mild temperature observed in France in fall 2011 may have favored a late activity of vectors (*9*). The date of first occurrence of seropositive red deer and the seroprevalence observed in each department (Table 2) were not strictly dependent on the distance from the Meurthe-et-Moselle department where the first domestic case (congenital form) had been confirmed on January 25, 2012 (*10*) (Figure). This result possibly arose because of uncontrolled variations in the sampling dates of red deer between the 9 departments and still unknown factors associated with SBV spread. Nevertheless, most of the departments that exhibited seropositive red deer from September 2011 to March 2012 had also reported clinical cases in domestic flocks during January–March 2012 (Figure).

In southwestern France (near the Pyrénées Mountains), a red deer seropositive for SBV was observed in the Hautes-Pyrénées department, whereas congenital clinical cases of SBV infection in domestic livestock (congenital malformations on kids) had been reported by March 30, 2012, in the neighboring Pyrénées-Atlantiques department (E. Bréard, pers. comm.) (Figure). These results suggest similar spread of SBV among red deer and domestic livestock during fall 2011 at the department level. In 2012, no evidence of abortions or malformations was reported in red deer or other native wildlife ruminant species within the populations monitored by wildlife biologists or zoo veterinarians in France (S. Rossi, A. Decors, A. Lécu, pers. comm.). However, specific studies exploring the effect of SBV on the reproductive success of wild species are still lacking.

Conclusions

This study provides a preliminary view of SBV spread among wild cervids in France during 2010–2012. Our data suggest that SBV spread quickly from northeastern to southwestern France (≈800 km) between October and

Table 2. Results of c-ELISA and indication of first seropositive result for Schmallenberg virus in red deer, by department, France, 2010–2012*

	Average distance to Meurthe-et-Moselle department, km†	No. positive samples/ no. tested in 2010–2011 (95% CI, %)	2011–2012		First positive result, 2011
Department			No. positive samples/no. tested	Mean prevalence, % (95% CI)	
Moselle	46		4/26	15 (2–29)‡	Nov 5
Haute-Marne	102		26/53	49 (36–63)‡	Nov 12
Bas-Rhin	103	0/41 (<7)§	8/53	15 (6–25)‡	Oct 25
Côte d'Or	184		11/37	30 (15–45)‡	Dec 3
Oise	282		26/69	38 (26–49)‡	Dec 19
Loir-et-Cher	375		11/132	8 (4–13)‡	Nov 25
Hautes-Pyrénées	789	0/14 (<19)§	1/12	8 (0–30)‡	Dec 10
Corsica	749		0/23	0 (<12)§	
Pyrénées Atlantiques	815		0/26	0 (<11)§	

*c-ELISA, competitive ELISA.
†Department where first domestic clinical cases were reported, January 25, 2012.
‡95% CIs were estimated by assessing a binomial distribution of seroprevalence.
§Upper value of the 95% CI was estimated according a hypergeometric distribution of the risk to detect at least 1 positive result (p = 1–exp(Ln(0,05)/N), with N being the sample size).

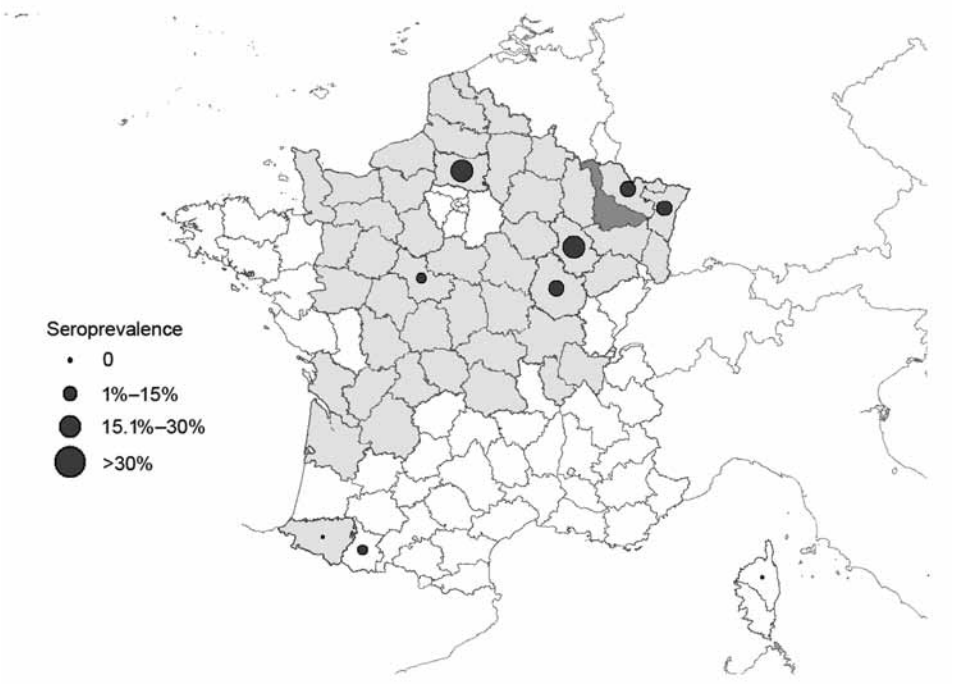

Figure. Sites where serum samples were obtained from red deer (9 departments), showing average seroprevalence for Schmallenberg virus, France, 2010–2012. Dark gray shading indicates Meurthe-et-Moselle department, where the first domestic case was found; light gray shading indicates departments where clinical cases were found during January–March 2012; and white indicates departments where no clinical cases occurred during January–March 2012.

December 2011. Our data also show the match of SBV spread among red deer and domestic flocks at the level of the department and highlight the perspective that red deer can be a sentinel of SBV spread for livestock. We also pinpointed the relevance of new competition ELISA for improving SBV surveillance in wildlife species, even though SNT remained the most reliable assay for SBV antibody detection in red deer. Further studies that encompass several years and include a larger number of species and localities would help provide a more complete picture of virus spread and risk factors in wildlife (*11*).

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Drug-Resistant Tuberculosis in High-Risk Groups, Zimbabwe

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To estimate prevalence of multidrug-resistant tuberculosis (MDR TB) in Harare, Zimbabwe, in 2012, we performed microbiologic testing on acid-fast bacilli smear-positive sputum samples from patients previously treated for TB. Twenty (24%) of 84 specimens were consistent with MDR TB. A national drug-resistance survey is needed to determine MDR TB prevalence in Zimbabwe.

Emergence of multidrug-resistant tuberculosis (MDR TB) in sub-Saharan Africa poses a major risk to further destabilization of regional TB control programs. Yet, fewer than half of the 46 countries in the World Health Organization (WHO) African Region have provided representative drug-resistance data, and only 10 have reported data since 2007 (*1*).

Zimbabwe has among the highest estimated TB incidence per capita (603/100,000 population) in the world (*2*). Sixteen percent of adults are HIV infected, and approximately three-quarters of active TB cases occur among persons with HIV (*3*). Increasing prevalence (*4*) and epidemic spread (*5*) of MDR TB in neighboring southern Africa countries, sociopolitical instability with economic collapse and severe health system disruptions in 2007–2008 (*6*), and anecdotal reports of MDR TB among Zimbabwean returnees (*7*) may herald an increase in MDR TB prevalence within the country. Formal national surveillance for drug-resistant TB has not been undertaken in Zimbabwe since 1995. To improve early detection and estimate the prevalence of MDR TB, we performed extensive microbiologic testing on samples from consecutive patients with presumptive drug-resistant TB in Harare, Zimbabwe.

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The Study

From November 15, 2011, to November 15, 2012, we prospectively recruited persons suspected of having drug-resistant pulmonary TB within Harare (metropolitan population 2.8 million, 2009), Zimbabwe. Those with suspected drug-resistant TB were defined as persons with cough, fever, night sweats, or weight loss and either 1) a history of ≥1 month of prior treatment (relapse, treatment after default, or treatment failure) (*8*), or 2) contact with a person with known or suspected drug-resistant TB. Because early identification of drug-resistant TB was an objective of the study, all persons who had smear-positive sputum samples between month 3 and month 5 of anti-TB treatment ("late smear conversion") were also enrolled. Subjects were recruited from outpatient clinics within Harare's 2 infectious diseases referral hospitals, health clinics in southern high-density suburbs, and Epworth (a locality outside Harare). For logistical reasons, not all polyclinics in Harare could be included. Compared with the number of notified sputum smear–positive retreatment patients in Harare in 2011 (the most recent year for which data are available), ≈60% of the population base was likely included in our sample.

Ethical approval was obtained from the Medical Research Council of Zimbabwe, the Institutional Review Board of the Biomedical Research and Training Institute, and Human Research Protection Program, University of California, San Francisco. Laboratory methods were undertaken as reported (*9*). In brief, 2 solid (Löwenstein-Jensen [LJ] media) and 2 liquid (BBL MGIT Mycobacterial Growth Indicator Tubes; Becton Dickinson, Sparks, MD, USA) cultures for mycobacteria were performed for each patient in an external quality-assured laboratory. Ziehl-Neelsen staining was used to confirm growth of mycobacteria in all test-positive tubes. Indirect drug susceptibility testing (DST) was performed on all *M. tuberculosis* isolates by using the absolute concentration method on LJ media. In addition, culture for mycobacteria and direct DST were also performed by using the microscopic-observation drug-susceptibility (MODS) assay (TB MODS Kit, Hardy Diagnostics, Santa Maria, CA, USA) in accordance with published procedures.

Of 240 recruited patients, 25 (10%) were from outside Harare provincial limits, and 2 had microbiologically confirmed MDR TB before enrollment; both patients had been referred from South Africa. Of the remaining 213 patients (Table), 203 (95%) had history of prior TB treatment, and 10 (5%) were new patients with known contact with a person who had suspected MDR TB. Of 211 patients, 157 (74%) were HIV infected. Most patients

Table. Characteristics of participants in study of MDR TB, Harare, Zimbabwe, 2011–2012*

Characteristic	MDR-TB, n = 25	Monoresistant TB, n = 14	Drug-sensitive TB, n = 90	Unconfirmed TB, n = 84	p value
Age, median (IQR)	34 (27–42)	35 (29–45)	37 (30–44)	39 (32–48)	0.67
Male, no. (%)	13 (52)	6 (43)	58 (64)	48 (57)	0.36
Retreatment category, no. (%)					
Treatment failure	9 (36)	0	9 (10)	21 (25)	<0.001
Late smear conversion	8 (32)	1 (7)	30 (33)	13 (16)	
Default	0 (0.0)	2 (14)	13 (14)	7 (8)	
Relapse	7 (28)	7 (50)	34 (38)	34 (41)	
New	1 (4)	4 (29)	1 (1)	4 (5)	
Sputum smear positivity, no. (%)	20 (80)	7 (50)	52 (53)	6 (7)	<0.001
HIV infection, no. (%)	18 (72)	13 (93)	57 (65)	69 (82)	0.02
Antiretroviral treatment, no. (%)	14 (78)	10 (77)	41 (72)	52 (75)	0.95
Time receiving TB treatment, median (IQR)	98 (4–175)	0 (0–3)	31 (2–103)	101 (5–186)	0.03
Prior treatment courses, no. (%)					
None	8 (32)	5 (36)	30 (33)	21 (25)	0.52
1	12 (48)	7 (50)	40 (44)	40 (48)	
≥2	5 (20)	2 (14)	20 (22)	22 (27)	

*Retreatment classification was defined according to international standards (*8*); late smear conversion was defined as sputum AFB smear-positivity after month 3 but before month 5 of treatment. Monoresistant TB was defined as resistance to either isoniazid or rifampin, but not both. The denominator for antiretroviral treatment included persons with HIV infection. MDR TB, multidrug-resistant tuberculosis; IQR, interquartile range; AFB, acid-fast bacilli.

(207/213; 97%) were enrolled at an outpatient setting; 40 (19%) had previously been hospitalized. Twenty-six (35%) of 75 patients had traveled outside of Zimbabwe in the previous 2 years. Neither prior hospitalization (p = 0.51) nor travel outside Zimbabwe (p = 0.94) was associated with MDR TB.

Among 84 TB case-patients with positive sputum-smear results, 20 (24%; 95% CI 15%–34%) had MDR TB. When patients with both smear-positive and smear-negative results were considered, 25 (12%; 95% CI 8%–17%) had MDR TB, 14 (7%; 95% CI 4%–11%) had monoresistant TB, and 90 (42%; 95% CI 36%–49%) had drug-sensitive TB. Three (12%) of 25 MDR TB patients reported prior TB treatment in South Africa. Among 84 patients (39%; 95% CI 33%–46%), *M. tuberculosis* could not be confirmed by culture on LJ media, manual MGIT, or MODS. Among 84 patients with unconfirmed TB, 21 (25%) had a reported sputum smear–positive specimen (any grade) within a public-sector laboratory in the 2 weeks before enrollment.

To our knowledge, this prospective study from the capital and largest city in Zimbabwe provides the first assessment of MDR TB in the country since 1995. Among a representative sample of retreatment patients in Harare, a high proportion of case-patients (24%) had MDR TB. Although these case-patients were not a random sample of the TB population, these data are worrisome, given regional immigration patterns and ongoing challenges within the health system.

Despite a 2009 World Health Assembly resolution to achieve "universal access to diagnosis and treatment of MDR TB and XDR TB [extensively drug-resistant TB]" by 2015, the extent and course of the MDR TB epidemic in the WHO African Region outside of South Africa (with the largest absolute number of MDR TB patients in the continent) remain poorly described. Although MDR TB prevalence has likely remained stable in Zambia, Malawi (*10*), and Mozambique (*11*), it has increased in Botswana (*4*) and Swaziland (*12*). The current WHO estimate of MDR TB prevalence among retreatment patients in Zimbabwe (8.3%; 95% CI 3%–22%) (*2*) is based on a 1995 subnational drug-resistance survey in which 3 of 36 sputum smear–positive patients had MDR TB. According to this estimate, 970 (95% CI 406–1,980) MDR TB cases occur annually, although only ≈10% are currently detected (*13*). In our sample, one-quarter of smear-positive, previously treated patients had MDR TB, suggesting that MDR TB cases in the country may exceed 1,000 among retreatment patients alone. Although drug-resistance surveys typically sample only those with sputum smear–positive results, we also enrolled retreatment patients with presumptive smear-negative results, given the known poor sensitivity of smear microscopy among HIV-infected patients in sub-Saharan Africa and the likelihood of underrepresentation of HIV-infected persons (*14*). Although fluoroquinolone resistance has been detected (A. Jindani, principal investigator of RIFAQUIN trial, pers. comm.), lack of further second-line DST has thus far precluded identification of extensively drug-resistant TB in the country.

Our report has limitations, however. Although a substantial proportion of nationally notified TB cases (≈15%) occur in Harare, regional variation in incidence and prevalence of MDR TB is known, in particular along Zimbabwe's border. Treatment with first-line drugs before microbiological investigation differentially selects for drug-resistant organisms. Most patients had been previously treated, and drug resistance within this group reflects a combination of acquisition, reinfection, and primary infection with a drug-resistant strain and subsequent treatment failure. A valid estimate of MDR TB prevalence among new patients without risk factors for drug resistance is not currently available,

although this group likely accounts for most MDR TB cases in the WHO African Region and globally (*1*). Finally, lack of genotyping data precludes identification of unsuspected transmission patterns, including transnational spread.

Conclusions

A representative drug-resistance surveillance study is urgently needed to estimate the prevalence of MDR TB in the general population of Zimbabwe. A comprehensive response is needed, including an increase in quality-assured laboratory capacity for culture and DST (including second-line drugs), reassessment of infection control practices, and expanded investigation of contacts with drug-resistant TB. Finally, overall strengthening of directly observed therapy, short course (DOTS), improvements in health service delivery, and political and socioeconomic stabilization are essential to prevent expansion of MDR TB in Zimbabwe.

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Epidemiology of Influenza A Virus among Black-headed Gulls, the Netherlands, 2006–2010

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We sampled 7,511 black-headed gulls for influenza virus in the Netherlands during 2006–2010 and found that subtypes H13 and H16 caused annual epidemics in fledglings on colony sites. Our findings validate targeted surveillance of wild waterbirds and clarify underlying factors for influenza virus emergence in other species.

Wild waterbirds of the orders Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, shore birds) are the ultimate source of influenza A viruses for domestic birds and mammals, including humans (*1*). Knowledge of the epidemiology of these avian influenza viruses (AIVs) among wild waterbirds is necessary to improve surveillance and better clarify underlying factors in host-switching of AIV. Epidemiology of AIV in wild waterbirds has been studied mainly among ducks (order Anseriformes) (*2*) but is poorly known among gulls, despite their abundance and close association with humans (*3*). Therefore, we studied the epidemiology of AIV in one of the most common gull species in western Europe, the black-headed gull (*Chroicocephalus ridibundus*).

The Study

Black-headed gulls (n = 7,511) were sampled year-round at multiple locations in the Netherlands during 2006–2010. Birds were captured by hand, leg-noose, or clap net; then, we determined their sex and age (first-year [FY] bird: nestling, fledgling; after first-year [AFY] bird) and weighed them. During the breeding season (April–July), 2,839 FY and 524 AFY birds were sampled at colony breeding sites. Three breeding sites were monitored annually during 2008–2010: Griend, De Kreupel, and Veluwemeer. At Griend, BHGU breeding success was also measured and used to compare breeding chronology to timing of infection (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/1/13-0984-Techapp1.pdf). Outside the breeding season, 1,200 FY and 2,948 AFY birds were sampled in meadows and cities. Cloacal and oropharyngeal swab samples were collected from each bird and tested for AIV by using matrix (M)–specific reverse transcription PCR (RT-PCR) and, if positive, for H5 and H7 subtypes by using hemagglutinin (HA)–specific RT-PCR. Virus culture was attempted on all M RT-PCR–positive samples by egg inoculation. Virus isolates were classified to HA subtype by hemagglutination inhibition assay and to neuraminidase (NA) subtype by using RT-PCR (*4*,*5*). Blood samples were collected from an arbitrary subset of 134 FY and 214 AFY birds and tested for anti-AIV antibody by nucleoprotein (NP)–specific ELISA (*6*). Statistics were performed by using software RStudio version 0.95.265 (www.r-project.org). Additional analyses on AIV prevalence among male versus female birds, dead versus live birds, recaptured birds, and capture bias were performed (online Technical Appendix).

Our results showed that AIV epidemics in black-headed gulls occurred annually during June and July, with a peak monthly prevalence of 47% during 2008 (Figure 1, Table 1). These epidemics were detected in FY birds only and were limited to subtypes H13 and H16; subtype H13 and H16 viruses represented 100% of all virus isolates and 55% of RT-PCR positive birds. In contrast, no AIVs were detected in 524 AFY birds sampled during the breeding season. Annual epidemics were detected in 2 of 3 colonies sampled annually during 2008–2010 (online Technical Appendix Table 1). More detailed investigation on Griend showed that, although H13 and H16 viruses were detected each year, H13 was the only (2008, 2009) or predominant (2010) subtype detected on the first day of virus detection of each breeding season (Figure 2, Table 2). In 2008 and 2009, H16 was detected the next sampling day, which was 1–2 weeks later. H16 was or became the predominant subtype during 2008–2010; H13 prevalence decreased during that period. The source of H13 and H16 viruses causing these epidemics is unknown. Possible sources are breeding or nonbreeding BHGU, other gull species at the colony sites, and freshwater ponds (if present) at the colony sites. Nonbreeding BHGU tend to wander among colony sites. BHGU that breed north of the Netherlands arrive in the Netherlands from July 1 onwards (F. Majoor, unpub. data).

Results from Griend also showed that these epidemics occurred after onset of fledging. The first detection of AIV on Griend (during the last week of June 2008, the first week

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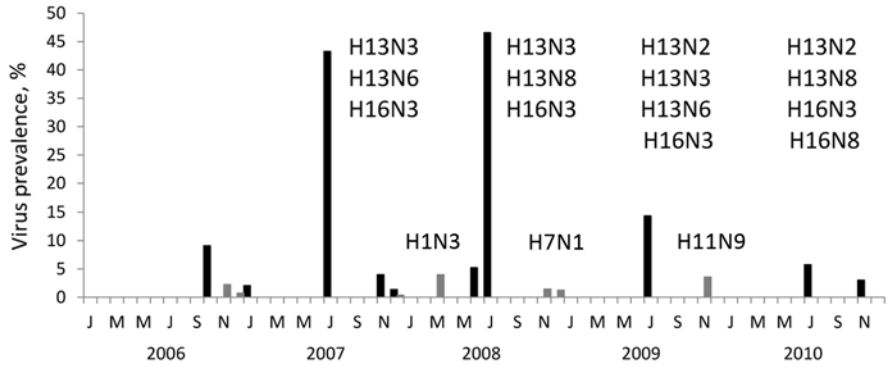

Figure 1. Avian influenza virus prevalence among 7,511 black-headed gulls, the Netherlands, 2006–2010. Cloacal and oropharyngeal samples were collected once from each gull for virus detection. Influenza virus subtypes detected are shown above virus positives. Bars indicate virus prevalence (No. PCR-positive samples/ no. gulls sampled per month). Black bars represent gulls in their first year (FY) of life, comprising nestling and fledgling stages; gray bars represent after-first year (AFY) gulls.

July 2009, and mid-July 2010) occurred 1–3 weeks after onset of fledging. Also, of 871 FY birds, AIV was detected only in FY birds with an average length of <200 mm, above which they are considered to be fledged (*7*). Possible explanations for timing of the epidemic could be increased mobility after fledging and, therefore, increased contact rate; access to water, facilitating more efficient virus transmission; and increased susceptibility of fledglings as a result of immature body condition and loss of maternal antibodies.

Body condition did not differ notably between virus-positive and virus-negative FY birds sampled on the same day ($p>0.05$, Mann-Whitney Wilcoxon test), except for during the third week of July during 2009 ($p = 0.046$) and 2010 ($p = 0.0004$), when virus-positive birds had lower body condition. This suggests that, overall, H13 and H16 virus infections are nonpathogenic for BHGU. Previous studies found no clinical signs (*8*) or histological lesions (*9*) in gulls naturally infected with H13 or H16 virus. No notable differences in virus prevalence were found related to gender, no consistent differences in virus prevalence were found related to capture method, and no AIVs in dead BHGU were detected outside epidemics (online Technical Appendix).

Outside the breeding season, AIV prevalence was much lower, and no H13 or H16 viruses were isolated; AIV were exclusively isolated from AFY birds and were typed as H1N3, H7N1, and H11N9 (Figure 1, Table 1). Additionally, a single H5 virus was detected by using H5 RT-PCR in an AFY gull sampled in December 2006. H13 viruses have been isolated from ring-billed gulls (*Larus delawarensis*) outside the breeding season (*10*). The lack of detection of H13 and H16 viruses in BHGU outside the breeding season in our study provides no support for virus circulation at low prevalence in overwintering FY birds. Our sample size of FY birds sampled outside the breeding season (n = 1,200) may be around the theoretical limit to detect the presence of these viruses in the population, assuming a virus prevalence of 0.5% in a homogeneously distributed population (*11*). However, a nonhomogeneous BHGU population structure outside the breeding season might support a situation in which susceptible FY gulls are present year-round and thus facilitate the circulation of AIV throughout the year at an even lower prevalence.

Prevalence of anti-AIV antibodies detected in FY birds sampled outside the breeding season was statistically more significant (15/59 [25.4%]) than in FY birds sampled

Table 1. Number of black-headed gulls sampled per month for detection of avian influenza virus among 7,511 black-headed gulls, the Netherlands, 2006–2010*

	No. sampled											
Y, Age	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2006												
FY	0	0	0	0	6	365	0	0	0	11	74	70
AFY	0	0	0	0	1	0	0	0	1	7	90	138
2007												
FY	96	28	1	0	0	167	37	0	1	6	100	73
AFY	72	39	0	1	34	2	0	1	1	4	153	275
2008												
FY	11	32	33	0	1	632	290	0	0	4	47	108
AFY	37	61	75	0	33	9	42	0	1	5	68	160
2009												
FY	169	43	0	0	0	295	383	0	0	0	45	57
AFY	740	172	3	0	31	82	55	0	0	0	56	288
2010												
FY	60	52	2	0	0	212	451	3	0	2	33	39
AFY	232	135	11	0	45	128	61	7	1	4	40	71

*FY, gulls in their first year of life, comprising nestling and fledgling stages; AFY, after first year.

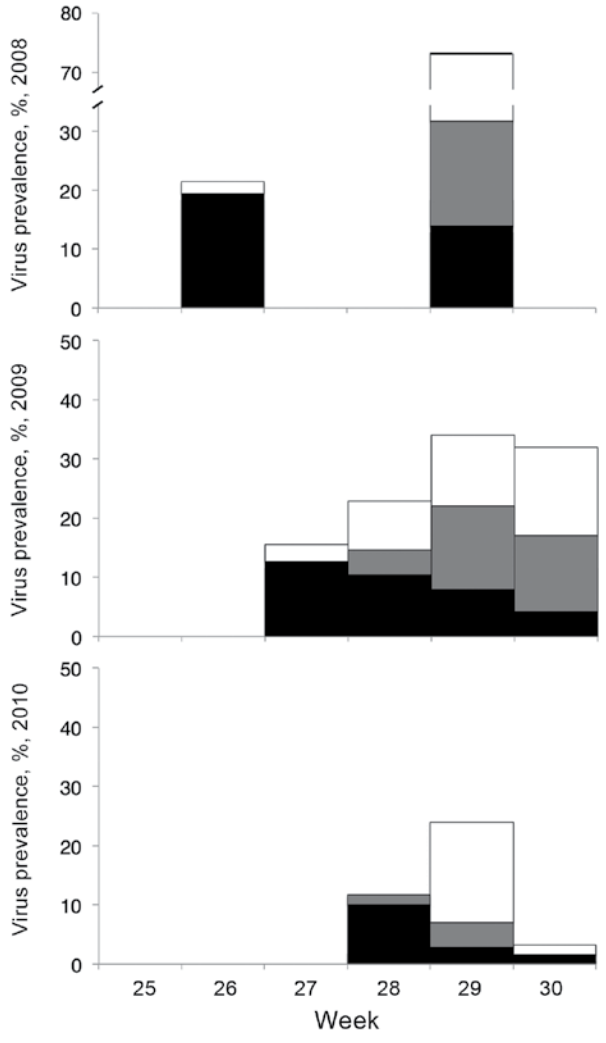

Figure 2. Avian influenza virus prevalence and hemagglutinin subtype (H) distribution of 871 first-year black-headed gulls sampled on the colony site of Griend during 2008–2010. Bars indicate virus prevalence (no. PCR-positive samples/no. sampled per week). Black bar sections, H13; gray bar sections, H16; white bar sections, unknown H subtype.

during the breeding season (4/75 [5.3%]) ($p<0.01$, Fisher exact test). The 4 seropositive FY birds were fledglings (n = 55); nestlings (n = 20) were seronegative. There was no statistically significant difference in the seropositivity of AFY gulls sampled during (40/101 [39.6%]) and outside (45/113 [39.8%]) the breeding season ($p>0.05$, Fisher exact test). These results suggest that FY birds during the breeding season are the most susceptible category to become infected with AIV.

Conclusions

We describe annual AIV epidemics in BHGU colonies. These epidemics were caused by AIV subtypes H13 and H16 and occurred in FY birds during the second half of the breeding season, with prevalence rates of up to 72% per week. On most sampling days, infected and noninfected FY birds had similar body conditions, suggesting H13 and H16 viruses are nonpathogenic for BHGU. These findings broaden our view on AIV dynamics in populations of gull species often closely associated with humans and facilitate more targeted sampling of colonial nesting waterbirds. Further research is needed to show if the same AIV dynamics apply to other gull species and other geographic areas and to clarify the epidemiology of AIV in wild birds and factors that influence emergence of influenza in domestic animals and humans.

Table 2. Number of 871 FY black-headed gulls sampled per week, Griend, the Netherlands, June–July 2008–2010*

Month and week	No. samples		
	2008	2009	2010
June			
25	0	46	44
26	98	70	33
27	0	71	74
July			
28	0	48	60
29	101	50	71
30	0	47	62

*FY, gulls in their first year of life, comprising nestling and fledgling stages.

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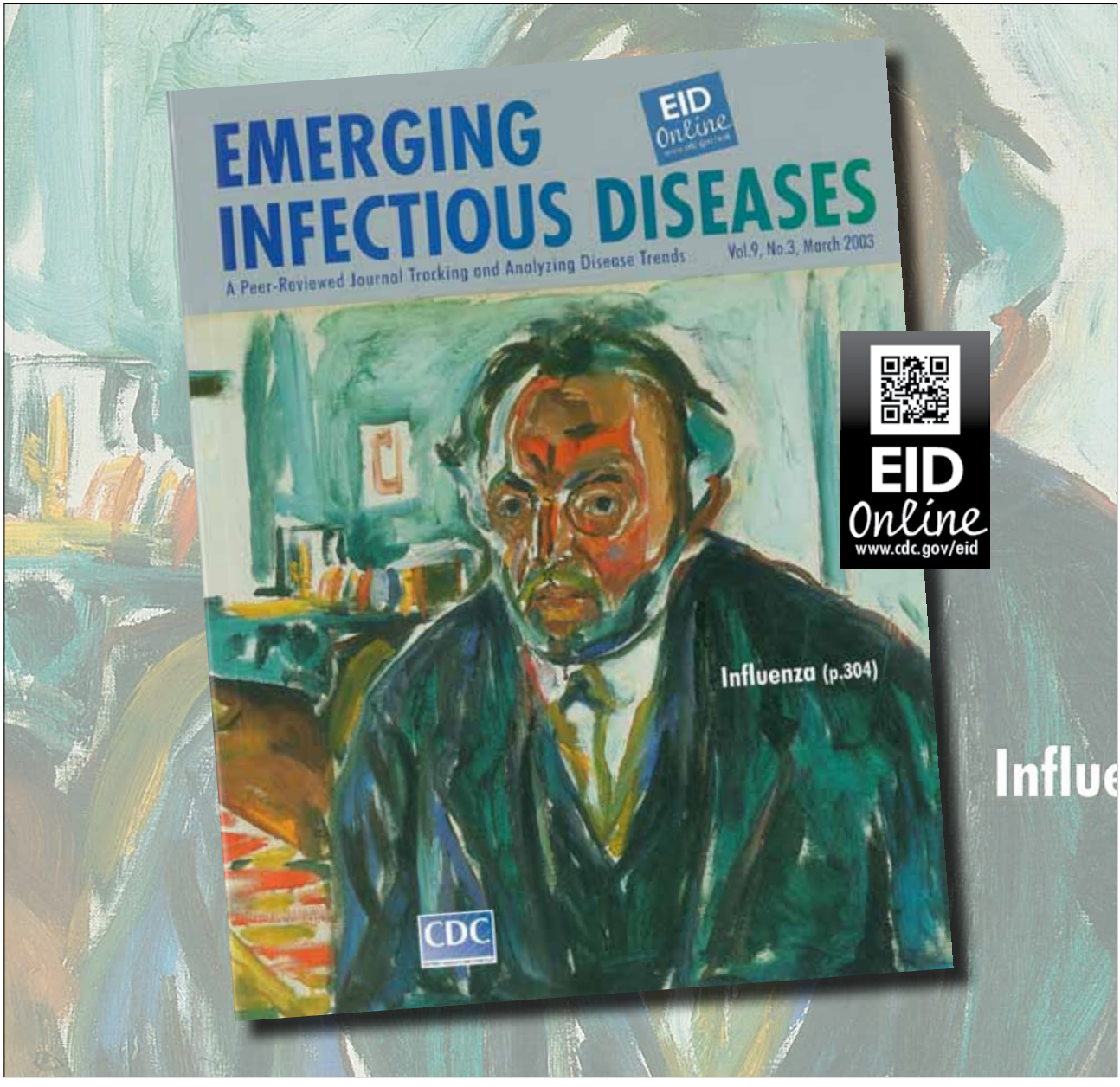

Resolution of Novel Human Papillomavirus–induced Warts after HPV Vaccination

Steffi Silling, Ulrike Wieland, Marko Werner, Herbert Pfister, Anja Potthoff, and Alexander Kreuter

Human papillomavirus (HPV) XS2 was isolated from warts on an immunosuppressed patient. After HPV vaccination, the warts resolved. HPVXS2 was also found in warts and normal skin of HIV-positive patients and rarely in HIV-negative controls. Further studies should elucidate the mechanisms that lead to wart clearance.

Human papillomaviruses (HPVs), small, double-stranded DNA viruses with a circular genome of ≈8,000 bp, are assigned to different genera and species on the basis of their major capsid protein gene (L1) nucleotide sequence, which reflects their tropism (cutaneous or mucosal) and potential to induce tumors. Most HPVs belong to genera alpha (e.g., genital and wart-associated types), beta, or gamma (cutaneous types) (*1*). HPV infections are common, and the prevalence of cutaneous viral warts is 3%–5% in children (*2*). Warts, benign HPV-induced lesions, usually regress spontaneously within several months. Immunodeficiency predisposes to persistent HPV infections and the development of generalized verrucosis (*2*,*3*).

We report the remission of cutaneous warts of prolonged duration in an immunosuppressed patient after HPV vaccination. The study was performed according to the declaration of Helsinki; written informed consent was obtained from the patient.

The Patient

In 1979, a 41-year-old, White woman received a diagnosis of B cell chronic lymphocytic leukemia and was treated with chlorambucil and prednisolone, followed by radiation therapy and splenectomy, resulting in a durable, complete remission of the leukemia. In October 2002, breast cancer was detected in the patient; the breast was surgically removed, and lymph node dissection was performed. Six cycles of chemotherapy were administered during November 2002–March 2003. In February 2010, after a 12-year history of slowly progressing cutaneous warts, the patient sought medical care for numerous, flat, erythematous warts that were coalescing into large plaques on her forearms, backs of hands, and fingers (online Technical Appendix Figure, panel A, wwwnc.cdc.gov/EID/article/20/1/13-0999-Techapp1.pdf). Immunophenotyping revealed a markedly decreased CD4/CD8 ratio (Table). During October 2005–December 2009, the patient received topical and ablative treatments for the warts (salicylic acid, podophyllotoxin, 5-fluorouracil cream, imiquimod 5% cream, cryosurgery, surgical

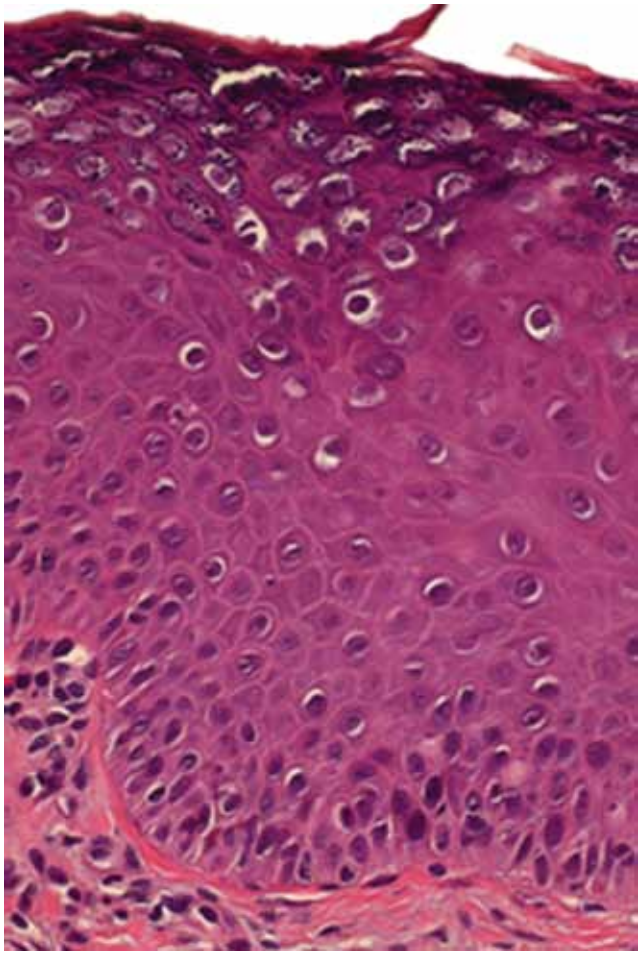

Figure 1. Histopathologic findings for a representative biopsy of skin lesions (erythematous warts) from a female patient before she was administered human papillomavirus vaccine. Analysis revealed features typical of benign cutaneous warts, including acanthosis, parakeratosis, and numerous koilocytes. Vacuolated granular cells show prominent keratohyalin granules, characteristic of human papillomavirus infection (hematoxylin-eosin staining; original magnification × 200)

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Table. Flow cytometric immunophenotyping results before and after human papillomavirus vaccination in a patient with splenectomy*

Cell type (reference range)	Results before vaccination†	Results after vaccination‡
CD3 cell count		
Absolute (690–2,540 cells/μL)	**2,729 cells/μL**	1,770 cells/μL
Relative (55%–84%)	**50%**	55%
CD4 cell count		
Absolute (410–1,590 cells/μL)	602 cells/μL	457 cells/μL
Relative (31%–60%)	11%	14%
CD8 cell count		
Absolute (190–1,140 cells/μL)	**2,149 cells/μL**	**1,339 cells/μL**
Relative (13%–41%)	40%	41%
CD4/CD8 ratio (0.8–2.0)	**0.28**	**0.34**

*__Boldface__ indicates results below or above the reference range. Vaccination was started on July 19, 2010, the second dose was given 2 months later in September 2010, and the third dose was given 4 months later in January 2011.
†February 2010.
‡March 2011.

curettage, electrocautery, and CO_2 laser therapy), but clinical improvement was not sustained.

Complete regression of cutaneous warts has been reported in persons after HPV vaccination (*4*,*5*); thus, we vaccinated the patient with the quadrivalent HPV (qHPV) vaccine (Gardasil, Sanofi Pasteur MSD SNC, Lyon, France), which contains L1 proteins of HPV types 6, 11, 16, and 18 as virus-like particles. Three doses were given during July 2010–January 2011. The patient's pre- and postvaccination CD4/CD8 counts did not differ substantially (Table). In April 2011, three months after the third injection, all skin lesions had resolved (online Technical Appendix Figure, panel B), and in July 2011 and March 2012, the patient was still in complete remission.

The Study

For virologic analyses, 20 biopsy specimens from the patient's fingers, backs of hands, and forearms and 1 specimen each from the cheek and back were available (all were collected before the patient received the first dose of qHPV vaccine). DNA extraction and HPV typing were performed as described (*6*,*7*). Histopathologic analysis revealed features typical of benign cutaneous warts, including acanthosis, parakeratosis, and numerous koilocytes (Figure 1), similar to warts caused by HPV-3 (*8*). A2/A4 PCR (*6*) was used to amplify HPV DNA from all biopsy specimens obtained before vaccination. Sequences of the PCR products were analyzed by using BLASTn (http://blast.ncbi.nlm.nih.gov) and were 100% homologous to a 261-bp fragment named HPVXS2 (*6*). Three overlapping PCR fragments covering the entire genome of HPVXS2 (7,830 bp; GenBank accession no. KC138720) were amplified by using Phusion HotStart II HF DNA Polymerase (Fermentas, St. Leon-Rot, Germany) and ligated into pJET1.2/blunt (Fermentas): fragment 1, XS2-M19fw 5′-GAATTGAGTCTTGCACCAGAGG-3′ and XhoIrev 5′-ATCTCGAGTCGCTGTCGCTTT-3′; fragment 2, XS2-M15fw 5′ -GTATCTAGCACACGAGAAGTAC-3′ and XS2 6413rev 5′-ATGGTGTCCCCGACAACCC-3′; fragment 3, XS2 6258fw 5′-CACCATGTAAACAGACTGCGTC-3′ and XS2-M8rev 5′-ACCCAAATTGTTCTTTAAACTTACC-3′.

MacVector software version 12.7.3 (MacVector, Inc. Cary, NC, USA) was used to determine the organization of the predicted open reading frames (ORFs) and perform phylogenetic analyses. The results showed grouping of HPVXS2 within the alpha-2 species (Figure 2), and in each case, the L1 ORF was <90% homologous to the closest relative. Thus, HPVXS2 can be considered a novel HPV type (*1*).

An HPVXS2-specific quantitative real-time PCR was established. In brief, a 20-μL reaction contained 10 μL of LightCycler 480 Probes Master (Roche, Mannheim, Germany), 0.1 μmol/L probe no. 46 (5′-ATGGCTGC-3′) of the Universal Probe Library (Roche), 0.2 μmol/L each primers XS2-L1fw 5′-CATTTGTCAGTCTGTTTGTAAATATCC-3′ and XS2-L1rev 5′-TCTGCGCAGGTAAAAGAACA-3′, and 2 μL extracted DNA (QIAamp DNA Mini Kit; QIAGEN, Hilden, Germany). Cycling conditions were 95°C for 10 min and 45 cycles at 95°C for 10 sec, 60°C for 30 sec, and 72°C for 5 sec. Virus load was expressed as HPV DNA copies per β-globin gene copy (HPV/β-globin) (*9*). In 17 of the patient's warts, HPVXS2 loads ranged from 903 to 99,571 (median 14,534) HPV/β-globin. Virus loads were much lower in a seborrheic keratosis from her back and a benign nevus from her cheek (1.424 and 0.012 HPV/β-globin, respectively). Two skin specimens obtained 14 months after the third qHPV vaccine dose were HPVXS2-negative.

To estimate the proportion of HPVXS2-positive specimens among archived, extracted DNA, we screened 62 skin warts from 17 immunocompetent and 24 immunosuppressed patients. HPVXS2-DNA was present in warts from 3 HIV-positive women. Two warts were co-infected with HPV-57, and HPVXS2 loads were low (0.0002 and 0.034 HPV/β-globin, respectively). One wart contained HPVXS2 only (virus load 6.853 HPV/β-globin). We also screened 449 swab samples collected for a previous study; the samples were of normal forehead skin from HIV-positive men and

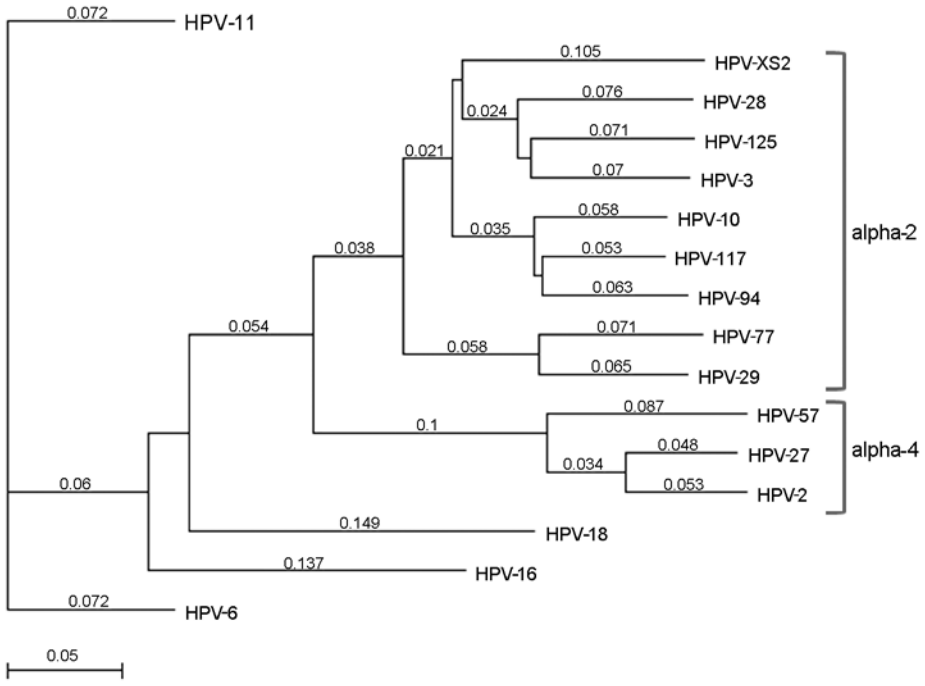

Figure 2. Phylogenetic tree based on selected human papillomavirus (HPV) major capsid protein gene (L1) open reading frames; the tree shows the grouping of HPVXS2. The phylogenetic analysis is based on the L1 open reading frames of all alpha-2 and alpha-4 papillomaviruses and on vaccine HPV types 6, 11, 16, and 18; the best tree was created by using the neighbor joining method with Tamura-Nei distances given. All L1 sequences were aquired from the Papillomavirus Episteme webpage (http://pave.niaid.nih.gov/#home). Scale bar indicates nucleotide substitutions per site.

HIV-negative male controls (*10*). HPVXS2 DNA was present in 16.2% (34/210) and 0.8% (2/239) of specimens from HIV-positive and HIV-negative men, respectively ($p<0.001$; χ^2 test, 2-sided). Among the HIV-positive men, those with CD4 counts of <350 cells/μL were more likely than those with CD4 counts of >350 cells/μ L to be HPVXS2-positive, but the difference was not statistically significant (25.0% vs. 13.5%, $p = 0.101$). Virus loads were low (0.014 and 0.023 HPV/β-globin) in the 2 HPVXS2-positive HIV-negative controls. Virus loads of >1.0 HPV/β-globin were found on normal skin of 10 of 34 HPVXS2-positive HIV-positive men (range 0.002–35.0 HPV/β-globin; median 0.107 HPV/β-globin; interquartile range 1.266 HPV/β-globin).

Conclusions

HPVXS2 fragments were previously identified in hyperkeratotic benign papillomas, squamous cell carcinomas, and dermatitis on 3 renal transplant recipients at consecutive visits and in different parts of the body (*6*). In our study, HPVXS2 was identified in disseminated warts from a patient who had had a splenectomy and in benign skin warts from 3 HIV-positive patients. HPVXS2 DNA loads were well above 1.0 HPV/β-globin in the patient who had received a splenectomy and in an HIV-positive woman with HPVXS2 monoinfection. These virus loads are in line with those in warts induced by other HPV types (*11*). HPVXS2 was found more frequently on normal skin of HIV-positive than HIV-negative men. Thus, immunocompromised persons seem to have difficulties in clearing HPVXS2. However, virus loads remain low to moderate, and the infection in immunocompromised persons is clinically inapparent in most cases.

The patient in our study was free of warts 3 months after the last dose of qHPV vaccine, even though HPVXS2 is not closely related to the vaccine virus types (Figure 2). This observation correlates with published case reports (*4*,*5*,*12*,*13*), but it is still surprising, given that clinical improvement was not seen in 5 patients with HPV-6–positive condylomas who received the same vaccine (*14*). The qHPV vaccine was shown to induce type-specific humoral and cellular immune responses in an immunodeficient patient (*15*), but the mechanisms leading to regression of skin warts associated with heterologous HPV types have not been analyzed. One explanation could be that vaccination led to a general stimulation of the immune system, and innate immunity destroyed virus-infected cells.

We report a single observation; however, correlation does not necessarily imply causation, and a placebo effect is possible. Considering our findings, immunologic studies elucidating the mechanisms that lead to wart clearance and controlled clinical trials should be initiated.

Acknowledgments

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Note added in proof: According to the International Human Papillomavirus Reference Center, HPVXS2 is a variant of the hitherto unpublished HPV-78 (de Villiers EM. Crossroads in the classification of papillomaviruses. Virology. 2013;445:2–10).

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Mother-to-Child Transmission of Congenital Chagas Disease, Japan

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We report a patient with congenital Chagas disease in Japan. This report reemphasizes the role of neglected and emerging tropical diseases in the era of globalization. It also indicates the need for increased vigilance for detecting Chagas disease in non–disease-endemic countries.

Chagas disease, which is caused by the pathogenic protozoa *Trypanosoma cruzi*, was previously endemic only to Central and South America but is now estimated to affect up to 10 million persons worldwide (*1*). Recent unprecedented trends in globalization have been accompanied by the migration of ≈14 million persons from disease-endemic regions to North America, Europe, Japan, and Australia. Consequently, and as predicted, sporadic reports of patients with chronic Chagas disease have emerged, and documented cases have presumably been caused by chronically infected persons who migrated from disease-endemic countries (*2*). Despite the wide geographic spread of patients with Chagas disease, cases of congenital transmission in non–disease-endemic countries have been documented (Table 1) (*3*)

It is estimated that ≈300,000 immigrants from Latin America, to which Chagas disease is endemic, are currently living in Japan and that ≈34,000 births from these immigrants have occurred in the past 10 years. However, vertical transmission of the disease in Japan has not been detected, probably because of the lack of screening programs for at-risk pregnant women and the disregard for the silent clinical manifestation of congenital Chagas disease.

The World Health Organization recommends that each country should strengthen its national and regional capacity to prevent and control congenital transmission of infectious pathogens while improving case management (*4*). We report a patient with congenital Chagas disease in Japan. We also highlight the need for increasing awareness of congenital transmission and urge establishment of an appropriate diagnostic and treatment system for Chagas disease in nonendemic countries.

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The Patient

In October 2012, a 13-year-old boy in Japan was admitted to the National Defense Medical College Hospital in Saitama, Japan, for chronic constipation. His parents and grandparents were Bolivian nationals of Japanese descent who had lived in Chagas disease–endemic areas in Bolivia until 1992. In 1999, the boy was delivered full-term after an uncomplicated pregnancy in Japan but had a low birthweight. He was in excellent health and showed no signs of disease until 2 years before his admission, when he began to report chronic constipation. At that time, he had a medical examination at a Catholic church because most hospitals in Japan could not make a definitive diagnosis of Chagas disease. He underwent serodiagnostic screening for *T. cruzi* infection. The boy and his mother were seropositive for *T. cruzi*.

After admission, he reported extreme constipation and explained that he defecated only once per week. Results of laboratory tests at admission, including those for serum brain natriuretic peptide, were generally within reference ranges. However, abdominal radiography showed major distension of the colon that extended 65 mm (Figure).

The definitive diagnosis, including effectiveness of antiparasitic treatment, was confirmed by using serologic, genomic, and parasitologic methods (Table 2). An ELISA (ORTHO *T. cruzi* ELISA Test System; Ortho-Clinical Diagnostics, Raritan, NJ, USA) was performed according to the manufacturer's protocol. A nested PCR that amplifies a DNA fragment of a repetitive TCZ sequence was performed as described (*5*). The parasite was also isolated by blood culture on Novy, McNeal, and Nicolle agar (*6*) and examined by light microscopy and real-time PCR. All tests showed positive results. It was later determined that the boy's mother was also seropositive for Chagas disease. The boy was given a diagnosis of congenital Chagas disease accompanied by megacolon.

The patient was treated with oral benznidazole (5 mg/kg/d for 60 days) and showed no adverse effects. Parasitemia and DNA of *T. cruzi* in peripheral blood could not be detected by the end of treatment. To ensure successful treatment and cure, we intend to clinically follow up the patient for several decades until serologic results eventually become negative (*7*).

Table 1. Patients with congenital Chagas disease in non–disease-endemic countries*

Country	No. patients	Mother's country of origin	Age, y, at time of diagnosis	Symptoms at birth
Sweden	1	Chile	5	Asymptomatic
Spain	7	Argentina (2), Bolivia (5)	At birth (5), 2 (1), after death (1)	Asymptomatic (5), symptomatic (2)
Switzerland	2	Bolivia (2)	At birth (2)	Asymptomatic (2)
United States	1	Bolivia	At birth	Symptomatic
Japan	1†	Bolivia	13	Asymptomatic

*Values in parentheses are no. patients.
†Patient in this study.

Conclusions

Chagas disease is usually regarded as one of the most serious health problems in rural areas of Central and South America. However, recent successful vector control programs to reduce vector-borne transmission have dramatically changed the epidemiology of this disease (*8*). Mass migration of chronically infected and asymptomatic persons has caused globalization of Chagas disease, and has made nonvectorial infection, including vertical and blood-borne transmission, more of a threat to human communities than vectorial infection (*9*).

On the basis of local and limited serologic surveys, the presumptive number of chagasic patients living in Japan is currently 4,500, compared with >100,000 in the United States and >6,000 in Spain (*2*). Sporadic imported cases have been recognized and reported in Japan in the past decade, but the exact incidence is unknown. Most cases were diagnosed only after patients had critical complications, including severe cardiac involvement (*10*). It is also conceivable that chagasic patients with less severe cardiac symptoms or gastrointestinal involvement have sought treatment at local hospitals in Japan, where the potential for missing or misdiagnosing the disease would likely be high. The difficulty in making a correct diagnosis of Chagas disease is compounded in Japan by low awareness and recognition of the disease by medical staff; scarcity of epidemiologic or statistical data; and lack of diagnostic tools, resources, and facilities available to help with the differential diagnosis.

There is currently no laboratory test–based screening system for donated blood to detect Chagas disease in Japan. Instead, a questionnaire is used to determine if donors have any connections with disease-endemic regions. As of October 2012, to avoid transmission through transfusions, Japanese Red Cross Blood Centers no longer use donated blood for transfusions or producing blood products if the donor or donor's mother has spent ≥4 weeks in Latin America. Therefore, before 2012, it is difficult to estimate how many contaminated blood donations were overlooked in Japan.

The estimated vertical transmission rate from an infected mother to her newborn is ≈5% in Bolivia (*11*). If one considers that 34,000 children were born to Latin American women during the past decade in Japan and that the seroprevalence of *T. cruzi* is estimated to be 1.8%, the number of infected newborns in the past decade is ≈30. However, there are no current screening programs for Chagas disease in Japan to detect chronically infected persons, including pregnant women and newborns.

The patient in this study had congenital chagasic infection, accompanied by advanced gastrointestinal complications. The delay in diagnosis for this patient case was caused by the absence of a screening program in Japan, a problem which also makes it impossible to determine the precise number of pregnant women and newborns with *T. cruzi* infection in this country. In Spain, the most affected country in Europe, a specific program was developed to focus on migrants from Latin American woman of childbearing age. Since its introduction, the program has contributed not only to the early diagnosis of Chagas disease but also to improvements in the quality of life and prognosis for patients (*12*).

Because the therapeutic efficacy of treatment, including benznidazole, for infection with *T. cruzi* is >90% in infants

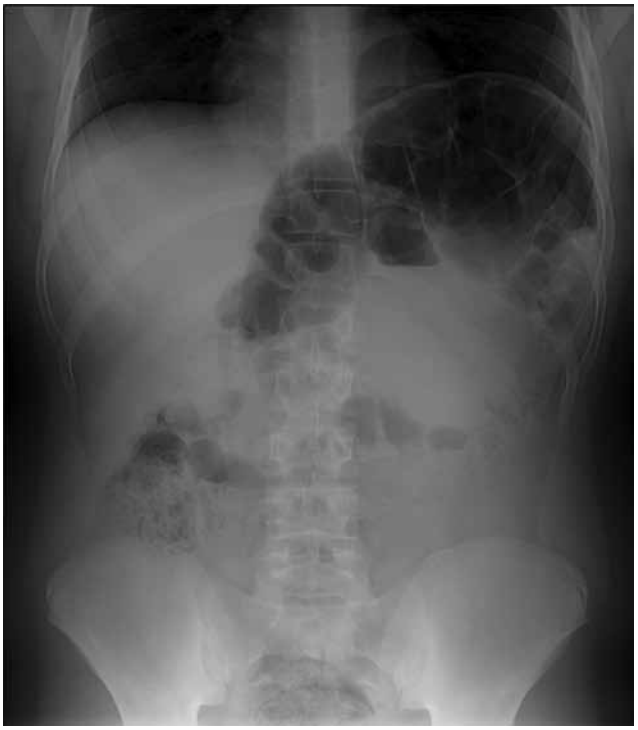

Figure. Abdominal radiograph of a 13-year-old boy with congenital Chagas disease, Japan, showing megacolon and marked dilatation at the splenic flexure.

Table 2. Clinical course of a 13-year-old boy with congenital Chagas disease, Japan, after treatment with benznidazole

Characteristic	Before treatment	Days after starting treatment		
		30	60	180
Antibody titer*	160	160	160	160
Nested PCR result	+	+	–	–
Blood culture result	+	–	–	–

*Antibodies against *Trypanosoma cruzi*.

with congenitally transmitted Chagas disease if treated during the first year of life (*13*), it would be ideal for all pregnant women entering Japan from disease-endemic countries to be screened for the presence of serum antibody against *T. cruzi*. This report indicates the urgent need for implementing proper measures to prevent the vertical transmission of *T. cruzi* in non–Chagas disease–endemic countries, including Japan.

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Avian Hepatitis E Virus in Chickens, Taiwan, 2013

Ingrid W.-Y. Hsu and Hsiang-Jung Tsai

A previously unidentified strain of avian hepatitis E virus (aHEV) is now endemic among chickens in Taiwan. Analysis showed that the virus is 81.5%–86.5% similar to other aHEVs. In Taiwan, aHEV infection has been reported in chickens without aHEV exposure, suggesting transmission from asymptomatic cases or repeated introduction through an unknown common source(s).

Avian hepatitis E virus (aHEV) was first isolated from chickens with big liver and spleen disease or hepatitis-splenomegaly syndrome (*1*,*2*). aHEV infection in chickens can cause death and reduce egg production, resulting in economic losses in the poultry industry (*3*). The zoonotic characteristic of aHEV have not been verified with certainty (*4*); however, the virus may have public health implications related to the consumption of contaminated poultry eggs and meat, the use of poultry viscera as a culinary delicacy, and the handling of poultry.

In Taiwan, the prevalence of aHEV in avian livestock has been increasing, but the causative strain has not been known. To increase our knowledge of this growing problem, we determined the seroprevalence of aHEV antibody in chickens in Taiwan and then isolated the infecting virus and sequenced and phylogenetically analyzed its full genome to better determine the origin and evolutionary status of the virus.

The Study

In 2013, we analyzed serum samples from 1,326 chickens in 61 flocks throughout Taiwan to study the prevalence of aHEV antibodies. In addition, we collected bile samples from 150 chickens among the 4 commercial egg-layer flocks in Pingtung County, Taiwan, to isolate and identify the causative aHEV strain. All chickens appeared to be healthy and ranged in age from 30.1 to 62.8 (mean 43.9) weeks for breeders and from 19.0 to 65.1 (mean 53.1) weeks for layers (Table 1). We tested serum samples for aHEV antibodies by using an ELISA (BioChek, Reeuwijk, the Netherlands) essentially as described by the manufacturer. aHEV antibody seroprevalence was 40.57% (538/1,326) among the chickens and 95.08% (58/61) among the flocks (Table 1).

We used reverse transcription PCR to isolate the aHEV RNA genome from chicken bile, and 3 sets of degenerative primers were designed to amplify a specific region of the genome. The first and the second sets of degenerative primers were designed on the basis of multiple sequence alignments derived from the helicase gene in open reading frame (ORF) 1 and the capsid gene in ORF2 (*5*), respectively. Primers based on ORF1 were AHEV F-1/SD, 5′-TGTTATYACACCCACCAARACGYTG-3′ for positions 2,524–2,548; Helic R-1, 5′-CCTCRTGGACC-GTWATCGACCC-3′ for positions 2,975–2,954; AHEV F-2/SD, 5′-GCCACGGCTRTTACACCYCAYGT-3′ for positions 2,573–2,595; and Helic R-2, 5′-GACCCRG-GRTTCGACTGCTT-3′ for positions 2,958–2,939. Primers based on ORF2 were AHEV ORF2/F-1/SD, 5′-TCGC-CYGGTAAYACWAATGC-3′ for positions 5,473–5,492; AHEV ORF2/R-1/SD, 5′-GCGTTSCCSACAGGYCG-GCC-3′ for positions 5,750–5,731; AHEV ORF2/F-2/SD, 5′-ACWAATGCYAGGGTCACCCG-3′ for positions 5,485–5,504; and AHEV ORF2/R-2/SD, 5′-ATGTACT-GRCCRCTSGCCGC-3′ for positions 5,726–5,707.

The third primer set was designed on the basis of 5 multiple alignments of complete or nearly complete aHEV sequences of other aHEV strains (GenBank accession nos. AM943647, GU954430, AM943646, EF206691, and AY535004) for the aligned results near the end of ORF2. The primers included nt5883/F, 5′-GGAYTATGGGAAY-CAGCATG-3′ for positions 5,862 5,881; nt6579/R, 5′-ATCACAATAAATTAAACATAGGG-3′ for positions 6,600–6,578; nt6216/F, 5′-TGGGGRCCYCAGGGC-GCTG-3′ for positions 6,196–6,214; and nt6498/R 5′-GAGGGGAATGTYYTACTAAG-3′ for positions 6,515–6,496.

Following the primer walking strategy, we designed sequencing primers as detailed in Table 2. We then sequenced the complete genome (6,653 bp) of the aHEV strain isolated from chickens in Taiwan (TWNaHEV; GenBank accession no. KF511797) and determined that it is 1 base pair shorter than that of the prototype aHEV (*6*). The cloned sequence of TWNaHEV RNA is composed of the noncoding region at the 5′ end (1 25 nt); ORF1 (26–4,618 nt), including methytransferase (191–742 nt), helicase (2,429–3,124 nt), and RNA-dependent RNA polymerase (3,167–4,618 nt); ORF3 (4,652–4,915 nt); ORF2 (4,705-6,525 nt); and the noncoding region at the 3′ end (6,525 6,653 nt).

We compared the sequence of strain TWNaHEV with complete or near-complete sequences in GenBank for 7 other aHEVs that had been isolated from chickens; TWNaHEV shared 81.5%–86.5% sequence identity (GenBank accession nos. AM943647 and JN997392, respectively) with

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Table 1. Results of avian hepatitis E antibody testing in serum samples from chickens, Taiwan, 2013*

Type of chicken, mean age, wk	No. flocks tested	No. (%) positive	No. chickens tested	No. (%) positive
Breeders, 43.9	41	39 (97.22)	857	294 (34.31)
Layers, 53.1	20	19 (95.00)	469	244 (52.03)
Total, 47.1	61	58 (95.08)	1,326	538 (40.57)

*ELISA was used to test samples for antibody.

the other aHEVs. Phylogenetic analysis using the maximum likelihood method (*7*) with 1,000 bootstraps, based on the sequence variations in nucleotides, indicated high support for a close relationship (98%) between the aHEV genotype IV strains from Hungary and Taiwan (GenBank accession nos. JN997392 and KF511797, respectively) (Figure) (*8*). In addition, the aHEV genotype IV strains are close to the genotype III strains, which are represented by another strain from Hungary and a strain from China (GenBank accession nos. AM943646 and GU954430, respectively), but this relationship is supported by a bootstrap value of only 55%. Moreover, previous studies (*9*–*13*) suggested an association of genetic types of aHEV with geographic regions. In contrast, we found a mixture of strains from Taiwan, China, and Hungary (GenBank accession nos. KF511797; GU954430; and JN997392 and AM943646, respectively) classified into the separate genotype III and IV clades (Figure).

Conclusions

Several cases of aHEV in chickens without aHEV exposure have been reported in Taiwan. No apparent full-scale outbreaks of acute or chronic aHEV disease have occurred, yet the estimated high seroprevalence of aHEV antibodies among chickens in Taiwan indicates that the disease is now endemic. This finding suggests the possibility of aHEV transmission from asymptomatic cases or repeated introduction through an unknown common source(s). Studies on public health issues related to aHEV; the geographic prevalence and genetic diversity of aHEV; and cross-species infection with aHEV are lacking, and studies on the zoonotic properties of aHEV are incomplete but underway. Knowledge of the diffusion pattern of aHEV around Taiwan is also lacking, although it is known that horizontal, but not vertical, transmission of aHEV is possible (*12*,*14*). Given these facts, hepatitis surveillance is essential in Taiwan.

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Table 2. Primers used in the generation of the complete genome of the Taiwan avian hepatitis E virus strain*

Primer name†	Primer sequence, 5′→3′	Direction
TWN6490–6470	CTAGAAGTCGGCGTGTCTCAG	R
TWN6469–6448	GTGACTGGTCCTCAGGTGCTTG	R
TWN6244–6270	CAGGAGTGGATCTATTTCCTTCAGAAC	F
TWN6243–6262	CCAGGAGTGGATCTATTTCC	F
TWN6215–6241	GATACTTCTATCAGTACAACAACACAC	F
TWN5705–5686	CGCAGCAGCGTGGATGGTAG	R
TWN5506–5526	GTTAAGGTGACTGCTCCGCAC	F
TWN5043–5023	GTCCTGAAGTGGCATGAGCGG	R
TWN4784–4763	CTTCCGGCTGGGAGCGTTTGGG	R
TWN3421–3441	CCATTGTCGCCTGGCTGCACC	F
TWN3227–3247	GACGGGTTATTGGATATACCG	F
TWN2952–2932	GGGTTCGACTGCTTGGCCACC	R
TWN2930–2909	GAGTAAACACAATTTTTTGGCC	R
TWN2569–2588	CGGGGCTGTTGCGATTACGC	F
TWN1657–1635	CATAATGTGCAACGATGGCGGCG	R
TWN1607–1582	CAGCAAGCTCTTTAAGTGTGAGTAGC	R
TWN1570–1546	GAGGTCAATCAAATTCTCAGTGCTG	R
TWN1145–1123	CAGCAATGGCAACAGCCGTCAGC	R
TWN1114–1091	CTCAGGCTGCCAACCCTCATTGGC	R
TWN1048–1026	GTAGGTCAGCAAGCGCGAGCAGC	R
TWN670–646	CTTGTCCGTTGTATTTACGGTATTG	R
TWN607–584	CTCCTCTGGTAAGTGCAACACGAC	R
TWN580–557	CAGTGTCCGCATATTATGGCGGGC	R
HaHEV1–26‡	GCGGCCGCTCTAGCTGCAGCGAATAC	F
HaHEV1410–1433‡	CATCCGTGCGGGTACTAAATCTGC	F
Anchored-oligo (dT)18 primer	NVTTTTTTTTTTTTTTTTTT	NA
Oligo d(T)-anchor primer	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV	NA

*The annealing temperature was set at 60°C for each primer combination. R, reverse; F, forward; NA, not applicable.
†Numbers following TWN in primer names is the nucleotide position extracted by the respective primers.
‡Primers were designed on the basis of the sequence from Hungary (GenBank accession no. JN997392).

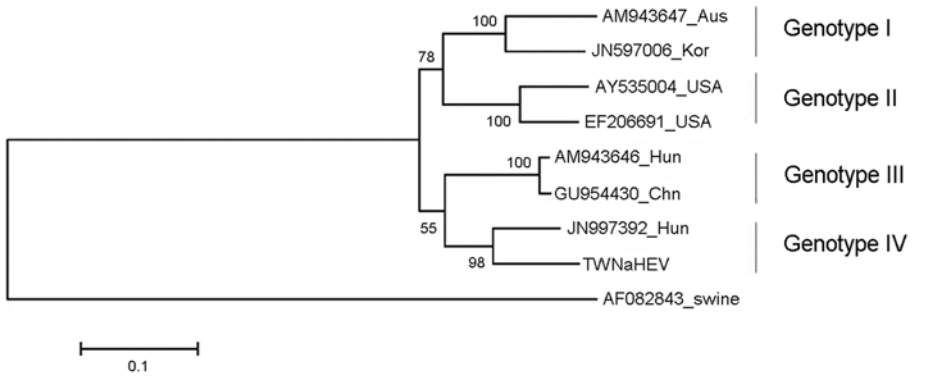

Figure. Phylogenetic tree was aligned by using the maximum likelihood method (1,000 bootstraps) for 8 complete or nearly complete avian hepatitis E (aHEV) sequences and a swine HEV outgroup. GenBank accession numbers, country abbreviations, and avian genotype are indicated. Scale bar indicates nucleotide substitutions per site.

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Dr Tsai is a professor in the School of Veterinary Medicine, National Taiwan University, and is director general of the Animal Health Research Institute, Council of Agriculture. His research interests are in the emerging and zoonotic diseases, especially in avian pathology and public health.

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Endemicity of *Opisthorchis viverrini* Liver Flukes, Vietnam, 2011–2012

To the Editor: Fishborne zoonotic trematodes are highly prevalent in many Asian communities (*1*,*2*). Although presence of the liver fluke *Clonorchis sinensis* is well documented in Vietnam (*3*), evidence of the presence of the more common liver fluke of Southeast Asia, *Opisthorchis viverrini,* is only circumstantial. Surveys of human fecal samples have frequently reported *O. viverrini* fluke eggs in humans in southern and central Vietnam (*4*); however, identifications based on fecal eggs are notoriously unreliable for differentiating species of liver and intestinal flukes (*5*). The few reports of surgical recovery of adult *O. viverrini* flukes from humans do not eliminate the possibility of infection having been acquired during travel in neighboring fluke-endemic countries.

Metaceraria from fish in the Mekong Delta have been tentatively identified as *Opisthorchis* spp., but this identity has not been confirmed (*6*). Specific identification is necessary for an understanding of the liver fluke diversity in Vietnam, especially because *O. lobatus* flukes, a related species that infects ducks, have been reported from nearby Laos (*7*).

To clarify the status of fishborne liver flukes in Vietnam, during 2011–2012, we conducted a survey for liver fluke metacercariae in fish from Phu Yen Province. We selected this province because the local populations have a strong preference for raw fish and because previous surveys of human fecal samples conducted there indicated high prevalence of fishborne parasites (*4*). We chose to investigate metacercariae in fish to avoid the uncertainty of identifications based on fecal eggs and because of the availability of recent molecular methods for species identification of *Opisthorchis* fluke metacercariae (*7*).

Fish were collected from Tuy Hoa City and from the districts of Hoa Xuan Dong, Tuy An, and Song Hinh; these 3 districts are areas of large aquaculture production of freshwater fish. Fresh fish from ponds, rice fields, rivers, and swamps were purchased at local markets from April 2011 through March 2012. The fish sellers provided information about the source of the fish (e.g., type of water body). Fish were transported live with mechanical aeration to the Research Institute for Aquaculture No. 3 in Nha Trang, where they were examined for metacercariae by use of whole individual fish pepsin digestion (*8*).

Recovered metacercariae were examined microscopically, and those identified morphologically as *Opisthorchis* spp. flukes (*9*) were isolated. A subset of these metacercariae were fixed in 70% alcohol and examined by PCR and sequence analysis of the CO1 gene (*7*) at the Department of Helminthology, Mahidol University, Bangkok. For the purpose of obtaining adult worms, 3 hamsters were inoculated with the *Opisthorchis* metacercariae (15, 30, or 45 metacercariae/hamster). The adult worms were recovered from the infected hamsters 25–30 days after infection and were fixed and stained for morphologic determination of species (*10*).

A total of 4 fish species were infected with *O. viverrini* metacercariae (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/20/1/13-0168-Techapp1.pdf). Metacercariae prevalence was highest (28.1%) among crucian carp (*Carasius auratus*). Specific identification was confirmed by morphologic appearance of adult worms recovered from hamsters (Figure) and PCR and sequence analysis of the partial metacercarial CO1 gene, amplified by CO1-OV-Hap-F&R primers (*7*). Infected fish originated predominantly from so-called wild water (i.e., swamps, rice fields, rivers). The prevalence of *O. viverrini* metacercariae in crucian carp varied seasonally (online Technical Appendix Table 2).

Crucian carp are cultured in some countries but not in Vietnam. However, the high prevalence and mean intensity of *O. viverrini* metacercariae (28.3 metacercariae/fish) is of public health concern because wild species such as crucian carp are often eaten raw, marinated, or lightly cooked. In contrast, infected barb (*Puntius brevis*) and rasbora (*Rasbora* spp.) fish (online Technical Appendix Table 1) are not eaten raw. However, barb fish are invasive in farm fish ponds and can persist as a self-recruiting species; the presence of barb is an indication that pond management is insufficient to prevent invasive species of fish. Furthermore, barb fish are often

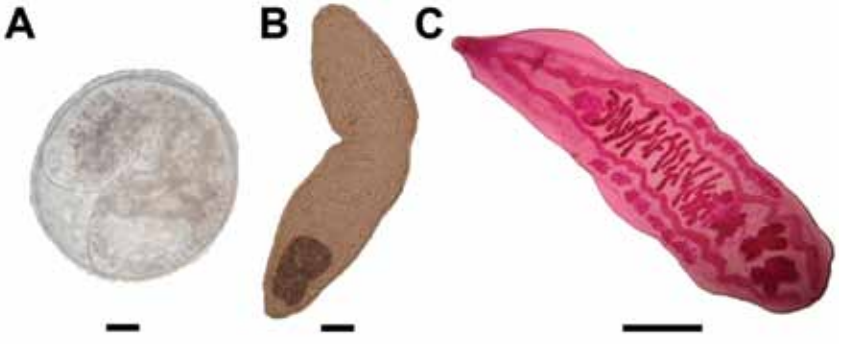

Figure. Morphologic appearance of different stages of *Opisthorchis viverrini* flukes. A) Encysted metacercariae. Scale bar indicates 30 μm. B) Metacercariae released from cyst. Scale bar indicates 30 μm. C) Adult worm from experimentally infected hamster. Scale bar indicates 1 mm.

fed to farm cats, which are major reservoir hosts for fishborne liver and intestinal trematodes. Infections (prevalence 8.3%) in snakehead fish (*Channa* spp.) also represent a food safety risk, because snakehead fish are cultured in Vietnam and are sometimes eaten raw or inadequately cooked. In addition to *O. viverrini* flukes, metacercariae of the zoonotic intestinal flukes *Centrocestus formosanus, Haplorchis taichui*, *and H. yokogawi* were recovered from snakehead and barb fish (online Technical Appendix Table 1), all of which are common throughout Southeast Asia (*1*).

The results of this study demonstrate that the human liver fluke *O. viverrini* is endemic to Vietnam and that it is being naturally transmitted to fish species that are often consumed raw or inadequately cooked. For determination of the prevalence, distribution, and epidemiology of *O. viverrini* flukes in fish, humans, and reservoir hosts (e.g., cats and dogs), these results need to be extended, especially because aquaculture is a growing industry in Vietnam.

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etymologia

Opisthorchis

[o″pis-thor′kis]

From the Greek *opisthen* (behind) and *orchis* (testicle), *Opisthorchis* is a genus of trematode flatworms whose testes are located in the posterior end of the body. Rivolta is generally credited with discovering the first opisthorchid, which he named *Distoma felineus*, in a cat in Italy in 1884. However, the fluke may have been mentioned by Rudolphi in 1819, and in 1831, Gurlt published a textbook that included a drawing of a fluke that was almost certainly *Opisthorchis*. By the end of the 19th century, *Distoma* contained so many species that Blanchard introduced the genus *Opisthorchis* for elongated flat flukes with testes in the posterior end of the body. He chose Rivolta's *Opisthorchis felineus* as the type species.

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Foodborne Trematodiasis and *Opisthorchis felineus* Acquired in Italy

To the Editor: Opisthorchiasis comprises diverse clinical manifestations caused by infections with *Opisthorchis felineus* or *O. viverrini* liver flukes, which are transmitted by eating infected raw or undercooked fish and other aquatic products. In regions outside Western Europe where human opisthorchiasis is endemic, the disease is mainly described as being chronic and asymptomatic. Recent studies indicate cases of *O. felineus* infection in the Mediterranean region, particularly Italy (*1*–*4*). Patients with acute infection have signs/symptoms ranging from fever to hepatitis-like signs/symptoms (e.g., pain in upper right abdominal quadrant, weakness, fatigue, loss of appetite, diarrhea, weight loss); sign/symptom onset occurs ≈2 3 weeks after infection, depending on the number of ingested flukes (*2*–*4*).

Acute opisthorchiasis is a feature of *O. felineus* infection that is not often reported for other trematode infections. Opisthorchiasis is characterized by hepatosplenomegaly, abdominal tenderness, eosinophilia, chills, and fever (*2*); left untreated, it can lead to obstructive jaundice, cholangitis, cholecystitis, and intra-abdominal masses (*1*,*2*,*4*).

Transmission of *O. viverrini* mainly occurs in Southeast Asia, but *O. felineus* transmission expands further westward to parts of Western and Central Eurasia (*1*,*2*,*4*). Recent outbreaks of *O. felineus* infection have been described in Italy (*5*–*7*). In 2010, two travelers from the Netherlands who ate raw tench near Lake Bolsena in Tuscany, Italy, were infected (*8*). We describe 3 additional cases of *O. felineus* infection in Dutch travelers who ate raw fish near Lake Bolsena.

In August 2011, a 54-year-old woman in the Netherlands with no relevant medical history sought medical care for fever, chills, and myalgia lasting 2 weeks. Symptoms began after the patient returned from a vacation in Tuscany. Physical examination showed no abnormalities; her temperature was 37.4°C. Laboratory examinations showed eosinophilic leukocytosis, an elevated C-reactive protein level, and elevated liver enzyme levels (Table).

Opisthorchiasis was suspected because of the patient's travel history and report of eating carpaccio (Italian dish made with raw fish/meat) near Lake Bolsena (*8*). A fecal sample examined by microscopy was negative for eggs, cysts, and helminths. A serum sample was tested at Leiden University Medical Center by using an in-house immunofluorescence assay and ELISA with *Fasciola* spp. antigens, which are likely to show cross-reactivity with other liver flukes (*9*). The immunofluorescence assay result was positive, but the ELISA result was negative.

To confirm the diagnosis of opisthorchiasis, we obtained another fecal sample 1 month later, and low numbers of *Opisthorchis* eggs were seen by microscopy. The sample was sent to Leiden University Medical Center, where in-house real-time PCR was performed using primers (Of350F 5′-CTC CGT TGT TGG TCC TTT GTC-3′ and Of418R 5′-AAA CAG ATT TGC ATC GAA TGC A-3′) and a detection probe (Opis372 FAM-5′-TGC CAA CAC TGG AGC CTC AAC CAA-3′-BHQ1) designed from the O. felineus internal transcribed spacer 2 sequence (GenBank accession no. DQ513407). This PCR amplifies and detects a 69-bp fragment within the *O. felineus* internal transcribed spacer 2 sequence. Simultaneous isolation, amplification, and detection of a standard amount of phocid herpesvirus were used for internal control of inhibition (*10*). The *O. felineus* real-time PCR was positive (cycle threshold 24.7).

Table. Clinical parameters and results of opisthorchiasis diagnostic tests for 3 Dutch travelers after visiting Tuscany, Italy, in 2011

Patient	Symptom severity	Eosinophilia	Liver enzyme level	Microscopy finding	*Fasciola* spp.†		*Opisthorchis felineus* real-time PCR
					IFA IgG	ELISA IgG	
Index patient	Severe	Present	Elevated	*Opisthorchis* egg	1:128	<1:40	Positive, C_t 24.7
Travel companion 1	Moderate	Present	Elevated	*Opisthorchis* egg	1:64	<1:40	Positive, C_t 25.1
Travel companion 2	Moderate	Present	Elevated	Negative	<1:32	1:160	Negative

*IFA, immunofluorescence assay; C_t, cycle threshold.
†Cut-off values for *Fasciola* spp. serology: IFA IgG 1:40, ELISA IgG 1:32.

The patient had 2 travel companions with similar, but less severe, symptoms. Both had serology test results positive for *Fasciola* spp. For 1 traveler, microscopic examination revealed an *Opisthorchis* egg in a fecal specimen, and *O. felineus* real-time PCR was positive (threshold 25.1). All 3 patients were treated with praziquantel (25 mg/kg orally 3 times/d for 2 d) and completely recovered.

Foodborne trematodiasis is reemerging and occurring in developed regions (*1*–*3*). A total of ≈8.4 million persons worldwide have opisthorchiasis, of whom ≈325,000 are in Europe (*1*). Earlier reports of human infections around Lake Bolsena did not result in complete transmission control in the region, as illustrated by the current cases.

The reference standard for diagnosing opisthorchiasis is observation of eggs in feces by microscopy. However, the sensitivity of microscopy is low, particularly in the early disease stage because egg production starts 1–3 months after exposure (*4*,*9*), and the similarity of eggs of different trematodes hampers species-specific differentiation (*1*). In addition, sensitivity of microscopy is highly observer-dependent and varies with the microscopist's level of experience. Because most opisthorchiasis cases in Europe have low numbers of worms, at least 3 separate fecal samples should be obtained and thoroughly examined to rule out a positive diagnosis (*4*,*9*). If test results are negative, a fecal examination should be repeated after several weeks.

Specific *Opisthorchis* spp. serology tests are not available within the Netherlands, but because of known serologic cross-reactivity, antibody detection for *Fasciola* spp. can be performed if opisthorchiasis is suspected (*9*). For confirmation, an *O. felineus*–specific real-time PCR can be performed.

Although opisthorchiasis is not frequently reported in Europe, it should be considered in cases of unexplained acute febrile eosinophilic syndrome with cholestasis, especially when patients confirm the ingestion of raw or undercooked aquatic products. Furthermore, opisthorchiasis should be considered even without a relevant travel history to regions outside Europe where the disease is endemic.

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Hepatitis E Virus Genotype 4, Denmark, 2012

To the Editor: Hepatitis E virus genotype 4 (HEV4) is most commonly reported in China and Japan; it has primarily been categorized as a zoonotic virus because it has been found in humans and several other animal species (*1*). In Europe, HEV4 has been identified in 6 countries, in humans and other animals. The first cases of HEV4 infection in a human and an animal (pig) were detected through strain surveillance activities in Germany (*2*) and Belgium (*3*), respectively. In May 2009, France reported an isolated case of HEV4 infection in a human; however, the source of infection could not be determined. Consumption of contaminated food was deemed unlikely, but receipt of contaminated blood during transfusion was possible (*4*). In 2011, an outbreak of HEV4 in France was associated with consumption of figatelli, a liver sausage that is traditionally consumed uncooked (*5*). Also in 2011, an outbreak in Italy was reported, in which contaminated water was suggested as a possible source of infection (*6*). HEV4 has also been detected in a patient in the United Kingdom. This patient, however, had just returned from India, so the infection was associated with travel (*7*). Transmission was suspected to be zoonotic because the sequence was most closely related to isolates from swine in India.

From January 2010 through December 2012, the Department of Microbiological Diagnostics and Virology of the Statens Serum Institut received 1,112 samples from 823 patients with clinically suspected hepatitis for HEV diagnostic serologic and PCR testing. Blood samples were tested for HEV IgM and IgG by use of the DS-EIA-ANTI-HEV-M and DS-EIA-ANTI-HEV-G kits (DSI, Saronno, Italy) according to the manufacturer's instructions. Diagnostic real-time reverse transcription PCR (RT-PCR) amplifying a 79-bp fragment of open reading frame 3 (*8*) was conducted by using a QIAGEN OneStep RT-PCR Kit (Hilden, Germany). Samples from 59 (7%) patients were HEV positive according to serologic testing; of these, 20 (34%) were positive for HEV RNA according to RT-PCR.

Genotyping was conducted by amplifying 804 bp of open reading frame 2. By sequencing of PCR products, 13 (65%) samples were successfully genotyped; 3 in 2010, 7 in 2011, and 3 in 2012. In 2010 and 2011, HEV genotypes 3 and 1, respectively, were detected in 5 patients. In 2012, a total of 3 patients were infected with HEV genotype 4.

The 3 sequences obtained in this study (GenBank accession nos. KC928081–KC928083) were subjected to phylogenetic analysis, along with reference sequences that included the sequences from the France and Italy outbreaks and the strain from the HEV4 virus identified in a pig in Belgium in 2008. All sequences were aligned by using the Simmonic sequence editor (*9*). Phylogenetic trees were constructed by using MEGA5 (*10*) with the maximum-likelihood method and the Jukes-Cantor algorithm with 500 bootstrap replicates.

Of the 3 patients from Denmark, the HEV4 sequence from 1 patient (March 2012) resembled the sequence from Italy, and these 2 strains grouped together with strains from humans and other animals from China (Figure). This patient reported having traveled to China; thus, zoonotic infection acquired while abroad is suggested. The other 2 patients from Denmark (June 2012) reported no travel history, and the viruses detected in these patients

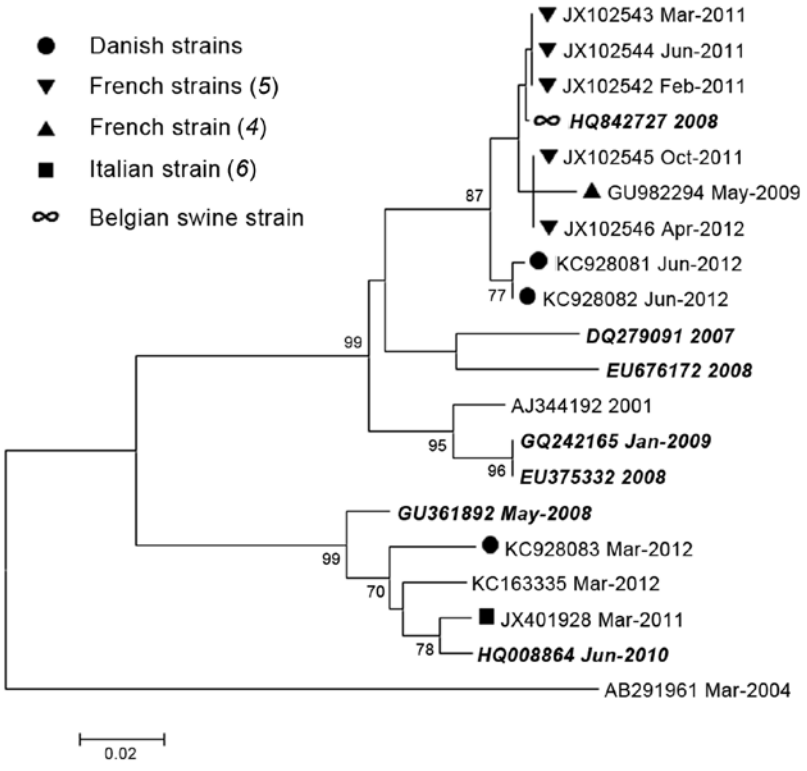

Figure. Maximum-likelihood phylogenetic analysis of hepatitis E virus genotype 4. Reference sequences are identified by their GenBank accession numbers; animal strains are indicated in **boldface *italics***. Months (when available) and years of sample collection are indicated after the sequence names. Countries from which strains were isolated are indicated by symbols. Strain AB291961 is a human genotype 3 reference strain included as an outgroup. Scale bar indicates nucleotide substitutions per site.

were almost identical; only 2 nt differences were found. No epidemiologic or geographic link connected these 2 patients. The only link was the date of sample collection. Both patients were ill during the summer, which suggests possible consumption of undercooked, or raw, contaminated food as the source of infection. The sequences from these 2 patients were most closely related to the sequences from patients involved in the outbreaks in France (Figure). These sequences all form a group with the HEV4 virus identified in the pig in Belgium in 2008, thereby suggesting a zoonotic origin.

Because Statens Serum Institut is the only laboratory in Denmark that offers diagnostic testing for HEV, we consider our national surveillance to be fairly complete. Prospective surveillance will show whether HEV4 becomes established within Denmark. To date, HEV4 has not been detected in animal populations in Denmark. In China, similarity of HEV4 data between strains from humans and other animals in the same geographic areas was high, which is highly suggestive of zoonotic transmission (*3*). Because of the rare detection of HEV4 in Europe, these types of data are not yet available for European countries. However, the close phylogenetic relationship between the strains from humans in Denmark and France and the strain from the pig in Belgium suggests a zoonotic origin for this genotype in these countries. This suggestion is further supported by the fact that some of the strains from France were associated with the consumption of pork liver sausage.

The emergence of autochthonous HEV4 infection in human populations in 4 European countries, and its detection in different years (2006/2007, 2008, 2009, 2011, and 2012), suggests that this genotype may be established in Europe. Thus, for the purpose of ensuring HEV4 detection, diagnostic and genotyping methods should be evaluated.

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Tour Leaders' Knowledge of and Attitudes toward Rabies Vaccination, Taiwan

To the Editor: Tour leaders accompany and care for the health, comfort, and safety of travelers in group tours, which remain a popular method of international travel in Asian countries, including Taiwan (*1*). In addition to travel agents and physicians, tour leaders can also play a key role in the prevention and management of travel-related infectious diseases during group tours.

Rabies is a viral, vaccine-preventable, zoonotic, infectious disease that occurs throughout the world; it is almost always fatal (*2, 3*). According to records of postexposure prophylaxis, ≈0.4% of all travelers have experienced 1 animal (at-risk) bite per month of stay in a rabies-endemic country; in the past 10 years, at least 22 confirmed cases of rabies among travelers have been reported (*4,5*). Given that rabies-endemic countries include many popular tourist destinations, rabies has become one of the most serious travel-related infectious diseases (*3*). In 2011, nearly half of the 9 million

travelers from Taiwan participated in group tours to Southeast Asia, a highly rabies-endemic area. Thus, tour leaders might be in a position to influence rabies risk among group travelers to high-risk destinations.

To determine tour leaders' knowledge of and attitudes toward rabies vaccination, we conducted a cross-sectional survey among those working in international tourism in Taiwan. A self-administered questionnaire was given to 191 tour leaders who attended 6 seminars in Taiwan during May–October 2010. This questionnaire (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/1/13-0673-Techapp1.pdf) comprised 3 sections: demographic information; attitude toward rabies vaccination; and knowledge about general rabies-related information, prevention, and postexposure management. The questionnaire was based on a literature review. Statistical analysis was performed by using SPSS for Windows 11.0 (SPSS, Chicago, IL, USA) and χ^2 test and stepwise logistic regression analysis; p value was set at 0.05.

A total of 175 (91.6%) tour leaders completed the questionnaire. Respondent mean age (± SD) was 44.5 ± 11.8 (range 20–71) years. Among them, 58.3% were women, and 82.3% had a college degree or above. A positive attitude toward preexposure rabies vaccination was reported by >90% of tour leaders (Table). Tour leaders who intended to receive vaccination showed higher willingness to recommend vaccination to group travelers. Most (46.3%) tour leaders indicated that the main factor influencing their intention to receive vaccination was disease severity. However, the mean percentage of accurate responses to rabies-related questions was only 52.4% (Table). Most (49.1%) tour leaders incorrectly thought that it often takes 1 day to 1 week for symptoms of rabies to develop after a person is infected. Only 44.6% of respondents knew that the mortality rate for rabies is >99% after symptoms appear. Regarding the question "Where is rabies present?" the most often chosen incorrect answer was Southeast Asia and mainland China only (32.0%). A positive attitude toward rabies vaccination and poor knowledge were noted regardless of tour leader age and education level. Multiple logistic regression analyses showed that the response to the question about mortality rate was a significant predicting variable regarding tour leaders' attitudes toward vaccination. Tour leaders who understood the high mortality rate associated with rabies tended to receive preexposure rabies vaccination (odds ratio 5.578, 95% CI 1.190–26.170, p = 0.029) and would recommend vaccination to group travelers (odds ratio 15.931, 95% CI 1.840–138.090, p = 0.012).

Our study revealed that tour leaders in Taiwan had a positive attitude toward rabies vaccination but a relatively low level of knowledge about rabies. Knowledge was poor regarding clinical manifestations, rabies-endemic areas, prevention, and management. We believe that the poor knowledge reflects insufficient information or education about rabies provided to the public or to tour leaders in Taiwan, which is a rabies-free area. Previous studies revealed that most animal-bitten travelers did not receive postexposure prophylaxis consistent with World Health Organization guidelines (*4,6*), possibly because travelers and local health practitioners were unfamiliar with the disease (*7,8*). Therefore, tour leaders with adequate knowledge about rabies might be able to provide immediate information to exposed travelers.

Knowledge of the high mortality rate associated with rabies was an independent factor influencing tour leaders' attitudes toward preexposure rabies vaccination. This finding was consistent with previous study findings that low preexposure vaccination rates among travelers might result

Table. Respondent's attitude and knowledge of rabies and vaccination (n = 175), Taiwan, 2010*

	Response, %					
Survey section, questions	Yes	No	No idea	Correct answer	Incorrect answer	Don't know
Section II: attitude toward rabies vaccination						
1. Do you intend to receive rabies vaccination before visiting a rabies-endemic area?	92.6	3.4	4.0	NA	NA	NA
2. Will you recommend rabies vaccination to travelers before they visit a rabies-endemic area?	94.3	1.7	4.0	NA	NA	NA
Section III: knowledge about rabies						
1. Transmission mode	NA	NA	NA	97.1	1.7	1.1
2. Infectious agent	NA	NA	NA	77.7	16.0	6.3
3. Particular symptom	NA	NA	NA	51.4	37.7	10.9
4. Incubation period	NA	NA	NA	25.1	65.8	9.1
5. Mortality rate	NA	NA	NA	44.6	34.3	21.1
6. Rabies-endemic area	NA	NA	NA	38.3	45.1	16.6
7. Preexposure vaccination protocol	NA	NA	NA	21.7	52.1	26.2
8. Postexposure vaccination protocol	NA	NA	NA	41.7	69.7	28.0
9. Postexposure management	NA	NA	NA	73.7	14.3	12.0

*NA, not applicable.

from the lack of knowledge among the travelers themselves or among their pretravel health care providers (*5,9*). In recent years, the World Health Organization and the GeoSentinel Surveillance Network recommended that persons planning to visit rabies-endemic areas receive preexposure prophylaxis before traveling (*6,10*). Understanding the factors influencing acceptance of vaccination could help governments develop and institute strategies for disease prevention. Thus, the Taiwan government should enhance tour leaders' knowledge about rabies, especially regarding the high mortality rate. Education of tour leaders could, in turn, increase vaccination rates and help with prevention and management of rabies.

The results of this study are relevant for countries other than Taiwan because many Asian tourists participate in group tours. We suggest that governments place more emphasis on tour leaders' education concerning travel medicine. Such education could not only improve the quality of group tours but also help prevent travel-related infectious diseases.

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Corynebacterium ulcerans in Ferrets

To the Editor: Infection with *Corynebacterium ulcerans* occurs sporadically throughout the world, and in the United Kingdom it has emerged as the most common cause of diphtheria-like disease (*1*). *C. ulcerans*, along with *C. diphtheriae* and *C. pseudotuberculosis*, can be lysogenized by diphtheria toxin–encoding bacteriophages; this process enables the organism to induce its characteristic sequela (the diphtheritic membrane) in the host. *C. ulcerans* in the environment has been a source of mastitis in cattle and a cause of diphtheria in humans who consume unpasteurized, contaminated milk. The organism has been isolated from various domestic, wild, and laboratory animals; additional definitive sources are dogs, cats, and pigs (*2*). *C. ulcerans* has been isolated from bonnet macaques with mastitis and from the cephalic implants of purpose-bred macaques used in cognitive neuroscience experiments (*3,4*). We report isolation of *C. ulcerans* from cephalic implants in 4 ferrets (*Mustela putorius furo*) and the oropharynx of 1 ferret, all used in imaging experiments in Massachusetts, USA, during 2007–2008.

All ferrets described here were purpose-bred, domestic ferrets, purchased from a commercial vendor. The index case occurred in a ferret with a cephalic implant. Microbiological culture of a purulent discharge

from the implant margin yielded a polymicrobial infection that included an organism identified as *C. ulcerans* by the API Coryne strip system (bioMérieux, Durham, NC, USA) (Table). This isolate and additional isolates from mixed infections of the implants of 3 other ferrets were subsequently identified as *C. ulcerans* by our diagnostic laboratory (Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA, USA) and by the Centers for Disease Control and Prevention (Atlanta, GA, USA) by use of the API Coryne strip system. The oropharyngeal isolate was originally identified by our laboratory as *C. pseudotuberculosis* (99.5%); the same test performed at the Centers for Disease Control and Prevention yielded ambiguous results (*C. ulcerans* [87.3%] and *C. pseudotuberculosis* [12.5%]).

Three isolates (2 implant isolates and the oropharyngeal isolate) were subsequently characterized by MALDI-TOF-MS (matrix-assisted laser desorption–ionization time-of-flight mass spectroscopy) Bruker Daltonics, Fremont, CA, USA) and by 16S rRNA sequencing (*5*) and partial *rpoB* (*6*) gene sequencing (Table). The conserved primers C2700F and C3130R from the *rpoB* gene were used to amplify the PCR products (*6*). All were confirmed to be *C. ulcerans*. The presence of toxin genes for diphtheria toxin (*tox*) (*7*) and phospholipase D (*pld*) (*3*) were evaluated by PCR. Diphtheria toxin production was evaluated by a modified Elek test (*4*). None of the isolates produced diphtheria toxin or contained the diphtheria toxin gene; all isolates were phospholipase-D positive for the 720-bp product.

To determine the source of the isolates, we tested the ferret isolates along with 3 select isolates from our macaque colony by BOX PCR and random amplified polymorphic DNA analysis. Neither type of analysis of the ferret and macaque *C. ulcerans* strains identified any common patterns (data not shown). Ferrets and macaques were housed in separate rooms in the same vivarium; animal care technicians were dedicated to 1 of the 2 species during any particular month. The prevalence of *C. ulcerans* in our macaque population and the precedence of its isolation from those animals more than a decade ago strongly suggests that the isolates are of macaque origin (*3,4*). More exhaustive comparison of the ferret isolates with archived macaque isolates might provide a match. The possibility also exists that newly acquired ferrets arrived infected with *C. ulcerans* or contracted it from an animal technician, veterinarian, or researcher. These possible sources of *C. ulcerans* infection have not been investigated.

An organism recently isolated from the lung, liver, and kidney tissue of a ferret that died of sepsis has been designated as a novel species, *C. mustelae* (*8*). *C. mustelae* is 96.78% related to *C. ulcerans* in 16S rRNA gene sequence similarity and is the first member of the genus to be implicated in disease of ferrets. *C. ulcerans* must now also be considered a potential pathogen of ferrets, although the mixed nature of these implant infections precludes definitive etiologic statements. Implant infection and oropharyngeal carriage in ferrets potentially represent additional zoonotic sources of this organism, underscoring the need for accurate and complete characterization of coryneform bacteria. Notably, the API Coryne test was unable to definitively identify the oropharyngeal isolate, a result reported by our group for other studies and by other investigators (*4*). The results of additional characterization modalities were all concordant. The *C. ulcerans* isolates from this study were nontoxigenic, and their potential for causing classical diphtheria is unlikely (Table). In contrast, a non–diphtheria toxin–producing *C. ulcerans* skin infection mimicking cutaneous diphtheria in a 29-year-old man was recently reported (*9*). Although the source of *C. ulcerans* was not definitively determined, nontoxigenic *C. ulcerans* was later isolated from the oral cavity of the patient's pet cat. Identity of these 2 isolates was not confirmed by molecular identification techniques (*9*). In another case, strain identity was

Table. Identification of *Corynebacterium ulcerans* strains isolated from ferrets*

MIT accession no.	CDC API code	CDC interpretation (confidence limit, %)	Isolate source	CDC MALDI-TOF-MS (score)†	16S rRNA (confidence limit, %), GenBank accession no.	*rpoB* (confidence limit, %), GenBank accession no.
07–3331	0101326‡	*C. ulcerans* (87.3), *C. pseudotuberculosis* (12.5)	Oropharynx	*C. ulcerans* (2.13)	*C. ulcerans* (99.5) KF564646	*C. ulcerans* (99.5) KF539859
08–0584	0111326	*C. ulcerans* (99.7)	Cephalic implant	*C. ulcerans* (2.35)	*C. ulcerans* (99.5) KF564647	*C. ulcerans* (99.5) KF539860
07–3276	0111326	*C. ulcerans* (99.7)	Cephalic implant	*C. ulcerans* (2.24)	*C. ulcerans* (99.7) KF564645	*C. ulcerans* (99.5) KF539858

*Identification was performed by use of the API Coryne strip system (bioMérieux, Durham, NC, USA), MALDI-TOF-MS (matrix-assisted laser desorption–ionization time-of-flight mass spectroscopy) (Bruker Daltonics; Fremont, CA, USA), and gene sequencing. Percentages after the API identification refer to confidence limits. Percentages after the gene sequencing results refer to percentage identities with a reference strain (*4*). MIT, Massachusetts Institute of Technology; CDC, Centers for Disease Control and Prevention.
†2.000–2.299, secure genus identification, probable species identification; 2.300–3.000, highly probable species identification (*4*).
‡The API code generated at MIT was 0101320, interpreted as *C. pseudotuberculosis* (99.5%).

established between a toxigenic isolate cultured from a woman with clinical diphtheria and the same organism cultured from her asymptomatic cat (*2*). Toxigenic and nontoxigenic isolates of *C. diphtheriae* have been reported to cause the cutaneous form of this disease (*10*).

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Bat Lyssaviruses, Northern Vietnam

To the Editor: Bats have been associated with a wide diversity of viruses, including lyssaviruses, which can cause rabies. Currently, 12 distinct species of lyssaviruses have been classified worldwide; 3 of these were isolated from bats in northern and central Asia (*1*). In addition, 3 putative novel bat lyssaviruses (Boklob, Ikoma, and Leida) have recently been described and are awaiting taxonomic assessment (*1*,*2*). Surveys for lyssaviruses in bat reservoirs in several countries in Southeast Asia, such as the Philippines, Cambodia, and Thailand, showed that bat lyssaviruses are naturally circulating in insectivorous and frugivorous bats (*3*–*5*).

Rabies is endemic to Vietnam, and ≈100 human deaths caused by rabies are reported annually; most are attributable to canine rabies (*6*). Although bat-associated rabies cases have not been reported in humans or animals in Vietnam, this finding might be caused by lack of a suitable reporting system. The limited understanding of the extent of lyssavirus circulation in Vietnam and its potential effect on public and animal health prompted this surveillance study.

This study was approved by the ethics committee of The National Institute of Hygiene and Epidemiology, and all capture and experimental procedures complied with institute guidelines for bat capture and use. During May–September 2011, a total of 926 bats were collected from 6 northern provinces in Vietnam (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/20/1/13-0813-Techapp1.pdf). Bats were classified by using a gross morphology key (*7*). Blood and brain samples were obtained after anesthetizing bats by intramuscular injection with 0.05–0.1 mg ketamine hydrochloride.

All bat brains were tested for lyssavirus by using reverse transcription

PCR (*8*) and for lyssavirus antigens by using a direct fluorescence antibody test (*9*) using fluorescein isothiocyanate–conjugated monoclonal antibodies (Fujirebio Diagnostic, Inc, Malvern, PA, USA). The mouse inoculation test was conducted by using brains of 13 bats that died during capture and transport (*9*).

A total of 789 bat serum samples were of sufficient quality and quantity to be screened for neutralizing antibodies against rabies virus (RABV) strain CVS-11, European bat lyssavirus-1 (EBLV-1), and Duvenhage virus (DUVV). We also tested for neutralizing antibodies against Lagos bat virus (LBV) and Mokola virus in 535 samples by using a modified rapid fluorescent focus inhibition test (*10*). A sample was defined as positive for neutralizing antibodies if at a serum dilution of 1:10 a ≥90% reduction was observed in the number of infectious fields in comparison with the virus control.

All 926 bats collected were identified to 25 species. Of these species, 23 were Microchiropteran species and 2 were Megachiropteran species (Table). None of the 926 bat brain samples showed evidence of lyssavirus antigens or virus RNA by direct fluorescence antibody test and reverse transcription PCR, respectively. No virus was isolated by the mouse inoculation test.

Of the 789 bat serum samples tested, 193 (24.5%) were positive for neutralizing antibodies against lyssaviruses. Ninety (11.4%) of 789 bat serum samples had neutralizing antibodies against RABV, 71 (9.0%) against DUVV, 142 (18.0%) against EBLV-1, and 4 (0.75%) against LBV. No bat serum was positive for neutralizing antibodies against Mokola virus. Neutralizing antibodies against the 5 lyssavirus genotypes tested were found in 16 Microchiropteran and 1 Megachiropteran species (Table).

Of the 193 serum samples positive for neutralizing antibodies against lyssaviruses, 65 (33.7%) also neutralized ≥1 of the remaining viruses tested. Twenty-five samples that were positive for RABV were negative for the other viruses; 103 samples were negative for RABV but positive for ≥1 of EBLV-1, DUVV, and LBV. Different titers of neutralizing antibodies against different lyssaviruses were found in some bats (online Technical Appendix Table).

This study provides serologic evidence of lyssavirus-neutralizing antibodies in bats in northern Vietnam. Because no virus was isolated, we could not conclude to which virus or viruses these bats had been exposed. Positive results for antibodies to multiple lyssaviruses, including RABV, found in some bats might have been caused by cross-neutralization of other viruses. The absence of consistent reactivity patterns suggests exposure of these bats to the tested lyssaviruses

Table. Screening of bat serum samples for neutralizing antibodies against lyssaviruses, northern Vietnam*

Bat species (no.captured)	Virus strains				Total, no. positive/no. tested†
	RABV	DUVV	EBLV-1	LBV	
Microchiropteran				0	NA
Aselliscus stoliczkanus (45)	0	0	1	0	1/29
Hipposideros alongensis sungi (19)	0	3	2	0	5/16
Hipposideros armiger (11)	0	0	3	0	3/9
Hipposideros larvatus (138)	26	55	53	0	63/126
Hypsugo sp. 1 (16)	0	0	3	0	3/12
Hypsugo sp. 2 (17)	0	7	2	0	7/15
Ia io (46)	4	1	6	0	8/44
Miniopterus cf. *fuliginosus* (27)	0	0	2	0	2/23
Miniopterus sp. (13)	0	0	0	0	0/10
Myotis sp. 1 (13)	1	0	2	0	3/8
Myotis sp. 2 (11)	0	0	0	0	0/9
Pipistrellus sp. (19)	0	0	0	0	0/10
Rhinolophus affinis (11)	0	0	0	0	0/7
Rhinolophus cf. microglobosus (40)	0	1	2	0	2/34
Rhinolophus cf. *pearsonii* (21)	0	0	0	0	0/16
Rhinolophus cf. *pusillus* (9)	0	0	0	0	0/6
Rhinolophus macrotis (large) (18)	0	0	0	0	0/14
Rhinolophus macrotis (small) (16)	0	3	1	0	3/6
Rhinolophus pusillus (24)	0	1	2	0	2/19
Tadarida plicata (65)	17	0	9	1	19/55
Taphozous cf. *melanopogon* (223)	9	0	22	0	22/203
Taphozous sp. (25)	0	0	1	0	1/19
Taphozous theobaldi (74)	30	0	31	3	45/74
Megachiropteran					NA
Eonycteris spelaea (9)	3	0	1	0	4/9
Rousettus sp. (16)	0	0	0	0	0/16
Total	90	71	142	4	193/789
No. positive/no. tested (%)	90/789 (11.4)	71/789 (9)	142/789 (18)	4/535 (0.75)	193/789 (24.5)

*RABV, rabies virus CVS 11; DUVV, Duvenhage virus; EBLV-1, European bat lyssavirus-1; LBV, Lagos bat virus; NA, not applicable.
†Values in rows may be higher than those in the total because of reactivity of individual serum samples against >1 lyssavirus.

or another unknown lyssavirus. These findings are similar to findings reported from other parts of Asia (*3*–*5*).

Information on lyssavirus circulation in bat populations in Vietnam should be made available to public health authorities, clinicians, and the general public to increase awareness of the risk for rabies transmission from bats; improve recognition, documentation, and reporting of bat exposure to rabies surveillance systems; and increase consideration of the need for post exposure prophylaxis after receiving a bat bite. Our data suggest that several lyssaviruses are circulating among bats in northern Vietnam, and a substantial proportion have neutralizing antibodies to RABV. Further investigations are required, particularly of sick and dying bats, to determine the implications of these findings for human health.

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Co-Production of NDM-1 and OXA-232 by *Klebsiella pneumoniae*

To the Editor: New Delhi metallo-β-lactamase 1 (NDM-1) and OXA-48-group β-lactamase have been increasingly reported as carbapenemases responsible for carbapenem resistance in *Enterobacteriaceae* worldwide (*1*). However, in the United States, *Klebsiella pneumoniae* carbapenemase (KPC)–type β-lactamase is the most common carbapenemase among *Enterobacteriaceae*, especially *K. pneumoniae*. Isolates producing NDM-1 were first reported in the United States in 2010 (*2*), followed by several case reports and most recently a hospital outbreak in Colorado (*3*–*6*). As for OXA-48-group β-lactamase, 2 cases of infection with OXA-48–producing *K. pneumoniae* were recently reported from Virginia (*7*). We report *K. pneumoniae* co-producing NDM-1 and OXA-232, a variant of OXA-48, and *Escherichia coli* producing NDM-1 that were isolated from the same patient.

A 69-year-old woman was hospitalized in India for subarachnoid hemorrhage in January 2013. Her hospitalization was complicated by unsuccessful coil embolization and subsequent hydrocephalus. A ventriculoperitoneal shunt was inserted, and she was transferred to an acute care hospital in Pittsburgh, Pennsylvania, USA, for further management in February 2013. She underwent reinsertion of the shunt and was discharged to a long-term care facility (LTCF 1). She was readmitted to the same hospital because of fever in March 2013.

A urine culture collected at the time of readmission grew carbapenem-resistant *K. pneumoniae* and extended-spectrum β-lactamase–producing *E. coli*. Although production of KPC-type β-lactamase was initially suspected in *K. pneumoniae*, the unusually

high level of resistance to amikacin (MIC >32 μg/mL) and gentamicin (MIC >8 μg/mL) increased concern for presence of an NDM-1 producer, which is frequently highly resistant to aminoglycosides because of production of 16S rRNA methyltransferase (*8*). A modified Hodge test showed a positive result for carbapenemase production, and a metallo-β-lactamase Etest (bioMérieux, Marcy l'Etoile, France) showed a positive result for metallo-β-lactamase production.

PCR and sequencing identified NDM-1 and OXA-232, a 5-aa variant of OXA-48 recently reported in *K. pneumoniae* isolates from India (*9*). Presence of the gene for 16S rRNA methyltransferase (*armA*) was also confirmed by PCR and sequencing and accounted for the high-level aminoglycoside resistance. The isolate belonged to sequence type (ST) 14, as determined by multilocus sequence typing, and has been reported to be common among NDM-1–producing *K. pneumoniae* in Europe (*10*).

The patient was discharged to LTCF 1 but was readmitted because of recurrent fever. A urine culture collected at this admission grew carbapenem-resistant *K. pneumoniae* and carbapenem-resistant *E. coli*. This *E. coli* isolate belonged to ST95 and was positive for the NDM-1 gene but negative for the OXA-48 group and *armA* genes. The original extended-spectrum β-lactamase–producing *E. coli* isolate belonged to ST3865, which is distinct from ST95. Therefore, it is likely that the patient was already colonized by NDM-1–producing *E. coli* ST95 at the time of the first admission, but this colonization was not detected in a clinical culture at that time. All *K. pneumoniae* and *E. coli* isolates remained susceptible to fosfomycin and colistin.

The patient did not receive any antimicrobial drug therapy specific for these isolates because she was deemed to be only colonized with them in the urine. Enhanced contact precautions were also implemented at the time of PCR confirmation of the NDM-1 gene. These precautions included all components of contact precautions (handwashing, gowns, gloves, disinfected/dedicated equipment), and dedicated personnel monitored compliance with these measures around the clock.

The patient was eventually discharged to another long-term care facility (LTCF 2) in April 2013. A point surveillance testing for NDM-1–producing *Enterobacteriaceae* by using rectal swab specimens was conducted for all inpatients at the acute-care hospital and for all residents of the unit at LTCF 2. Testing did not identify any other patients colonized with NDM-1–producing *Enterobacteriaceae*.

In transformation and conjugation experiments, transformants carrying the OXA-232 gene were obtained from *K. pneumoniae*, but those carrying the NDM-1 gene could not be obtained by either method, suggesting that the 2 genes were not located on the same plasmid. For *E. coli*, transformants and transconjugants carrying the NDM-1 gene were obtained, which indicated that this gene was located on a self-conjugative plasmid.

Detection of NDM-1– or OXA-48-group–producing *Enterobacteriaceae*, in particular *K. pneumoniae*, poses a diagnostic challenge in regions to which KPC-producing *K. pneumoniae* is endemic. In our case, recognition of resistance to multiple aminoglycosides by an automated instrument, which was confirmed to be high level by the disk diffusion method (i.e., no inhibition zone), prompted early detection and implementation of appropriate infection prevention measures. Production of 16S rRNA methyltransferase by KPC-producing *K. pneumoniae* is extremely rare, and no cases have been identified in the United States. Therefore, we propose that high-level resistance to amikacin and gentamicin can serve as a clue for suspecting potential NDM-1–producing isolates in clinical diagnostic laboratories.

Conversely, *Enterobacteriaceae* producing OXA-48-group carbapenemase, including variants such as OXA-232, do not have characteristic susceptibility patterns and may easily not be recognized in areas with a high background prevalence of KPC-producing organisms. Therefore, organisms producing OXA-48 or their variants might have already spread in the United States.

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Ground Beef Recall Associated with Non-O157 Shiga Toxin–producing *Escherichia coli*, United States

To the Editor: Shiga toxin–producing *Escherichia coli* (STEC) cause severe illness in humans, especially young and elderly persons. In previous decades, prevention and control measures focused on STEC O157:H7; however, in recent years, non-O157 STEC–related outbreaks and illnesses have been detected more frequently. In the United States, 6 serogroups (O26, O45, O103, O111, O121, and O145) account for ≈75% of the reported non-O157 STEC illnesses (*1*).

On August 4, 2010, the Maine Center for Disease Control and Prevention (Maine CDC) investigated 2 isolates of nonmotile STEC O26 that were indistinguishable by pulsed-field gel electrophoresis (PFGE). Both case-patients had diarrhea and abdominal cramps, shopped at grocery stores in the same town, and reported consumption of ground beef. Case-patient 1 purchased ground beef at Store A; a shopper card used for the purchase was shared with investigators. Case-patient 2 consumed ground beef purchased from 2 stores (Stores B and C); neither shopper cards nor receipts were available.

On August 5, a Maine Department of Agriculture, Food and Rural Resources (Maine DoA) inspector visited Stores A and B. On June 25, case-patient 1 had purchased 90% lean ground beef at Store A; the beef was produced by a parent company with multiple establishments. Inspectors cross-referenced this purchase with meat grinding logs from Store B, which revealed that the parent company that supplied ground beef to Store A also supplied beef to Store B. Maine DoA notified the United States Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS), of a common manufacturer.

On August 9, the New York State (NYS) Department of Health contacted Maine CDC regarding a third case-patient with an STEC O26 isolate that was indistinguishable by PFGE from the other 2 isolates. Case-patient 3 had handled 90% lean ground beef purchased from the grocery store chain used by case-patient 1 (Store A). Shopper card information indicated that the beef was purchased on June 17. Ground beef was the only common exposure among the 3 case-patients.

During August 18–26, Maine DoA, NYS Department of Agriculture and Markets, and USDA-FSIS conducted a traceback of ground beef (Figure). Traceback revealed that for >10 years, Store A had been purchasing 90% lean ground beef from Establishment X (1 of many establishments within the parent company). Further investigation revealed that implicated ground beef from Store A locations in Maine and New York had come from the same lot at Establishment X. USDA-FSIS conducted ground beef traceback at Stores B and C; source materials were received from multiple establishments, but Establishment X was the only common supplier (Figure). On August 28, Establishment X recalled ≈8,500 pounds of ground beef that had been produced on June 11.

On September 2, the NYS Department of Health Public Health Laboratory tested leftover hamburger patties purchased by case-patient 3. The samples were confirmed as STEC O26 with a PFGE pattern indistinguishable from the strains isolated from case-patients.

On November 17, USDA-FSIS completed an assessment at Establishment X and determined that the company's food safety system was adequate to control pathogens of concern. Follow-up testing of beef trim samples at Establishment X were negative for STEC O26 and O157:H7.

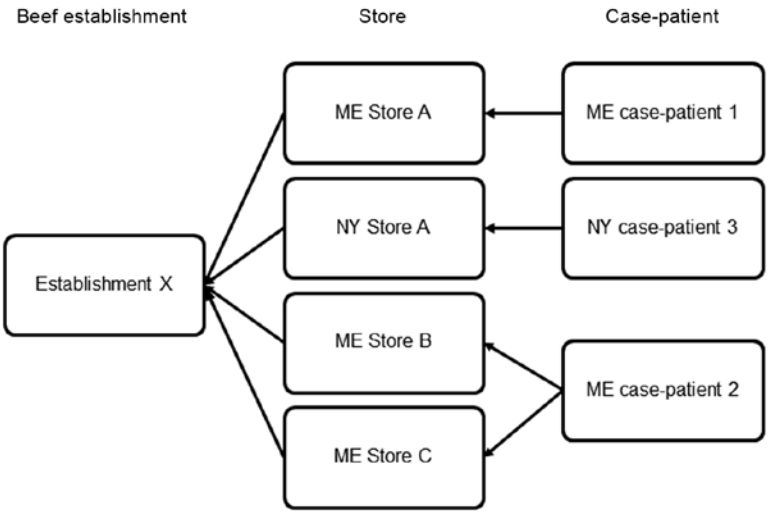

Figure. Supplier traceback for ground beef associated with a cluster of *Escherichia coli* O26 infections in Maine (ME) and New York (NY), 2010.

The Council to Improve Foodborne Outbreak Response guidelines emphasize the importance of timely involvement of all members in outbreak investigations (*2*). A review of enteric disease investigations by Hedberg et al. (*3*) concluded a need to increase timeliness of case investigation and to reduce delays during outbreak investigations. While waiting for PFGE confirmation, Maine CDC notified Maine DoA of case-patients who purchased ground beef in the same city. Within 48 hours, an inspector visited grocery stores where case-patients purchased ground beef and notified USDA-FSIS of the illness investigation. Ten days after the shopper card information from case-patient 3 was available, USDA-FSIS convened a recall committee. Quick action by all agencies led to timely investigation, traceback, and recall. This well-characterized outbreak of only 3 cases of STEC O26 infection led to a recall of ground beef.

Foodborne illness investigations increasingly rely on purchase records from shopper cards, which record information such as purchase dates, brands, and product types that are valuable for traceback and identification of common exposure to a food item. After a thorough record review, investigators in this outbreak were able to narrow the purchased beef to 1 production date. This finding emphasizes the importance of recordkeeping at retail stores and meat processing establishments to determine production dates in the event of a recall. Shopper cards are used more frequently during investigations, so safeguards to protect the consumers' personally identifiable information are needed to prevent inappropriate disclosure and accidental breeches of confidentiality.

In an effort to reduce human illnesses, USDA-FSIS developed policy on non-O157 STEC in raw beef products to declare 6 serogroups of pathogenic STEC as adulterants in nonintact raw beef (*4*). The Agency began implementing routine testing for these serogroups in June 2012.

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Salmonellosis and Meat Purchased at Live-Bird and Animal-Slaughter Markets, United States, 2007–2012

To the Editor: *Salmonella* spp. cause ≈1.2 million human illnesses annually in the United States (*1*). Infections are primarily acquired through exposure to contaminated food or infected animals (*1*,*2*). Since 2007, state and local health departments and the Centers for Disease Control and Prevention have investigated multiple salmonellosis outbreaks linked to meat purchased at live-bird markets (LBMs) and live-animal markets (LAMs), where poultry and livestock are sold for onsite slaughter. These markets typically operate in large cities and serve populations of diverse ethnic backgrounds (*3*).

In 2007, an outbreak involving 62 case-patients infected with 1 of 3 *S. enterica* serotype Schwarzengrund strains was investigated in Massachusetts; 61% were children <5 years of age, including 14 (23%) infants <1 year of age, and 96% were Asian (Table). A case-patient was defined as a person infected with *S. enterica* who had a pulsed-field gel electrophoresis *Xba*I restriction enzyme pattern indistinguishable from the outbreak strain. Exposure to poultry purchased at LBMs was reported, and environmental sampling at an implicated LBM identified 6 *S. enterica* serotypes, including 1 outbreak strain.

Three subsequent investigations of *S. enterica* serotype Schwarzengrund infections were conducted: a 2009 outbreak of 50 cases in New York, New York; a 2010–2011 multistate outbreak of cases predominantly in New York, New Jersey, and Massachusetts; and a 2012 multistate outbreak of cases mostly in Illinois and Michigan. Most case-patients in these outbreaks were of Asian race or Hispanic ethnicity, but 3/5 case-patients in Michigan reported Arab ethnicity; >50% were infants or children <5 years of age.

Among case-patients with available information, exposure to poultry from LBMs was reported by 88% of case-patients in the 2009 New York investigation, 35% in the 2010–2011 multistate investigation, and 50% in the 2012 multistate investigation. In Michigan, the outbreak strain was isolated from chicken purchased at an LBM and collected from households of 2 case-patients.

During 2011–2012, the Centers for Disease Control and Prevention investigated a nationwide increase in *S. enterica* serotype I,4,[5],12:i- infections (pulsed-field gel electrophoresis *Xba*I restriction enzyme pattern JPXX01.1314). Although no single vehicle was implicated, clusters linked to LAMs were identified. In Minnesota, 14 illnesses were linked to meat from 3 neighboring LAMs. Environmental sampling identified the outbreak strain from an animal-holding pen at 1 of the markets. Seven case-patients were infants <1 year of age, and 10 reported Hmong ethnicity. In California, 10 illnesses likely associated with pork, lamb, and beef purchased at 3 LAMs were identified; case-patients reported Ethiopian and Hmong ethnicity. The outbreak strain was isolated from a pork leg collected from the freezer of a case-patient.

LBMs and LAMs appear to be preferred by certain populations for cultural, culinary, or religious reasons. Exposure to meat from these markets is being increasingly recognized as a potential source of salmonellosis. The

Table. Characteristics of outbreaks of human *Salmonella enterica* serotype Schwarzengrund infections linked to meat purchased at live-bird markets, United States, 2007–2012*

				No. yes/total no. (%)‡				
Year	Location	Outbreak strain†	No. cases	Children <5 y of age§	Infants <1 y of age	Asian race	Hispanic ethnicity	Exposure to meat purchased at live-bird markets
2007	Massachusetts	JM6X01.0240, JM6X01.0225, JM6X01.0118	62	38/62 (61)	14/62 (23)	53/54 (98)	NA	8/10 (80)
2009	New York, NY	JM6X01.0240	50	37/50 (74)	15/50 (30)	7/20 (35)	9/17 (53)	14/16 (88)
2010–2011	Multistate¶	JM6X01.0240	233	105/209 (50)	19/209 (9)	26/72 (36)	29/72 (40)	28/80 (35)
2012	Multistate#	JM6X01.0323	15	8/15 (53)	3/15 (20)	1/12 (8)	5/12 (42)	6/12 (50)

*NA, not available.
†Defined by pulsed-field gel electrophoresis *Xba*I restriction enzyme pattern.
‡Denominators are dependent on number of case-patients interviewed and may vary from case counts.
§Includes infants <1 y of age.
¶Case distribution includes New York (91, 39%), New Jersey (52, 22%), and Massachusetts (44, 19%).
#Case distribution includes Illinois (8, 53%) and Michigan (5, 33%).

cause is uncertain, but one factor may be an increased number of markets: in New York, New York, the number of LBMs nearly doubled from 44 to >80 during 1994–2002 (*4*). Most case-patients in these outbreaks had minimal direct contact with poultry or livestock at these markets; many case-patients were infants or young children who had not visited the markets or consumed meat. Therefore, one risk factor appears to be living in a household where the meat purchased from these markets is handled or consumed.

Several factors could make meats from these markets more risky for acquiring salmonellosis. Although LBMs and LAMs must meet sanitation requirements and prevent product adulteration (*5*–*7*), most are exempt from Food Safety and Inspection Service pathogen reduction performance standards (*8*,*9*) and probably do not require suppliers to use pathogen control measures on the farm or employ them during slaughter. Regulatory oversight by state agencies varies. Investigation findings, including environmental sampling, indicate that these markets could be heavily contaminated with *S. enterica*.

Preliminary results of a Massachusetts study found that fresh-killed chickens from LBMs had higher *Salmonella* and *Campylobacter* spp. contamination rates than those for chickens purchased at grocery stores (*10*; T. Stiles, unpub. data). High-risk cultural preferences identified in these outbreaks included consuming raw or undercooked meat and cooking parts (e.g., feet, intestines) that are more likely to harbor *Salmonella* spp. Further processing (e.g., de-feathering, butchering) conducted inside homes could lead to cross-contamination in the household environment. Because of language and cultural barriers, existing food safety messages may not have been effective.

The number and type of LBMs and LAMs, the populations these markets serve, and regulatory authority vary considerably by state, and many case-patients and market owners have been reluctant to speak with public health authorities. Therefore, illness prevention requires a local, targeted approach. To strengthen regulations, some states have created guidelines and begun regular inspection of these markets. Educational outreach has included distribution of posters, flyers, and magnets with safe food handling messages in multiple languages; collaboration with community groups; and education of market owners and workers. Given the various communities who use LBMs and LAMs, multifaceted interventions, including collaboration between human and animal health agencies, are needed to reduce disease risk among market patrons and their families.

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MLB1 Astrovirus in Children with Gastroenteritis, Italy

To the Editor: Astroviruses are notable agents of gastroenteritis in many mammalian and avian hosts. Astroviruses are nonenveloped RNA small, round, viruses (SRVs) with a single-stranded, positive sense RNA of 6.1 to 7.9 kb (*1*). The genome contains 2 nonstructural genes, open reading frame (ORF) 1a and 1b, and a capsid gene, ORF2, with short 5′ and 3′ untranslated regions. Human astroviruses, a major cause of gastroenteritis, are classified in the human astrovirus species, comprising 8 serotypes (*1*). Recently, astroviruses genetically unrelated to canonical human astroviruses have been identified in human stools in several countries. These unusual astroviruses form 2 main genetic clades. One clade contains MLB1, MLB2, and MLB3 (*2–4*). The second clade contains VA1, VA2, VA3 (also known as HMO-C, HMO-A, and HMO-B, respectively) and VA4 (*5,6*). More recently, a VA1/HMO-C–like virus was detected in brain tissue from an immunocompromised child with encephalitis (*7*). The discoveries of these viruses provide novel candidate agents of human disease and raise concerns inherent of possible zoonotic implications. Here we describe the detection and genome characterization of MLB1-like astrovirus in a 4-year-old male child hospitalized with severe gastroenteritis during January 2007 at the University Hospital of Parma, Italy. Clinical signs included vomiting and severe diarrhea, with moderate dehydration. The child was treated with rehydration and maintenance therapy (balanced glucose-electrolyte solutions) and completely recovered after 3 days.

Fecal samples collected at admission were subjected to routine virologic (electron microscopy [EM], cell cultures, latex agglutination, and reverse transcription PCR) and bacteriologic (culturing with selective and differential media) examinations. Fecal samples tested negative for common bacterial (*Clostridium difficile, Shigella* spp., *Salmonella* spp., *Yersinia enterocolitica, Staphylococcus aureus,* and *Campylobacter* spp.) and viral (adenovirus, rotavirus, norovirus, human astrovirus, enterovirus and sapovirus) enteric pathogens. However, through EM, SRV particles were observed in the feces of the patient (Figure, panel A). Despite several efforts with additional consensus primer sets for calicivirus and enterovirus, it was not possible to identify the SRVs detected by EM, and the case was archived as undiagnosed. However, beginning in 2008, several astroviruses genetically unrelated to canonical human astroviruses have been described (*2*). Broadly reactive consensus primers for astrovirus (*8*) spanning the RNA-dependent RNA polymerase (RdRp, ORF1b), along with sets of specific primers for these novel astroviruses (*2*), have been designed. By using these sets of primers, astrovirus RNA was detected in the sample. Upon sequence analysis of a small ORF2 fragment, the astrovirus strain (ITA/2007/PR326) displayed up to 97.8% nucleotide identity to MLB1-like viruses. Fragments of the genomic RNA in ORF1a (1,173 nt), ORF1b, and the genome 3′ end (2,930 nt), including the full-length ORF2, were sequenced by using primers specific for MLB1-like astroviruses and

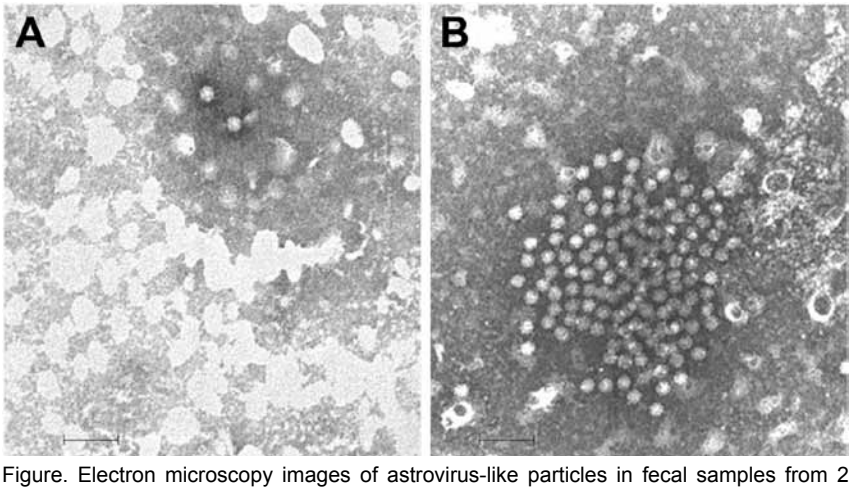

Figure. Electron microscopy images of astrovirus-like particles in fecal samples from 2 patients in Italy: A) strain ITA/2007/PR326, from a 4-year-old child hospitalized in January 2007; and B) strain ITA/2008/PR3147, from a 14-month-old child hospitalized in November 2008. The viral particles are ≈28–30 nm in diameter. Scale bars indicate 100 nm.

RACE 3′ protocols; total genome coverage was 66.5% (4,103/6,169 nt). In all the genome regions sequenced, the virus displayed the highest sequence identity to MLB1-like astroviruses (97.4%–98.2%). The partial ORF1b, full-length ORF2, and 3′ UTR sequence was submitted to GenBank under accession no. KF417713.

We hypothesized that the MLB1-like astrovirus was the causative agent of the acute gastroenteric disease observed in the child, because this virus was the only infectious agent identified in samples from this patient. The virus was also present in fecal samples at high titers, detectable by using EM, without using immune-precipitating sera. However, in a case–control study for MLB1-like astroviruses in India, no clear association was established between MLB1-like viruses and diarrhea, and no differences were observed in the viral load between symptomatic and asymptomatic subjects (*9*). We cannot rule out the presence of other, yet unrecognized, viruses in the sample, because we did not perform massive sequencing.

To assess whether the reported case was sporadic or if other infections by MLB1-like astroviruses occurred, we screened fecal samples of 3 other patients hospitalized with gastroenteritis at University Hospital of Parma during 2007–2010 that were negative for common bacterial and viral enteric pathogens and that contained SRVs upon EM observation. In this screening, an additional MLB1-like astrovirus strain (ITA/2008/PR3147) was identified (Figure, panel B). The patient was a child, 14 months of age, who was hospitalized for 4 days with severe gastroenteritis during November 2008. Sequence analysis of the small ORF2 fragment targeted by the MLB1-specific primers (*2*) revealed that the MLB1-like astrovirus was highly similar (99.7% nt identity) to strain ITA/2007/PR326.

These unusual astroviruses have been identified in fecal samples of persons in Australia and in countries of Asia, Africa, and North America (*2–6*,*10*). In this study, MLB1-like astroviruses were identified in children with severe gastroenteritis, thus extending information on geographic diffusion of these viruses to the continent of Europe. Yet, the epidemiologic information on these viruses is limited and scattered. Structured virologic and serologic studies and effective diagnostic tools are necessary to assess more properly the epidemiology and role of these viruses in humans.

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Indigenous Hepatitis E Virus Genotype 1 Infection, Uruguay

To the Editor: Hepatitis E virus (HEV) is the only virus in the genus *Hepevirus*, which is the only member of the family *Hepeviridae*. HEV is the causative agent of acute hepatitis E, a moderately severe enteric disease with death rates ≤4% in the general population and ≥30% in pregnant woman (*1*). The virus is transmitted primarily by the fecal-oral route associated with consumption of contaminated drinking water (*2*). HEV infection is of public health concern because it causes large epidemics and endemic waterborne outbreaks in Asia, Africa, and Latin America (*2*,*3*). During the past decade, an increasing number of locally acquired cases have been reported in industrialized and previously non–HEV-endemic countries, where evidence of zoonotic transmission has been discovered (*4*,*5*).

Human HEV sequences are classified into genotypes 1–4, which are subdivided into subtypes. Genotype 1 was isolated in Asian and African countries and from Cuba and Venezuela in Latin America. Genotype 2 was described in Mexico and Africa. These genotypes have been isolated from human samples and are mostly associated with large epidemics and outbreaks of HEV. Genotype 3 is distributed worldwide, and genotype 4 is found in Asia and central Europe. Genotypes 3 and 4 have been isolated from humans and animal reservoirs, mainly pigs and wild boars, and are commonly found in persons with sporadic acute hepatitis cases (*6*). Thus, zoonotic transmission of HEV also poses a danger to public health

Several countries in South America, including Argentina, Brazil, Bolivia, Venezuela, and Uruguay, have reported the detection and characterization of HEV strains. In these studies, 2 Venezuelan strains were classified within genotype 1; the remaining 70 published strains were indigenous isolates belonging to genotype 3 (*7*). However, little data regarding molecular epidemiology of HEV infection in South America exists. Here, we report the identification and molecular characterization of a genotype 1 strain detected in Uruguay, isolated from a person with locally acquired HEV infection.

The patient was a male, 26 years of age, and a member of the National Army Force, who had symptoms and paraclinical findings compatible with acute hepatitis: malaise, fever, choluria, jaundice, and elevated levels of bilirubin and liver enzymes. Syphilis, leptospirosis, hepatitis A–C, cytomegalovirus, Epstein-Barr virus, and HIV infection were ruled out by laboratory testing. No risk factor for HEV infection arose from the formal questionnaire, including travel out of Uruguay during the 40 days before the onset of the symptoms. Dietary habits of the patient were also investigated. Shellfish intake and water consumption from potentially contaminated supplies were not reported.

A blood specimen was tested for IgM and IgG against HEV by using Line immunoassay (recomLine HEV IgG/IgM, Mikrogen Diagnostics, Germany); results were positive. To confirm the presence of HEV, RNA was extracted from 140 mL serum and fecal suspension by using QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany), then retrotranscribed with Superscript II (Life Technologies, Grand island, NY, USA). HEV infection was confirmed by using nested PCR amplification of a conserved sequence within the HEV open reading frame (ORF) 2–3 overlapping region (*8*). For genotyping and phylogenetic analysis, a nested PCR was then performed for the ORF1 RNA dependent RNA polymerase (RdRp) region (*9*). PCR products obtained from serum and fecal samples were cloned into pJET Vector (Thermo Scientific); 3 positive clones for each sample were sequenced. Sequences from 6 clones of both specimens were identical. The isolate, He_Uy 16, was deposited into GenBank under accession no. KF680001.

Phylogenetic reconstruction of the ORF2–3 overlapping region showed that the HEV isolated in Uruguay clustered in genotype 1 with high bootstrap values (Figure, panel A). The same was observed from the phylogenetic reconstruction of the ORF1 RdRp region (Figure, panel B). Furthermore, these analyses revealed a very close relationship of this strain isolated in Uruguay to a strain isolated in India, Yam 67 (99.5% nucleotide identity). Additionally, within the ORF1, the strain shares a high percentage of nucleotide identity (99%–100%) with the genotype 1 strains isolated in Cuba (Cub 11 and 13) and Venezuela (VNZ792), the 2 countries in Latin America in which genotype 1 has been reported to be associated with autochthonous cases. This relationship between these strains from South America and the Yam 67 strain from India warrants further investigation. In Uruguay, we have recently reported the full molecular characterization of a set of HEV strains isolated from patients with 9 sporadic cases in a 2-year period; all strains were genotype 3 (*10*). Here, we describe the detection and phylogenetic analysis of a locally-acquired indigenous case of HEV infection associated with genotype 1 in Uruguay. Although the detection of genotype 1 in 1/10 cases might have occurred by chance, this result supports an endemic circulation of HEV in Uruguay. Data presented here, together with recent advancements in molecular epidemiology of HEV infection in South America, reveal an epidemiologic picture more complex than we initially assumed.

Acknowledgements

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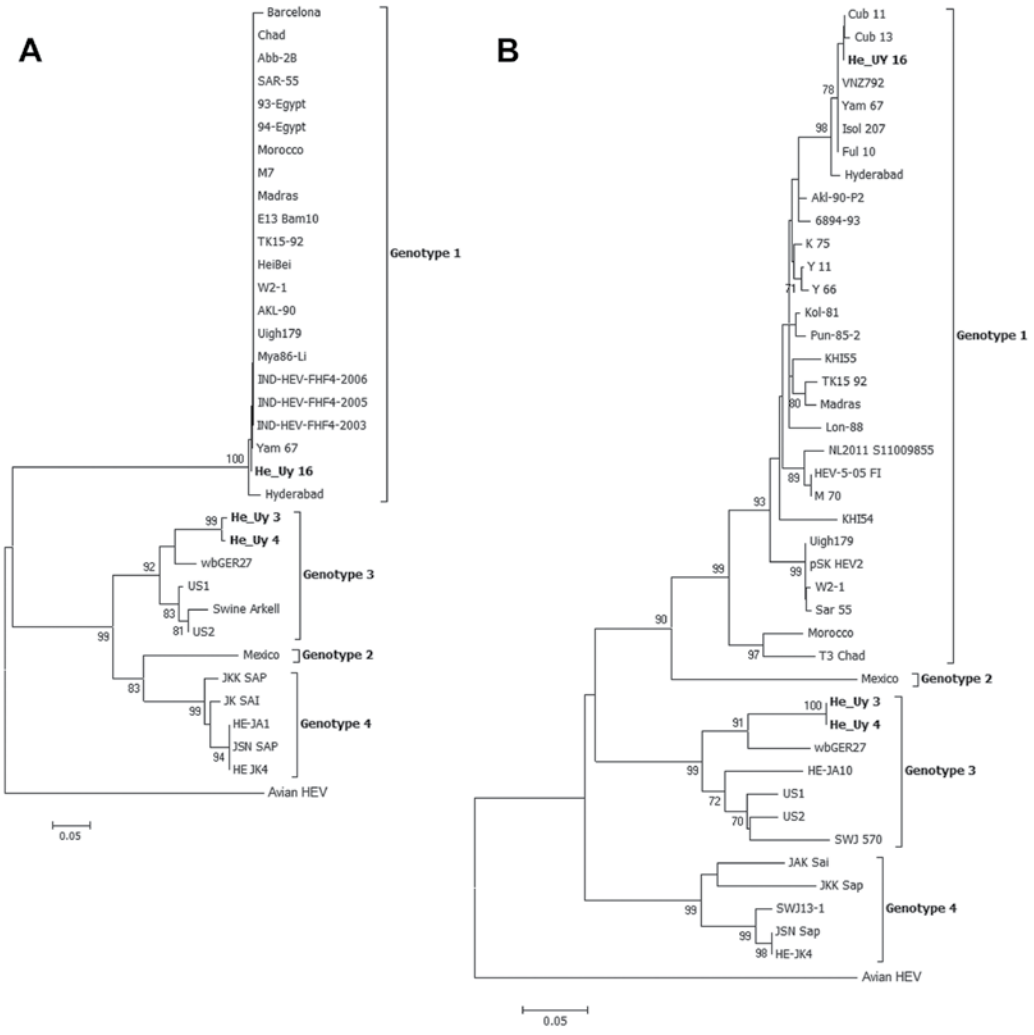

Figure. Phylogenetic tree of the Hepatitis E virus constructed on the basis of A) the partial 137-nt open reading frame (ORF) 2–3 overlapping region and B) the 244-nt sequence of the RNA dependent RNA polymerase within ORF1. Trees were generated by using the neighbor-joining method with the Kimura 2-parameter as the substitution model. The robustness of the trees was determined by bootstrap for 1,000 replicates. Values ≥70% are shown. Strains isolated in Uruguay are shown in **boldface**. The isolate of genotype 1 detected in this study (He_Uy 16) clustered (98% bootstrap value) with genotype 1 strains from India and Latin America (Cub 11 and 13 and VNZ792). Scale bars indicate nucleotide substitutions per site.

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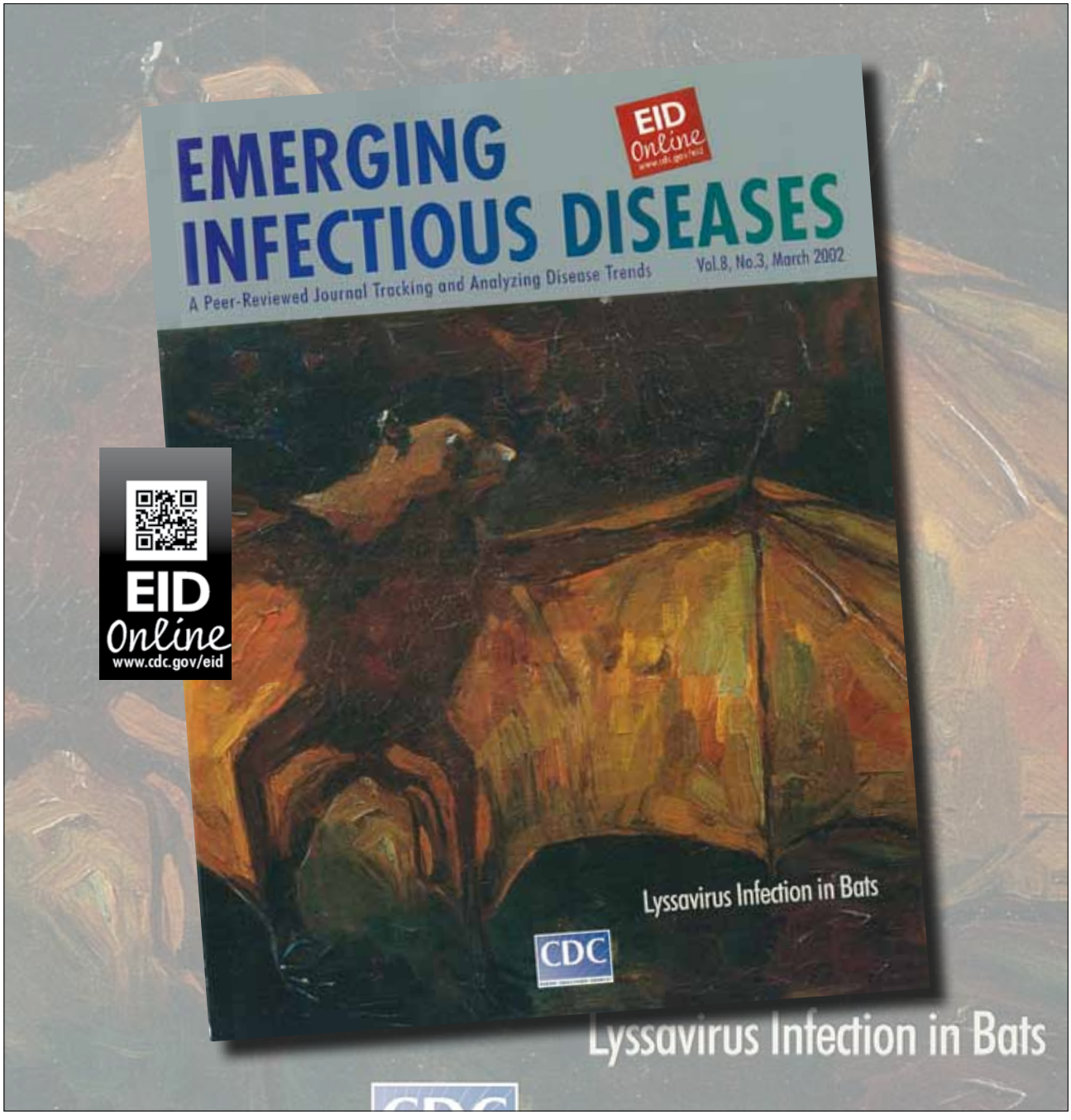

Prions: Current Progress in Advanced Research

Akikazu Sakudo, Takashi Onodera, editors

Caister Academic Press, Norfolk, UK, 2013

ISBN: 978-1-908230-24-9
Pages: 134; Price: US $240 (hardback)

This 134-page book provides a brief but wide-ranging overview of current research into prions, the infectious proteinaceous agents believed to be responsible for a range of fatal, neurodegenerative diseases including "mad cow" disease and Creutzfeldt-Jakob disease. Divided into 11 chapters, the book covers topics such as prion biology, disease pathogenesis, and strategies for vaccination and treatment. The history and current status of animal prion diseases, including bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD), and scrapie, are also described.

The book delivers on its title of "current progress," providing information on a range of recent developments, including diagnostic advances such as real-time quaking-induced conversion, and novel disease descriptions including variably protease sensitive prionopathy. The sections on atypical BSE are particularly enlightening and emphasize that, while the incidence of classical BSE may be decreasing, surveillance and research are still essential.

The format of the book leads to inevitable redundancies: the authors of different chapters provide similar background information. More distracting, however, is the occasionally imperfect English; although the reader can usually determine the author's intent, grammatical errors can make already abstruse subject matter even more difficult to comprehend. Additionally, the book's breadth, although generally a strength, results in an oversimplification of certain topic areas. A reader with limited knowledge of prion diseases, for example, could get the impression that CWD readily transmits to cattle and goats, because the text states that these animals are "CWD-susceptible" without clarifying that such transmissions have only been achieved through intracerebral inoculation.

Prions: Current Progress in Advanced Research is an excellent introduction to the many facets of these unusual agents and the challenges confronted by those studying them. I recommend it to students and researchers wishing to gain some insight into this unique field. However, supplementation with peer-reviewed prion literature is also warranted, as both a means to obtain additional background information and as a way to remain informed about rapidly developing scientific advances.

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Article Title

Raw Milk Consumption among Patients with Non–Outbreak-related Enteric Infections, Minnesota, USA, 2001–2010

CME Questions

1. You are seeing a pair of brothers, ages 4 and 10 years, for a 2-day history of fever and loose stools. The boys' mother explains that they had been drinking raw milk for the past week. What should you consider regarding foodborne illness associated with the consumption of raw milk?

A. Outbreaks of illness account for many more cases of foodborne illness associated with raw milk compared with sporadic cases
B. The risk for illness with raw dairy products is approximately 150 times that associated with pasteurized products
C. Public health records accurately describe the prevalence of sporadic foodborne illness
D. Less than 1% of cases in the current study reported raw milk exposure before their infection

2. What was the most common infection associated with raw milk consumption in the current study?

A. *Cryptosporidium* species
B. *Campylobacter* species
C. *Salmonella* species
D. *Escherichia* coli

3. As you evaluate these patients, what should you consider regarding the clinical presentation of patients with illness related to raw milk in the current study?

A. Infections affected both genders equally
B. One-quarter of affected individuals were younger than 5 years
C. Hispanic adults were particularly at risk for infection
D. Cases were spread equally throughout the year

4. What was the principal source of raw milk among individuals infected in the current study?

A. Urban farmers' markets
B. Unlicensed roadside farm stands
C. Their own dairy farm or a relative's dairy farm
D. Daycare or school

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue

February 2014

Poxvirus Viability and Signatures in Historical Relics

Genomic Variability of Monkeypox Virus among Humans, Democratic Republic of the Congo

Human Antibody Responses to Avian Influenza A(H7N9) Virus

7-valent Pneumococcal Conjugate Vaccine and Nasopharyngeal Microbiota in Healthy Children

Fungal Endophthalmitis Associated with Compounded Products

Monitoring Emergence of Babesiosis among Humans through Surveillance of Vectors, New England

Lymphocytic Choriomeningitis Virus Outbreak among Employees and Mice at a Multipremises Feeder Rodent Operation, 2012

Melioidosis Caused by *Burkholderia pseudomallei* in Drinking Water, Thailand, 2012

Genetic Characterization of Coronaviruses from Domestic Ferrets, Japan

Reemergence of Rift Valley Fever, Mauritania, 2010

Co-circulation of West Nile Virus Sequence Variants, Arizona, USA, 2010

Andes Hantavirus Variant in Rodents, Southern Amazon Basin, Peru

Crimean-Congo Hemorrhagic Fever Virus, Greece

Lethal Factor and Anti-Protective Antigen IgG Levels Associated with Inhalation Anthrax, Minnesota, USA

Complete list of articles in the February issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

January 27–29, 2014
ASM BIODEFENSE and Emerging Diseases Research Meeting
Washington Marriott Wardman Park
Washington, DC
http://www.asmbiodefense.org

April 2–5, 2014
16th International Congress on Infectious Diseases
Cape Town, South Africa
http://www.isid.org/icid/

April 9–11, 2014
9th Conference Louis Pasteur Emerging Infectious Diseases
Paris, France
http://www.clp2014.org/

May 17–20, 2014
114th General Meeting
American Society for Microbiology
Boston, Massachusetts
http://www.asm.org/asm2014/

June 24–27, 2014
EMBO conference on "Microbiology after the genomics revolution–Genomes 2014"
Institut Pasteur, Paris
http://www.genomes-2014.org

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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).

Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

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Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

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

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.