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April 2015

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EMERGING INFECTIOUS DISEASES

April 2015

On the Cover

Pam Longobardi (1958–)

"Ghosts of Consumption/Archaeology of Culture (for Piet M.)"

2011 Found ocean plastic, steel pins, silicone.

110 x 75 x 5 in. / 279 x 191 x 13 cm.

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Perspective

Synopses

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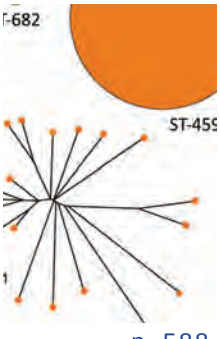

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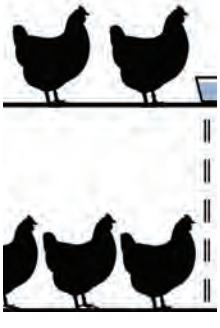

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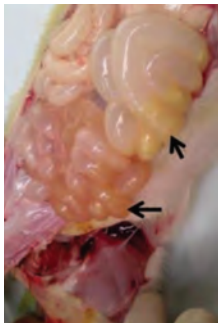

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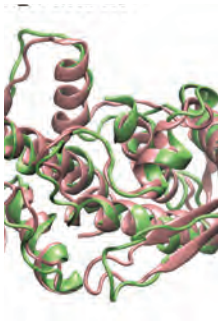

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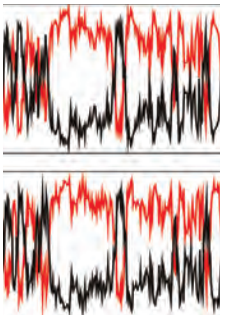

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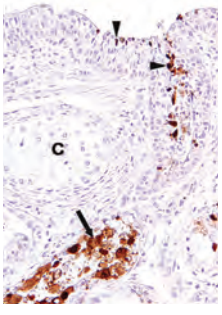

Book Review

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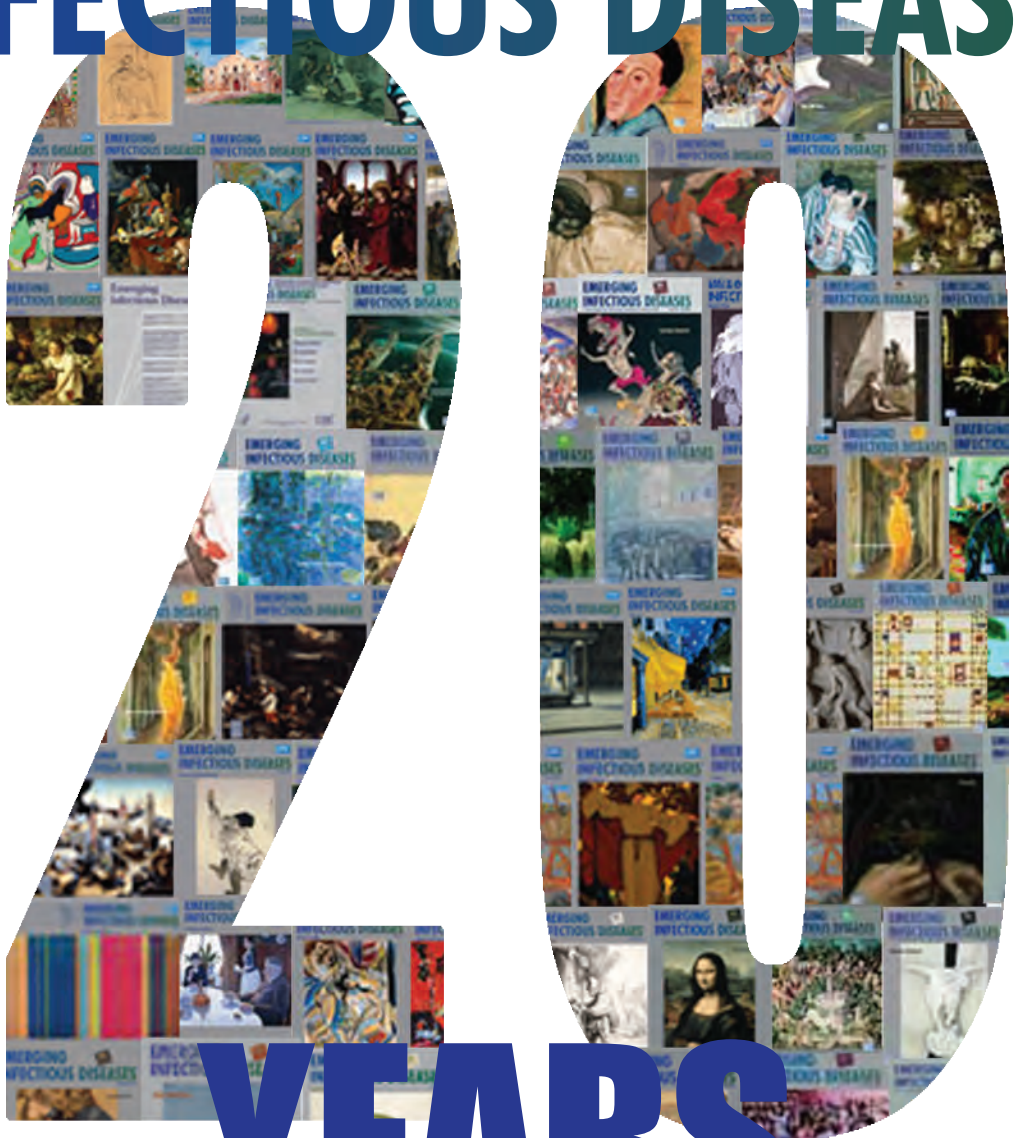

20 YEARS

Presenting the ongoing challenges that emerging microbial threats pose to global health

Reappearance of Chikungunya, Formerly Called Dengue, in the Americas

Scott B. Halstead

After an absence of ≈200 years, chikungunya returned to the American tropics in 2013. The virus is maintained in a complex African zoonotic cycle but escapes into an urban cycle at 40- to 50-year intervals, causing global pandemics. In 1823, classical chikungunya, a viral exanthem in humans, occurred on Zanzibar, and in 1827, it arrived in the Caribbean and spread to North and South America. In Zanzibar, the disease was known as kidenga pepo, Swahili for a sudden cramp-like seizure caused by an evil spirit; in Cuba, it was known as dengue, a Spanish homonym of denga. During the eighteenth century, dengue (present-day chikungunya) was distinguished from breakbone fever (present-day dengue), another febrile exanthem. In the twentieth century, experiments resulted in the recovery and naming of present-day dengue viruses. In 1952, chikungunya virus was recovered during an outbreak in Tanzania, but by then, the virus had lost its original name to present-day dengue viruses.

Chikungunya has returned to the Americas after an absence of ≈200 years. The return of this viral exanthem was first recognized on St. Martin, in the Caribbean, in December 2013, and as of January 9, 2015, the US Centers for Disease Control and Prevention reported that the disease had been identified in 42 countries or territories in the Caribbean, Central America, South America, and North America. A total of 1,094,661 suspected and 26,606 laboratory-confirmed chikungunya cases have been reported (http://www.cdc.gov/chikungunya/geo/americas.html). The return of chikungunya virus to the Americas provides an opportunity to revisit the epidemiology of this zoonotic togavirus from Africa and to contrast it with that of dengue viruses, flaviviruses that are maintained as zoonoses in Southeast Asia. All of these viruses can be transmitted by *Aedes aegypti* and *Ae. albopictus* mosquitoes in an urban cycle. In the course of history, a remarkable name change has taken place because of the similarities between the clinical syndromes caused by dengue and chikungunya virus infections. The story of how the term dengue was originally applied to what we now call chikungunya and then subsequently applied to what we now call dengue should be well known by persons who deal with these 2 similar, but crucially different, global diseases. For more details on the name switch, the reader should consult the historical account by Carey (*1*).

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The Chikungunya Epidemic of 1827–1828

According to a contemporary medical observer of the chikungunya epidemic of 1827–1828, S. Henry Dickson, Professor of Medicine, Medical College of South Carolina,

> "[A]n arthritic fever with cutaneous exanthema [(*2*)]… appeared first in the island of St. Thomas, the chief town of which it invaded in September, 1827, attacking in rapid succession almost every individual in a population of about 12,000. Towards the end of October, it passed over to the neighbouring island of St. Croix. We hear of it, in November, in St. Bartholomew's, and in Antigua in January, 1828. It prevailed at Havanna [sic] in the succeeding April, at New Orleans in May and June; and in July and August affected very generally the inhabitants of Charleston, South Carolina, and reached Savannah (Georgia) in September and October." (*3*)

On clinical evidence, this outbreak was caused by chikungunya virus. However, that clinical evidence is supplemented by the eyewitness report and the epidemiologic detective work of James Christie, physician to His Excellency Syud Bargash, Sultan of Zanzibar, 1865–1873. In his report, published in the British Medical Journal in 1872, Christie described the onset in July 1870 on Zanzibar of an acute febrile exanthem that rapidly achieved epidemic proportions (*4*). He himself was sick and in early convalescence experienced "pain on rising from my chair [that] was very severe after a short interval of rest....I suffered severely [from joint pain] for more than two months afterward" (*4*). From older patients in his practice, Christie learned that there had been a similar epidemic on Zanzibar 48 years earlier that was known by the Swahili term kidinga pepo (also called kidenga or kidyenga pepo). In this phrase, "ki… simply means 'a kind of,'" the word

"dinga or dyenga… means sudden cramp-like seizure," and "pepo, means wind and also a spirit… so that the full designation of the term signifies a disease characterized by a sudden cramp-like seizure, caused by an evil spirit" (*5*).

Origin of the Term Dengue

Christie explicitly linked the 1827–1828 epidemic of kidinga pepo in the Americas to the 1823 epidemic on Zanzibar. He noted that published reports indicated that the 1823 epidemic soon spread from Zanzibar to Gujarat, India, and then to Calcutta, India, and by 1824 it had spread to Rangoon in present-day Myanmar. In 1827, there were reports of a similar disease in St. Thomas in the West Indies. Christie stated, "I am of the opinion that both the disease and its designation were imported in the West Indian Islands direct from the East Coast of Africa" (*5*). It was in the West Indies, as Christie observed, that the medical term dengue was introduced. Dumaresq, an observer of the dengue epidemic in New Orleans, Louisiana, USA, in 1828 commented, "The disease alluded to is supposed to have been brought from Africa, with some slaves imported into the Havana. In that place it obtained the name of Dingee, Dengue, Danga, etc. It was there very prevalent, and also in Barbadoes [sic], where it received the name of Dandy fever, from the stiffened form and dread of motion in patients" (*6*). In New Orleans, the disease "spread was so rapid among the inhabitants that in eight or ten days at least one third of the population was laboring under its influence, including persons of all ages and different sexes" (*6*). Dumaresq goes on to say,

> "A person on the disappearance of this fever would attempt to rise from bed, feeling not much loss of strength, and a consciousness of being able to move about and attend to a little to business; but how egregiously would he be mistaken when he assumed the upright posture! The joints felt as if fettered or anchylosed, and the advance of one foot or leg beyond the other, would cost more pain and effort than the purpose for which it may have been advanced was worth, —aye,—a thousand times told!" (*6*)

The arthritic component of this febrile exanthem is unique to epidemic human chikungunya infections. It has been variously called scarlatina rheumatica, exanthesis arthrosia, and an eruptive articular or rheumatic fever (*7*).

An interesting further insight into the colloquial Spanish meaning of dengue may contribute to an understanding why this term prevailed so quickly. In 1952, when Sabin inquired into the etymology of the term dengue, the standard Spanish dictionary meaning was affectation (*8*). Dengue researchers at that time were unable to make a connection between this term and characteristic signs and symptoms of dengue. However, an interesting connection does exist, but it is to the disease caused by what we now call chikungunya, not dengue. In 1828, contemporary observers were struck by the post-illness arthralgia and disability caused by dengue (i.e., present-day chikungunya), including the post-illness symptoms cited above in comments by Dumaresq (*6*). Stedman noted an even more extreme manifestation of dengue, reporting, "It is even said that when the disease first appeared in St. Thomas, several negroes, who, being all at once attacked with pain in the knees, had fallen down, were actually apprehended by the police for drunkenness" (*9*). Lehman, the lazaretto (i.e., quarantine station) physician for the port of Philadelphia, Pennsylvania, USA, interviewed a ship captain from Cuba who declared that, "It [dengue] is a vulgar phrase, and implies a 'staggering weakness,' and is somewhat similar in its import to our term of 'corned' [drunk] as applied to a man reeling about from intoxication" (*10*). The original meaning of kidinga pepo has been consistently maintained from Swahili to the colloquial eighteenth century Spanish term dengue as an apt name for a disease that produces a post-illness stagger.

Discovery of Chikungunya Pandemics

When Dr. Christie left Africa in 1876 to assume a post as a lecturer on public health at Anderson's College, Glasgow, he discovered reports in the medical literature of 3 pandemics of kidinga pepo. The epidemic of 1870–1880 had begun in Zanzibar and then spread to India and Southeast Asia. That epidemic had been preceded by one in 1823–1828 that originated in Africa and then spread to India, Southeast Asia, and the Americas, and that epidemic had been preceded by an even earlier one in 1779–1785 that was reported in Egypt, Africa, Arabia, India, and Southeast Asia (*5*). Of interest to contemporary observers, in 1872, an epidemic of kidinga pepo affected most inhabitants of low-lying areas of Réunion Island, the site where the chikungunya pandemic of 2005–2006 was first recognized (*11*). Dr. Christie suspected that the illness in all 3 pandemics was kidinga pepo because he had personally observed the 1870 epidemic spread from Zanzibar to the entire Indian subcontinent and progress on to Southeast Asia. Then, from a published report, he learned of an epidemic of kidinga pepo in Cairo in 1779. This report was followed by others that reported outbreaks in Arabia, India, and Southeast Asia. This epidemic reached Indonesia in 1779, where another astute physician, David Bylon, municipal surgeon for the city of Batavia (now Jakarta, Indonesia), acquired the disease. Dr. Bylon described the epidemic in a classic account, which has been widely cited as the initial clinical description of dengue fever:

> "It was last May 25, in the afternoon at 5:00 when I noted while talking with two good friends of mine, a growing pain in my right hand, and the joints of the lower arm, which step by step proceeded upward to the shoulder and then continued onto all my limbs; so much so that at 9:00 that same evening I was already in my bed with a high fever.… It has now been three weeks since I… was stricken by the illness, and because of that had to stay home for 5 days; but even until today I have continuously pain and stiffness in the joints of both feet, with swelling of both ankles; so much so, that when I get up in the morning, or have sat up for a while and start to move again, I cannot do so very well and going up and down stairs is very painful." (*12*, as translated by K. DeHeer)

Carey, who rediscovered Christie's work, noted that chikungunya pandemics originating in eastern Africa had crossed the Indian Ocean at roughly 40- to 50-year intervals: the 1770s, 1824, 1871, 1902, 1923, and 1963–1964 (*1*). To those cycles we now can add 2005–2014. The last 2 trans–Indian Ocean pandemics occurred in the modern virologic era and have been documented by the isolation of virus. In 1963–1964, a chikungunya epidemic swept down the eastern coast of India from Calcutta to Sri Lanka (*13*–*15*). It was this epidemic that resulted in the recognition of the pronounced clinical differences between syndromes caused by dengue viruses and chikungunya virus. During the 1964 epidemic in Vellore, in southern India, most of the 275 patients with virologically or serologically confirmed chikungunya were adults (*16*). The patients had "sudden onset… of fever, headache and severe pains in the joints, these last being the dominant complaint. The pains mainly affected the small joints of the hands, wrist and feet, but frequently occurred in the knees as well" (*16*). After 1964, chikungunya virus gradually disappeared from India, with the last isolates recorded in 1972 (*17*,*18*). During 2005–2006, a chikungunya epidemic that originated in eastern Africa was observed on Réunion Island and then in Mauritius, Madagascar, Mayotte, and Seychelles (*19*); the epidemic soon spread to India and Southeast Asia (*20*,*21*). The Réunion Island outbreak was noteworthy because *Ae. albopictus* mosquitoes were efficient vectors that were aided by a genetic mutation in the virus (*22*). This virus was subsequently introduced into Europe by tourists returning from visits to Réunion Island or India, resulting in modest outbreaks of autochthonous *Ae. albopictus* mosquitoborne chikungunya in southeastern France and northeastern Italy (*23*,*24*).

History of Disease Caused by Dengue Viruses

The first clinical description of a syndrome likely to have been caused by a dengue virus was one by Benjamin Rush, who in 1789 described an epidemic of a disease he called bilious remitting fever (*25*). The epidemic occurred from mid-August through September 1780 in Philadelphia, principally among residents living along the Delaware River waterfront. According to Rush (*25*):

> "The fever generally came on with rigor… In some persons it was introduced by a slight sore throat…. The pains which accompanied this fever were exquisitely severe in the head, back and limbs. The pains in the head were sometimes in the back parts of it and sometime occupied only the eyeballs…. A few complained of their flesh being sore to touch… the disease was sometimes believed to be a rheumatism. But, its more general name among all classes of people was *breakbone fever*…. A nausea universally, and in some instances, vomiting, accompanied by a disagreeable taste in the mouth, accompanied this fever…. A rash often appeared on the third and fourth days."

Rush's description of bilious remitting fever was well known to physicians who attended to patients during the 1828 outbreak in the Caribbean. However, at the same time, George Stedman, a former president of the Royal Medical Society of Scotland, who practiced medicine on St. Croix, felt that the 1828 dengue was quite different from bilious remitting fever. He observed, "I think that it will be evident to everyone who pays the least attention to the symptoms, that the diseases, though somewhat alike in a few symptoms, are essentially different" (*9*). The principal distinctions made by Stedman were in the suddenness of the onset and the nature and duration of the after-pains of dengue (present-day chikungunya) (*9*). Christie also recognized the existence of 2 distinct febrile exanthems, 1 with and 1 without post-illness arthritis. He cited a description of dengue "with an almost entire absence of the articular pains"; this description of illness during an 1853–1854 epidemic in Calcutta was from The Science and Practice of Medicine (*26*), an authoritative text authored by William Aitken (*4*,*27*).

History of Chikungunya Name Change

Why then did chikungunya lose and dengue gain a name? Throughout the nineteenth century, astute observers of outbreaks in the Americas and India recognized the clinical differences between dengue and breakbone fever, principally the duration of fever and the occurrence of post-illness arthritis (*27*–*29*). The term dengue was in use to describe an epidemic that reached India in 1871 from Zanzibar and eastern Africa (*30*–*32*). However, once Reed and coworkers identified *Ae. aegypti* mosquitoes as the vector of

yellow fever, the epidemiologic similarities between dengue and yellow fever led researchers in Lebanon, Australia, and the Philippines to investigate the etiology of dengue and the mode of transmission of dengue virus (*33*–*36*). At that time, by coincidence, dengue but not chikungunya viruses were endemic at these 3 sites. Two groups, one in Australia and the other in the Philippines, apparently successfully transmitted virus from sick humans to healthy volunteers through the bite of infected *Ae. aegypti* mosquitoes (*35*) and *Culex fatigans* (now called *C. quinquefasciatus*) mosquitoes (*36*). Ashburn and Craig successfully infected human volunteers by inoculating them with diatomaceous earth–filtered blood from patients with dengue, thereby proving a viral etiology for the disease (*36*). It remained for Siler and Simmons and co-workers in the Philippines in 1923 and 1929 to definitively demonstrate that *Ae. aegypti* mosquitoes, but not *C. quinquefasciatus* mosquitoes, are a biological vector of dengue virus (*37*–39). During the first half of the twentieth century, many experimental infections with dengue viruses were studied in human volunteers, and the clinical features of the infections were recorded in detail; all authors referred to the disease under study as dengue. In 1952, decades after these experiments were begun, a virus was recovered from an outbreak of an exanthematous febrile disease in Southern Province, Tanganyika Territory (now in Tanzania). The virus was called chikungunya, which in the Makonde language (spoken by an ethnic group in southeast Tanzania and northern Mozambique) means that which bends up (*40*). The name change was complete.

Acknowledgment

I thank Koon DeHeer for his translation of David Bylon's paper (*12*) from original archaic Dutch.

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SYNOPSIS

Hantavirus Pulmonary Syndrome, Southern Chile, 1995–2012

Raúl Riquelme, María Luisa Rioseco, Lorena Bastidas, Daniela Trincado, Mauricio Riquelme, Hugo Loyola, Francisca Valdivieso

Hantavirus is endemic to the Region de Los Lagos in southern Chile; its incidence is 8.5 times higher in the communes of the Andean area than in the rest of the region. We analyzed the epidemiologic aspects of the 103 cases diagnosed by serology and the clinical aspects of 80 hospitalized patients during 1995–2012. Cases in this region clearly predominated during winter, whereas in the rest of the country, they occur mostly during summer. Mild, moderate, and severe disease was observed, and the case-fatality rate was 32%. Shock caused death in 75% of those cases; high respiratory frequency and elevated creatinine plasma level were independent factors associated with death. Early clinical suspicion, especially in rural areas, should prompt urgent transfer to a hospital with an intensive care unit and might help decrease the high case-fatality rate.

Since the first cases described in United States in 1993, hantavirus pulmonary syndrome (HPS) has been reported in the United States, Argentina, Bolivia, Brazil, Chile, Ecuador, Paraguay, Panama, Uruguay, and Venezuela (*1*). Several types of New World hantaviruses (family *Bunyaviridae*) have been recognized. Their distribution is determined by the density of rodent populations serving as specific reservoirs of each virus type.

In Chile, Andes virus is the only identified hantavirus (*2*). It was first reported in 1995 during an outbreak in Argentina and is carried by the murid rodent *Oligoryzomyslongicaudatus* (i.e., long-tailed mouse or "colilargo") in southern Argentina and central and southern Chile (*3*).

In Chile, where HPS is subject to immediate mandatory reporting to health authorities, a total of 786 cases occurred during 1995–2012. Regional and seasonal incidences varied from 0.17 to 0.53 cases per 100,000 inhabitants (*4*). Despite such low incidence, HPS is of public health concern because of its severity and its high case-fatality rate (CFR) (20%–60%).

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We examined the clinical and epidemiologic features of HPS during 17 years in the provinces of Llanquihue and Palena, which had the highest incidences of this disease in Chile. This geographic area is served by the Health Service of Reloncaví (HSR) in Puerto Montt city, which has its 420-bed reference center at the Hospital of Puerto Montt in Puerto Montt.

Material and Methods

Study Site and Population

The provinces of Llanquihue and Palena are located in southern Chile, on the western edge of South America. Together they comprise 30,178 km^2 and 340,464 inhabitants. These 2 provinces are subdivided into 13 communes (Figure 1).

Our study comprised all HPS cases reported to HSR during 1995–2012. All were confirmed by serologic tests performed at the National Reference Centers at the Public Health Institute (Santiago) or Universidad Austral (Valdivia). These tests are ELISAs for IgM and IgG that use hantavirus Sin Nombre antigen provided by the US Centers for Disease Control and Prevention (Atlanta, GA, USA).

Data Collection

We obtained data from 3 sources. First, we used epidemiologic records from all cases reported during 1995–2012. Data included patient age, sex, occupation, residence, site of probable infection, contact with other HPS patients, dates of hospitalization, and outcome.

Second, we reviewed clinical records of all patients admitted to Hospital of Puerto Monttwith confirmed HPS during the same period. Data recorded were age, sex, probable mechanism of infection, incubation period (only for patients for whom precise information about the time of rodent exposure and onset of symptoms was available), and medical history. On admission, presence of dyspnea, fever, asthenia, headache, myalgias, chills, cough, abdominal pain, and cyanosis and blood pressure, pulse, temperature, and respiratory frequency were recorded. During hospital stay, the following data were collected: presence of bleeding, alterations in renal and hepatic functions, admissions to intensive care unit (ICU), oxygen support,

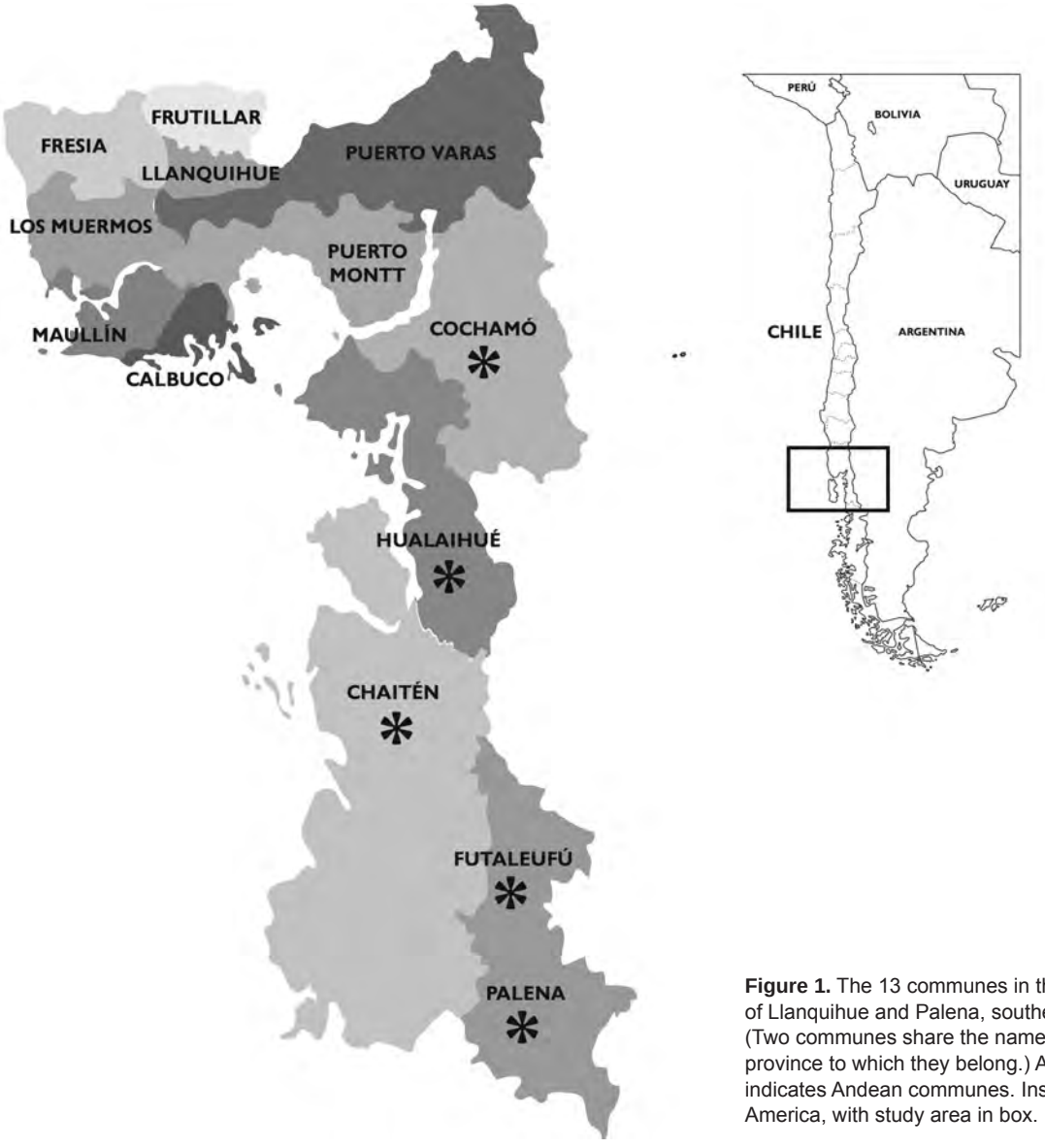

Figure 1. The 13 communes in the provinces of Llanquihue and Palena, southern Chile. (Two communes share the name of the province to which they belong.) Asterisk indicates Andean communes. Inset: South America, with study area in box.

arterial oxygen tension/inspiratory oxygen fraction (PAFI) index, steroid administration, mechanical ventilation (MV) (specifying timing of connection), and circulatory shock. Shock was defined as systolic blood pressure <90 mm Hg that did not improve with fluid administration or that required the use of vasoactive drugs and abnormalities in tissue perfusion manifested by alteration of consciousness, oliguria, and lactate acidosis (*5*). One of the authors (R.R.) analyzed chest radiographs and classified the infiltrates as alveolar, interstitial, or mixed pattern and unilateral or bilateral; number of compromised quadrants and presence of pleural effusion were recorded. Results of laboratory tests performed on admission and during illness were also recorded. Each case was classified into 1 of 3 groups: grade I (mild disease) when patients had only prodromal symptoms without pulmonary involvement; grade II (moderate disease) when patients had interstitial pulmonary infiltrates or required supplemental oxygen but were hemodynamically stable; and grade III (severe disease) when patients required MV or had hemodynamic instability (*6*). Final outcome (death or survival) was also recorded.

Finally, we reviewed reports of epidemiologic inspections to homes, workplaces, and probable sites of infection (dwellings and their surroundings) at the time of case report to HSR. A survey administered to each patient or to close relatives asked about HPS risk during the 6 weeks before symptom onset. Visited places were classified as urban, rural, or semirural. We stratified the infection risk in visited dwellings according to a 5-parameter scale, each

with 1 point assigned to absence of foundations, presence of holes, poor ventilation and lighting, presence of trash without adequate container inside the dwelling, and grain-storage, flour, and other food packaging Risk was considered high for scores 4–5, moderate for 2–3, low for 1, and absent for 0. We similarly classified dwelling surroundings according to presence of droppings, rodent pathways, rubbing stains, gnawing signs, rodent nests or holes, or observation of rodents themselves.

Statistical Analyses

We used the Student *t* test to compare parametric variables and χ^2 and Fisher exact tests to compare discrete variables when necessary. A p value <0.05 was considered statistically significant. Incidence rate of HPS per commune was calculated from information provided by Chilean Census 2002 (*7*).

Results

During 1995–2012, a total of 103 confirmed HPS cases were reported to HSR. Mean age of patients was 35 ± 17 years (range 3–80 years); 71 (69%) were men. Overall CFR was 32% (33/103); CFR for the 80 HPS patients admitted to Hospital of Puerto Montt was 30% (24/80).

Epidemiologic Characterization

We identified 52 rural locations as probable infection sites for 100 patients. For the remaining 3 patients, infection site could not be determined because of exposure to several risky sites.

Infection most likely was acquired through farming and forestry work for 44% of patients and was associated with recreational activities for 13%. For the remaining patients, infection-associated activity was not determined because of similar risk at home and at work.

HPS incidence per 100,000 inhabitants varied widely among communes. The highest rates occurred within Andean mountainous areas, mainly Palena and Cochamó communes (350 and 364 cases per 100,000 inhabitants, respectively). Incidence for the aforementioned communes was 8.5 times higher than that for the rest of the region (Table 1; Figure 1).

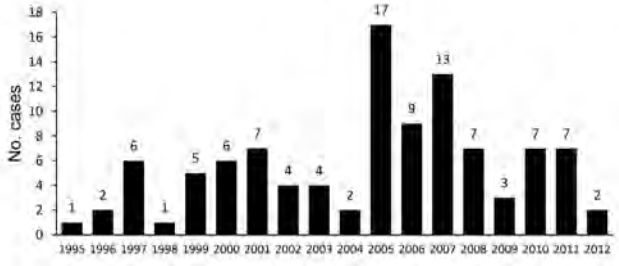

Figure 2. Number of hantavirus pulmonary syndrome cases in provinces of Llanquihue and Palena, southern Chile, 1995–2012.

Table 1. Incidence of hantavirus pulmonary syndrome, Health Service of Reloncaví, Chile, 1995–2012

Commune	No. patients, n = 103	Population*	Incidence rate†
Cochamo‡	16	4,399	363
Palena‡§	6	1,715	350
Chaiten‡	12	7,290	164
Fresia	10	12,861	77
Los Muermos	13	17,004	76
Futaleufu‡	1	1,849	54
Maullin	4	15,205	26
Calbuco	8	32,792	24
Hualaihue‡	2	8,464	24
Frutillar	3	16,504	18
Puerto Varas	6	35,590	16
Puerto Montt	19	196,561	10
Llanquihue§	0	17,228	0
Unknown	3	Not applicable	Not applicable
Andean area	37	23,717	156
Not Andean area	63	343,165	18

*Census 2002 (http://www.ine.cl/cd2002/).
†Per 100,000 inhabitants.
‡Communes of Andean area.
§Palena and Llanquihue communes have the same name as the province to which they belong.

Yearly incidence varied widely during 1995–2012. Most cases occurred during 2005–2007 (Figure 2). In the region studied, incidence was highest during winter. By contrast, in the rest of the country, incidence was highest during summer and autumn (Figure 3).

For 23 patients, HPS occurred in related persons and made up a total of 8 clusters, 5 with 3 cases each and 4 with 2 cases each. All patients in each cluster shared both environmental risk factors and family relationship; included in the clusters were 6 cohabitating couples.

Epidemiologic reports on home or workplaces were available for 42 patients. Thirty-one percent of houses, 43% of housing environments, and 39% of working environments had high or moderate risk for rodent infestation.

Clinical Characteristics

The 80 HPS patients admitted toHospital of Puerto Montt during 1995–2012 represented 78% of cases reported to HRS during that period. Seventy percent were men. Patients were 35.7 ± 16 years of age (range 4–73 years), and 90% were rural inhabitants. Their main occupational activities were farming or forestry (35%), housework (25%), student (15%), and fishery or marine harvesting (11%). Of these patients, 25 (31%) had a history of contact with rodents or rodent droppings. Infection was attributed to occupational exposure for 35 (44%) patients and traveling to a high incidence zone for 10 (12%).

Mean interval from appearance of symptoms to hospitalization was 5.7 ± 3 days (range 2–17 days). Incubation period, estimated from the analysis of 20 patients, was 10 ± 7 days (range 2–28 days).

For patients admitted during 1995–2004, HPS was considered 1of the admission diagnoses for only 7 (27%)

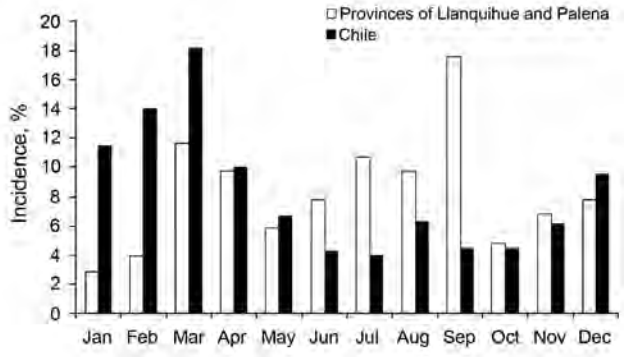

Figure 3. Seasonal incidence of hantavirus pulmonary syndrome, provinces of Llanquihue and Palena, Chile (n = 103), and entire country (n = 785), 1995–2012.

of 26 case-patients. This presumptive diagnosis increased to 76% (37/49) during 2005–2012 (Tables 2, 3).

Duration of hospitalization for HPS patients was 5.2 ± 4.6 days (range 1–25 days). Sixty-three (79%) case-patients were admitted to the ICU; 72 (90%) required oxygen administration; and 40 (50%) were connected to MV for 4.2 ± 4.7 days (range 1–17 days).

Mean PAFI at admission was 216 ± 107 (range 40–508). Five (6%) patients had no pulmonary involvement. Shock occurred in 37 (46%) patients, all of whom received vasoactive drugs as prescribed. Hemorrhagic manifestations occurred in 31 (39%) patients: hematuria in 15 patients; cutaneous or puncture sites bleeding in 12 patients, hemoptysis in 5 patients, metrorrhagia in 4 patients; and epistaxis gingivorrhagia, rectorrhagia, and epidural hematoma after lumbar puncture in 1 patient each.

For 95% of patients, platelet counts were $<100 \times 10^3/\mu L$(reference range 140–44010^3/μL) at a given time; for 34%, platelet counts were $<35 \times 10^3/\mu L$. The mean platelet count was 50 ± 39 (range 8–238) $\times 10^3/\mu L$. In 48% of hospitalized patients, creatinine increased >1.2 mg/dL (reference range 0.5–0.9 mg/dL); 4 (5%) of these patients required hemodialysis. Hepatic enzymes were elevated in 57 (71%) patients.

Thirty-one patients received steroids. Methylprednisolone was administered to 20 patients in accordance with a published protocol (*8*): 1 g intravenously per day for 3 days, followed by 16 mg orally per day for 3 days, 8 mg per day for 3 days, and 4 mg per day for 3 days. Eleven other patients were enrolled in a clinical trial and received methylprednisolone 1 g intravenously per day for 3 days (*9*). Nevertheless, we observed no difference in CFR between patients who did and did not receive methylprednisolone.

According to their clinical course, 5 (6%) patients were classified as having grade I HPS; 34 (42%) as having grade II HPS, and 41 (51%) as having grade III HPS. Twenty-four (30%) hospitalized patients died; for 21 (88%) of these, death was attributed directly to HPS. Shock was considered the cause of death for 18 (75%) patients, respiratory failure for 2 (8%), multiorgan failure for 2 (8%), and secondary sepsis for 2 (8%) (1 gram-negative sepsis and 1 *Staphylococcus aureus* sepsis).

Table 2. Clinical characteristics on admission to Hospital Puerto Montt for 80 patients with confirmed hantavirus disease, Puerto Montt, Chile, 1995–2012

Symptoms/signs	No. (%) patients
Main symptoms	
Fever	73 (91)
Myalgia	57 (71)
Headache	39 (49)
Respiratory distress	36 (45)
Abdominal pain	35 (44)
Cough	32 (40)
Malaise	31 (39)
Vomiting	19 (24)
Diarrhea	17 (21)
Anorexia	17 (21)
Chills	8 (10)
Rare symptoms	
Generalized rash	1 (0.1)
Low back pain	4 (0.5)
Bloody sputum	2 (0.3)
Confusion	2 (0.3)
Sore throat	1 (0.1)
Urinary	1 (0.1)
Signs on chest radiograph	
Infiltrates	75 (94)
Interstitial pattern	53 (66)
Alveolar pattern	12 (15)
Mixed pattern	10 (13)
Bilateral	71 (95)
Infiltrates in 4 quadrants	39 (49)
Opacity progress by ≥50% within 48 h	24 (30)

Fifteen (63%) of 24 patients died during the first 24 hours after admission, and 22 (92%) died during the first 72 hours after admission. All deaths occurred among patients with grade III disease. Independent factors associated with death were respiratory frequency ≥30 breaths/minute and creatinine ≥1.3 mg/dL on admission (Table 4). CFR did not differ by sex, abnormal hepatic test results, or chest radiographic images progression >50% in 48 hours. Furthermore, we found no relation to CFR for patients with hematocrits >45% or >50%; platelet counts <100, <50 or $<35 \times 10^3/\mu L$ at admission; or PAFI <250, <200, <150, or <120 at admission.

Discussion

HPS is endemic in southern Chile, and human–rodent contact is considered the main mechanism for transmission. Several factors can explain the occurrence of human HPS in each of the 17 years of this study; these include a favorable habitat for rodent populations, which enables circulation of the virus between them (*10*), and high percentages of rural population (Llanquihue 27.5% and Palena 60%) for whom agriculture and forestry as the main occupational activities. Consequently, humans invade the rodents' natural habitat, the temperate rain forest and its residues.

Table 3. Vital signs and laboratory results for 80 hantavirus pulmonary syndrome patients admitted to Hospital Puerto Montt, Puerto Montt, Chile, 1995–2012

Parameter	Median ± SD (range)
Laboratory value*	
Hematocrit, %, n = 74	47 ± 7 (32–72)
Platelets × 10^3/μL, n = 75	67,505 ± 42,717 (14,000–238,000)
Leukocytes × 10^3 cells/μL, n = 74	13,520 ± 9,971 (2,800–59,400)
Creatinine, mg/dL, n = 70	1.41 ± 0.97 (0.6–6.6)
Bilirubin, mg/dL, n = 64	0.62 ± 0.70 (0.03–5.9)
Aspartate amiontransferase, U/L, n = 66	245.2 ± 178.2 (26–705)
Alanine aminotransferase, U/L, n = 44	172.2 ± 134.7 (11–536)
Prothrombin, %, n = 56	81.1 ± 22.4 (13–100)
Vital signs	
Temperature, °C, n = 75	37.7 ± 1.1 (35–40.5)
Pulse, beats/min, n = 76	109,13 ± 22(61–158)
Respirations, breaths/min, n = 63	30.6 ± 9.5 (16–60)
Systolic blood pressure, ≤90 mm Hg, n = 79	22.5 %

*Reference values are as follows: hematocrit 37%–47%, platelets 140–440× 10^3/μL, leucocytes 4,1–10.9× 10^3 cells/μL, creatinine 0.5–0.9 mg/dL, bilirubin 0.05–1.0 mg/dL, glutamic-oxalacetic transaminase 10–50 U/L, glutamic-pyruvic transaminase <35 U/L, prothombin 70%–120%.

Epidemiologic visits also found evidence of inadequate rural housing and peridomestic and workplace conditions that permitted rodent invasion; for 87% of patients, those places were considered the probable infection site. These findings reflect the usual conditions of life of the rural population and provide evidence that humans are exposed to hantavirus at home and in their workplaces. This contact is expected to increase if rodent population augments.

The incidence of cases varied in time. In Chile and southern Argentina, disease incidence has increased coincidence with the synchronic flowering and seeding of the shrub *Chusqueaquila*, a perennial bamboo that occurs in long inter annual cycles and provides abundant food for the granivorus rodent *O. longicaudatus*. Large outbreaks of rodents (known as "ratadas") are associated with this cyclic phenomenon (*10*,*11*), with existing chronicles as old as the conquest and Spanish colonization of the country (*12*).

In Chile, reported cases peaked in 2001, but in our study, cases peaked in 2005–2007. A possible explanation for the increase in HPS cases during 2005–2007 (Figure 3), without evidence of "ratada," is that other native trees (olivillo, *Aextoxiconpunctatum*; avellano, *Gevuinaavellana*; and others), shrubs ("murtilla"), and herbs also have cycles of seed production not clearly known because they are not under surveillance and therefore not reported. These seasonal cycles could be associated with localized and minor differences in rodent population density (*13*). More research on the characteristics of the reservoir species and its habitat is needed to better understand the epidemiology of human disease.

In the United States, HPS displays a strong seasonal distribution. Most cases occur in May, June, and July; the fewest occur in December, January, and February (*14*). In Chile, HPS incidence is also highest during the Southern Hemisphere summer (*4*) (Figure 3); the same has been described in Argentina (*15*). During previous years, brief periods of observation found this seasonal distribution in the study region (*10*). Seasonal distribution has been suggested to be a consequence of increasing human recreational and occupational activities in rural areas (*10*,*16*). However, we found that most cases occurred during autumn and winter, in coincidence with the increase in *O. longicaudatus* mice, resulting from increased seed availability. Otherwise, this rodent goes out into open spaces in summer to reproduce, favoring human contact on vacations and outdoor activities (*17*).

In our study, the increase in HPS cases during autumn and winter suggests a particular form of contagion. In the provinces studied here, humans live and work in the invading rodents' habitat during times when rodents are more abundant.

Hantavirus seroprevalence in rodents varies by season and geography. Captures in our region during 1998–2001 showed seroprevalence rates of 7.2%–13.5%, which is higher than in the rest of the country (1.5%–3.2%). This prevalence could be even larger in *O. longicaudatus* mice captured in a patient's home and peridomestic storage buildings (*10*).

The native landscape fragmentation caused by forestry and agriculture has favored the overgrowth and wider distribution of *C. quila* bamboo and the movement of *O. longicaudatus* mice between patches of vegetation, which increases the chance of human–rodent contact (*18*,*19*). This observation could explain the higher incidence of HPS in communes of our region exhibiting higher densities of *C. quila* bamboo and in the Andean region next to El Bolson, Argentina, where the first HPS cases in South America were reported, followed by the communes of Los Muermos and Fresia in the Cordillera de la Costa (Table 1; Figure 1).

Since identification of the first cases of HPS in HSR, clinicians have improved their initial diagnostic accuracy from 27% during 1995–2004 to 76% during 2005–2012. Accurate diagnosis is important because HPS is an unusual disease, even in a zone to which it is endemic, and early suspicion enables timely and effective management.

Table 4. Study variables with significant differences between hantavirus pulmonary syndrome patients who did and did not survive, Hospital Puerto Montt, Puerto Montt, Chile, 1995–2012

Variable	Survivors, no. (%), n = 56	Nonsurvivors, no. (%), n = 24	Odds ratio (95% CI)	p value
Systolic blood pressure ≤90 mm Hg*	8 (14)	10 (41.7)	4.0 (1.3–12.1)	0.014
Respirations ≥30 breaths/min*	13 (23)	19 (79.2)	15.3 (3.8–61.5)	0.000
Pulse >120 beats/min*	14 (25)	14 (58.3)	4.0 (1.4–11.1)	0.008
Bleeding manifestations	15 (27)	17 (70.8)	6.8 (2.3–19.7)	0.000
Creatinine ≥1.3 mg/dL*	13 (23)	13 (54.2)	3.7 (1.2–10.6)	0.017
Admitted to intensive care unit	39 (70)	24 (100)		0.001
Mechanical ventilation	16 (29)	24 (100)		0.000
Shock	13 (23)	24 (100)		0.000
Infiltrates in 4 quadrants in chest radiograph*	21 (38)	18 (75)	5.0 (1.6–15.5)	0.006
PAFI <100*	2 (4)	5 (20.8)	7.1 (1.2–41.2)	0.008
Classified as grade III (severe)	17 (30)	24 (100)		0.000

*On admission. PAFI, arterial oxygen tension/inspiratory oxygen fraction.

The disease characteristics we observed—fever, myalgias, thrombocytopenia, increased hematocrit, leukocytosis, and elevated creatinine, followed by different degrees of pulmonary involvement, usually with rapidly evolving acute respiratory distress—confirm what we described previously among 25 cases (*6*) and matches HPS descriptions from the United States (*14*,*20*). However, in the US cases, hemorrhagic manifestations were not described; in Chile, hemorrhagic manifestations have been repeatedly reported (*6*,*8*,*21*,*22*), even though in this study they were less frequent than in 2003 (39% vs. 64%, respectively). The 32% CFR is similar to that reported in the United States (35%) (*14*).

On the basis of an open study suggesting the benefit of high-dose steroids for HPS (*8*), we administered that dose to 31 of the patients reported here. We discontinued high-dose steroids after a controlled trial showed its failure to decrease the severity of HPS (*9*).

In this study of a large number of cases, we confirmed the variable characteristics of hantavirus disease, from only mild prodromal symptoms without cardiopulmonary involvement to the severe cardiopulmonary syndrome, as observed in a small number of cases studied previously in our region (*6*). Five patients in this study showed no evidence of pulmonary involvement, which is consistent with seroprevalence studies identifying hantavirus-seropositive persons without history of severe disease in up to 10% of some groups of Andean inhabitants in Chile (*23*). Reports on hantavirus seropositivity in persons without pulmonary involvement also have been presented in Argentina and the United States, although at lower frequencies (*14*,*15*). As long as pulmonary symptoms are required for reporting of hantavirus infection to the US Centers for Disease Control and Prevention, these milder hantavirus infections will continue to go uncounted (*14*). Causes for different clinical characteristics of hantavirus infection are under investigation and can be related to the virus, the human host, or both (*24*).

Severe hantavirus disease is characterized by a rapid installation and progression of severe respiratory failure and shock, which requires urgent ICU management, which is not always available in a timely manner. Death occurs in most cases within 1–2 days after hospital admission. Respiratory frequency ≥30 breaths/minute and creatinine levels ≥1.3 mg/dLon hospital admission were independent factors associated with death (Table 3).

In US studies, platelet count was significantly lower in patients who died than in those who survived, but we did not confirm this finding. Low platelet count and high hematocrit are good indicators for suspecting the diagnosis, but we did not correlate them with death. The presence of these 2 elements with compatible clinical and epidemiologic background should prompt rapid transfer of the patient to a hospital with ICU facilities. A rapid test to detect hantavirus IgM based on recombinant N-protein of Puumala virus (IgM POC PUUMALA, Reagena Ltd, Toivala, Finland) was evaluated for Andes virus diagnosis and showed >90% sensitivity and specificity It is available in Chile and is of help in some cases for decision making (*25*). Early clinical suspicion of hantavirus disease, especially in small rural areas, must indicate urgent transfer to a hospital with ICU and may help decrease the high CFR observed in patients with HPS.

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Animal-Associated Exposure to Rabies Virus among Travelers, 1997–2012

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Learning Objectives

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

- Describe the demographic characteristics of travelers exposed to potentially rabid animals that might differentiate them from other ill travelers seeking medical care
- Describe the travel-related characteristics associated with potential exposure to rabid animals
- Discuss the clinical implications of the findings regarding travel-related characteristics associated with potential exposure to rabid animals

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Among travelers, rabies cases are rare, but animal bites are relatively common. To determine which travelers are at highest risk for rabies, we studied 2,697 travelers receiving care for animal-related exposures and requiring rabies postexposure prophylaxis at GeoSentinel clinics during 1997–2012. No specific demographic characteristics differentiated these travelers from other travelers seeking medical care, making it challenging to identify travelers who might benefit from reinforced pretravel rabies prevention counseling. Median travel duration was short for these travelers: 15 days for those seeking care after completion of travel and 20 days for those seeking care during travel. This finding contradicts the view that preexposure rabies vaccine recommendations should be partly based on longer travel durations. Over half of exposures occurred in Thailand, Indonesia, Nepal, China, and India. International travelers to rabies-endemic regions, particularly Asia, should be informed about potential rabies exposure and benefits of pretravel vaccination, regardless of demographics or length of stay.

Rabies causes ≈60,000 human deaths annually and is a public health concern in most countries in Asia and Africa (*1*). By contrast, it is rare among travelers; an average of 3.7 cases were documented each year during 2004–2012 (*2*). Nevertheless, bites to travelers by potentially rabid animals are relatively frequent; estimated incidence is 0.4% per month of stay, according to a meta-analysis of ≈1,270,000 travelers (*3*). By inference, expensive postexposure prophylaxis (PEP), which includes administration of rabies vaccine and rabies immunoglobulin, is probably provided to large numbers of travelers annually. Given the severity of rabies virus infection and the high costs associated with caring for large numbers of potentially exposed travelers, rabies pretravel preventive measures need to be reinforced. These measures include systematic pretravel counseling about animal bite avoidance, postexposure wound care and prophylaxis, and preexposure rabies vaccination for some travelers.

Generalizability of data regarding the epidemiology of travel-associated animal-related rabies virus exposures are limited because they come from studies that are small or single center or that focus on travelers returning from specific destinations. As such, travelers at highest risk for rabies cannot be reliably identified on the basis of available data (*3*,*4*). The decision as to which travelers should receive predeparture rabies vaccination is complex because of the combination of limited data defining rabies risk among travelers, the high cost of rabies vaccine and rabies immunoglobulin in some countries, and the occasionally limited rabies vaccine and rabies immunoglobulin availability because of production problems.

One way to assess the epidemiology of travel-associated illness in travelers and immigrants involves use of GeoSentinel, a global sentinel surveillance network established in 1995 through a collaborative effort from the International Society for Travel Medicine and the US Centers for Disease Control and Prevention (CDC) (*5*). We used the GeoSentinel database to assess geographic and demographic factors for a large number of patients who sought care at GeoSentinel sites for animal-related exposure (e.g., bite, scratch, lick on broken skin or mucous membrane) and required rabies PEP.

Methods

Data Source

GeoSentinel Surveillance participating sites are specialized travel or tropical medicine clinics in 24 countries on 6 continents; they systematically contribute point-of-care, clinician-based, sentinel surveillance data. Sites are staffed by clinicians recruited on the basis of their knowledge and experience in travel and tropical medicine (*6*). To be included in the database, patients must have crossed an international border within 10 years of the clinic visit and sought medical care for a presumed travel-related illness. Diagnoses are selected by the evaluating clinician from a standard list of ≈500 causative or syndromic diagnoses. Data about demographics, travel history, and presumed country of exposure are also collected. Region of travel is calculated from country of exposure by using the following modified regional groupings established by the United Nations Children's Fund: Australia/New Zealand, Caribbean, Central America, Eastern Europe, Middle East, North Africa, North America, North East Asia, Oceania, South America, South Central Asia, South East Asia, sub-Saharan Africa, and Western Europe (*6*). Institutional review board approval was not required because the GeoSentinel data collection protocol was reviewed at CDC and classified as public health surveillance and not human subject research.

Inclusion Criteria

We reviewed all records of patients who sought care at a GeoSentinel site from January 1, 1997, through December 31, 2012, and for whom data were entered into the GeoSentinel database. Analysis was limited to travelers with confirmed or probable final diagnoses of an animal exposure and receipt of rabies PEP. We excluded patients who reported animal exposure but did not receive rabies PEP (which probably includes those exposed to animals other than mammals as well as mammals in areas where rabies is absent) and patients who received rabies PEP but did not report animal exposure.

Statistical Analyses

First, we conducted a descriptive epidemiologic analysis. Eligible records were stratified by exposure animal (dog, bat, cat, nonhuman primate [NHP], and other mammal).

For patients seeking care after travel, duration of travel was calculated as the last day of the most recent trip minus the first day of the most recent trip. For patients seeking care during travel, travel duration was calculated as the date of the clinic visit subtracted from the trip departure date. Trips could have involved multiple countries; therefore, travel duration does not always represent time in the exposure country. Patients were excluded from this calculation if they did not list recent travel that included their country of exposure, if duration of travel could not be calculated or was invalid, or if they listed multiple trips to the country of exposure within the past 6 months.

Second, we conducted a subanalysis for temporal reporting trends in rabies risk exposure relative to total GeoSentinel reports among patients who received treatment during the final 10-year reporting period (2003–2012). For this analysis, we included only a subset of GeoSentinel sites contributing patient data for the entire 10-year period. A simple linear regression was used for this calculation. A 2-tailed p value of <0.05 was considered statistically significant. All analyses were performed by using SAS 9.3 (SAS Institute, Inc., Cary, NC, USA).

Results

Patient Characteristics and Animals Associated with Exposure

The analysis included 2,697 patients who had received rabies PEP at 1 of 45 GeoSentinel sites after an animal-related exposure during 1997–2012. These patients represented 1.5% of the 183,749 ill travelers entered into the database during the same 16-year period. Nearly all (99%) patients who reported animal exposure were evaluated in the outpatient setting; most (74%) travelers sought care in their country of residence after return from travel, and the others (25%) sought care during travel at GeoSentinel clinics in or near a destination country. The most frequent region of residence was Western Europe (32%), followed by northeastern Asia (17%), Australia/New Zealand (17%), Southeast Asia (14%), and North America (10%); 8% had emigrated from their country of birth to another country. A pretravel encounter with a health care provider was recorded for 32% of patients, no pretravel consultation was reported by 42%, and this information was unknown or missing for 26%. Information about pretravel rabies vaccination status was available for 756 (28%) patients, 83 (11%) of whom were vaccinated before traveling.

The animal species associated with exposure was recorded for 2,637 (98%) patients (Table 1). The most common species were dog (60%) and NHP (24%), followed by cat (10%) and bat (2%). Among patients in this analysis, about half were male; however, male patients accounted for slightly more than half of the exposures to dogs and less than half to NHPs, cats, and other mammals. The median age of patients was 30 years (range birth–90 years). Overall, the proportion of children <15 years of age was 11%, but children were overrepresented among cat exposures (19%) and underrepresented among bat exposures (4%). The most common reason for travel was tourism (71%), followed by visiting friends and relatives (12%) and business (10%). Tourists made up a disproportionately large proportion (92%) of those exposed to NHPs.

The region in which the animal exposure occurred was recorded for 2,645 (98%) patients; most exposures occurred in Southeast Asia (42%), followed by other regions in Asia (32% for south-central and northeastern combined), Africa (9% for North Africa and Sub-Saharan Africa combined) and Latin America (7% for Central and South America combined, including Mexico) (Table 1). Although 42% of all exposures occurred in Southeast Asia, two thirds of all exposures to NHPs occurred there. A very small proportion of patients were exposed to animals in North Africa (5%) and the Middle East, (3%), but 17% and 14% of patients in those regions, respectively, were exposed to cats. Almost half of all bat exposures occurred in Latin America, whereas only 6% of patients overall were exposed there.

The country with the highest proportion of animal exposures was Thailand, followed by Indonesia, Nepal, China, and India (Table 2). Indonesia ranked first for NHP- and bat-related exposures. Among the top 5 countries for cat-related exposures were Turkey and Algeria, and among the top 5 countries for bat-related exposure, 4 were in Latin America (French Guiana, Peru, Mexico, and Suriname).

Seasonality

Overall, 801 (30%) patients receiving rabies PEP after an animal-related exposure received care at a GeoSentinel site during July–September (Figure 1). This seasonal pattern was most pronounced for those exposed to cats or bats. This finding is in contrast to all patients entered into the GeoSentinel database during the period of study with any diagnosis, 25% of whom received care during July–September.

Duration of Travel

Travel duration could be determined for 2,452 patients. Among these, median duration was 15 days (range 1–6,205 days) among 1,961 patients who sought care for an animal-related exposure after travel and 20 days (range 1–794 days) among 491 who sought care during travel.

Trends among Patients Receiving PEP

Of the 2,697 reported animal exposures, 83% occurred during 2007–2012. Among the 138,433 patients who sought care during 2003–2012 at sites that were active GeoSentinel members for that entire period, 1,490 (1.1%) received rabies PEP after an animal exposure at 23

Table 1. Characteristics of 2,697 patients who sought care for an animal exposure and received rabies postexposure prophylaxis at GeoSentinel Surveillance Network sites, January 1997–December 2012, by animal species*

Patient characteristic	Animal					
	Dog	NHP	Cat	Bat	Other†	Total‡
No. patients	1,618	638	271	46	126	2,697
Male sex, no. (%)	891 (55)	269 (42)	125 (46)	21 (46)	54 (43)	1,360 (51)
Age, y, no. (%)						
≤14	160 (10)	65 (10)	50 (19)	2 (4)	14 (11)	291 (11)
15-44	1,027 (64)	460 (72)	151 (56)	28 (61)	75 (60)	1,739 (65)
45-64	340 (21)	103 (16)	56 (21)	16 (35)	33 (26)	548 (20)
≥65	87 (5)	9 (1)	13 (5)	0	4 (3)	113 (4)
Reason for travel						
Tourism	1,016 (63)	590 (92)	183 (68)	31 (67)	89 (71)	1,908 (71)
Visiting friends/relatives	264 (16)	6 (1)	41 (15)	1 (2)	11 (9)	323 (12)
Business	206 (13)	18 (3)	25 (9)	2 (4)	13 (10)	264 (10)
Missionary/volunteer/researcher/ aid worker	82 (5)	15 (2)	14 (5)	7 (15)	10 (8)	127 (5)
Student	36 (2)	7 (1)	7 (3)	4 (9)	3 (2)	57 (2)
Other§	13 (1)	2 (<1)	1 (<1)	1 (2)	0	16 (1)
Region of exposure, no. (%)¶						
Southeast Asia	570 (36)	414 (66)	99 (37)	10 (22)	37 (30)	1,129 (43)
South-Central Asia	406 (26)	146 (23)	21 (8)	3 (7)	22 (18)	598 (23)
Northeastern Asia	217 (14)	13 (2)	25 (9)	0	6 (5)	261 (10)
North Africa	76 (5)	6 (1)	45 (17)	1 (2)	9 (7)	137 (5)
Latin America	121 (8)	15 (2)	7 (3)	21 (46)	10 (8)	174 (7)
Sub-Saharan Africa	55 (3)	18 (3)	16 (6)	1 (2)	16 (13)	106 (4)
Middle East	47 (3)	3 (<1)	38 (14)	0	2 (2)	90 (3)
Eastern Europe	40 (3)	2 (<1)	4 (2)	1 (2)	4 (3)	51 (2)
Western Europe	28 (2)	3 (<1)	6 (2)	4 (9)	5 (4)	46 (2)
Oceania	14 (1)	0	1 (<1)	2 (4)	1 (1)	18 (1)
North America	3 (<1)	1 (<1)	2 (1)	3 (7)	8 (6)	17 (1)
Caribbean	8 (1)	2 (<1)	2 (1)	0	3 (2)	15 (1)
Australia	1 (<1)	0	0	0	2 (2)	3 (<1)

*NHP, nonhuman primate. Data include 4 patients of unknown sex, 6 patients of unknown age, 4 patients of unknown country of residence, 52 patients whose region of exposure was unknown or unable to be ascertained, and 1 patient whose purpose of travel was unknown.
†Bear (n = 1), camel (n = 1), *Nasua* spp. coatis (n = 4), cow (n = 1), donkey (n = 2), fox (n = 1), hamster (n = 2), horse (n = 5), human (n = 1), lion (n = 2), mongoose (n = 1), meercat (n = 2), mouse (n = 5), opossum (n = 1), rabbit (n = 2), raccoon (n = 2), rat (n = 12), rodent (n = 4), squirrel (n = 11), tiger (n = 6), and other, unspecified (n = 60).
‡Two patients were exposed to >1 animal; 1 patient was exposed to cat and dog, and 1 patient was exposed to dog and other (tiger).
§This category includes immigration (n = 8), medical tourism (n = 8), and military (n = 1).
¶For explanation of GeoSentinel Surveillance Network regions, see Figure 2 (http://www.cdc.gov/mmwr/preview/mmwrhtml/ss6203a1.htm).

continuously reporting sites. In this group, the proportion of animal-associated exposures relative to total reports to GeoSentinel increased ≈0.1% per year over the 10-year period (β = 0.00149, 95% CI 0.00088 0.00210, p<0.001); the number of animal-associated exposures reported to GeoSentinel in 2012 was 4-fold greater than the number reported in 2003 (Figure 2).

Rabies Diagnoses

During the study period, 3 patients included in the GeoSentinel database received a diagnosis of rabies (Table 3). All 3 patients died.

Discussion

Our analysis is a comprehensive survey addressing the epidemiology of animal-related exposures leading to rabies PEP among international travelers. The number of patients (2,697), duration of the study (16 years), and multicenter design (45 sites) provided robust data for this analysis. We found a small but significant rise in the proportion of travelers who sought care at GeoSentinel sites during 2003–2012 and who required rabies PEP, even after we eliminated the bias of increased number of sites by including only continuously reporting sites. It is known from World Tourism Organization data that tourist destinations are becoming more diverse (*12*); increased proportions of international tourists are traveling to countries that have emerging and developing economies and where rabies is endemic. Because the trend toward more exotic travel destinations is predicted to continue well into the future (*12*), demand for rabies pharmaceuticals and postexposure wound care among international travelers will probably continue to grow.

During the study period, 2,697 travelers sought care for animal exposure at GeoSentinel surveillance clinical sites and received rabies PEP. The number of travelers who seek rabies PEP is known to be an underestimate of the actual number of travelers exposed. In a recent survey conducted among 7,681 international travelers leaving the Bangkok airport, two thirds of travelers who reported having been bitten by a potentially rabid animal during their trip sought no medical care (*13*). Although exact figures do not exist, it can be supposed that the prevalence of exposure to potentially rabid

Table 2. Countries with 5 highest levels of exposure among 2,697 patients who sought care for animal exposure and received rabies postexposure prophylaxis at GeoSentinel Surveillance Network sites, January 1997–December 2012, by animal species

No.	Animal, country of exposure, no. (%) exposures					
	Dog, n = 1,618	NHP, n = 638	Cat, n = 271	Bat, n = 46	Other, n = 126	Total, n = 2,697
1	Thailand, 294 (18)	Indonesia, 200 (31)	Thailand, 59 (22)	Indonesia, 7 (15)	Thailand, 16 (13)	Thailand, 534 (20)
2	Nepal, 198 (12)	Thailand, 166 (26)	Turkey, 31 (11)	French Guyana, 5 (11)	India,10 (8)	Indonesia, 314 (12)
3	China, 197 (12)	Nepal, 82 (13)	China, 25 (9)	Peru, 4 (9)	Indonesia, 10 (8)	Nepal, 295 (10)
4	India, 124 (8)	India, 43 (7)	Indonesia, 17 (6)	Mexico, 3 (7)	China, 6 (5)	China, 241 (9)
5	Indonesia, 80 (5)	Vietnam, 21 (3)	Algeria, 15 (6)	Surinam, 3 (7)	Nepal, 6 (5)	India, 185 (7)

animals among international tourists is substantial. The fact that 3 rabies diagnoses were entered into the GeoSentinel database during the study period confirms this supposition. More data defining the epidemiology of rabies exposure and disease among travelers are needed.

Our survey findings confirm those from an earlier GeoSentinel survey conducted among only 320 returned travelers (*14*). However, the inclusion criteria for the 2 analyses differed; the earlier analysis was conducted only among patients who sought care after travel, and 34% were patients who reported an animal exposure but did not receive rabies PEP. Additionally, the structure of the GeoSentinel network has evolved over time (*6*), so cautious interpretation of the comparison is warranted.

Most persons who report to GeoSentinel sites and require rabies PEP are young adult (15–44 years of age) tourists traveling from high-income regions to visit low- and low-middle–income regions. This profile corresponds to the overall traveler population seen at GeoSentinel sites (*15*). This apparent lack of distinction is important because the demographic characteristics of travelers exposed to potentially rabid animals did not differ from those of other ill travelers who seek medical care, which makes it challenging to identify specific travelers who might benefit from reinforced rabies pretravel preventive counseling. Although previous studies have found children to be at highest risk for animal bites requiring PEP (*1*), our results suggest that young adults may also be vulnerable and may also benefit from preventive counseling. We observed a small but significant seasonal pattern (especially for bat and cat exposures), which might be used to guide the pretravel advice given to summer tourist travelers.

The short median duration of travel (2 weeks) among returned travelers consulting for rabies PEP corroborates the World Health Organization recommendation that a travelers' assessment for risk of an animal bite should not be influenced by the duration of travel (*1*). Our results, however, are not consistent with the current CDC recommendations that preexposure rabies vaccine recommendations should be based, at least in part, on longer durations of stay (*16*), a position that is shared by many countries (*3*). Additionally, among those seeking care at GeoSentinel sites during travel, exposure occurred within a median of 3 weeks of arriving in the country of exposure, which suggests that rabies vaccination may also be indicated for patients embarking on shorter trips.

Of travelers consulting for rabies PEP at a GeoSentinel site, 70% had been exposed while in Asia, most in Southeast Asia. Rabies is endemic to most countries in Asia (*17*). Of 10 patients, 6 were exposed in 5 countries (Thailand, Indonesia, Nepal, China, and India). Large numbers of human rabies cases among the local population are reported from these 5 countries, with the exception of Thailand (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/4/14-1479-Techapp1.pdf), where only sporadic cases of rabies in humans are now reported (*1*,*17*–*25*; J.M. Shresta, 2012, pers. comm.). Rabies cases in humans are reported from almost all regions in India (rates are highest rates in Chhattisgarh, Uttar Pradesh, and Odisha states) and from almost all regions in China (rates are highest in Guizhou, Guangdong, Hunan, Guangxi, and Guangdong Provinces). In Thailand, rabies cases in humans show no specific geographic distribution. In Indonesia and Nepal, cases are concentrated in specific areas (*1*,*17*–*25*; P. Rupali, 2012, pers. comm.). Travel-associated rabies cases have been reported from all these countries except Indonesia; most such cases were acquired in India and China (*2*). Updated data about the incidence of rabies in many countries is difficult to find, which indicates a need for improved human rabies surveillance (*1*).

For travelers to these 5 countries, rabies vaccine is more accessible than rabies immunoglobulin. Tissue-cultured

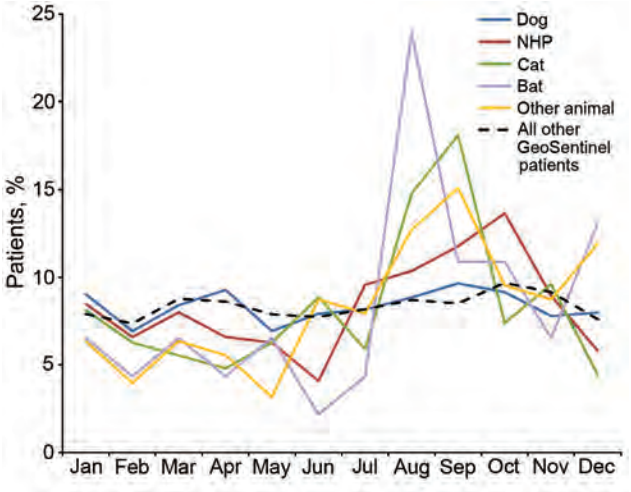

Figure 1. Monthly distribution of animal-related exposure cases requiring rabies postexposure prophylaxis, by exposure species, according to date of initial visit to GeoSentinel clinics, 1997–2012.

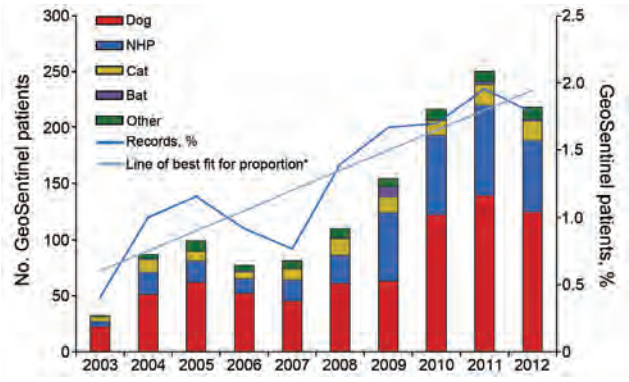

Figure 2. Number of patients requiring rabies postexposure prophylaxis for animal-related exposure, by exposure species and by year and line of best fit for proportion of all GeoSentinel records accounted for by animal-related exposure requiring postexposure prophylaxis, 2003–2012. Limited to patients treated at GeoSentinel sites that were active contributors for the entire listed period. NHP, nonhuman primate. *Linear regression was used to calculate a line of best fit of y = 0.0015x + 0.006.

vaccine is locally produced (China and India) or imported (all 5 countries) and may be available in most cities. Equine rabies immunoglobulin is available from most public hospitals in China and Thailand but may be difficult to find in smaller hospitals, notably in remote rural areas. In India, Indonesia, and Nepal, equine rabies immunoglobulin may be available from large cities only. Human rabies immunoglobulin is generally unavailable except in limited circumstances and at specialized centers (*26*–*32*; P. Rupali, 2012, pers. comm.). All recent studies addressing rabies PEP management in exposed travelers indicate that <1 in 10 travelers received rabies immunoglobulin in the country of exposure (*33*–*36*). In this study, among those who received rabies immunoglobulin after returning to their home country, there was a substantial delay between exposure and administration of rabies immunoglobulin. Some exposed travelers returned home to clinics in their own countries, having received the first dose of vaccine—without rabies immunoglobulin—in the country of exposure >7 days earlier; at this time, administration of rabies immunoglobulin may have reduced benefits. Equine rabies immunoglobulin carries a very low risk for anaphylaxis and is safe and effective (*37*); travelers should be encouraged to accept it when available and prescribed.

Although few patients in our analysis were exposed while in Vietnam or Philippines, these rabies-endemic countries are among the top 10 tourist destinations in Asia (*17*), so travelers to these countries should also be informed about potential rabies exposure and benefits of pretravel vaccination. Given the complex mix of high travel volumes, rabies endemicity, and inconsistent availability of rabies pharmaceuticals, Asia may be a region of considerable rabies risk for travelers.

Although dogs remain the leading animal responsible for exposure among travelers, NHPs account for one quarter of the exposures among patients seen at GeoSentinel sites; this proportion is even higher among tourists, female travelers, and travelers to Southeast Asia. Although rabies cases do occur in NHPs, they are less frequently reported in the literature than are cases in humans. The occurrence of documented transmission of rabies virus from NHPs to humans suggests that rabies PEP is indicated for patients exposed to NHPs in rabies-enzootic countries (*38*).

As found in previous studies (*3*,*4*), we found that a substantial proportion of exposed travelers did not receive pretravel advice. Our data also suggest that only a small proportion had received preexposure rabies vaccination. However, vaccination data were missing for many patients. Public health professionals should work toward increasing the proportion of travelers who receive pretravel medical care, including a selective proportion who receive preexposure rabies vaccination.

This analysis has limitations. The GeoSentinel Surveillance Network captures data only for persons who visit specialized travel or tropical medicine clinics after travel for a travel-related illness or concern; these data do not represent all international travelers. GeoSentinel Surveillance data cannot be used to calculate absolute risk. The composition of travelers included in this analysis probably overrepresents persons traveling to or residing in Asia, as well as those residing in Australia, and underrepresents those residing in North America or traveling to Latin America. Children may also have been underrepresented. In addition, generalizability could be affected by site-specific differences in referral patterns, clinic volumes, patient populations, and travel destinations.

Encouraging travelers to undergo a pretravel risk assessment and prevention counseling may help identify persons who will be at higher risk for a rabies exposure when traveling. The pretravel consultation should educate and warn higher risk travelers to rabies-endemic regions in Asia, Africa, and Latin America about their destination-

Table 3. Characteristics of 3 patients with travel-associated rabies, GeoSentinel Surveillance Network, January 1997–December 2012

Year (reference)	Age, y/sex	Citizenship	Reason for travel	Country (source) of exposure
2006 (*2*,*7*,*8*)	65/M	Japan	Business (expatriate)	Philippines (dog bite)
2012 (*2*,*9*,*10*)	41/M	Canada	Unknown	Island of Hispaniola (unknown)*
2012 (*2*,*11*)	34/M	Israel	Tourism	India (unknown)

*Although this patient's place of exposure was initially described as being the Dominican Republic, the exact location or source of exposure could not be definitively determined; Hispaniola comprises Haiti and the Dominican Republic.

and itinerary-specific rabies risk profile and the need to avoid contact with animals, notably dogs, NHPs, and cats. Pretravel vaccination against rabies is expensive in many countries (*37*), although the long-lasting resulting immunity may make this investment attractive for some patients in light of cumulative risk from iterative travels (*39*). Schedules of less expensive intradermal preventive vaccination are recommended by the World Health Organization, for travelers as well as others. Several preliminary studies have shown shorter, less expensive preexposure vaccine schedules to be effective (37,40). However, they are not yet widely available to travelers, and further large-scale studies are needed before any recommendation can be made. Travel-health specialists should work to identify those for whom pretravel vaccination is most strongly indicated on the basis of risk characteristics. Travelers to rabies-endemic regions, particularly those in Asia, should be well educated about their potential rabies exposure, the importance of avoiding contact with animals, and the potential benefit of pretravel rabies vaccination, regardless of travel duration and traveler demographics.

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Evolution of Ebola Virus Disease from Exotic Infection to Global Health Priority, Liberia, Mid-2014

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Over the span of a few weeks during July and August 2014, events in West Africa changed perceptions of Ebola virus disease (EVD) from an exotic tropical disease to a priority for global health security. We describe observations during that time of a field team from the Centers for Disease Control and Prevention and personnel of the Liberian Ministry of Health and Social Welfare. We outline the early epidemiology of EVD within Liberia, including the practical limitations on surveillance and the effect on the country's health care system, such as infections among health care workers. During this time, priorities included strengthening EVD surveillance; establishing safe settings for EVD patient care (and considering alternative isolation and care models when Ebola Treatment Units were overwhelmed); improving infection control practices; establishing an incident management system; and working with Liberian airport authorities to implement EVD screening of departing passengers.

> "Reviewing that first phase in the light of subsequent events, our townsfolk realized that they had never dreamed it possible that our little town should be chosen out for the scene of such grotesque happenings as the wholesale death of rats in broad daylight or the decease of concierges through exotic maladies."
>
> —Albert Camus, The Plague (1948)

The Ebola virus disease (EVD) epidemic in West Africa is recognized as the largest in history; more cases have been reported than in all previous EVD outbreaks combined (*1*). However, until the summer of 2014, the epidemic had not captured the world's attention.

The EVD epidemic began in Guinea in late 2013. In neighboring Liberia, EVD was first diagnosed in a patient in mid-March 2014. A team of epidemiologists from the US Centers for Disease Control and Prevention (CDC) began working with the Liberian Ministry of Health and Social Welfare (MOHSW) in early April. By April 9, a total of 12 EVD cases (6 laboratory-confirmed) had been identified in Liberia, but no additional cases were reported in April or during most of May, and it appeared that the outbreak had been contained locally. However, on May 25, a patient who had traveled from Sierra Leone died of suspected EVD in Lofa County in northern Liberia. Within days, additional EVD cases were reported in Lofa County and in Monrovia, the capital city of Liberia. The MOHSW initiated investigations into what was considered a second epidemic wave of EVD.

Background

Liberia is a West African country of ≈4 million that is bordered by Guinea, Sierra Leone, and Côte d'Ivoire (Figure 1). Administratively, the country is divided into 15 counties; Monrovia makes up most of Montserrado County and accounts for ≈25% of the country's population. Liberia ranks 175th of 187 countries in human development (*2*) and, reporting a gross domestic product per capita of US$454 per year, is 181st of 185 countries surveyed (*3*). During 1989–2003, civil war destroyed much of the country's infrastructure and left a generation without education: the adult literacy rate is 43% (*4*). Before the EVD epidemic, the country had <200 physicians (5).

At the request of the government of Liberia, CDC sent a second team to Liberia in mid-July with expertise in epidemiology, logistics, border health measures, and health education. Team members worked in the 6 counties in Liberia that had reported EVD cases at that point, as well as in 4 counties that had not yet been affected, to assess the extent of the outbreak, preparedness, and general needs. CDC staff worked with county health teams and facilities,

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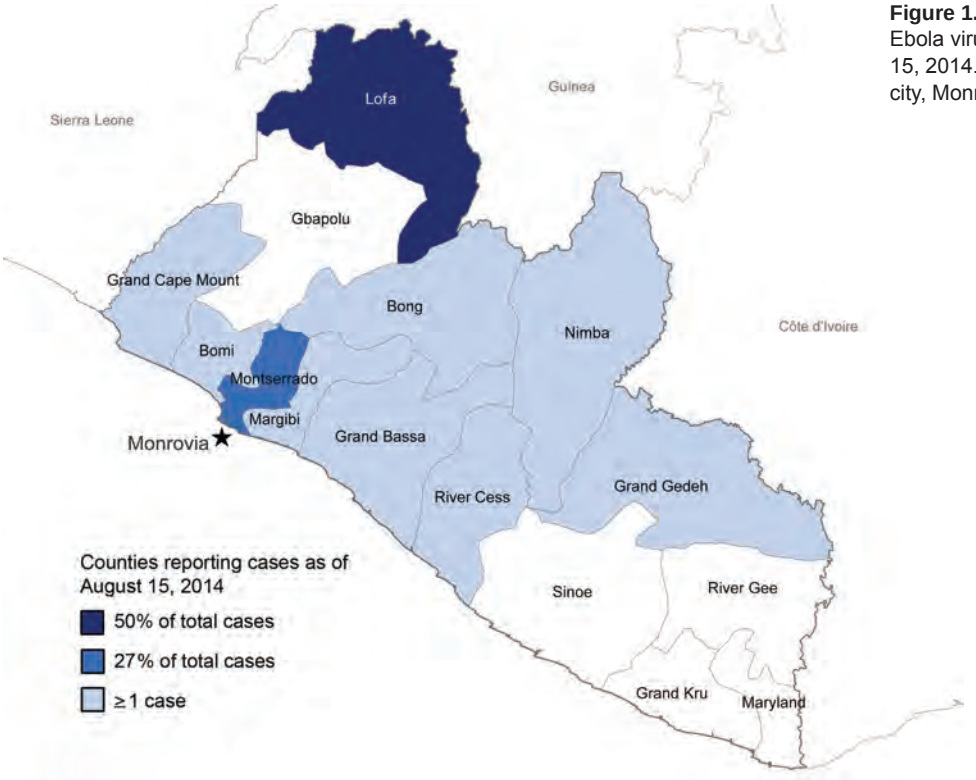

Figure 1. Counties in Liberia reporting Ebola virus disease cases as of August 15, 2014. Star indicates the capital city, Monrovia.

investigated clusters of EVD cases, and worked with the MOHSW to strengthen national surveillance for EVD and to support establishment of an incident management system. Team members also worked with Liberian airport authorities to implement Ebola virus (EBOV) screening of departing passengers. We describe the team's field observations from mid-July to mid-August, 2014.

Surveillance, Data, and Logistical Challenges

> "… so that he should not be one of those who hold their peace but should bear witness in favor of those plague-stricken people…"
>
> —Albert Camus, The Plague

The MOHSW compiled epidemic data, primarily received by telephone from county surveillance officers and supplemented by paper case report forms, into daily national situation reports. However, because of severely limited human and material resources, local health teams often prioritized case management, safe burial, and community education efforts over comprehensive data collection and reporting; therefore, official case counts underestimated disease incidence. In an extreme example, the daily national situation report listed 4 EVD cases from 1 county at a time when the county's health team was aware of >80 cases (M.A. Arwady, unpub. data). This county's surveillance officer had died; the public hospital had ceased operations after a large cluster of cases among its health care workers (HCWs); and the capabilities for both response and reporting had been overwhelmed. Improving EVD national surveillance was therefore a priority during July and August.

The MOHSW established public telephone hotlines, designed to aid in case finding by capturing information about ill or deceased persons with suspected EVD in the community. However, because <10 clinical and burial teams were available to dispatch to callers, assistance was often delayed by days, and the hotlines were overwhelmed by hundreds of calls daily from frustrated citizens.

Each day brought new local crises: counties exhausted their supplies of basic materials (gloves, body bags, and bleach); county employees evaluated threats from fearful and angry community members, searched for vehicles to carry bodies and transport patients, and worked to build isolation facilities and secure pay for health workers. In some settings, patients' families or entire communities refused to allow health care workers access, fearing the workers would bring illness.

The scale of the epidemic made the comprehensive data collection and data entry processes used in earlier EVD outbreaks impractical. In some instances, patient data could not be obtained because persons with EVD did not seek clinical care or were turned away from overburdened facilities. Occasionally, whole families died before public health workers could interview them. Particularly in rural areas, problems with copying and transporting paper forms limited

timeliness of reporting, and intermittent power supply and limited cell phone and internet service hindered connectivity.

Even with these known data limitations, the trajectory of the epidemic was clear. The number of counties reporting cases during July and August increased from 6 to 10. The MOHSW situation report dated July 15, 2014, described 173 EVD cases in detail (including suspect, probable, and laboratory-confirmed cases); over the next 30 days, a 4.8-fold increase in cases occurred, with a mean of 23 new cases and 12 deaths reported daily (range 3–60 new cases and 0–33 deaths per day). By August 15, 826 cases and 455 deaths (55.1% case-fatality rate) had been reported to the MOHSW (Figure 2). Of the reported cases, 23.5% were laboratory confirmed (Figure 3).

The second wave of the EVD epidemic in Liberia started in semi-rural Lofa County in late May 2014, as the epidemic spread in the cross-border area of Guinea, Sierra Leone, and Liberia. As of the August 15 MOHSW situation report, 409 (50%) of Liberia's 826 reported EVD cases were from Lofa County (Figure 1). EVD was likely introduced into Monrovia by infected persons traveling from Lofa County, Guinea, or Sierra Leone; Monrovia then became the second reservoir of infection in Liberia. By July 31, cases had been reported across much of the capital city (Figure 4). As of August 15, Montserrado County, which includes Monrovia, had reported 223 cases, accounting for 27% of total cases. Anecdotes suggested that many more cases were occurring in Monrovia than were being captured in official reports (M.A. Arwady, unpub. data). In the less-affected counties, infected travelers probably introduced localized outbreaks, and EVD was identified in health care settings in areas of Liberia where EVD had not been previously reported; EBOV infection was potentially propagated in these facilities. As of August 15, when approximately half of the country's cases had been reported in Lofa County and ≈25% in Montserrado County, the 8 other affected counties (Bong, Bomi, Grand Bassa, Grand Cape Mount, Grand Geddeh, Margibi, Nimba, and River Cess) together accounted for 194 cases (23%), with a range of 1–87 cases per county. Five counties, predominantly in the southeast, had at that point reported no cases.

EVD among Health Care Workers

> "It could be only the record of what had had to be done, and what assuredly would have to be done… despite their personal afflictions, by all who, while unable to be saints but refusing to bow down to pestilences, strive their utmost to be healers."
>
> —Albert Camus, The Plague

By August 14, a total of 97 EVD cases had been reported in HCWs in Liberia, representing 12% of the 810 reported EVD cases nationwide. Rates varied by county: HCWs represented 4% of EVD cases in Lofa County, 17% in Montserrado County, and 20% in the other affected counties combined. Many of the earliest cases in newly affected counties were identified in HCWs, after infected travelers sought care in hospitals that had not previously seen patients with EVD and where HCWs provided care with minimal or no personal protective equipment (PPE). The CDC team investigated 10 clusters of EVD among HCWs in 4 counties. The number of HCWs per cluster ranged from 2 to 22, with a median of 5. Nurses and nurse's aides accounted for 35% of infected HCWs; physicians and physician assistants accounted for 15%. Other occupations affected included laboratory workers, cleaners, hygienists, pharmacists, public health workers, and midwives.

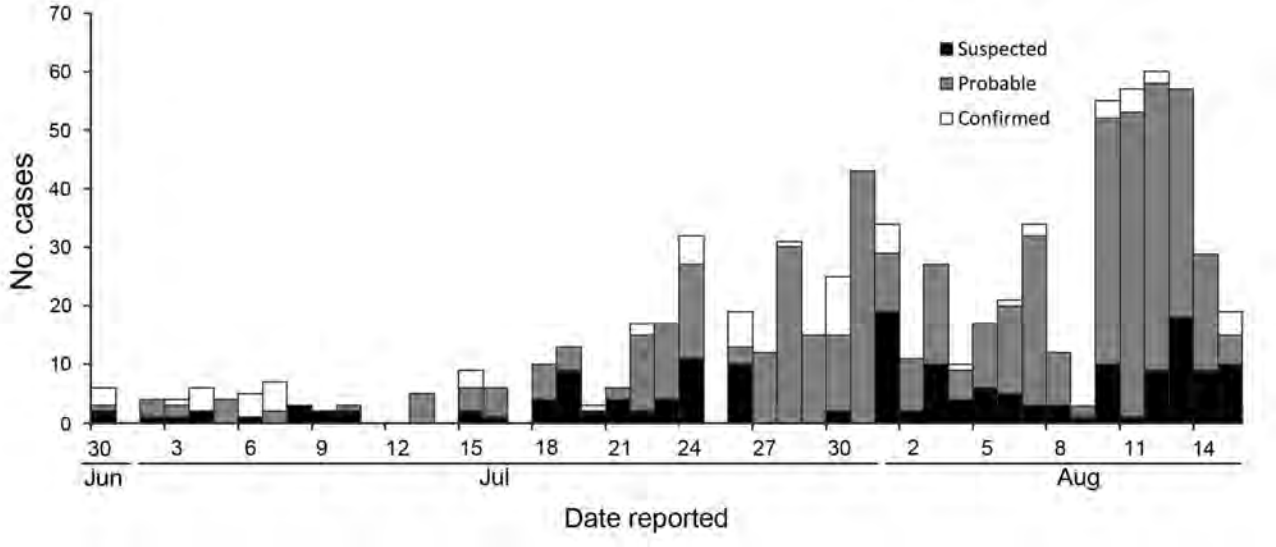

Figure 2. Reported Ebola virus disease cases by date, June 30–August 15, 2014, Liberia (n = 826).

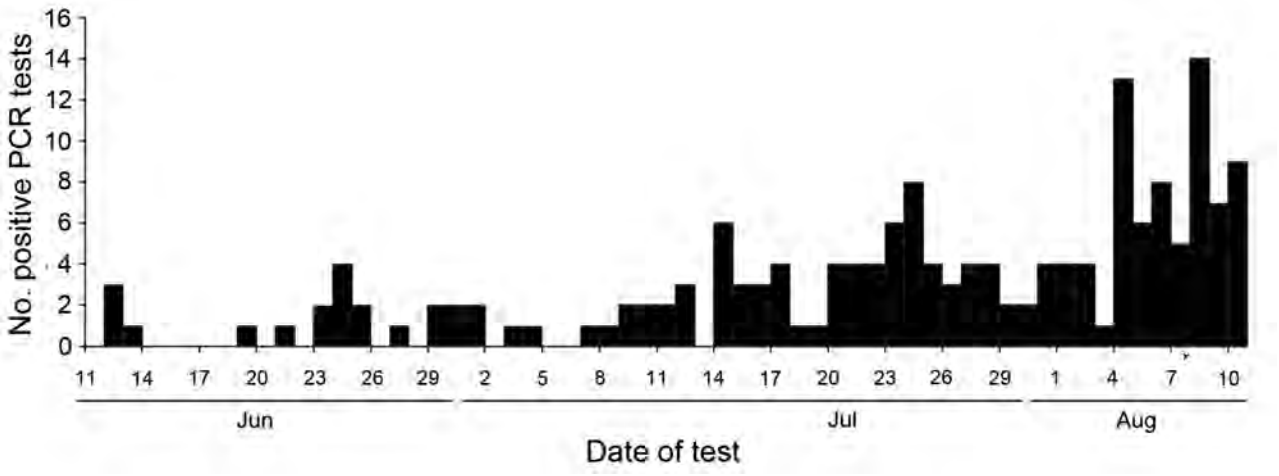

Figure 3. Positive PCR tests for Ebola virus infection, Liberia Institute for Biomedical Research, June 1–August 10, 2014 (n = 172).

In July and August, almost all health care facilities nationwide lacked standard infection control and preparedness procedures. Staff training was limited, and some at-risk workers such as cleaners had not received any training. PPE was generally not available in adequate quantities outside of the country's 2 Ebola treatment units (ETUs), and chlorine disinfectant was in short supply. Almost all hospitals lacked appropriate isolation facilities for suspected case-patients, although by August, many hospitals were beginning to construct or refurbish temporary isolation units.

Many referral hospitals and smaller health facilities ceased operations in July and August because staff and non-EVD patients abandoned facilities in which EVD cases had occurred. In many areas, emergency care and basic outpatient services including prenatal care, HIV/AIDS services, and vaccinations were no longer available. The effects of cessation of these services on overall health outcomes, such as infant or maternal death rates, have not been formally assessed, but it is likely that rates of illness and death from non-EVD illnesses increased. Although the MOHSW emphasized the importance of re-opening facilities to provide essential services, HCWs voiced the need for enhanced infection control, adequate PPE and training, and, in some cases, financial compensation for their increased level of risk.

Establishment of Liberia's Incident Management System

> "Everybody knows that pestilences have a way of recurring in the world; yet somehow we find it hard to believe in ones that crash down on our heads from a blue sky. There have been as many plagues as wars in history; yet always plagues and wars take people equally by surprise."
>
> —Albert Camus, The Plague

When the CDC team arrived in July 2014, the MOHSW was meeting daily with a group of in-country and international responders designated as the Ebola Task Force. The meetings were open, sometimes attracting nearly 100 persons, and were attended by technical staff involved in all aspects of the response, including staff from partner organizations such as the World Health Organization (WHO), Médecins Sans Frontières (MSF), and others (see complete list at the end of this article). Although the Task Force was intended to be national in oversight, meetings sometimes focused on local operational crises, such as a lack of vehicles for collecting bodies around Monrovia, and there was not a clear system for ensuring that agreed-upon actions had been implemented.

The MOHSW worked with the CDC team to establish an incident management system, which is a standardized tool for responding to emergencies under which personnel, resources, and logistic support are organized and managed according to specific objectives. The MOHSW appointed an incident manager, whose sole responsibility was to identify key objectives and lead the response, and a deputy incident manager to coordinate county activities. Task force meetings were reorganized to limit attendance to members of essential response committees that were empowered to make decisions and to include time for follow-up on outcomes of tasks assigned the day before. Key international partners, such as WHO, United Nations Mission in Liberia, CDC, and the United States Agency for International Development, also designated 1 representative each with decision-making capability. Documentation of daily assigned tasks and separation of national and county-level priorities improved organizational efficiency and effectiveness.

EVD Treatment Units, Laboratory Capacity, and the Provision of Care

> "As for the 'specially equipped' wards, he knew what they amounted to: two outbuildings from which the other patients had been hastily evacuated….The only hope was that the outbreak would die a natural death…"
>
> —Albert Camus, The Plague

The standard approach to controlling EVD epidemics depends on active case finding and isolation of patients, with identification and careful monitoring of their contacts and immediate isolation of any contacts who develop symptoms. Isolation units serve to remove infectious persons from the community and to provide patients with supportive care. In previous EVD outbreaks, ETUs have been established and staffed by international health workers with specialized expertise from organizations such as MSF, supported by local employees.

In late July, only two 20-bed ETUs were operating in Liberia: 1 in Foya, northern Lofa County, and the other adjacent to Eternal Love Winning Africa Hospital, known as ELWA, in Monrovia, Montserrado County. A third ETU at John F. Kennedy (JFK) Hospital in Monrovia closed in mid-July after infections were diagnosed in staff members and had not reopened a month later. Both operational ETUs were directed by Samaritan's Purse, an American missionary organization with a long history of providing health care in Liberia. Samaritan's Purse staff had been trained by MSF, which continued to provide technical support in both ETUs. In late July, Samaritan's Purse withdrew from Liberia after a cluster of EVD among staff members, and the ETUs then depended largely on MOHSW staff, with limited international support.

Increasing numbers of EVD cases overwhelmed the capacity of both ETUs, making them unable to accept patient transfers from other counties. On August 14, at the 20-bed ETU in Monrovia, >80 patients were on the premises, including ill patients lying on the grounds outside

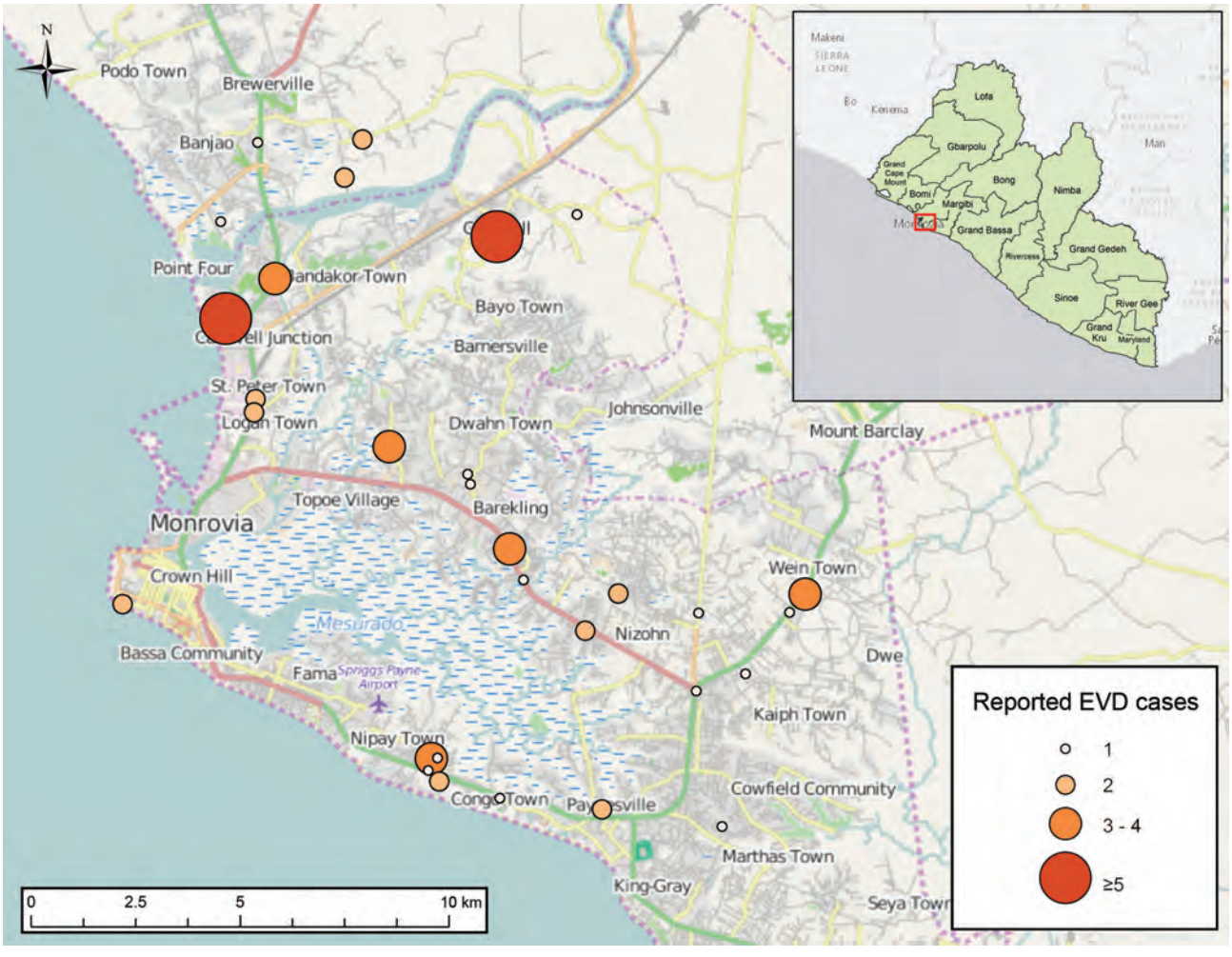

Figure 4. Reported Ebola virus disease cases, Montserrado County, Liberia, as of July 31, 2014.

the facility waiting for a bed. Fifteen bodies were awaiting collection and burial. MOHSW Surveillance Unit data from mid-August indicated that only 25% of all reported EVD case-patients had been admitted to ETUs, but given underreporting of cases, the actual proportion of persons with EVD who reached an ETU is likely even lower. A high case-fatality rate in the Monrovia ETU, possibly the result of inadequate staffing in the face of an overwhelming caseload and concurrent delays in patients seeking care, suggested that under these operational constraints, the units were providing little clinical benefit. The basics of EBOV containment (i.e., isolation of cases and contact tracing) failed because of the large number of cases, insufficient number of isolation beds, and inability to track all contacts or isolate them if they became ill.

Laboratory capacity was also limited. Until late August, the Liberia Institute for Biomedical Research laboratory outside Monrovia was the only laboratory in the country performing EBOV testing. At the initial staffing levels, only 30–40 specimens could be tested per day; in addition, the laboratory was a full day's drive from many outlying areas, and counties had few vehicles for transporting laboratory specimens or patients. Specimens from the ETU in Lofa County were sent across the border to Guéckédou, Guinea, for testing in the laboratory there, which was supported by the European Union. This arrangement was threatened in mid-August by an increase in the number of specimens requiring testing and by international border closures.

Priority for Global Health Security

> "…when the most pessimistic had fixed it at, say, six months; … a flash of foresight would suggest that, after all, there was no reason why the epidemic shouldn't last more than six months; why not a year, or even more?"
>
> —Albert Camus, The Plague

In the span of a few weeks in July and August, 5 sets of circumstances in West Africa changed perceptions of EVD from an exotic tropical disease to a priority for global health security. First, on July 20, a Liberian-American who had EVD traveled by air from Monrovia to Lagos, Nigeria. His arrival and subsequent care resulted in an EVD outbreak in Nigeria that resulted in 20 cases and required public health authorities to follow up on nearly 900 contacts to successfully contain the outbreak. After this exported EVD case, temperature screening and a health questionnaire for outbound passengers were instituted at Liberian airports. MOHSW, airport authorities, CDC, and private partners coordinated support to enhance safety and continuity of commercial air traffic into and out of Liberia as international attention increased.

Second, the late July diagnosis of EVD in 2 persons from the United States who were working in an ETU in Monrovia, their evacuation to the United States in early August, and their receipt of an investigational therapy aroused further international media interest. The situation highlighted the need for decisions concerning therapeutic and vaccine research during this epidemic and for defined policies by international organizations on evacuation of staff in the event of EBOV exposure or infection.

Third, local security concerns emerged in July and August. On July 23, for example, MOHSW employees and CDC team members had to evacuate the MOHSW after a relative of a person who died from EVD set the building on fire. In late July, a CDC team member and other international responders urgently crossed into Guinea from Lofa County after a burial team was attacked and its vehicle destroyed. On August 6, the president of Liberia declared a national state of emergency, and later in August, a nationwide curfew was established. On August 20, looting of an isolation facility in West Point, Monrovia, led to police gunfire and a death.

Fourth, responders recognized that adequate isolation facilities and a county-specific or community-specific containment approach were essential, and that these needs required additional resources and new approaches. With the resources available in July and August, medical relief, public health, and other organizations were unable to provide the trained personnel and specialized resources required to establish new ETUs in all places where they were needed, and other models of isolation and care had to be considered. Preliminary discussions were held with MOHSW, MSF, CDC, WHO, and other organizations about establishing lower level isolation units in community settings (e.g., schools) and the provision of home-based care for patients unable or unwilling to be evacuated.

Fifth, during this time period it became clear that, to have any chance of containment, the response would have to be increased by several orders of magnitude. By August, MSF had concluded that it could not provide the usual level of care given in other EVD outbreaks with the available resources and repeatedly stressed the inadequacy of the global response. On August 8, the Director-General of WHO declared that conditions for a public health emergency of international concern had been met (http://who.int/mediacentre/news/statements/2014/ebola-20140808/en/). Even as the global response increased, adverse epidemiologic trends seen in July and August worsened, and case counts through September increased exponentially. The sheer number of cases continued to outstrip efforts at active case finding and contact tracing, and ill persons continued to be turned away from hospitals and ETUs that had no beds. By September, EVD was widespread across the country, and cases doubled nationwide every 15–20 days.

Discussion

The unprecedented scale of this epidemic has indirectly affected both the health system and daily life in Liberia. Beginning in July 2014, the government of Liberia closed all schools, canceled sporting and community recreation events, and restricted public gatherings to limit the spread of infection. Because hospitals with EVD cases closed or were abandoned and many health clinics were temporarily shut, many Liberians avoided health care settings out of fear of infection. The long-term effect on public health will become evident with time, although adverse effects must be expected, including deterioration of core public health services such as routine immunizations, tuberculosis programs, HIV/AIDS treatment, and maternal and child health services. As hospitals reopen for regular care, adequate patient triage, PPE, and HCW training remain essential. Enhanced infection control must continue to be a major component of the national response, and protecting HCWs must be of paramount importance. The vulnerability from lack of proper infection control practices and inadequate PPE in health care settings is a strong lesson for other African countries as they prepare for potential EVD introduction.

Thousands of people in West Africa continue to work tirelessly to fight this epidemic. In Liberia, the MOHSW has continued to adjust strategies and organizational structures as it leads the response. Other CDC teams replaced the July–August team in Liberia; the West African EVD response is already the largest international outbreak response in CDC's history (*6*). However, in the same way that Liberia has isolated certain areas internally, the country itself faces isolation from the rest of the world. Fears around exposure to EBOV in social settings and lack of available medical care for conditions other than EVD prompted many expatriates and privileged Liberians to leave the country. Despite ongoing passenger screening programs, many airlines ceased flying into Monrovia, and some countries are refusing entry of travelers from Liberia. The economy has visibly contracted, prices of commodities and certain food items have increased, and national food security is an ongoing concern.

A "before and after" moment, before the West African EVD epidemic and after, has occurred in global health. The EVD epidemic in Liberia and other parts of West Africa reinforces the reality of global interconnectedness and common vulnerability from the weakest links in the chain, and remains a formidable challenge to the political and humanitarian solidarity of Africa and of the world. Continued support is required for efforts to implement and sustain effective border health measures to facilitate continued travel to the region, which is essential for the necessary flow of humanitarian aid.

Other than MOHSW, WHO, and MSF, principal collaborating organizations in the early response included, alphabetically: Action Contre La Faim; the International Committee of the Red Cross; the International Federation of the Red Cross; Rebuilding Basic Health Services; Samaritan's Purse; Tiyatien Health; the United Nations Mission in Liberia; United Nations Children's Fund; the United States Agency for International Development and its Office of Foreign Disaster Assistance; the United States Department of Defense; the United States Department of State through its Embassy in Liberia; and the United States National Institutes of Health.

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Dr. Arwady is a physician specializing in internal medicine and pediatrics. She is currently in CDC's Epidemic Intelligence Service and continues to work on the Ebola response. Her research interests include improving access to care and reducing health disparities in the United States and globally.

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Population Structure and Antimicrobial Resistance of Invasive Serotype IV Group B *Streptococcus*, Toronto, Ontario, Canada

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We recently showed that 37/600 (6.2%) invasive infections with group B *Streptococcus* (GBS) in Toronto, Ontario, Canada, were caused by serotype IV strains. We report a relatively high level of genetic diversity in 37 invasive strains of this emerging GBS serotype. Multilocus sequence typing identified 6 sequence types (STs) that belonged to 3 clonal complexes. Most isolates were ST-459 (19/37, 51%) and ST-452 (11/37, 30%), but we also identified ST-291, ST-3, ST-196, and a novel ST-682. We detected further diversity by performing whole-genome single-nucleotide polymorphism analysis and found evidence of recombination events contributing to variation in some serotype IV GBS strains. We also evaluated antimicrobial drug resistance and found that ST-459 strains were resistant to clindamycin and erythromycin, whereas strains of other STs were, for the most part, susceptible to these antimicrobial drugs.

Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae*, are a major cause of neonatal sepsis and meningitis and are increasingly being associated with invasive infections in nonpregnant adults (*1*–*3*). For instance, in 2011, adult cases accounted for nearly 90% of the burden of GBS disease in the United States (*4*). Elderly persons and those with preexisting conditions (e.g., diabetes mellitus, cirrhosis, cancer, and compromised immunity) are most at risk (*5*). The clinical features of GBS disease in adults range from localized tissue infection to severe bacteremia with shock (*1*). Less common clinical syndromes, such as endocarditis and meningitis, are associated with considerable illness and death (*1*).

GBS are classified into 10 serotypes (Ia, Ib, and II–IX) on the basis of a serologic reaction against capsular polysaccharide. The most common GBS serotypes causing invasive disease in the United States and Canada in adults and neonates are Ia, III, and V (*2*,*3*,*6*–*9*). However, recent reports have shown that serotype IV GBS is emerging in pregnant carriers and causing infections in neonates and adults in North America and several other regions (*10*–*14*). This emergence is of concern because GBS conjugate vaccines that are being developed to prevent invasive disease may protect only against serotypes Ia, Ib, II, III, and V, or combinations thereof (*15*,*16*).

Ferrieri et al. reported different genetic backgrounds among serotype IV GBS strains, which have been shown to include sequence types (STs) ST-452 and ST-459 (*11*). Horizontal gene transfer occurs frequently in GBS. For example, capsular switching from serotype III to IV has been recently reported and found to be the result of recombination involving the entire *cps* locus (*8*,*17*,*18*). GBS are considered to be universally susceptible to penicillins; therefore, these are the primary drugs used for prophylaxis and treatment of GBS disease (*19*,*20*). However, macrolide and lincosamide antimicrobial drugs, such as erythromycin and clindamycin, are often used to treat GBS infections in patients allergic or suspected to be allergic to β-lactam drugs. Acquisition of resistance against these drugs by horizontal gene transfer has been frequently described in GBS (*2*,*21*).

We recently reported a relatively high prevalence (6.2%) of serotype IV strains among GBS causing invasive infections in Toronto, Ontario, Canada (*8*). In the current study, we used whole-genome sequencing (WGS) to characterize the population structure of serotype IV GBS isolates. We describe a genetically heterogeneous population of isolates that differ in their antimicrobial drug resistance profiles.

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Methods

Bacterial Strains, Culture Conditions, DNA Preparations, Serotyping, and Pilus Typing

We used 37 serotype IV GBS strains collected during 2009–2012 by the Toronto Invasive Bacterial Disease Network, a population-based surveillance system for invasive bacterial diseases in metropolitan Toronto and the Peel region in Ontario, Canada (total population under surveillance ≈5.5 million persons in 2011). Laboratory-based surveillance involves the 28 hospitals providing care to and the 25 laboratories processing sterile site cultures for residents of the population area; laboratory personnel submit all GBS isolates from sterile sites to the central study laboratory. The sample was composed of all available serotype IV GBS strains determined by latex agglutination and corresponded to 6.2% of GBS identified during the study by the program (*8*). Only 1 strain per patient was included in the collection; there was no clear evidence of localized outbreaks caused by serotype IV GBS during the collection period. Strains are listed in online Technical Appendix Table 1 (http://wwwnc.cdc.gov/EID/article/21/4/14-0759-Techapp1.pdf).

Bacteria were grown at 37°C in an atmosphere of 5% CO_2 on Columbia agar plates supplemented with 5% sheep blood. Genomic DNA was obtained by using the QIAamp DNA Minikit (QIAGEN, Toronto, Ontario, Canada) according to the manufacturer's protocol for gram-positive organisms. The presence of pilus islands PI-1, PI-2a, and PI-2b was determined by using PCR as described (*22*).

WGS, Bioinformatics Methods, and Phylogenetic Analysis

WGS libraries were prepared for all 37 isolates by using Nextera XT Kits (Illumina, San Diego, CA, USA) and sequenced as paired-end reads with either Illumina HiSeq 2500 (100 bp + 100 bp) or MiSeq (150 bp + 150 bp) instruments (data are available at the Sequence Read Archive under accession no. SRP040805). Parsing of the multiplexed sequencing reads and removal of barcode information was performed by using onboard software. STs were derived directly from short reads by using SRST2 (https://github.com/katholt/srst2). Ambiguous or novel alleles were confirmed by PCR and Sanger sequencing.

Neighbor-joining phylogenetic trees (1,000 bootstrap replications) were then generated by using SplitsTree4 (*23*) and the concatenated sequences of the 7 loci used in the GBS multilocus sequence typing (MLST) scheme. For WGS-based phylogenies, we sequenced to closure the genomes of ST-459 strain NGBS061 and ST-452 strain NGBS572 (GenBank accession nos. CP007631 and CP007632, respectively) by using single-molecule real-time (SMRT) sequencing (Pacific Biosciences, Menlo Park, CA, USA). Two SMRT cells of sequence were generated for each isolate, which generated >220 Mb of data in reads >3 kb (37,218 reads, average length 6.1 kb for NGBS061; 54,803 reads, average length 5.6 kb for NGBS572). In brief, we used HGAP version 2 (*24*) to correct the long reads and Celera Assembler 7.0 (*25*) to assemble the corrected reads, followed by 2 rounds of polishing with Quiver (https://github.com/PacificBiosciences/GenomicConsensus). Coverage of final assemblies in reads >3 kb was 84× for NGBS061 and 111× for NGBS572.

To assess base-calling accuracy in the Pacific Biosciences assembly, Illumina short reads for the 2 isolates were aligned to their respective assemblies by using BLAT (*26*). Both genome assemblies were completely concordant with full-length perfectly aligning Illumina short-reads, except for NGBS061 in a region of increased AT richness, which had reduced Illumina coverage. For consistency, both genome assemblies were formatted to begin at the first nucleotide of the intergenic region immediately preceding the gene encoding the chromosomal replication initiation protein (*dnaA*; Genbank accession no. YP-328728).

Illumina short reads of all other isolates were aligned to the reference genomes, and coverage relative to the reference genome was calculated by using Mosaik (https://code.google.com/p/mosaik-aligner/). Polymorphisms were identified by using VAAL (*27*). A matrix file containing genotypes of all strains at each polymorphic locus was created from the VAAL polymorphism output data by using a custom script. Then, for each individual strain, single-nucleotide polymorphisms (SNPs) were concatenated in order of occurrence relative to the genome of the corresponding reference strain (see Results). Neighbor-joining phylogenetic trees (1,000 bootstrap replications) were then generated with SplitsTree4.

The A5 pipeline was used for de novo assembly of Illumina-sequenced GBS strains (*28*). Areas of recombination of the ST-196 strains NGBS447 and NGBS472 were defined by using BratNextGen software (*29*) run with 20 iterations and 100 replicates, using a p value of 0.05 as the significance cutoff. Genome visualizations were created by using BRIG (*30*) and edited by using Adobe Illustrator (Adobe Systems Inc., San Jose, CA, USA).

Antimicrobial Drug Susceptibility

MICs for penicillin, ampicillin, cefotaxime, levofloxacin, tetracycline, erythromycin, and clindamycin were determined by using the agar dilution method according to the Clinical and Laboratory Standards Institute (*31*). We also used SRST2 and a database of 1,913 variants of genes encoding antimicrobial drug resistance (https://github.com/katholt/srst2) to test for genetic determinants of antimicrobial drug resistance in genomes of serotype IV strains.

Results

Isolate Source and Age Distribution of Patients

The 37 serotype IV GBS isolates used in this study represent 6.2% of 600 GBS strains isolated in the Toronto–Peel metropolitan area of Ontario during 2009–2012 (*8*). One isolate was collected in 2009, 15 isolates in 2010, 9 isolates in 2011, and 12 isolates in 2012. Most isolates were from invasive cases in adults (19–59 years of age, n = 14) or older adults (≥60 years of age, n = 21) patients. We did not observe serotype IV GBS in neonates with early-onset disease (<7 days of age). However, 1 isolate was obtained from a child with late-onset disease (7–89 days of age), and an additional strain was isolated from a child 188 days of age. Most (34/37) serotype IV isolates were collected from blood; only 1 isolate was collected from soft tissue, and 2 isolates were collected from synovial fluid.

Genetic Diversity of Serotype IV GBS Strains

To assess genetic diversity of serotype IV GBS strains in our collection, we sequenced the genomes of all 37 strains and used SRST2 to derive MLST information directly from the short-read WGS data. We identified 6 STs representing 3 clonal complexes (CCs), which is indicative of a relatively high level of genetic diversity among our isolates. Two isolates were ST-291 included in hypervirulent CC17; 11 isolates were ST-452 (CC23); 2 isolates were ST-3 (CC1); and 1 isolate was a novel ST-682 included in CC1. Most (19/37) strains belonged to ST-459 (CC1). We also identified 2 strains of ST-196, a closely related single locus variant (*sdhA* gene) of ST-459. The 2 STs comprising most strains (ST-452 and ST-459) were also the most genetically distant, as shown by neighbor-joining phylogenetic analysis of concatenated sequences of alleles at all MLST loci (Figure 1, panel A).

To further understand genetic differences between these 2 STs, we arbitrarily chose 1 ST-452 strain (NGBS572) and 1 ST-459 strain (NGBS061) and sequenced their genomes to closure by using Pacific Biosciences SMRT technology. The genomes were circular chromosomes of 2,061,426 bp and 2,221,207 bp, respectively (online Technical Appendix Figure 1). We did not find evidence of any associated plasmids in either isolate. However, we used an SMRT sequencing protocol with a DNA size-selection step for DNA fragments ≥20 kb, which might have removed small plasmids that could have been present. The difference in genome size between the strains was explained primarily by the smaller number and size of mobile genetic elements found in NGBS572 than in NGBS061 (online Technical Appendix Figure 1). The core genomes of NGBS572 and NGBS061 (i.e., ≈1.9-Mbp portion of the genome lacking mobile genetic elements that is conserved in gene content between both strains) differed by 13,158 biallelic SNPs and 852 insertions or deletions (indels).

We next investigated genetic diversity within the ST-452 and ST-459 groups. Both groups were relatively homogeneous. On average, ST-452 strains differed from the ST-452 reference strain NGBS572 by only 45 SNPs and 10 indels in the core genome. On average, ST-459 strains had only 68 SNPs and 16 indels relative to the core genome of the ST-459 reference strain NGBS061. Neighbor-joining phylogenetic trees constructed by using concatenated SNPs in the core genome showed additional but limited diversity among isolates within each ST and some clustering within ST-459 (Figure 1, panels B,C).

Other STs in our collection had either 1 or 2 strains. Thus, we chose not to sequence these genomes to closure to assess genetic diversity but instead identified polymorphisms relative to the ST-452 and ST-459 reference strains. Both ST-3 strains showed a comparable number of polymorphisms against each reference strain (online Technical Appendix Table 2). Similar results were observed for each of the ST-291 strains (i.e., both strains differed from the ST-452 and ST-459 reference strains by a comparable number of polymorphisms) (online Technical Table 2). In contrast, the 2 ST-196 strains greatly differed between themselves in the number of polymorphisms separating them from the reference strains (online Technical Appendix Table 2). One ST-196 strain, strain NGBS447, had 384 core SNPs and 60 core indels relative to the ST-459 reference strain, where the second ST-196 strain, NGBS472, had 1689 core SNPs and 124 core indels relative to the ST-459 reference strain. Further analysis discovered that the excess polymorphisms in NGBS472 were not evenly distributed across the genome. Instead, some discrete regions of the genome showed an overabundance of polymorphisms, which is suggestive of recombination.

Using BratNextGen, we precisely defined these regions of recombination (online Technical Appendix Figure 2). When these regions of recombination were excluded from the analysis, strain NGBS447 had 107 SNPs and 16 indels relative to the ST-459 reference strain and NGBS472 had 281 SNPs and 128 indels relative to the same reference genome. Genome regions having undergone recombination in strain NGBS472 included genes encoding major virulence factors, such as the 2-component system *csrRS* (*32*) and pili (*33*). Pili in GBS are encoded by genes found in 2 distinct genome locations and organized in so-called pilus island 1 (PI-1) and PI-2. PI-2 has 2 variants (PI-2a or PI-2b) (*33*). One of the ST-196 strains had a pilus profile of PI-1 + PI-2a, and the other had a profile of PI-1 + PI-2b (online Technical Appendix Figure 3). Except for these ST-196 strains, strains of the different STs had consistent pilus island profiles (online Technical Appendix Figure 3).

Antimicrobial Drug Susceptibility of Serotype IV GBS Strains

All serotype IV GBS strains were susceptible to all β-lactams, vancomycin, and levofloxacin. We observed resistance to erythromycin and clindamycin in 20/37 (54%) and 19/37 (51%) of the isolates, respectively; 23 (62%) of the isolates were resistant to tetracycline. The proportion of resistance and susceptibility, as well as 50% MICs and 90% MICs, are shown in online Technical Appendix Table 3. All ST-459 isolates were resistant to clindamycin and erythromycin; ST-452 isolates were susceptible to these antimicrobial drugs (Figure 2; online Technical Appendix Table 4). Strains of other STs were susceptible to these 2 drugs, except 1 of the ST-291 strains, which was resistant to erythromycin but susceptible to clindamycin (Figure 2; online Technical Appendix Table 4). Macrolide resistance in ST-459 strains is probably attributable to the action of *ermTR*, a gene that was detected in all ST-459 strains (online Technical Appendix Table 1). We also identified the *ermT* gene in the genome of the erythromycin-resistant ST-291 strain (online Technical Appendix Table 1). Resistance to tetracycline correlated with presence of the *tetM* gene (online Technical Appendix Table 1) and was observed among strains of all STs except ST-452. However, we identified 3 ST-459 strains that were susceptible to tetracycline (Figure 2; online Technical Appendix Table 4). One of these had the *tetM* gene, but genome data showed a 7-nt deletion in the gene, which was predicted to result in early termination of translation. We did not detect *tet* genes in the other 2 ST-459 strains (online Technical Appendix Table 1).

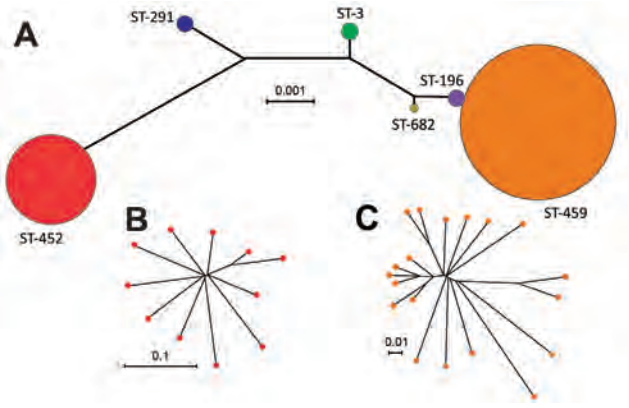

Figure 1. Inferred genetic relationships of invasive serotype IV group B *Streptococcus* (GBS), Toronto, Ontario, Canada. A) Neighbor-joining phylogenetic tree constructed by using the concatenated sequences of the 7 gene loci (*sdhA*, *adhP*, *tkt*, *glcK*, *atr*, *pheS*, and *glnA*) used in the multilocus sequence typing (MLST) scheme for GBS. Each circle represents a single MLST sequence type (ST); circle colors differentiate the 6 STs found among strains in our collection and their sizes are proportional to the number of isolates. B) Neighbor-joining phylogenetic analysis based on concatenated sequences of 316 nonredundant single-nucleotide polymorphism (SNP) loci identified by whole-genome sequencing relative to the core genome ST-452 reference strain NGBS572, which shows further diversity within ST-452. C) Diversity within ST-459 serotype IV GBS shown by neighbor-joining phylogenetic analysis based on concatenated sequences of 526 nonredundant SNP loci relative to the core genome of ST-459 reference strain NGBS061. Scale bars indicate nucleotide substitutions per site.

Discussion

The prevalence of serotype IV GBS is increasing globally among pregnant women; infections in neonates and invasive infections in adults are also increasing. Large proportions of serotype IV GBS isolates have been reported among carrier isolates in the United Arab Emirates (26.3%) and Turkey (8.3%) (*34*,*35*). In Zimbabwe, serotype IV strains accounted for 5.1% of GBS isolates from carrier pregnant women and 4.0% of all GBS strains isolated from hospitalized patients (*36*). A recent report found a relatively high percentage (16%) of serotype IV strains among cases of GBS early-onset disease in Minnesota (*11*). In a longitudinal study in the United States, the proportion of this serotype among all GBS strains isolated from nonpregnant adults increased from 0.2% in 1998–1999 to 5.7% in 2005–2006 (*3*).

In Canada, a country-wide survey conducted in 1996 found no serotype IV GBS cases in 79 nonpregnant adult women (*9*). However, we recently reported that serotype IV GBS in the greater Toronto area during 2009–2012 accounted for 6.2% of all invasive GBS infections (*8*). Cases in children were rare, and most (94%) serotype IV GBS invasive infections in Toronto were in adults ≥19 years of age. It may be possible that the low incidence of serotype IV in neonates, and the absence of cases of early-onset disease, is a reflection of a low prevalence of colonizing serotype IV GBS in women of childbearing age. Future studies to determine the prevalence of serotype IV GBS in pregnant women will be useful in elucidating the reasons for this apparent absence of serotype IV GBS neonatal invasive disease.

Given the restricted geographic and temporal origin of our collection of invasive serotype IV GBS strains, we hypothesized that their emergence might represent the successful expansion of a single clone. However, other GBS serotypes usually encompass strains with different genetic backgrounds that might have different tissue tropism and virulence potential (*37*). Thus, an alternative hypothesis was that our serotype IV GBS collection was composed of strains of diverse genetic backgrounds and that ≥1 clones underwent expansion.

To begin to differentiate between these and other hypotheses, we conducted WGS and evaluated the population structure of our collection. Results showed a relatively high level of genetic diversity among the 37 serotype IV

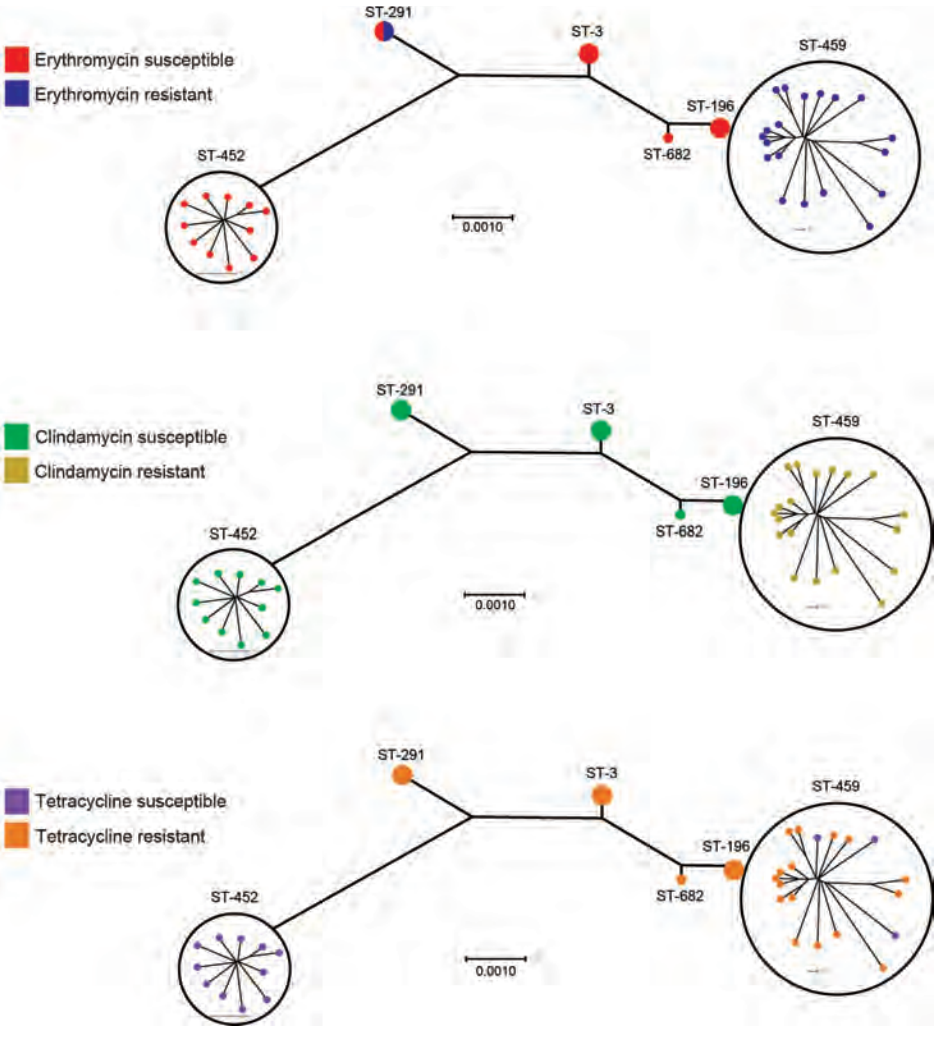

Figure 2. Susceptibility to erythromycin, clindamycin, and tetracycline among serotype IV group B *Streptococcus* and sequence types (STs), Toronto, Ontario, Canada. All ST-459 strains and 1 ST-291 strain were resistant to erythromycin; we detected *ermTR* and *ermT* genes in all erythromycin-resistant strains (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/4/14-0759-Techapp1.pdf). Resistance to clindamycin was observed only among ST-459 strains. Resistance to tetracycline was common among all STs except ST-452 and correlated with presence of the *tetM* gene in isolates (online Technical Appendix). Scale bars indicate nucleotide substitutions per site.

GBS isolates, which were assigned to 6 MLST STs and 3 CCs. Two of our invasive serotype IV isolates belonged to ST-291, a member of the hypervirulent CC17. Previous studies have shown that several strains of this ST have acquired a serotype IV capsule by recombination involving exchange of the full capsule-encoding *cps* locus (*8*,*17*). We also found 2 ST-3 strains and a novel ST-682 strain, both included in CC1, one of the major CCs associated with human GBS disease. Our collection contained 2 strains of ST-196 (CC1). WGS showed that the genomes of these 2 ST-196 strains differed extensively, and further analysis indicated that these genome differences were likely caused by recombination. These findings highlight the major role of horizontal gene exchange of extensive regions of DNA in GBS diversification (*38*) and expose the limitations of MLST to fully identify GBS diversity.

ST-452 (CC23) and ST-459 (CC1) were the 2 most common STs in our collection. These 2 STs have recently been described and were found in invasive serotype IV strains that caused disease in Minnesota (*10*,*11*). The reasons why these 2 highly divergent genomic backgrounds seemingly thrive among serotype IV GBS strains that caused invasive disease in Toronto remain to be investigated. However, SNP analysis showed a low level of genetic diversity among strains of each ST-452 and ST-459, which is compatible with recent emergence and ongoing clonal expansion. These STs were also the most phylogenetically distant groups in our collection. We showed that ST-452 and ST-459 genomes differ greatly in size, largely because they have different mobile genetic elements, such as prophages and different pilus islands. The core genomes of these 2 most prevalent STs also differed considerably, as shown by SNP analysis.

Another difference among the groups was that ST-459 strains were resistant to clindamycin and erythromycin, and for the most part to tetracycline, whereas ST-452 isolates were susceptible to these drugs. We associated resistance to macrolides with presence of the *ermTR* gene in

all ST-459 strains. The increase in the number of cases of invasive disease caused by serotype IV isolates and concurrent emergence of clindamycin resistance among a successful clone of this serotype could be an early indication of the beginning of clinical problems similar to those observed for serotype V GBS (*39*). Resistance to erythromycin was observed in 1 ST-291 strain and correlated with presence of the *ermT* gene. Tetracycline resistance was observed in 23 (62%) of the serotype IV GBS isolates, and in most instances correlated with presence of the *tetM* gene.

Capsular switching has been described frequently in GBS (*22*,*40*). At least in part, our results suggest that different lineages of serotype IV GBS have independently acquired a serotype IV capsule from unidentified donors by recombination. As the global presence of serotype IV continues to increase, monitoring of this emerging serotype is warranted. Conjugate vaccines that are being developed should include polysaccharide and/or associated GBS virulence proteins of this serotype, or they risk not being completely protective against the full range of GBS disease.

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Norovirus Genotype Profiles Associated with Foodborne Transmission, 1999–2012

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Worldwide, noroviruses are a leading cause of gastroenteritis. They can be transmitted from person to person directly or indirectly through contaminated food, water, or environments. To estimate the proportion of foodborne infections caused by noroviruses on a global scale, we used norovirus transmission and genotyping information from multiple international outbreak surveillance systems (Noronet, CaliciNet, EpiSurv) and from a systematic review of peer-reviewed literature. The proportion of outbreaks caused by food was determined by genotype and/or genogroup. Analysis resulted in the following final global profiles: foodborne transmission is attributed to 10% (range 9%–11%) of all genotype GII.4 outbreaks, 27% (25%–30%) of outbreaks caused by all other single genotypes, and 37% (24%–52%) of outbreaks caused by mixtures of GII.4 and other noroviruses. When these profiles are applied to global outbreak surveillance data, results indicate that ≈14% of all norovirus outbreaks are attributed to food.

Noroviruses are a leading cause of gastroenteritis worldwide. They belong to the family *Caliciviridae* and consist of an ≈7.5-kb genome in 3 open reading frames (ORFs). The first ORF (ORF1) encodes a polypeptide; ORF2 encodes the viral capsid protein (VP1); and ORF3 encodes a minor structural protein (VP2). Noroviruses are classified into at least 6 genogroups, GI–GVI (*1*). According to a recent unified proposal for nomenclature, genogroups are further subdivided into at least 38 genetic clusters (genotypes) (*2*). Noroviruses are environmentally stable (*3*) and can be transmitted by different routes (e.g., foodborne, personborne, waterborne, and environmental). Determining the transmission route during an outbreak investigation is complicated because transmission can occur by multiple routes in a single outbreak. After primary introduction of the virus through food, secondary person-to-person and environmental transmission can rapidly take over, making it hard to trace the disease back to contaminated food. Another complexity is that foodborne transmission can follow different routes as well; food can be contaminated during production (*4*) or during handling by an infected food handler (*5*).

Different exposure attribution methods (i.e., epidemiologic investigations, microbiological typing/subtyping, intervention studies, and expert elicitations) have been used to estimate the foodborne proportion of the overall disease incidence caused by a pathogen. Each approach has its advantages and disadvantages, and therefore the use of multiple methods has been recommended (*6*). Information about pathogen strain or subtypes may be of value for attribution but is dependent on substantial amounts of contextual data. For example, a method commonly used to attribute *Salmonella* spp. infections to a specific source uses strain collections representative of the pathogen in each of these sources (*7*).

For noroviruses, genogroup-specific differences have been reported with regard to environmental persistence (*8*), sensitivity to removal (*9*), and binding to receptors (*10*). These biological differences may underpin strain-specific epidemiologic patterns, suggesting a potentially useful approach for norovirus attribution. Such an approach was recently developed in a norovirus attribution study, which showed that the proportion of foodborne and person-to-person outbreaks differed between genotypes; the GI genotypes were more likely to be foodborne, and the II.4 genotype was more likely to be personborne (*11*). These findings indicate that genotype profiles may help distinguish which outbreaks are more likely to be foodborne than personborne. Also, a recent study on norovirus outbreaks in the United States showed that GI.3, GI.6, GI.7, GII.3, GII.6, and GII.12 were the norovirus genotypes most often associated with foodborne outbreaks and that, of the outbreaks with a known transmission route,

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16% were foodborne (*12*). Norovirus infections, however, are a global problem, and efforts are under way to estimate the global social and economic costs of foodborne norovirus illness (*13*,*14*). To estimate the proportion of outbreaks attributed to foodborne transmission from a global perspective, we used aggregated norovirus outbreak data and genotyping information from different outbreak surveillance systems and from peer-reviewed literature.

Methods

Data from 4 sources were available for comparison: 3 laboratory-based norovirus outbreak surveillance network databases and 1 systematic review of norovirus outbreaks in the peer-reviewed published literature. Laboratory surveillance networks aim to link norovirus outbreaks that may be caused by common sources (such as food), monitor genotype trends, and identify emerging norovirus strains. Relevant data from these networks included transmission route(s) and norovirus genotyping performed by sequence analysis of the polymerase (ORF-1) and capsid (VP1/ORF-2) regions of the norovirus genome. Polymerase and capsid sequences were not available for all samples, requiring separate analyses.

Databases

Foodborne Viruses in Europe Network/Noronet

The Foodborne Viruses in Europe (FBVE) network was started in 1999 as a European Union–funded combined laboratory and epidemiological network of 13 European countries sharing norovirus outbreak data (*15*). Since 1999, the FBVE network has maintained a joint database in which members have shared their data. In 2009, Noronet was started as a continuation of the FBVE network (*13*). Noronet is an informal network of scientists working in public health institutes or universities that share virologic, epidemiologic, and molecular data on norovirus. The network now includes laboratories in countries outside Europe. Although all laboratories use PCR-based methods for norovirus detection, the detected parts of the norovirus genome may differ between laboratories; consequently, genotyping is not necessarily based on the same part of the genome. Sequence data shared in the FBVE/Noronet database include regions A and B (polymerase) and/or regions C and D (capsid) of the genome (*15*). Data from 5,583 outbreaks that occurred from January 1999 through December 2012 are included in the analyses.

CaliciNet

The US Centers for Disease Control and Prevention developed CaliciNet in 2009 (*16*). CaliciNet is a national norovirus outbreak surveillance network of public health and food regulatory laboratories that submit epidemiologic information and PCR-based norovirus detection and typing information, including regions C and D (capsid) sequences of the genome, to a national database by using standardized protocols (*12*,*16*). As of November 2013, participants from 32 public health laboratories in 28 states have been certified to participate in CaliciNet. Data from 3,094 outbreaks reported from September 2009 through December 2012 are used in the analyses.

Institute of Environmental Science and Research–EpiSurv

In New Zealand, gastroenteritis outbreaks are reported by public health units that submit epidemiologic information to the EpiSurv database, a surveillance system operated by the Institute of Environmental Science and Research (ESR) for the New Zealand Ministry of Health. As part of the New Zealand norovirus surveillance, the ESR Norovirus Reference Laboratory conducts norovirus detection testing by using PCR-based methods (*17*) followed by genotyping of at least 1 case per outbreak. Sequence data are obtained from region B and/or region C of the genome (*18*). Combined data from the ESR Norovirus Reference Laboratory and EpiSurv are summarized in annual outbreak reports (https://surv.esr.cri.nz/surveillance/annual outbreak). Data from 819 outbreaks reported from January 2008 through December 2012 are included in the analyses.

Systematic Literature Review

In 2012, Matthews et al. (*19*) published results of a systematic review of all norovirus outbreaks reported in the scientific literature from 1993 through 2011. We updated this systematic review to include norovirus outbreaks published in 2012 (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/4/14-1073-Techapp1.pdf). During September 2012, we searched the literature in the EMBASE, Medline, Web of Science, and Global Health databases for the term "norovirus" and related terms (*19*). Two independent reviewers screened titles and abstracts for relevance. We included articles published in 2012 that fit the following criteria designated by Matthews et al.: 1) full article, 2) published in English, 3) describes human norovirus outbreaks, 4) used PCR-based diagnostics for at least 1 case per outbreak (*19*). We excluded articles describing sporadic norovirus cases and articles describing outbreaks among immunocompromised patients (e.g., transplant recipients). Separate outbreak-level data were extracted from articles reporting multiple outbreaks, but the articles' citations were not referenced for outbreak reports. Genogroup and genotype information (and if available, the PCR target region) was extracted.

Definitions

Transmission Routes

To determine the proportion of outbreaks that were caused by foodborne transmission of the virus (hereafter called the

foodborne proportion) we accepted the route(s) assigned in the various databases to each outbreak, based on previous investigation of the FBVE database (*11*) and based on the local public health investigation and with the assistance of standardized guidance materials to provide some consistency (http://www.cdc.gov/mmwr/preview/mmwrhtml/rr6003a1.htm). If the primary or secondary mode of transmission was reported as foodborne, this outbreak was considered a foodborne outbreak (includes foods contaminated during production or during handling). If person-to-person transmission was reported to be one of the possible routes, the outbreak was considered a person-to-person outbreak. We excluded outbreaks for which transmission route was listed as environmental or waterborne only (1 for ESR-EpiSurv, 0 for CaliciNet, 68 for FBVE/Noronet).

Genotype Profiles

Each of the surveillance databases used genotypes as previously proposed (*2*). For the purpose of attribution, the proportion of outbreaks listed as being transmitted by the foodborne route was calculated for each polymerase genotype (P-type) or capsid genotype (C-type) individually. The proportions of all individual genotypes that were transmitted by the foodborne route, on the basis of a single data source, were termed genotype profiles and were aggregated into categories of profiles according to the analyses described in the following sections.

Analyses

Comparison of Data to Test Robustness of Genotype Profiles

First, to test whether the genotype profile–based data from 1999–2012 could be generalized, we analyzed genotype data for differences in time (i.e., yearly). This analysis for robustness over time was performed for the FBVE/Noronet database only. Second, to test whether the genotype profiles based on these 2 genomic regions (P-type and C-type) for typing could be combined, we analyzed genotypes based on polymerase sequences if they matched with genotype profiles based on capsid sequences. Because the FBVE and ESR-EpiSurv data had capsid and polymerase sequences available, the comparison was performed for these datasets. Third, we tested whether the analysis by individual genotypes could be aggregated into larger categories on the basis of how well their foodborne proportions corresponded. For this purpose, the profile of each individual genotype was compared with a profile consisting of sequences that belonged to 1 of 4 categories (genogroup aggregation): GI, GII genotype 4 (GII.4), GII but not GII.4 (GIInon4), and mixed (genotypes belonging to multiple genogroups). Categories were further aggregated into larger categories if the aggregation did not significantly affect the results. Thus, aggregation into categories was performed in the following 3 steps: 1) if the foodborne proportions of an individual genotype did not statistically differ from 1 of the 4 categories, genotypes were merged into this category; 2) if categories did not statistically differ from each other, these categories were merged into larger categories; and 3) if profiles of different data sources did not statistically differ (on the basis of their CIs), these profiles were merged and considered as a profile from 1 data source.

Comparison of Worldwide Data and Estimating the Foodborne Proportion

We compared the outcomes after the genotypes were aggregated into the proposed categories of all 4 datasets. We used the resulting profiles to estimate the foodborne proportion among outbreaks for which genotype was known but transmission mode was not known and thereby to estimate the foodborne proportion of norovirus outbreaks in different parts of the world.

Statistical Analyses

For each genotype in the surveillance systems, we estimated the fraction of outbreaks for which origin was known to be foodborne or person-to-person on the basis of the foodborne proportion of all foodborne plus person-to-person outbreaks for each genotype. We used the estimated proportion of foodborne outbreaks of all foodborne plus person-to-person outbreaks in each genotype to estimate the probability that outbreaks with unknown transmission mode were foodborne. We calculated 95% CIs by using the Monte Carlo simulation with 10,000 random draws from the β distributions, which are the posterior probabilities of the proportions (*20*). If 95% CIs overlapped, the genotypes were considered not statistically significantly different and genotypes were aggregated into profiles of larger groups. Data were analyzed by using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA).

Results

The FBVE/Noronet database included 5,583 norovirus outbreaks reported from 22 countries during 1999–2012 (Figures 1, 2). Of these, C-type sequence information was available for 4,580 outbreaks, P-type for 2,195 outbreaks, and both types for 1,192 outbreaks. The CaliciNet database included information about 3,094 outbreaks that occurred in the United States during 2009–2012; C-type sequence information was available for all outbreaks. The ESR-EpiSurv database included 818 outbreaks reported from New Zealand during 2008–2012, of which C-type and P-type information was available for 813 and 685, respectively. Our updated systematic review (online Technical Appendix) provided information on 966 norovirus outbreaks, of which genotype and transmission mode information was available for 608 (127 C-type, 107 P-type, 374 both) from

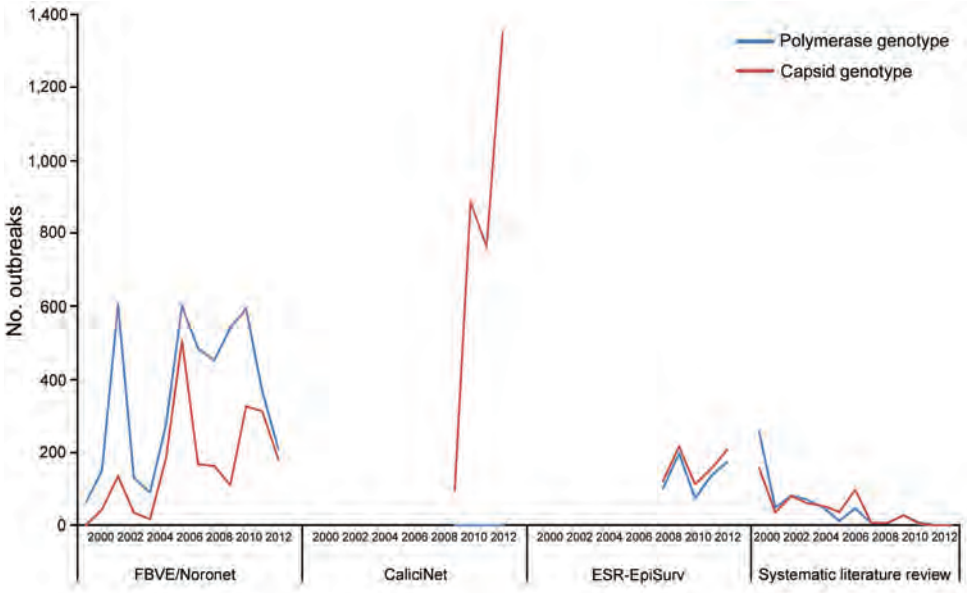

Figure 1. Norovirus data for which the genotyped region and transmission mode were reported in different surveillance systems: FBVE/Noronet, 5,583 outbreaks in 22 countries (1999–2012); CaliciNet, 3,094 outbreaks in 1 country (2009–2012); ESR-EpiSurv, 818 outbreaks in 1 country (2008–2012); and systematic literature review, 808 outbreaks in 61 countries (1993–2011). ESR, Institute of Environmental Science and Research; FBVE, Foodborne Viruses in Europe.

61 countries during 1983–2010 (Figures 1, 2). Our updated systematic literature search yielded reports of 320 outbreaks in Japan, 113 in the United States, 500 in other countries, and 18 in multiple countries; country information was missing for 15 outbreaks.

Robustness of Genotype Profiles

Initial analysis showed substantial differences for the estimated proportion of foodborne outbreaks caused by each genotype, depending on the dataset used, the time of reporting, and the method of typing, most likely because of low numbers of outbreaks for some genotypes (online Technical Appendix Table). These differences were no longer observed when the data were aggregated into 4 categories (GI, GII.4, GIInon4, and mixed genotype outbreaks) and later into 3 categories (GII.4, all other single genotypes, and mixed genotype outbreaks).

With respect to time trend, the comparison of the yearly profiles based on FBVE/Noronet data showed variation in the proportions of outbreaks with foodborne origin per category over time (Figure 3). Because of this finding, together with the knowledge that norovirus is known for its emerging new variants (*13*), the synchronization of time frames of different surveillance databases was considered necessary.

Our analyses showed differences in foodborne proportions for outbreaks for which the C-type was determined and for outbreaks for which the P-type was determined (online Technical Appendix Table). This difference is possibly a surveillance artifact caused by multiple countries contributing to FBVE/Noronet, because this effect was not seen in the ESR-EpiSurv profiles (online Technical Appendix Table), in which P and C types were both available for 685 outbreaks. For this reason the profiles for each genomic region were kept separately.

With respect to the genotypes versus aggregated categories, for FBVE/Noronet and ESR-EpiSurv data, aggregation of individual genotypes into the GI and GIInon4 categories showed that these cannot be statistically distinguished but that they differ from the categories of the GII.4 genotype and mixed outbreaks. GII.4 and mixed outbreaks show statistical difference and need to be treated as separate categories (online Technical Appendix Table). For CaliciNet and the updated systematic review data, results were similar to those of FBVE/Noronet and ESR-EpiSurv but with one difference (i.e., that mixed outbreaks could not be statistically distinguished as a separate group in the CaliciNet dataset). On the basis of these results, we used the following criteria for subsequent profile comparisons: 1) assignment into 3 categories (GII.4, all other single genotypes, and mixed genotypes); 2) separate analysis of data based on P-type and C-type; and 3) synchronized periods for different surveillance systems (2009–2012).

Database Comparisons

For outbreaks reported during 2009–2012, the P-type profiles from the New Zealand surveillance system, the European surveillance system, and from other countries contributing to Noronet were comparable (Table). Attribution profiles from CaliciNet showed similar values for the categories of GII.4 and nonGII.4 outbreaks but different values for the group of mixed outbreaks. However, this finding was based on 31 (1.0%) of 3,094 outbreaks. Because of this low number and the knowledge that how these outbreaks are ascertained in the respective surveillance systems might differ, we considered aggregation of the mixed outbreaks

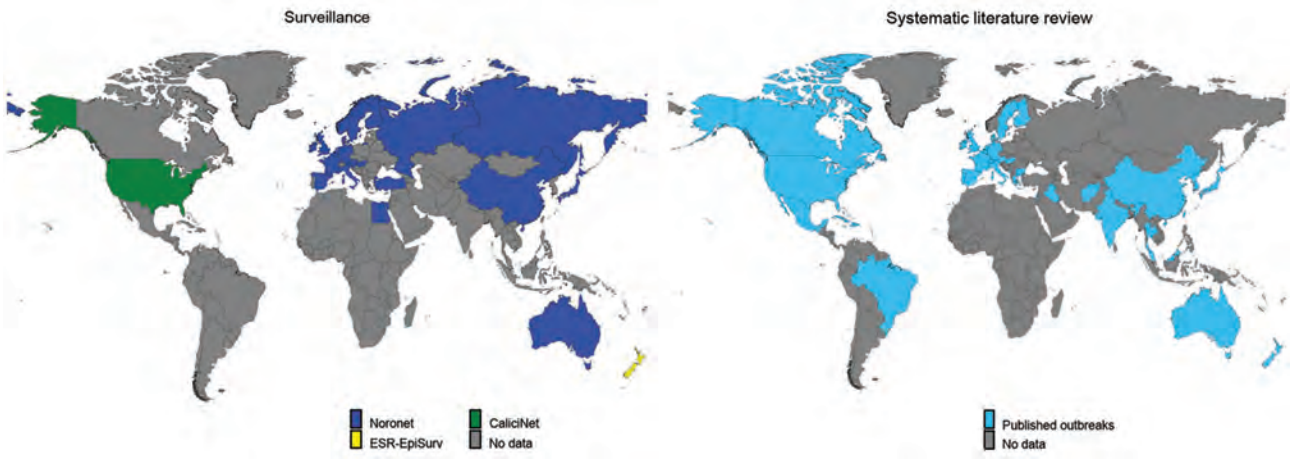

Figure 2. Countries from which norovirus outbreak reports were included in analyses of norovirus genotype profiles associated with foodborne transmission, according to Foodborne Viruses in Europe/Noronet (1999–2012), CaliciNet (2009–2012), ESR-EpiSurv (2008–2012), or systematic literature review (1993–2011). ESR, Institute of Environmental Science and Research.

category of the 3 surveillance databases justified. This consideration is strengthened by the finding that foodborne proportions are similar for the other 2, far more common, categories (GII.4, GIInon4).

The profiles resulting from the updated systematic review differed, but not significantly, probably because only 8 outbreaks were selected for the included time span of 2009–2012. This finding resulted in no distinguishable categories, and therefore this dataset was excluded from further calculations. On the basis of this analysis, for food attribution purposes, the profiles from the United States, New Zealand, Europe, and other countries contributing to Noronet can be merged into a global profile (Table).

Foodborne Attribution of Norovirus Outbreaks

Applying the profiles per surveillance database, the proportion of outbreaks attributed to foodborne transmission varied slightly (12% for FBVE/Noronet, 13% for ESR-EpiSurv, and 16% for CaliciNet), the aggregated global estimate was 13.7%. Overall, 10% (range 9%–11%) of all GII.4 norovirus outbreaks, 27% (25%–30%) of outbreaks caused by all other single genotypes, and 37% (24%–52%) of outbreaks with mixtures of GII.4 and other genotypes were attributed to foodborne transmission. Applying the global attribution profile to all outbreaks reported worldwide in surveillance systems in 2009–2012, of the outbreaks with an unknown mode of transmission, 193 (14.5%) of 1,332 outbreaks could be attributed to food.

Discussion

This analysis of aggregated surveillance datasets of norovirus outbreaks suggests that genotyping can provide useful information for attribution. At 14%, the estimated proportion of outbreaks attributed to foodborne transmission is comparable across the 3 independent datasets. We found that the proportion of outbreaks caused by foodborne transmission was lower for outbreaks caused by GII.4 norovirus than for those associated with all other genotypes (Table). This finding is consistent with previous findings (*11*). GII.4 viruses are notorious for their potential to spread easily from person to person and their rapid evolution (*21*). GII.4 viruses are more often described as causing outbreaks in (semi-)closed settings (*12*,*18*,*22*–*24*), implying a higher proportion of person-to-person spread as well. Although proportionally low, the absolute contribution of GII.4 foodborne outbreaks to the social and economic costs of outbreaks caused by noroviruses is considerable, given the numbers of outbreaks caused by this genotype.

Although the calculations for mixed infections were based on low numbers, mixed norovirus infections were less frequently associated with foodborne outbreaks in the United States than in the other countries for which data were available. Mixed infections have been associated with sewage-related outbreaks (*25*). Further research is needed to confirm whether our findings reflect true differences or differences in laboratory and investigative practices.

The use of internationally collected surveillance data and published outbreak reports is potentially associated with different kinds of biases. Therefore, our study has some limitations.

First, the differences between the surveillance data and the data reported in the literature gathered by Matthews et al. (*19*) (and in our update in the online Technical Appendix) in terms of foodborne proportion suggests the possibility of publication bias in favor of foodborne outbreaks. This bias has been described; reports of larger foodborne outbreaks with severe outcomes are more likely to be published (*26*). This hypothesis is supported by the

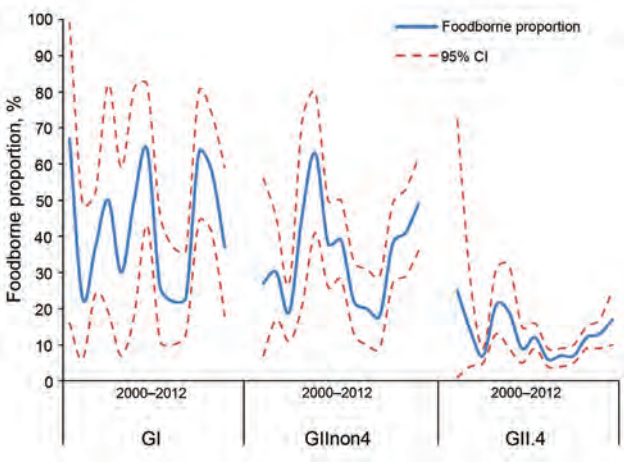

Figure 3. Genotype profiles. Foodborne proportion per genotype group per year, as reported to Foodborne Viruses in Europe/Noronet, with polymerase genotypes (n = 4,580) or, if missing, capsid genotypes (n = 1,003).

higher proportion of foodborne outbreaks caused by GII.4 and nonGII.4 found in the literature review.

Second, combining retrospective data from countries that differ in surveillance setup, coverage, and continuity is difficult. Individual cases of norovirus infection are not nationally notifiable in most of Europe, the United States, or New Zealand. Ascertainment and reporting of foodborne outbreaks probably varies substantially by setting (*27*) or between countries because of differing priorities and the complexity of the food chain. Despite this limitation, there was no feasible way to verify the transmission designation for every sample. Nevertheless, previous investigation of assignment of transmission modes within the FBVE network (*28*) showed that 2 of 13 countries used personborne transmission as a diagnosis of exclusion. Therefore, we consider that our estimate of the foodborne proportion is probably diluted and thus conservative. Ideally, data would be collected prospectively worldwide in a systematic approach, but until that is feasible, we consider our approach of using the data available from surveillance systems acceptable as best practice.

Third, differences in results based on P and C typing may reflect biases. This limitation mainly involves the FBVE/Noronet database, which includes data from multiple countries. For some countries, like the Netherlands, polymerase genotyping is the standard but capsid genotyping is performed for more thoroughly investigated outbreaks. Examples of reasons for additional capsid sequence typing are to find transmission chains (*29*,*30*), to confirm whether a new GII.4 variant is emerging (*22*), or to look for recombinants when suspected (*31*). Therefore, the FBVE/Noronet profiles based on capsid genotyping may be biased toward more unusual outbreaks and thereby possibly more often toward foodborne outbreaks. The FBVE network previously investigated the country-specific approaches (*27*,*28*) showing that, for example, Denmark has a surveillance system strongly focused on foodborne outbreaks, whereas in the United Kingdom, outbreaks in institutional settings are more often reported. This difference was reflected in the genotype profiles of these countries, thus confirming the different genotype profiles. In the United States, capsid sequencing is the standard, regardless of the outbreak circumstances; polymerase genotypes are not routinely uploaded to CaliciNet. In New Zealand, genotyping is based on the polymerase and capsid regions (if both can be determined), regardless of the outbreak circumstances. This systematic approach to genotyping regardless of the outbreak circumstances may explain why the US capsid profile and both the ESR-EpiSurv capsid and polymerase profiles are most similar to the FBVE/Noronet polymerase profile; these profiles are the result of systematic surveillance. A recent illustration of the value of performing both polymerase and capsid genotyping is the global emergence of the GII.4 Sydney 2012 variant (*32*). This variant is a GII.4 recombinant (*33*) with a GII.4 C-type and a GII.e P-type (*34*). Distinction of GII.4 versus nonGII.4 would not be possible if only polymerase-based genotype profiles were used.

Despite the shortcomings in working with surveillance data or data available in the public domain, our study showed that the proportion of norovirus outbreaks attributed to foodborne transmission is comparable in different parts of the world. The proportion of norovirus outbreaks attributed to foodborne transmission is in the same order of magnitude as the 17% found in an expert elicitation study from the Netherlands (*35*) and 11% in the United Kingdom as estimated from outbreak surveillance data (*36*). This similarity strongly

Table. Proportion of foodborne outbreaks per category, estimated according to different databases for 2009–2012*

	Database				
Genotype	FBVE/Noronet, n = 1,715	ESR-EpiSurv, n = 584	CaliciNet, n = 3,094	Systematic literature review, n = 8	Global profile, n = 5,393
GII.4	*0.07 (0.06–0.09)*	*0.09 (0.06–0.11)*	*0.12 (0.10–0.14)*	*0.12 (0.00–0.40)*	0.10 (0.09–0.11)
Other genotypes	*0.31 (0.25–0.37)*	*0.25 (0.17–0.33)*	*0.26 (0.23–0.30)*	*0.67 (0.16–0.97)*	0.27 (0.25–0.30)
Mixed outbreaks	*0.75 (0.48–0.94)*	*0.50 (0.20–0.75)*	**0.16 (0.05–0.33)**	CNBC	0.37 (0.24–0.52)
Foodborne proportion†	0.12	0.13	0.16	CNBC	0.14

*FBVE/Noronet polymerase profile was used to compare with ESR-EpiSurv polymerase, systematic literature review polymerase, and CaliciNet capsid profiles. CNBC, could not be calculated; ESR, Institute of Environmental Science and Research; FBVE, Foodborne Viruses in Europe. Estimates in italics do not statistically differ from the FBVE/Noronet polymerase profile as a reference; estimates in boldface do statistically differ.
†Proportion of outbreaks with an unknown mode of transmission estimated to be attributed to food.

indicates that this microbiology-based attribution method is robust, albeit in need of continued refinement (*6*). With ≈1 in 7 norovirus outbreaks being attributable to food, the foodborne transmission route represents a major target for intervention, particularly given the possibility of widespread exposures and the possibility of preventing not only primary but also secondary cases if contaminated foods are recalled from the market. Given the high incidence (*37*) and prevalence (*38*) of norovirus infections, norovirus has become a major cause of foodborne illness worldwide. To improve estimates of the social and economic costs of norovirus illness, future research should be aimed at filling the data gaps and should include nonindustrialized countries while aiming for global coverage of norovirus surveillance data.

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Deaths Associated with Respiratory Syncytial and Influenza Viruses among Persons ≥5 Years of Age in HIV-Prevalent Area, South Africa, 1998–2009[1]

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Release date: March 12, 2015; Expiration date: March 12, 2016

Learning Objectives

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

- Distinguish general trends in rates of respiratory death in South Africa
- Discuss trends in influenza-related mortality in South Africa
- Analyze trends in mortality related to respiratory syncytial virus in South Africa

CME Editor

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[1]Data from this study were presented in part at the Options for the Control of Influenza Conference VIII; 2013 Sep 5–10; Cape Town, South Africa.

We estimated deaths attributable to influenza and respiratory syncytial virus (RSV) among persons ≥5 years of age in South Africa during 1998–2009 by applying regression models to monthly deaths and laboratory surveillance data. Rates were expressed per 100,000 person-years. The mean annual number of seasonal influenza–associated deaths was 9,093 (rate 21.6). Persons ≥65 years of age and HIV-positive persons accounted for 50% (n = 4,552) and 28% (n = 2,564) of overall seasonal influenza-associated deaths, respectively. In 2009, we estimated 4,113 (rate 9.2) influenza A(H1N1) pdm09–associated deaths. The mean of annual RSV-associated deaths during the study period was 511 (rate 1.2); no RSV-associated deaths were estimated in persons ≥45 years of age. Our findings support the recommendation for influenza vaccination of older persons and HIV-positive persons. Surveillance for RSV should be strengthened to clarify the public health implications and severity of illness associated with RSV infection in South Africa.

Influenza virus and respiratory syncytial virus (RSV) infections cause substantial numbers of illness and deaths globally each year; the highest rates are in young children and persons ≥65 years of age (*1*–*4*). However, national estimates of deaths caused by these infections remain scarce in Africa.

Available data suggest that the severity of influenza and RSV illness is higher among HIV-positive persons (*5*–*11*). During 2009 in South Africa, ≈5.1 million HIV-positive persons ≥5 years of age were reported (*12*). The highest prevalence was in the 20–44-year age group, among whom HIV prevalence increased from 10% in 1998 to 24% in 2009. Coverage with highly active antiretroviral therapy (HAART) among HIV-positive persons slowly increased from 2004 to reach a plateau of ≈14% in 2009 (*12*). The lowest reported occurrence of HIV infection in the country was in persons ≥75 years of age; data showed a 0.6% prevalence and 26% HAART coverage for this group in 2009 (*12*). Pneumonia ranked within the top 5 leading causes of death among persons ≥15 years of age in South Africa in 2009 (*13*).

Clarification of the annual number of deaths associated with influenza and RSV in South Africa could assist with the prioritization of interventions. Because influenza virus and RSV infections are rarely confirmed by laboratory diagnosis and related deaths may be attributed to co-morbid conditions or secondary infections, we applied modeling approaches (*14*) to estimate seasonal and pandemic influenza- and RSV-associated deaths among persons ≥5 years of age during 1998–2009.

Methods

Mortality Data and Population Denominators

We obtained data on underlying causes of death for persons ≥5 years of age during 1998–2009 from Statistics South Africa (*15*). We used codes from the International Classification of Diseases, 10th Revision (ICD-10), to compile an age-specific (5–19, 20–44, 45–64, 65–74, and ≥75 years) monthly mortality data time series for all-cause (ICD-10: any); all respiratory (ICD-10: J00–J99); all circulatory (ICD-10: I00–I99); and pneumonia and influenza (P&I) (ICD-10: J10–J18), a subset of all respiratory deaths. During the study period, underreporting of deaths was estimated to be <5% (*13*). Population denominators were obtained from Statistics South Africa (*16*); we obtained year- and age-specific estimates of HIV prevalence in the population and HAART coverage among HIV-positive older children and adults from the Actuarial Society of South Africa AIDS and Demographic Model (*12*).

Influenza and RSV Surveillance Data

For the study period before 2002, we obtained influenza virus data, including types and subtypes, from influenza-like illness surveillance implemented by the National Institute for Communicable Diseases, a division of the National Health Laboratory Service, South Africa (*17*), and RSV data from a cohort study (*10*). For the years after 2002, we acquired virologic data on influenza and RSV from the National Health Laboratory Service corporate data warehouse, a national database that includes all patients tested for respiratory viruses in the public sector in South Africa. We considered an influenza type or subtype to be dominant during the influenza season if it represented >50% of the circulating viruses.

Estimation of Influenza- and RSV-Associated Deaths

We conducted a 2-stage analysis. In the first stage, we estimated the annual number of deaths associated with influenza and RSV in South Africa; in the second stage, we estimated the proportion of these deaths that were experienced by HIV-positive and HIV-negative persons. During the first stage, to estimate the number of deaths associated with seasonal and pandemic influenza and RSV, we fitted age-specific generalized regression models with a Poisson distribution and an identity link to the number of monthly deaths as previously described (*14*). The full model (model 1) included co-variates for time trends, seasonal variation, and proxies for viral circulation. Model specification, selection procedures and sensitivity analysis are provided in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/21/4/14-1033-Techapp1.pdf). Separate models were fitted for each age group and cause of death.

In South Africa, a diagnosis of AIDS is rarely coded on the death certificate (*13*), which hinders direct estimation of respiratory virus–associated deaths by HIV status. To assess the proportionate number of deaths associated

with influenza and RSV among HIV-positive and HIV-negative persons, we used a previously developed methodology (*14*) that leverages the increasing trend in HIV prevalence in South Africa. The rationale is that if HIV is a risk factor for influenza- or RSV– associated death, influenza- or RSV– related mortality rates should increase over time proportionately to the observed increase in HIV prevalence. Our approach also controls for increasing HAART coverage, which may decrease the severity of influenza or RSV in HIV-positive patients (*18*). In this second-stage analysis, we fitted regression models (model 2) to annual estimates of influenza- and RSV-associated excess mortality rates by age group, including data for HIV prevalence and HAART coverage, time trends, and dominant influenza subtypes (for the influenza model) (*14*). Model specifications are provided in the online Technical Appendix.

Subsequently, we obtained mortality rates associated with HIV status by dividing the estimated influenza-associated deaths by HIV status from model 2 by the mid-year population estimates within each category (i.e., HIV-positive and HIV-negative persons). Mortality rates were expressed per 100,000 person-years. We used log-binomial regression (*14*) to estimate age-specific and age-adjusted relative risk for influenza- and RSV-associated death related to HIV infection by comparing influenza- or RSV-associated mortality rates among HIV-positive and HIV-negative persons. We used STATA version 12 (StataCorp, College Station, Texas, USA) to implement the statistical analysis.

Sensitivity Analysis of Influenza- and RSV-Associated Deaths among Persons ≥45 Years of Age

In contrast to previous studies (*3*,*4*), our models estimated that influenza, but not RSV, was associated with excess deaths in South Africa among persons aged ≥45 years. We hypothesized that differences in the timing of the RSV and influenza seasons may explain these discrepancies because RSV and influenza rarely co-circulate in South Africa, but they do in other temperate settings (*3*,*4*). This difference could potentially confound burden of illness estimates for these pathogens. We implemented a sensitivity analysis to explore this hypothesis by artificially shifting the RSV surveillance time series so that it overlapped with the influenza season and repeating model 1 calculations (online Technical Appendix).

Ethics

This analysis used only publicly available mortality data and deidentified and aggregated laboratory data. Therefore, the study was considered to be exempt from human subjects ethics review.

Results

Deaths and Mortality Rates

South Africa had a population of ≈44.8 million persons ≥5 years of age in 2009; persons 5–64 years of age accounted for 85% of this population. During the study period, a mean of 463,594 deaths occurred annually among persons from South Africa ≥5 years of age, of which 101,450 (22%) were attributable to respiratory and 112,716 (24%) to circulatory causes of death (online Technical Appendix Table 1). The mean annual mortality rate for all-cause death increased from 112 for persons 5–19 years of age to 9,732 for persons ≥75 years of age. Similar patterns were observed regarding the other underlying causes of death evaluated in this study. Among persons 20–44 years of age, for which the HIV burden is greatest (24% HIV prevalence in 2009 [*12*]), the annual mortality rate for all respiratory deaths increased from 78 in 1998 to 310 in 2004, subsequently declining to 233 in 2009 (monthly trends provided in the Figure, panel A). In contrast, no evident secular trend for all respiratory mortality rates was observed among persons ≥75 years of age, for whom HIV incidence is lowest (<0.01% HIV prevalence in 2009 [*12*]) (Figure, panel B).

Influenza and RSV Laboratory Surveillance

A mean of 3,403 (range 227–15,321) and 1,810 (range 578–5,247) respiratory specimens were tested annually for influenza virus and RSV, respectively. The mean annual number of specimens that tested positive was 937 (27%) for influenza virus and 356 (20%) for RSV. During the study period, the influenza season peaked between May and August (winter in South Africa); 10 of the 12 years showed peak activity during June–July (Figure, panel C). In 2009, an epidemic of influenza A(H3N2) peaked in June, and influenza A(H1N1) pdm09 activity peaked in August. RSV peak activity was observed during March and April (autumn in South Africa) in 8 of the 12 years. Early or late peaks were observed in February or May in the remaining years.

Influenza- and RSV-Associated Deaths

During 1998–2009, the estimated annual number of all-cause seasonal influenza-associated deaths in persons ≥5 years of age (model 1) ranged from 6,450 to 11,012 (rate 16.7–24.5) (online Technical Appendix Table 2). In the same population, estimated annual all-cause RSV-associated deaths ranged from 292 to 626 (rate 0.7–1.4) (online Technical Appendix Table 2).

The estimated mean seasonal influenza–associated mortality rate for all-cause deaths increased progressively from 0.8 for the 5–19-year age group to 379.2 for the ≥75-year age group. Similar trends were observed for the other causes of death evaluated in this study (Table 1). Overall, the estimated mean seasonal influenza–associated mortality

rate for all-cause deaths was higher for HIV-positive persons than for those who were HIV-negative (age-adjusted relative risk [aRR] 7.9, 95% CI 7.1–8.9). Overall, 28% (2,564/9,093) of estimated all-cause seasonal influenza–associated deaths occurred among HIV-positive persons.

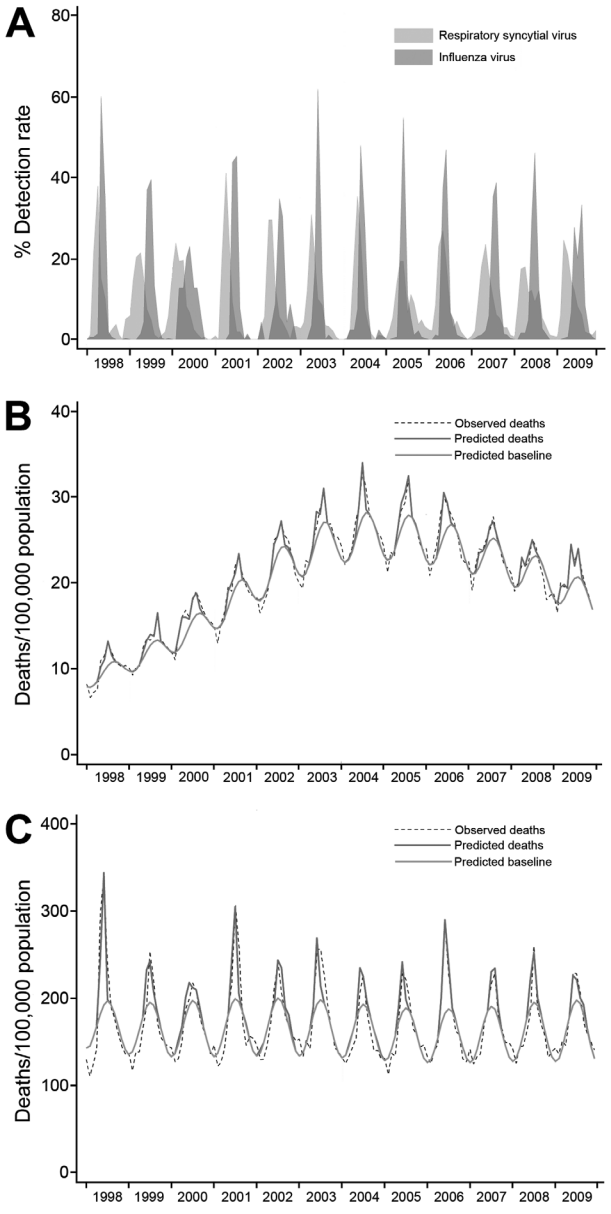

Figure. Monthly mortality and detection rates for influenza and respiratory syncytial virus in South Africa, 1998–2009.
A) Observed respiratory deaths, predicted deaths, and predicted baseline by month (model 1) of persons 20–44 years of age.
B) Observed respiratory deaths, predicted deaths, and predicted baseline by month (model 1) in persons ≥75 years of age.
C) Detection rate (i.e., monthly number of positive specimens divided by annual number of specimens tested) of influenza and respiratory syncytial virus (all ages). A color version of this figure is available online (wwwnc.cdc.gov/eid/article/21/4/14-1033-f.htm)

In 2009, we estimated 4,113 (rate 9.2) all-cause influenza A(H1N1)pdm09–associated deaths among persons ≥5 years of age (Table 2). The mortality rate associated with influenza A(H1N1)pdm09 in 2009 in persons 5–19 years of age was 5.4 times higher than the mean for prepandemic years. In contrast, persons ≥75 years of age experienced ≈100 times lower influenza A(H1N1)pdm09 rates than expected in typical nonpandemic years. A similar trend was observed for other causes of death evaluated in this study.

The estimated RSV-associated mortality rate for all-cause deaths was 0.4 for the 5–19-year age group and 2.4 for the 20–44-year age group (Table 3). However, no RSV-associated deaths were estimated among persons ≥45 years of age. Among persons 5–44 years of age, the RSV-associated mortality rate for all causes of death was considerably higher for HIV-positive than HIV-negative persons (aRR 66.1, 95% CI 26.0–167.8). Similar trends were observed for the RSV-associated mortality rate among all respiratory and P&I deaths. A nonsignificant RSV-associated mortality rate (mean annual deaths: 8) was identified among circulatory deaths only in the 5–19-year age group. Overall, 89% (455/511) of all-cause RSV-associated death occurred among HIV-positive persons.

Sensitivity Analysis of Influenza- and RSV-Associated Deaths among Persons ≥45 Years of Age

On sensitivity analysis, we applied an artificial incremental shift of the RSV laboratory surveillance time series to make the influenza and RSV seasons more synchronous, resulting in a progressive increase in estimated RSV-associated deaths (online Technical Appendix Table 3). We found that annual all-cause RSV-associated deaths peaked at 3,661 among persons ≥45 years of age (compared with 0 in the main analysis) when the RSV and influenza seasons coincided in most years (2 months incremental shift of the RSV season). Thereafter, the estimated mean annual all-cause RSV-associated deaths decreased again to 0 when the peak circulation of the 2 pathogens were farther apart (5-month incremental shift of the RSV season). This trend was observed for all causes of death evaluated in this study. Conversely, the estimated influenza-associated deaths remained stable throughout sensitivity analyses, without regard to the shift in RSV season. Specifically, the estimated all-cause influenza-associated deaths among persons ≥45 years of age remained within 10% of its original value (online Technical Appendix Table 3).

Discussion

We reported estimates of influenza- and RSV-associated deaths in persons ≥5 years of age in a high HIV prevalence setting in Africa. The number of seasonal influenza–associated deaths was substantial and observed across age groups and underlying causes of death evaluated

Table 1. Seasonal influenza virus mean annual excess deaths and relative risk for death related to HIV infection among persons ≥5 y of age, South Africa, 1998–2009*

Cause of death by age, y	Mean annual excess deaths							Relative risk, HIV+ vs HIV–
	Total			HIV+		HIV–		
	No.	Rate†	% Death over model baseline	No.	Rate†	No.	Rate†	
All causes								
5–19	127	0.8	2.4	52	23.6	75	0.5	46.6
20–44	1,966	10.7	3.0	1,851	56.3	114	0.7	72.8
45–64	2,447	37.3	5.7	661	163.9	1,785	29.3	5.6
65–74	1,664	115.4	8.1	NED	NED	1,664	115.4	NA
≥75	2,888	379.2	10.7	NED	NED	2,888	379.2	NA
≥5	9,093	21.6	5.7	2,564	64.7	7,189	18.9	7.9‡
All respiratory								
5–19	96	0.6	9.8	55	24.6	41	0.3	87.7
20–44	778	4.2	5.0	722	21.5	56	0.4	57.1
45–64	1,106	16.8	11.4	380	93.9	725	11.9	7.9
65–74	626	43.4	14.3	NED	NED	626	43.4	NA
≥75	1,005	132.3	17.3	NED	NED	1,005	132.3	NA
≥5	3,613	8.5	10.0	1,157	28.7	2,455	6.4	11.1‡
All circulatory								
5–19	28	0.2	6.2	NED	NED	28	0.2	NA
20–44	252	1.4	4.0	226	7.2	26	0.2	41.2
45–64	854	13.2	6.9	258	66.3	596	9.9	6.6
65–74	749	52.1	8.3	NED	NED	749	52.1	NA
≥75	1,270	167.4	10.1	NED	NED	1,270	167.4	NA
≥5	3,153	7.5	7.8.	484	12.0	2,669	6.9	6.8‡
Pneumonia and influenza								
5–19	86	0.6	13.7	50	22.4	36	0.2	91.1
20–44	569	3.1	5.4	522	15.5	47	0.3	48.5
45–64	612	9.3	12.5	279	67.1	336	5.5	12.2
65–74	299	20.8	16.1	NED	NED	299	20.8	NA
≥75	620	83.0	21.2	NED	NED	620	83.0	NA
≥5	2,186	5.2	10.8	848	20.9	1,341	3.5	17.3‡

*Estimated from model 1 (excess deaths irrespective of HIV status) and model 2 (excess deaths by HIV status). HIV, human immunodeficiency virus; NED, no estimated deaths; NA, not applicable. An expanded version of this table that includes 95% CIs is available online (http://wwwnc.cdc.gov/EID/article/21/4/14-1033-T1.htm).
†Mortality rates per 100,000 person-years.
‡Age-adjusted relative risk.

in this study, irrespective of the person's HIV status. However, the seasonal influenza-associated mortality rates were highest among persons ≥65 years of age and HIV-positive adults 5–64 years of age. The seasonal influenza–associated deaths in these groups accounted for 50% and 28%, respectively, of the total influenza-associated deaths among persons ≥5 years of age. Conversely, a moderate number of deaths associated with RSV infection was found mainly among HIV-positive persons 5–44 years of age; the model did not estimate RSV-associated deaths for persons ≥45 years of age.

Previous studies have reported elevated influenza-associated mortality rates among the elderly (*3,4,19–21*) and HIV-positive persons (*6,8,9,11,14*). We did not find an excess risk for seasonal influenza–associated death due to HIV infection among persons ≥65 years of age across the underlying causes of deaths evaluated in this study. This finding may reflect the low HIV prevalence among elderly persons that may have hindered our ability to estimate the extent of disease in this group using our described method.

Among persons ≥5 years of age years in South Africa, the number of deaths associated with pandemic influenza A(H1N1)pdm09 during 2009 was approximately half that of an average influenza season in prepandemic years. However, pandemic-related mortality rates were higher in the 5–19-year age group and lower in the ≥45-year age group compared with typical seasons. Other studies have reported overall lower mortality rates associated with the first year of circulation of the 2009 pandemic virus, compared with that of seasonal influenza, but have found a higher disease burden for children and young adults (*4,21–25*). Our estimates are similar to the lower-bound estimates for South Africa from a global influenza A(H1N1)pdm09 mortality model (*26*).

For persons ≥5 years of age in South Africa, ≈90% of RSV-associated deaths were estimated to have occurred among HIV-positive persons 5–44 years of age, although our model did not estimate RSV-associated deaths among persons ≥45 years of age, an age group in which the HIV infection rate is low (1.3% in 2009) (*12*). This finding suggests that HIV infection may be a major risk factor for RSV-associated death for persons ≥5 years of age, consistent with our high estimate of HIV as a risk factor for RSV-associated death (aRR 66.1, 95% CI 26.0–167.8). Other

Table 2. Influenza A(H1N1)pdm09 excess deaths among persons ≥5 y of age, South Africa, July–September 2009*

Cause of death by age, y	Influenza A(H1N1)pdm09 excess deaths in 2009			Mortality rate ratio‡
	No.	Rate†	% Death over model baseline	
All causes				
5–19	682	4.4	12.7	5.4
20–44	1,820	9.3	2.6	0.9
45–64	1,301	17.2	2.5	0.5
65–74	279	17.6	1.2	0.2
≥75	31	3.6	0.1	0.01
≥5	4,113	9.2	2.6	0.4§
All respiratory				
5–19	626	4.1	61.6	6.9
20–44	936	4.8	6.0	1.2
45–64	729	9.6	6.3	0.6
65–74	159	10.1	3.3	0.2
≥75	16	1.8	0.2	0.01
≥5	2,466	5.5	7.1	0.7§
All circulatory				
5–19	7	0.05	1.7	0.2
20–44	252	1.3	4.1	1.0
45–64	404	5.3	3.0	0.4
65–74	75	4.7	0.8	0.1
≥75	13	1.5	0.09	0.01
≥5	751	1.7	1.7	0.2§
Pneumonia and influenza				
5–19	449	2.9	73.1	5.5
20–44	548	2.8	5.7	0.9
45–64	421	5.6	7.3	0.6
65–74	90	5.7	4.3	0.3
≥75	3	0.4	0.08	0.005
≥5	1,511	3.4	3.7	0.7§

*Estimated from model 1 (excess deaths irrespective of HIV status). An expanded version of this table that includes 95% CIs is available online (http://wwwnc.cdc.gov/EID/article/21/4/14-1033-T2.htm).
†Mortality rates per 100,000 person-years.
‡Mortality rate ratio: 2009 influenza A(H1N1)pdm09 vs. 1998–2009 mean annual seasonal influenza.
§Age-adjusted rate ratio.

studies have reported an increased risk for RSV-associated death among HIV-positive persons (*5,7*).

Our findings differ from those of similar studies from the United States and England, where RSV-associated deaths have been reported across age groups (including persons referred to as elderly in the respective studies) and where the influenza and RSV seasons are, in most cases, synchronous (*3,4*). However, there are notable geographic variations in the timing of RSV circulation across the United States (*27*). In southern Florida, where the RSV season precedes the influenza season by several weeks as in South Africa, 1 peak of pneumonia hospitalizations among persons ≥65 years of age could be detected concurrently with the influenza season. In contrast, among children <5 years of age, 2 distinct peaks of pneumonia hospitalizations were observed concomitantly with the RSV and influenza seasons (*27*). Further, a study that used a methodology similar to ours and evaluated data from a large hospital group in South Africa estimated elevated RSV-associated hospitalizations among children <5 years of age but no RSV-associated hospitalizations among adult and elderly persons (*28*). These results are similar to a previous study conducted in South Africa that found influenza- and RSV-associated mortality among children <5 years of age (*14*), but no RSV-associated mortality was estimated among elderly persons in our study.

We performed a sensitivity analysis to test whether the overlap between the RSV and influenza seasons affected mortality estimates. We found that RSV mortality estimates increased substantially among persons ≥45 years of age when the RSV season was artificially shifted to later in the year, so that it coincided with influenza activity. However, influenza estimates remained within 10% of their main analysis values, solidifying our influenza results. This finding suggests that careful interpretation of the results of time series excess mortality models is needed when used to simultaneously estimate the mortality attributable to co-circulating pathogens, particularly for RSV.

Severe illness and death among laboratory-confirmed RSV-infected adults has been reported (*29,30*), even though the clinical association between pathogen detection and disease remains difficult to interpret in the absence of comparison groups (i.e., RSV prevalence among adults without respiratory illness). Studies conducted in Kenya and South Africa that compared the RSV prevalence among patients hospitalized with severe acute respiratory illness (SARI) to control groups found that RSV infection was associated with hospitalization among

Table 3. Respiratory syncytial virus mean annual excess deaths and relative risk of death related to HIV infection among persons ≥5 y of age, South Africa, 1998–2009*

Cause of death by age, y	Mean annual excess deaths							Relative risk, HIV+ vs. HIV–
	Total			HIV+		HIV–		
	No.	Rate†	% Death over model baseline	No.	Rate†	No.	Rate†	
All causes								
5–19	61	0.4	0.9	25	11.6	36	0.2	58.4
20–44	449	2.4	0.6	430	13.1	19	0.1	98.3
45–64	NED	NED	NED	NED	NED	NED	NED	NA
65–74	NED	NED	NED	NED	NED	NED	NED	NA
≥75	NED	NED	NED	NED	NED	NED	NED	NA
≥5	511	1.2	0.7	455	12.1	55	0.1	66.1‡
All respiratory								
5–19	39	0.3	3.6	22	10.1	16	0.1	90.2
20–44	389	2.1	2.3	369	11.1	20	0.1	81.7
45–64	NED	NED	NED	NED	NED	NED	NED	NA
65–74	NED	NED	NED	NED	NED	NED	NED	NA
≥75	NED	NED	NED	NED	NED	NED	NED	NA
≥5	429	1.0	1.1	392	9.8	37	0.1	85.4‡
All circulatory								
5–19	8	0.05	1.4	NED	NED	8	0.05	NA
20–44	NED	NED	NED	NED	NED	NED	NED	NA
45–64	NED	NED	NED	NED	NED	NED	NED	NA
65–74	NED	NED	NED	NED	NED	NED	NED	NA
≥75	NED	NED	NED	NED	NED	NED	NED	NA
≥5	8	0.05	1.4	NED	NED	8	0.05	NA
Pneumonia and influenza								
5–19	35	0.2	5.2	20	9.2	15	0.1	92.2
20–44	257	1.4	2.3	243	7.2	14	0.1	73.4
45–64	NED	NED	NED	NED	NED	NED	NED	NA
65–74	NED	NED	NED	NED	NED	NED	NED	NA
≥75	NED	NED	NED	NED	NED	NED	NED	NA
≥5	292	0.7	1.3	263	6.6	29	0.1	82.7‡

*Estimated from model 1 (excess deaths irrespective of HIV status) and model 2 (excess deaths by HIV status). HIV, human immunodeficiency virus; NED, no estimated deaths; NA, not applicable. An expanded version of this table that includes 95% confidence intervals is available online (http://wwwnc.cdc.gov/EID/article/21/4/14-1033-T3.htm).
†Mortality rates per 100,000 person-years.
‡Age-adjusted relative risk.

children <5 years of age, but no association was found among persons ≥5 years of age (*31,32*). This finding may suggest that, although RSV is detected among older children and adults, it may play a less important role as a pathogen in this group. However, both studies were underpowered to look specifically at disease association in persons ≥65 years of age. Studies conducted in Egypt, Guatemala, Kenya, and Thailand, where patients of all ages hospitalized with acute lower respiratory tract infections were systematically enrolled and tested using PCR techniques, reported RSV detection rates of <1%–5% among persons ≥50 or ≥65 years of age, compared with RSV detection rates of >20% in infants and young children (*31,33–35*). Reinfection with RSV during life has been reported (*36*), but titers of serum-neutralizing antibodies ≥6 (log2 scale) have been associated with 3 times lower risk for RSV-associated hospitalizations (*37*). Adults reinfected with RSV may have high levels of serum-neutralizing antibodies that have potential to lower the prevalence and severity of RSV-associated hospitalizations in this age group.

In South Africa during 2009–2010, the RSV detection rate among patients hospitalized with SARI decreased from 26.8% among infants <1 year of age to 0.9% among persons ≥65 years of age. In the same study population, the influenza detection rate across age groups was 8%–12% (*38*). The low RSV detection rate among adults and elderly persons with SARI suggests a lower rate of RSV-associated hospitalization than that for influenza for this group (and as a result, a potentially low number of RSV-associated deaths).

Although RSV-associated deaths among persons ≥45 years of age are expected to occur in South Africa, our modeling approach may fail to statistically estimate a small number of cases. Ecologic studies conducted in settings similar to ours, where influenza and RSV peak activities are not synchronous, may assist in better differentiating the relative impact of these pathogens, especially in adults. In addition, results obtained from ecologic models should be interpreted along with findings from case-based studies and the strengths and weaknesses of both approaches should be evaluated.

Our study has limitations that warrant discussion. First, the lack of weekly mortality statistics and the paucity of virologic data before 2002 may have hindered the ability

to accurately estimate the relative contribution of RSV and influenza virus on number of associated deaths. Second, the lack of influenza incidence data (such as influenza-like illness indicators) hampered our ability to consider more refined indicators of respiratory virus activity in our time series models as reported by Goldstein et al. in 2012 (*39*). Third, because of poor records of HIV infection in the death register documenting the early years of our study, we used indirect methods to estimate the number of deaths associated with respiratory viruses among HIV-positive and HIV-negative persons. Although the HIV epidemic in South Africa is considered to be a major factor responsible for the increased mortality rates observed over the years (*40*), the lack of time series data for other potential co-occurring conditions and risk factors may have resulted in overestimation of the increased risk for death associated with HIV infection. Last, we could not estimate the influenza A(H1N1)pdm09–associated mortality by HIV status because our method requires availability of HIV prevalence data over several years of A(H1N1)pdm09 circulation.

In conclusion, we report a substantial risk for death associated with seasonal influenza virus infection, especially for persons ≥65 years of age and HIV-positive adults 20–64 years of age. The risk for death associated with RSV was mainly found among HIV-positive persons 5–44 years of age; our model did not identify excess RSV-associated deaths in persons ≥45 years of age. We also report low to moderate numbers of RSV-associated deaths among persons ≥5 years of age; however, clinical diagnosis and surveillance for RSV should be continued and strengthened to better describe the consequences and severity associated with RSV infection in this age group.

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Sequence Variability and Geographic Distribution of Lassa Virus, Sierra Leone

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Lassa virus (LASV) is endemic to parts of West Africa and causes highly fatal hemorrhagic fever. The multimammate rat (*Mastomys natalensis*) is the only known reservoir of LASV. Most human infections result from zoonotic transmission. The very diverse LASV genome has 4 major lineages associated with different geographic locations. We used reverse transcription PCR and resequencing microarrays to detect LASV in 41 of 214 samples from rodents captured at 8 locations in Sierra Leone. Phylogenetic analysis of partial sequences of nucleoprotein (NP), glycoprotein precursor (GPC), and polymerase (L) genes showed 5 separate clades within lineage IV of LASV in this country. The sequence diversity was higher than previously observed; mean diversity was 7.01% for nucleoprotein gene at the nucleotide level. These results may have major implications for designing diagnostic tests and therapeutic agents for LASV infections in Sierra Leone.

Lassa fever (LF) belongs to a group of viral hemorrhagic fevers characterized by a febrile syndrome and high case-fatality rates (*1*). LF differs from most viral hemorrhagic fevers in that it is endemic to a large geographic area of sub-Saharan Africa. Human cases of LF have been reported in (or imported from) Guinea, Sierra Leone, Liberia, Mali, Burkina Faso, and Nigeria; however, LF outbreaks seem to be restricted to Guinea, Sierra Leone, Liberia (the Mano River Union region), and Nigeria (*2*–*4*). In some areas of Sierra Leone and Guinea, more than half of the population has antibodies against Lassa virus (LASV; family *Arenaviridae*), the etiologic agent of LF (*5*,*6*). According to various estimates, 300,000–500,000 cases of LF result in 5,000–10,000 deaths annually in West Africa (*6*,*7*). An analysis based on seroepidemiologic data suggested that the number of cases might be much higher, reaching 3 million cases and 67,000 fatalities per year (*8*). Overall, the population at risk might include as many as 200 million persons living in a large swath of West Africa from Senegal to Nigeria and beyond (*4*).

LASV can cause infection in the multimammate rat (*Mastomys natalensis*), a natural host and reservoir of this pathogen (*9*,*10*). The multimammate rat is a commensal rodent ubiquitous in Africa (*11*,*12*). Although the routes of LASV infection are poorly characterized, humans probably get infected by eating contaminated food (*13*), by inhaling virus-contaminated aerosols (*14*), or while butchering infected rat meat (*15*). Person-to-person transmission of LASV is well documented, mostly in the form of nosocomial outbreaks (*13*).

Like other arenaviruses, LASV is an enveloped virus with a bisegmented single-stranded RNA genome encoding 4 proteins using an ambisense coding strategy (*16*). The small segment contains genes for the glycoprotein precursor (GPC) and nucleoprotein (NP), which serves as the main viral capsid protein. The large segment encodes the small zinc-binding protein (Z), which contains a RING motif, and another gene (L) containing the RNA-dependent RNA polymerase domain.

Complete genome sequences are available for several LASV strains, as are a considerable number of partial sequences from isolates originating from humans and rodents (*17*–*20*). Their analysis revealed the existence of high sequence diversity (up to 27% nt) and 4 major lineages of LASV, which correlate with geographic location (*17*). Lineages I, II, and III, and the greatest diversity of LASV strains, were found among isolates from Nigeria, whereas strains from Guinea, Sierra Leone, and Liberia seemed to be more closely related and belong exclusively to lineage IV. Sequence of the AV strain (*21*) and recently published sequences from rodent LASV isolates from Mali (*18*)

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suggest the existence of an additional clade (proposed as lineage V) (*22*). LASV sequences of isolates from humans and rodents are found interspersed throughout the phylogenetic tree, which is consistent with the notion that human cases typically result from transmission from rodents (*17*).

The high degree of sequence divergence of LASV genomes is a major problem affecting the development of molecular and immune-based diagnostic technologies, vaccines, and possibly antiviral drugs (*13*,*16*,*17*,*23*–*25*). Forty-seven unique partial LASV sequences from Sierra Leone were available in GenBank at the time of this analysis, which included fragments of NP (27 sequences), GPC (9 sequences), L (9 sequences), and Z (2 sequences) genes plus full sequences of small and large segments of 2 strains—Josiah and NL. Most of these sequences are from isolates collected >30 years ago; only 2 more recent sequences (GPC and L gene fragments) from strain SL06-2057 were isolated in 2006 (*17*,*19*).

To fill this gap, we investigated the sequence diversity of strains circulating among small rodents captured in peridomestic settings in Sierra Leone. In 2014, we screened 214 samples collected during 2009 from several species of rodents trapped in villages where LF was reported in humans. We used diagnostic reverse transcription PCR (RT-PCR) and high-density resequencing microarrays to detect LASV and amplify fragments of NP, GPC, and L genes. The obtained amplicons were sequenced and compared with previously published sequences from Sierra Leone to obtain a more complete and updated picture of the strains circulating in this country.

Methods

Rodent Sample Collection

The rodent samples collected were part of a separate project (L.M. Moses, unpub. data). Thirteen locations were selected for study in the LF-endemic region of eastern Sierra Leone. The geographic coordinates of the sampling locations and details of rodent trapping methods are available in the online Technical Appendix (Technical Appendix Table 1, Rodent Trapping Procedures, http://wwwnc.cdc.gov/EID/article/21/4/14-1469-Techapp1.pdf). Traps with captured small animals were processed in remote areas outside of the villages according to approved guidelines (*26*). The animals were anesthetized with isoflurane, and their morphometrics recorded. Animals were euthanized by exsanguination using cardiac puncture or cervical dislocation, and necropsies were performed. Spleen sections were stored in RNALater or TRIzol for RNA extraction (Life Technologies, Grand Island, NY, USA). Rodents were identified to the genus level in the field. Animals identified as *Mastomys* sp. were further identified down to species level by using molecular methods as described previously (*27*).

Nucleic Acid Extraction

RNA from 10 mg of spleen of each rodent was extracted with TRIzol following the manufacturer's recommendations. The samples were stored at –80°C.

RT-PCR and Sequencing

RNA were reverse-transcribed by using the SuperScript III Reverse Transcriptase kit (Life Technologies) according to the manufacturer's instructions, and RT products were stored at –20°C. Specific oligonucleotide primer pairs were used for the PCR targets of interest (Table 1) at final concentrations of 0.25 μM each. For PCR, 2 μL of RT reaction was used as template in 25 μL reactions containing 1.25 mM dNTPs, 1× Taq buffer, 0.2 μM each of primers, and 1.25 U FastStart Taq enzyme (Roche Diagnostics, Indianapolis, IN, USA). NP targets were amplified by using an initial 2-min denaturation at 95°C, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. Some specimens produced poor PCR products, with low yields or multiple bands when we used published primer pair 1010C/OW1696R (*17*); 1 μL of PCR product from those specimens was amplified in nested PCR by using the primer pair LAS_NP_F_1/LAS_NP_R_1 and the same thermal cycling program to generate DNA fragments suitable for sequencing (Table 1). GPC targets were amplified by using 36E2 and LVS339-rev primers (*24*) and a PCR profile consisting of 2-min denaturation at 95°C, followed by 45 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min. L gene targets were amplified by using modified primers, LVL3359-F and LVL3754-R, based on published sequences (*28*) and a PCR program consisting of 2-min denaturation at 95°C followed by 45 cycles of 95°C for 30 sec,

Table 1. PCR and sequencing primers used in study of Lassa virus, Sierra Leone*

Primer name	Sequence, 5′ → 3′	Target gene	Amplicon size	Reference
1010C	TCIGGIGAIGGITGGCC	NP	670	(*17*)
OW1696R	AIATGAIGCAGTCCAIIAGTGCACAGTG			(17)
LAS_NP_F_1	GGGTGGCCATAYATTGCATC		650	This study
LAS_NP_R_1	GTCCAGGAGTGCACAGTGAG			This study
36E2	ACCGGGGATCCTAGGCATTT	GPC	317	(*24*)
LVS339-rev	GTTCTTTGTGCAGGAMAGGGGCATKGTCAT			(*24*)
LVL3359-F	AGAATYAGTGAAAGGGARAGCAATTC	L	394	(*28*)
LVL3754-R	CACATCATTGGTCCCCATTTACTRTGATC			(*28*)

*GPC, glycoprotein precursor; L, polymerase; NP, nucleoprotein.

53°C for 30 sec, and 72°C for 1 min. PCR amplicons were size-confirmed by electrophoresis by using 1.2% FlashGel DNA cassettes (Lonza, Walkersville, MD, USA) and purified on Zymo DNA Clean & Concentrator columns (Zymo Research, Irvine, CA, USA). All DNA sequencing was performed by Eurofins MWG Operon (Huntsville, AL, USA). The sequences were deposited into GenBank under the following accession numbers: NP sequences, KM406518–KM406556; GPC sequences, KM406590–KM406623; and L sequences, KM406557–KM406589.

RPM-TEI Microarray Analysis

The resequencing pathogen microarray (RPM) analysis was conducted by using Tropical and Emerging Infections microarrays (RPM-TEI v. 1.0; TessArae, Potomac Falls, VA, USA). The RPM-TEI microarray enables detection of 84 biothreat agents, including all lineages of LASV (*29*). Sample preparation was conducted as previously described (*29*). Pathogen identification was performed using the "C3 Score" identification algorithm (*30*).

Phylogenetic Analysis

We conducted the sequence alignment using the MUSCLE algorithm implemented in the MEGA 6.0 software package (*31*). In addition to partial NP, GPC, and L sequences obtained in this study, we included in the alignments all homologous sequences from these genes in samples collected in Sierra Leone (or clustering with Sierra Leone sequences) available in GenBank. Twenty-seven NP, 10 GPC, and 8 L sequences were available that meet these criteria. To root the trees, sequences from more distantly related, lineage IV isolate Z-158, which originated from Macenta district in Guinea, were used as an outgroup on the basis of the previous phylogenetic analyses (*17*). We also used MEGA 6.0 to perform statistical selection of the nucleotide substitution model for each sequence collection. We selected the Tamura 3-parameter model with discrete γ-distributed rate variation as the best-fitting model for NP and L sequence sets and the Kimura 2-parameter model with a fraction of evolutionary invariant sites for GPC sequences. The phylogenies were inferred by using the Bayesian, Markov Chain Monte Carlo method, as implemented in MrBayes v3.2.2 (*32*). The analysis was run without an assumption of a molecular clock. The resulting phylogenies were presented as 50% majority rule consensus trees in which the branches with posterior probability <0.5 were collapsed into polytomies. We manually adjusted the trees using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

Results and Discussion

We collected 681 small mammals during the field survey. Of these, we analyzed 214 for this study on the basis of RNA availability at the time of the study (online Technical Appendix Table 2). These samples were obtained from rodents captured at 13 locations in 3 districts within the southern and eastern provinces of Sierra Leone (Figure 1; online Technical Appendix Table 1). The rodents belonged to 6 genera; 199 were identified as *M. natalensis*, which is consistent with published data on the ubiquitous presence of this species in domestic environments in West Africa (*11*,*12*). The other rodents (identified to genus level only) were *Rattus* sp. (9 [4.2%] rodents); *Cricetomys* sp. (3 [1.4%] rodents); and *Mus* sp., *Praomys* sp., and *Hylomyscus* sp. (1 [0.5%] rodent each).

We screened all samples for LASV by RT-PCR using pan–Old World arenavirus (OWA) primers (*17*), which amplify a 670-nt section of NP gene. Because of poor results of NP amplification using OWA primers (inefficient amplification, multiple bands), we modified the screening protocol to include a second nested PCR using primers internal to the OWA amplification product, designed to amplify 650-nt segment of NP sequence and be more specific for lineage IV NP gene sequences. We obtained sequencing-quality NP amplicons from 39 samples using this protocol. For all of these NP-positive samples, we attempted to obtain RT-PCR amplicons for fragments of GPC and L genes using previously published or modified primers (*17*,*24*,*28*). The GPC and L amplifications were successful for most NP-positive samples and failed only in 5 and 6 samples, respectively (Table 2).

In addition, we screened a randomly selected subset of 51 samples (representing 8 collection sites and 4 rodent genera; online Technical Appendix Table 2) for nucleic acids of 84 different pathogens using RPM-TEI v. 1.0. Of the 51 samples tested using RPM-TEI, 9 were positive for LASV, and no other pathogens were detected in the analyzed samples. Although the percentage of positive samples was similar to that from RT-PCR results, the RPM-TEI failed to detect LASV in 3 samples that were positive for the NP gene by 2-step RT-PCR (LM34, LM58, and LM68). All these samples originated from the same village (Bumpeh), and no sample from this site was RPM-TEI positive, which suggests that detection failures might have been caused by inefficient amplification of the sequence variant of LASV circulating in the Bumpeh area with the RPM-TEI primers. On the other hand, RPM-TEI detected viral RNA in 2 RT-PCR–negative samples (LM591 and LM649), bringing to 41 the number of samples positive for LASV nucleic acids (Table 2).

In summary, 41 LASV-positive samples were obtained from animals captured in 8 locations: Barlie (13 samples), Largo (8 samples), Bumpeh (7 samples), Ngiehun (4 samples), Koi and Yawei (3 samples each), Taiama (2 samples), and Saama (1 sample) (Figure 1). No LASV RNA was detected in samples collected from Gouma, Joru, Kenema, Panguma, or Segbwema. Lack of LASV detection in

Table 2. Results of Lassa virus detection among rodent samples with ≥1 positive test result, Sierra Leone*

Sample	Date collected	Collection site		PCR†			RPM‡
		Village/town	District	NP	GPC	L	
LM0034	2009 Jan 27	Bumpeh	Kenema	+	+	+	Neg
LM0036	2009 Jan 27	Bumpeh	Kenema	+	+	+	NT
LM0047	2009 Jan 28	Bumpeh	Kenema	+	+	Neg	NT
LM0054	2009 Jan 28	Bumpeh	Kenema	+	+	+	NT
LM0058	2009 Jan 29	Bumpeh	Kenema	+	+	+	Neg
LM0064	2009 Jan 30	Bumpeh	Kenema	+	+	+	NT
LM0068	2009 Jan 30	Bumpeh	Kenema	+	+	Neg	Neg
LM0087	2009 Feb 3	Largo	Kenema	+	Neg	Neg	NT
LM0091	2009 Feb 3	Largo	Kenema	+	+	+	+
LM0092	2009 Feb 3	Largo	Kenema	+	+	+	NT
LM0093	2009 Feb 3	Largo	Kenema	+	+	+	NT
LM0111	2009 Feb 4	Largo	Kenema	+	+	+	NT
LM0122	2009 Feb 5	Largo	Kenema	+	+	+	NT
LM0123	2009 Feb 5	Largo	Kenema	+	+	+	NT
LM0124	2009 Feb 5	Largo	Kenema	+	+	+	NT
LM0224	2009 Feb 18	Koi	Kenema	+	+	+	NT
LM0250	2009 Feb 19	Koi	Kenema	+	+	+	NT
LM0273	2009 Feb 20	Koi	Kenema	+	+	+	NT
LM0395	2009 Jul 22	Ngiehun	Kenema	+	+	+	+
LM0396	2009 Jul 22	Ngiehun	Kenema	+	Neg	+	+
LM0434	2009 Jul 23	Ngiehun	Kenema	+	+	+	+
LM0473	2009 Jul 24	Ngiehun	Kenema	+	+	+	NT
LM0513	2009 Aug 1	Saama	Kenema	+	Neg	Neg	NT
LM0582	2009 Aug 8	Barlie	Bo	+	+	+	NT
LM0591	2009 Aug 8	Barlie	Bo	Neg	Neg	Neg	+
LM0610	2009 Aug 8	Barlie	Bo	+	+	+	+
LM0619	2009 Aug 8	Barlie	Bo	+	Neg	+	NT
LM0645	2009 Aug 8	Barlie	Bo	+	+	+	NT
LM0649	2009 Aug 8	Barlie	Bo	Neg	Neg	Neg	+
LM0657	2009 Aug 8	Barlie	Bo	+	+	+	NT
LM0660	2009 Aug 8	Barlie	Bo	+	+	+	NT
LM0661	2009 Aug 8	Barlie	Bo	+	+	+	NT
LM0676	2009 Aug 8	Barlie	Bo	+	+	+	NT
LM0677	2009 Aug 8	Barlie	Bo	+	+	+	NT
LM0678	2009 Aug 8	Barlie	Bo	+	+	+	NT
LM0680	2009 Aug 8	Barlie	Bo	+	+	+	NT
LM0714	2009 Aug 14	Yawei	Kenema	+	+	Neg	NT
LM0716	2009 Aug 14	Yawei	Kenema	+	+	+	+
LM0729	2009 Aug 15	Yawei	Kenema	+	Neg	Neg	+
Z0005	2009 Dec 17	Taiama	Kenema	+	+	+	NT
Z0007	2009 Dec 17	Taiama	Kenema	+	+	+	NT

*GPC, glycoprotein precursor; L, polymerase; Neg, negative; NP, nucleoprotein; NT, sample not tested; RPM, resequencing pathogen microarray; +, positive.
†Results of PCR detection using appropriate diagnostic primers.
‡Results of RPM-TEI detection of LASV.

Kenema, Panguma, and Segbwema, areas well known to have regular LASV transmission, might be due to the small number of traps used. The town of Joru was extensively trapped, and no LASV was found. This finding is not surprising because Joru is south of the area where LASV is usually found.

All positive samples came from multimammate rats, which is considered the sole vector species for LASV (*13*). The results of LASV detection using several different RT-PCR strategies and a broad-range resequencing microarray (RPM-TEI v. 1.0) showed that none of the techniques applied alone detected viral RNA in all positive samples. This result underscores the difficulty of developing a truly universal diagnostic assay for this highly variable virus, even in the case of closely related strains belonging to lineage IV.

The analysis of the new sequences of LASV strains circulating in rodents in Sierra Leone indicated that the viral genome diversity is higher than previously estimated (*17*). For all available Sierra Leone sequences (including this study) the mean difference calculated for partial NP, GPC, and L sequences was 7.01% nt, 8.92% nt, and 9.83% nt, respectively, and 2.82% aa, 4.06% aa, and 0.71% aa, respectively (Table 3). These differences are higher than the reported 4.6% nt and 1.7% aa differences based on partial NP sequences in a study with fewer isolates (*17*). The L gene fragment seemed to vary the most at the nucleotide level, followed by GPC and NP, which is consistent with previous observations (*33*). However, at the amino acid level, the GPC gene varied most, followed by the NP and L genes. The high conservation of the protein sequence of L

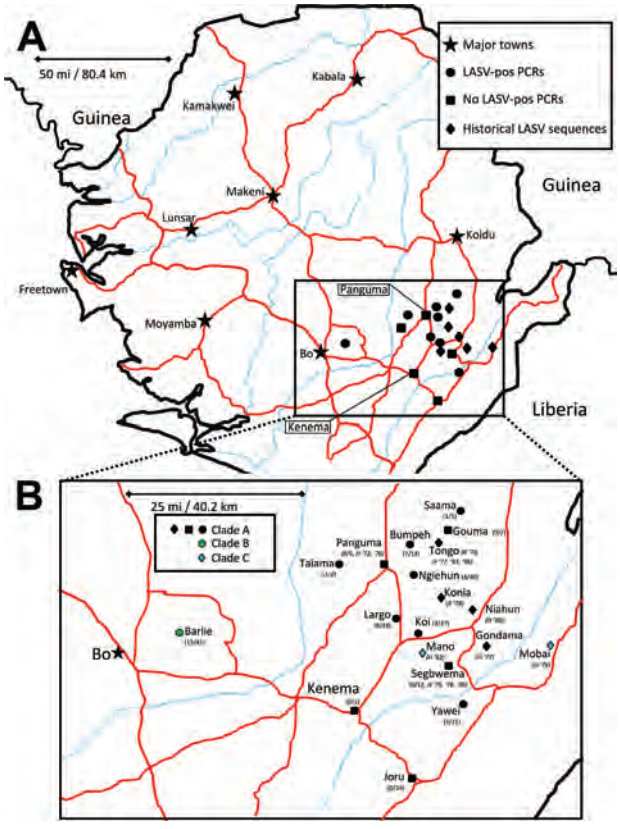

Figure 1. A) Locations of origin for Lassa virus (LASV) nucleic acid sequences, Sierra Leone. B) Enlarged view of region from which rodent specimens were collected. Major roads (red) and waterways (blue) are indicated. Symbols indicate major cities and towns (stars); sites in this study with rodent samples that were PCR positive for LASV (circles); sites in this study from which all samples from mulitmammate rats were PCR negative for LASV (squares); and sites from which published LASV sequences originated (diamonds). The color of the symbols in panel B indicates the clade for nucleoprotein sequence: black, clade A; green, clade B; blue, clade C. Fractions indicate, for each site included in this study, number of PCR-positive samples and total number of samples. Other designations for published sequence sites indicate type of isolate (H, human; R, rodent) and year(s) of isolation. No published information about geographic origin was available for the following strains: 807875, 331, 523, IJ531, Josiah, NL, SL06–2057, SL15, SL20, SL21, SL25, SL26, SL620.

gene fragment analyzed in this study (0.71% mean difference) seemed have resulted from selection of a highly conserved part of L gene (located in RNA polymerase domain) when the diagnostic assay was designed (*28*).

The analysis of phylogenetic trees constructed by using all available partial sequences of NP, GPC, and L genes from Sierra Leone confirmed previous findings that the strains circulating in this country belong to lineage IV and are closely related to each other (*17*,*19*). The topology of the largest NP-based tree (Figure 2) strongly supports the hypothesis that the isolates from Sierra Leone belong to at least 3 distinct major clades (posterior probability 1.00 in all cases): the first clade (A), including a large cluster of strains originating from a group of villages to the north and east of Kenema in the Eastern Province (Bumpeh, Gondama, Koi, Konia, Largo, Ngiehun, Panguma, Segbwema, Taiama, Tongo, and Yawei; Figure 1); the second clade (B), including several strains isolated from rodents captured in Barlie (located a few kilometers southeast of Bo) and 1 isolate from Saama (located northeast of Kenema); and the third clade (C) represented by just 2 older human isolates from Mano and Mobai.

Table 3. Estimates of average evolutionary divergence of NP, GPC, and L gene fragments for Lassa virus strains, Sierra Leone*

Gene, grouping	Difference†	
	Nucleotide	Amino acid
NP		
Overall	7.01	2.82
Clade A	5.03	2.06
Clade B	0.62	0.77
Clade C	6.44	2.42
GPC		
Overall	8.92	4.06
Clade A	6.26	2.60
Clade B	0.68	0.49
Clade D	7.49	3.29
L		
Overall	9.83	0.71
Clade A	6.59	0.58
Clade B	0.89	0.00
Clade D	0.88	0.00

*GPC, glycoprotein precursor; L, polymerase; NP, nucleoprotein.
†The numbers of nucleotide and amino acid differences per site from averaging over all sequence pairs or all sequence pairs within a clade multiplied by 100 are shown. All positions containing gaps and missing data were eliminated. Values for clade E defined for GPC and L sequences were not calculated because clade E contained only 1 sequence.

Phylogenetic trees based on GPC and L sequences (Figures 3, 4) had similar topology and supported existence of clades A (with posterior probabilities 0.74 and 1.00, respectively) and B (with posterior probabilities 1.00 for both trees). However, the clade C was not present because the sequences for GPC and L gene fragments were not available for the strains forming this cluster in the NP-based tree. In addition to clades A and B, GPC- and L-based trees suggested existence of 2 additional and distinct clades. Clade D was represented by 2 sequences from human isolates SL25 and SL26, which formed a separate cluster (posterior probability 1.00 for both trees), and clade E represented by sequences obtained from a single strain isolated in 2006 (SL06–2057). These clades are defined by a very small number of sequences, and the GPC- and L-based trees disagree on the order of their separation from other clades. In addition, no data have been published on geographic origin of clade D and E samples. More data are needed (including corresponding NP sequences) to establish the existence and position of clades D and E with more certainty.

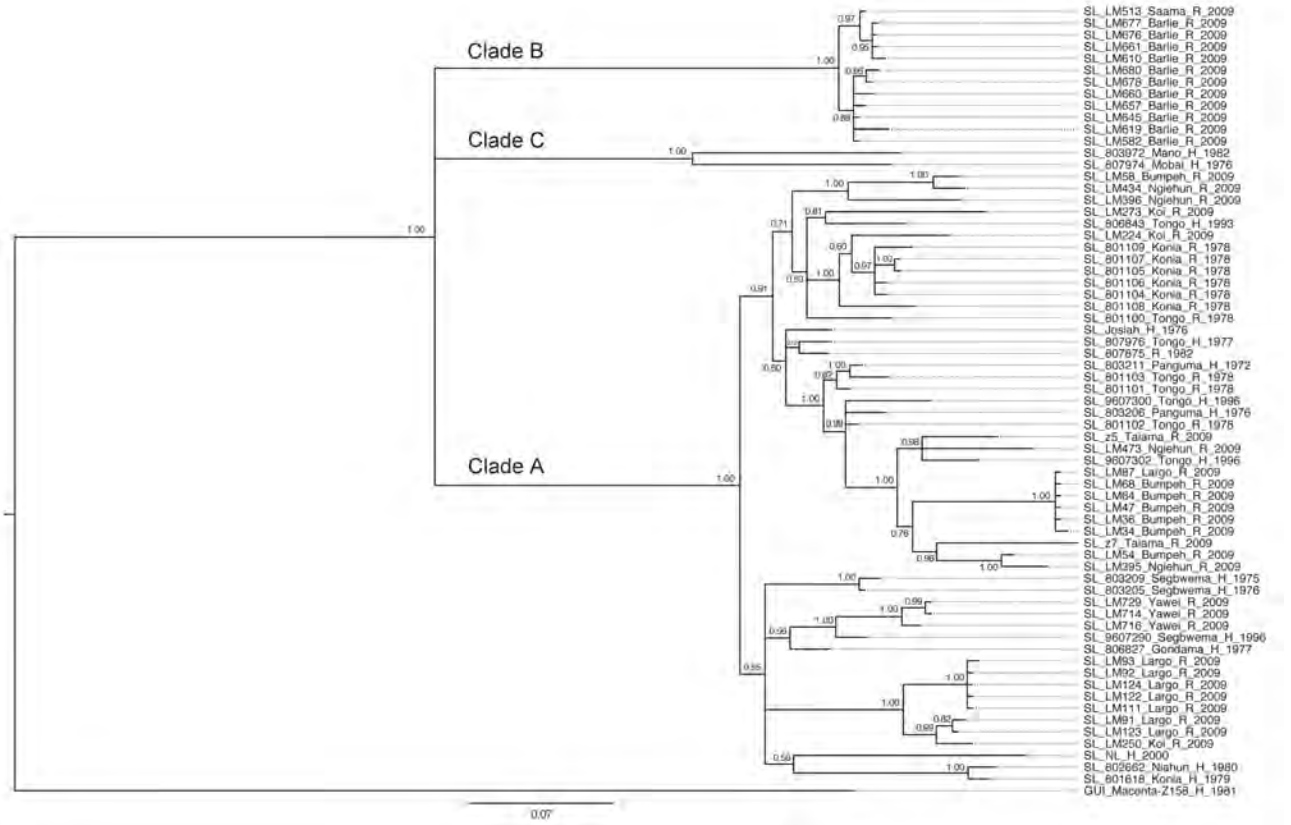

Figure 2. Phylogenetic analysis of Lassa virus isolates from Sierra Leone based on partial nucleoprotein (NP) gene sequences. The homologous NP fragments of 621 nt were aligned. The isolate Z-158, which originated from Macenta district in Guinea, was used as outgroup based on the previous phylogenetic analyses to root the tree. The 50% majority rule consensus tree was estimated by using Bayesian Inference method implemented in MrBayes software (*32*) using the Tamura 3-parameter substitution model with discrete γ-distributed rate variation. The strain labels contain information on the country of origin (SL, Sierra Leone; GUI, Guinea), strain designation, village or town of origin, type of isolate (H, human; R, rodent), and year of isolation. The numbers next to the branches indicate the posterior probability of particular clades. The clades as defined in this study (clades A, B, and C) are also indicated next to the appropriate branches. Scale bar indicates substitutions per site.

All of the trees indicate a high degree of geographic clustering of the strains. This kind of clustering has been reported previously over large geographic distances and is believed to have resulted from limited dispersal and migration of the host species (*17*,*19*). Results of this study show that this phenomenon also can be observed over relatively short distances. Isolates originating from multimammate rat specimens obtained in a particular location tended to cluster, and conversely sequences present in specific branches of the trees in many cases originated from a single location or few locations not far from each other. This kind of clustering could be observed especially well in samples from Barlie, Largo, Bumpeh, Konia, and Yawei (Figures 2–4).

In addition to the general pattern of geographic clustering, in several cases single isolates clustered with strains from different locations. For example, the sequence from a single sample from Saama (LM513) was closely related to that of strains from Barlie (based on NP sequence analysis). In another example, 1 GPC sequence originating from Liberia (523) clustered with Sierra Lone clade A sequences. In some cases (e.g., Saama sample LM513), such unusual clustering patterns may be explained by cross contamination or mislabeling of the samples. They also might result from relative proximity of all sampling sites and inadvertent anthropogenic transfer of rodents. Massive population movements that occurred in Sierra Leone during the 1991–2002 civil war could contribute to the process of mixing multimammate rat subpopulations carrying different LASV strains (*34*).

The geographic location of human cases at such a fine spatial scale can be problematic because humans can move large distances after exposure before disease is detected. For the human isolates, the clustering inconsistent with geographic location might have resulted from recording of the hospital location or patient's current location as strain's origin instead of the actual location of rodent–human transmission. For example, the NP-based phylogenetic tree indicates that human isolates from Segbwema and Gondama

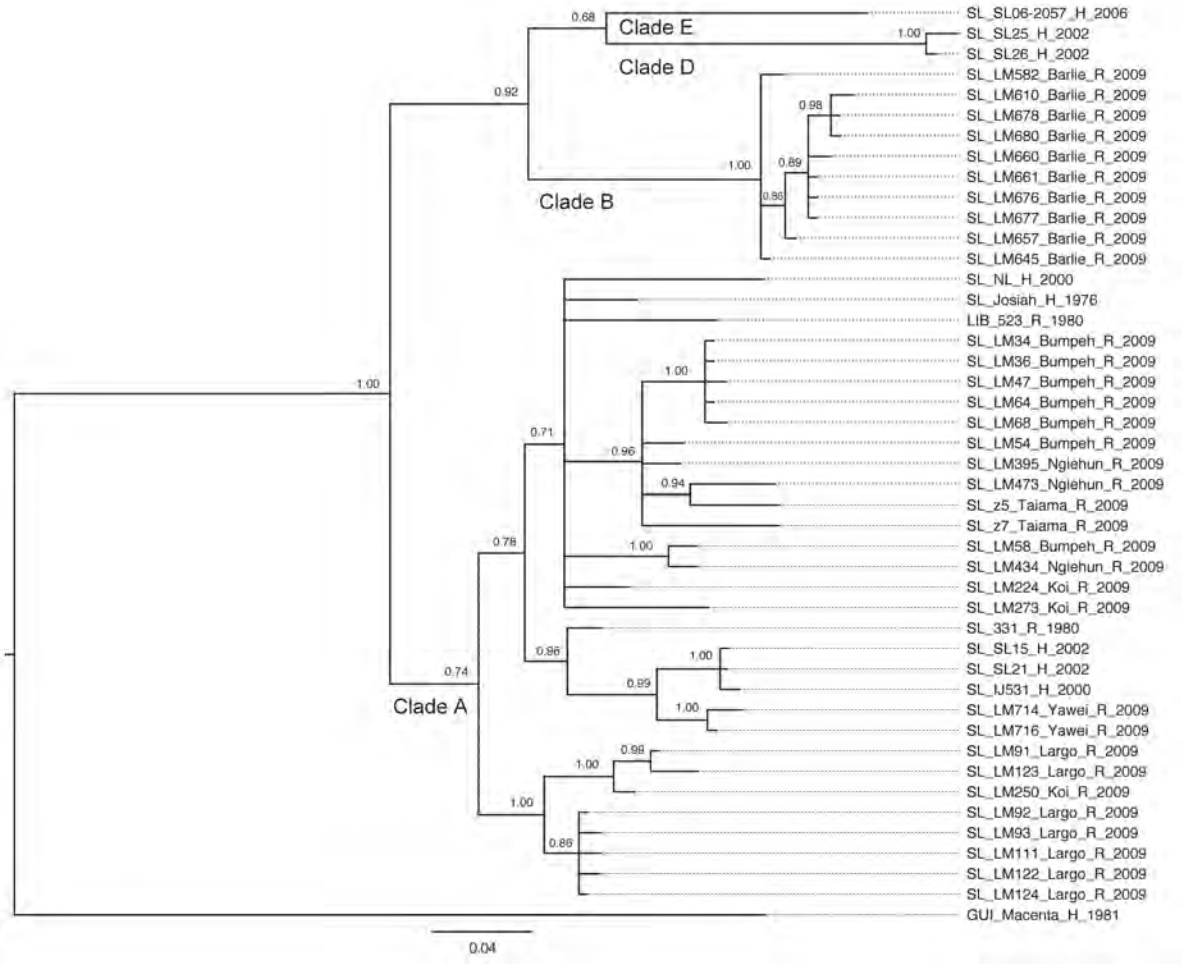

Figure 3. Phylogenetic analysis of Lassa virus (LASV) isolates from Sierra Leone based on partial glycoprotein precursor (GPC) gene sequences. The homologous GPC fragments of 284 nt were aligned. The isolate Z-158, which originated from Macenta district in Guinea were used as outgroup based on the previous phylogenetic analyses to root the tree. The 50% majority rule consensus tree was estimated by using Bayesian Inference method implemented in MrBayes software (*32*) using the Kimura 2-parameter substitution model with a fraction of evolutionary invariant sites. The strain labels contain information on the country of origin (SL, Sierra Leone; GUI, Guinea; LIB, Liberia), strain designation, village or town of origin, type of isolate (H, human; R, rodent), and year of isolation. The numbers next to the branches indicate the posterior probability of particular clades. The clades as defined in this study (clades A, B, D, and E) are also indicated next to the appropriate branches. Scale bar indicates substitutions per site.

(obtained in 1996 and 1977, respectively) most likely originated from the Yawei village area because they cluster closely. A few other human isolates (SL15, SL20, and SL21) for which no location information is available also clustered with Yawei isolates on the basis of GPC and L sequences, suggesting their origin in the same area. These sequences were obtained in 2002 from United Nations peacekeepers stationed in this part of Sierra Leone (*28*,*35*).

Recent epidemiologic data show that LF was detected in 10 of 13 districts in Sierra Leone, which suggests that the infection is much more common that previously recognized (*36*). Phylogenetic analysis of the sequences revealed that strains circulating in districts to the west of the traditional hyperendemic area from which most sequence information is available differ significantly (clade B), which suggests that these could be distinct LASV strains that circulated in local multimammate rat populations for a long time since diverging from a common ancestor and are unlikely to have resulted from recent expansion of this rodent to new areas, as was recently suggested to explain emergence of cases from districts in which LF was not previously reported (*36*). Furthermore, the presence of LASV in Barlie with such high prevalence was surprising because this area historically has had few reports of LASV until 2 human LF cases reported in 2009 (L.M. Moses, unpub. data). The lack of reported LF cases from this area leads to speculation that clade B may be a less pathogenic form of LASV, and transmission to humans might have occurred previously but went unrecognized because of milder, nonhemorrhagic symptoms. In fact, the

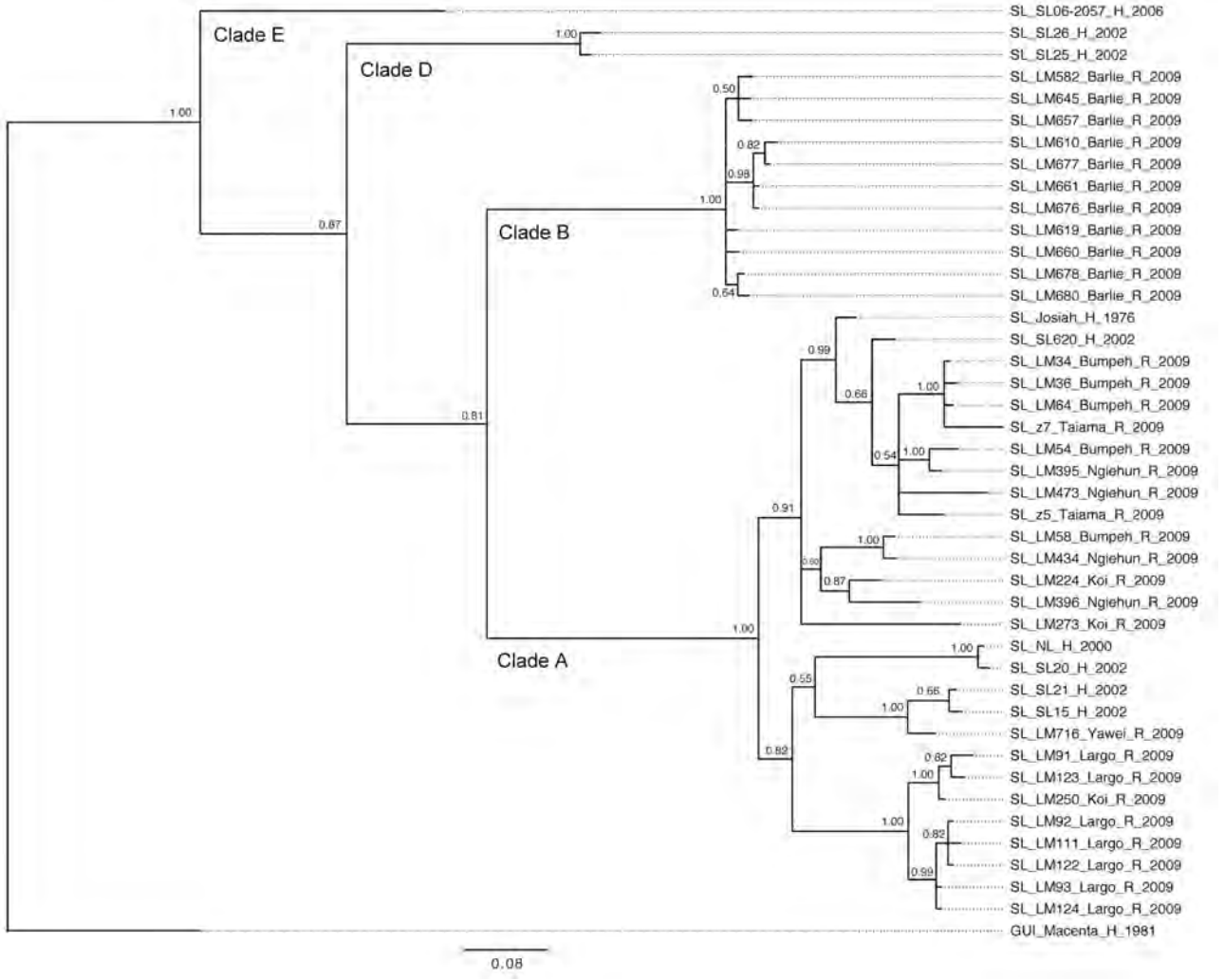

Figure 4. Phylogenetic analysis of Lassa virus (LASV) isolates from Sierra Leone based on partial polymerase (L) gene sequences. The homologous L fragments of 373 nt were aligned. The isolate Z-158, which originated from Macenta district in Guinea, was used as outgroup based on the previous phylogenetic analyses to root the tree. The 50% majority rule consensus tree was estimated by using Bayesian Inference method implemented in MrBayes software (*32*) using the Tamura 3-parameter substitution model with and a fraction of evolutionary invariant sites. The strain labels contain information on the country of origin (SL, Sierra Leone; GUI, Guinea), strain designation, village or town of origin, type of isolate (H, human; R, rodent), and year of isolation. The numbers next to the branches indicate the posterior probability of particular clades. The clades as defined in this study (clades A, B, D, and E) are also indicated next to the appropriate branches. Scale bar indicates substitutions per site.

idea of broader area of LASV endemicity in Sierra Leone is consistent with results of serosurveys conducted during the 1980s by McCormick, who found seroprevalence levels ranging from 8% in southern coastal areas to 15% in villages in Northern Province (*6*).

Molecular characterization of isolates from a wider geographic area of the country is needed to fully understand the diversity of the LASV strains in Sierra Leone and its impact on disease distribution and risk. Such information would be useful for developing efficient viral detection technologies, for example, enabling design of PCR primers and antibodies specific for a broad range of LASV types. These diagnostic tests are extremely relevant to disease surveillance and monitoring and evaluation of interventions to prevent primary LASV infection in humans. More extensive information about sequence diversity affecting the antigenicity of the virus or the function of its RNA-dependent RNA polymerase may help in the development of vaccines and antiviral drugs. It will also lead to deeper understanding of the biology and pathogenesis of LASV.

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Influenza A(H7N9) Virus Transmission between Finches and Poultry

Jeremy C. Jones, Stephanie Sonnberg, Richard J. Webby, Robert G. Webster

Low pathogenicity avian influenza A(H7N9) virus has been detected in poultry since 2013, and the virus has caused >450 infections in humans. The mode of subtype H7N9 virus transmission between avian species remains largely unknown, but various wild birds have been implicated as a source of transmission. H7N9 virus was recently detected in a wild sparrow in Shanghai, China, and passerine birds, such as finches, which share space and resources with wild migratory birds, poultry, and humans, can be productively infected with the virus. We demonstrate that interspecies transmission of H7N9 virus occurs readily between society finches and bobwhite quail but only sporadically between finches and chickens. Inoculated finches are better able to infect naive poultry than the reverse. Transmission occurs through shared water but not through the airborne route. It is therefore conceivable that passerine birds may serve as vectors for dissemination of H7N9 virus to domestic poultry.

In spring 2013, novel avian influenza A(H7N9) viruses emerged in eastern China (*1*). These viruses are reassortants of subtype H7 and H9N2 viruses from wild birds and poultry (*2*,*3*) and were detected in humans and subsequently in chickens, ducks, pigeons, water, and soil at bird markets (*4*,*5*). H7N9 virus does not induce clinical signs in poultry (*6*), and genetic analyses show a monobasic cleavage site in the hemagglutinin (HA) protein (*1*); H7N9 virus is therefore classified as a low pathogenicity avian influenza virus (LPAIV). However, the virus can infect humans and cause severe disease (*7*). Human infection with H7N9 virus was first reported in China in March 2013 (*8*). By October 2, 2014, a total of 453 confirmed cases and 175 associated deaths had been reported (http://www.who.int/influenza/human_animal_interface/influenza_h7n9/riskassessment_h7n9_2Oct14.pdf?ua=1). Despite their avian genetic background, some H7N9 viruses have HA and polymerase protein mutations that confer a replication advantage in mammals (*1*). Human infection has been associated with exposure to poultry or live poultry markets (*7*,*9*); market closings likely contributed to infection declines in mid-2013 (*10*). Nevertheless, H7N9 virus persists in poultry, and human infections surged in the late 2013, demonstrating that this virus is an ongoing public health threat (*11*).

The polymerase acidic (PA) and polymerase basic 2 genes derived from A/Anhui/1/2013 (H7N9)–like virus are homologous to those from A/brambling/Beijing/16/2012 (H9N2) (*1*,*8*), a strain isolated from a brambling (*Fringilla montifringilla*, a small passerine bird). In addition, during surveillance in 2013, the influenza strain A/tree sparrow/Shanghai/01/2013 (H7N9) was identified in a tree sparrow (*Passer montanus,* a passerine bird) found at a site where migratory and local birds congregate (*12*).

We previously showed that society finches (*Lonchura striata domestica*), zebra finches (*Taeniopygia guttata*), sparrows (*P. domesticus*), and parakeets (*Melopsittacus undulates*) are susceptible to H7N9 virus and shed virus into water (*13*). The birds used in those experiments are examples of passerine and psittacine birds, which include individual species that are migratory, peridomestic, and domesticated. The interaction of wild birds, humans, and domesticated animals may contribute to the maintenance and spread of H7N9 virus. To further address the contribution of passerines to the ecology of H7N9 virus, we modeled potential interspecies virus transmission by using society finches (a passerine bird) and poultry (bobwhite quail and chickens) and determined the route of virus transmission.

Methods

Viruses and Facilities

For the experiments, we used A/Anhui/1/2013 (H7N9) (hereafter referred to as Anhui/1) from an index human patient (*14*–*18*) and a poultry isolate, A/chicken/Rizhao/867/2013 (H7N9) (hereafter referred to as Ck/Rizhao), from an original swab sample. Anhui/1 and Ck/Rizhao (provided by Huachen Zhu [Shantou University, Shantou, China] and Yi Guan [University of Hong Kong, Hong Kong, China]) were propagated and titrated in embryonated chicken eggs (*13*). Pooled allantoic fluid was used as virus stock, and the viruses were passaged 3 times in eggs. The genomic sequence of the Anhui/1 sample corresponded to those of an isolate from GISAID

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(Global Initiative on Sharing Avian Influenza Data; accession no. EPI_ISL_138739), and genomic sequences of the Ck/Rizhao sample corresponded to those of an isolate from GenBank (accession nos. KF260954, KF259043, and KF259731). Experiments were performed under Animal Biosafety Level 3+ conditions as defined in US Department of Agriculture regulatory documents 9 CFR part 121 and 7 CFR part 331 (http://www.aphis.usda.gov/programs/ag_selectagent/downloads/FinalRule3-18-05.pdf).

Animals

Study birds were of both sexes and included 3- to 6-month-old society finches (*L. striata domestica*) (Birds Express, South El Monte, CA, USA); 5-week-old white leghorn hens (*Gallus gallus domesticus*) (McMurray Hatchery, Webster City, IA, USA); and 16-week-old bobwhite quail (*Colinus virginianus*) (B&D Game Farm, Harrah, OK, USA). The birds were quarantined for 1 week, and prechallenge swab samples were confirmed influenza virus–negative by egg isolation. Food and water were provided ad libitum. Animal experiments were approved by the St. Jude Children's Research Hospital Animal Care and Use Committee and complied with all applicable US regulations.

Inoculation and Sampling

Birds were inoculated intranasally, intraocularly, or orally with 10^5 $\log_{10}$ 50% egg infectious doses (EID_{50}) of virus in 100 mL (finches) or 500 mL (poultry) of phosphate buffered saline. Oropharyngeal and cloacal swab samples were collected on days postinoculation (dpi) 2, 4, 6, 8, 10, and 13. Water samples (500 mL) were obtained 1–4 and 8 dpi. Samples were titrated in eggs (*13*).

Interspecies Transmission Study Design

Birds were cohoused in a cage-within-a-cage setup. Poultry (n = 3) were housed in a 97 cm × 58 cm × 53 cm cage that contained a 30 cm × 41 cm × 41 cm cage housing finches (n = 4 or 5). This setup was used in duplicate for each experiment, and the data obtained from each set of cages were combined. Waterborne transmission was examined by sliding a water pan (15 cm × 25 cm) halfway into a notched hole in the finch cage (Figure 1, panel A); birds shared water but did not have physical contact. For airborne transmission experiments, an air-permeable barrier separated poultry from the finch cage, and water sources were separate (Figure 1, panel B). Each day, 1 L of filtered, nonchlorinated water was provided by topping off the supply remaining in the water pans; every 96 h, the full water supply in the pans was replaced.

Necropsy

Necropsies were performed on birds that died during the study. Trachea and/or lung and intestine samples were harvested (Table 1) and homogenized in 1 mL of saline–antimicrobial drugs. Virus was isolated and titrated in eggs (*13*).

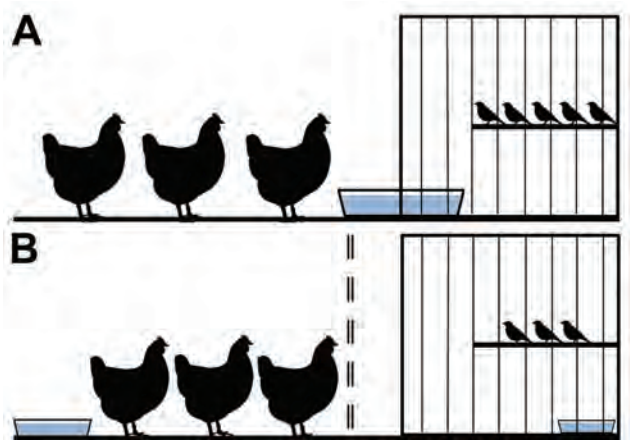

Figure 1. Design model for an interspecies study of influenza A(H7N9) virus transmission. Birds were housed in a cage-within-a-cage setup with a 30 cm × 41 cm × 41 cm finch cage placed within a 97 cm × 58 cm × 53 cm poultry cage. A) Waterborne transmission was examined by sliding a 15 cm × 25 cm pan containing ≈1 L water halfway into a notched hole in the finch cage. All birds had shared access to the water, but poultry and finches were excluded from physical contact with each other. B) Airborne transmission was examined by inserting an air-permeable cage barrier (represented by double-dashed line) between the poultry and the finch cage and providing separate water supplies so that poultry and finches had no direct physical contact and did not share food or water resources.

Serologic Testing

Before beginning the experiments, we tested ≥5 birds from each species for influenza A virus antibodies; all results were negative. On dpi 16, we collected blood samples from the surviving birds and tested them for H7N9 virus seroconversion by using the IDEXX AI MultiS-Screen Ab Test (IDEXX Laboratories, Westbrook, ME, USA) according to the manufacturer's instructions.

Statistical Analyses

Mean infectious titers were compared by using the 1-tailed Student *t*-test in Excel (Microsoft, Redmond, WA, USA) or GraphPad Prism v5 (La Jolla, CA, USA). Area under the curve (AUC) analysis for cumulative shedding was performed by using GraphPad Prism v5.

Results

Waterborne Transmission between Society Finches and Chickens

Waterborne virus transmission between finches and chickens was investigated by inoculating 1 species (donors) with 10^5 $\log_{10}$ EID_{50} units of virus and pairing the donor birds

Table 1. Virus isolation from organs of dead birds in an interspecies study of influenza A(H7N9) virus transmission*

Bird species	Time of death, dpi	Influenza virus exposure	Transmission		Virus titer, $\log_{10}$ EID_{50}/mL†	
			Route	Direction	Trachea and/or lung	Intestine
Naive contact						
Finch	4	A/Anhui/1/2013 (H7N9)	Airborne	Chicken → Finch	0	0
Finch	5	A/Anhui/1/2013 (H7N9)	Waterborne	Chicken → Finch	4.3 (combined)	0
Finch	5	A/chicken/Rizhao/867/2013 (H7N9)	Waterborne	Chicken → Finch	6.5 (combined)	0
Quail	15	A/Anhui/1/2013 (H7N9)	Waterborne	Finch → Quail	4.7 (trachea); 5.5 (lung)	ND
Quail	15	A/Anhui/1/2013 (H7N9)	Waterborne	Finch → Quail	7.3 (trachea); 8.3 (lung)	ND
Quail	10	A/chicken/Rizhao/867/2013 (H7N9)	Waterborne	Finch → Quail	6.5 (trachea); 7.5 (lung)	ND
Inoculated						
Finch	6	A/Anhui/1/2013 (H7N9)	Waterborne	Finch → Chicken	4.5 (combined)	0
Finch	2	A/Anhui/1/2013 (H7N9)	Waterborne	Finch → Quail	2.5 (trachea); 2.3 (lung)	ND
Quail	15	A/Anhui/1/2013 (H7N9)	Airborne	Quail → Finch	3.3 (trachea); 4.5 (lung)	ND

*dpi, days postinfection; EID_{50}, 50% egg infectious dose; ND, not determined.
†Assessed in embryonated chicken eggs.

with the naive bird species (water contacts) (Figure 1, panel A). We previously observed little to no shedding in society finches via the cloaca (*13*); thus, in this study, we collected swab samples at a single time point (4 dpi). We obtained oropharyngeal and cloacal swab samples from poultry at each time point.

All donor finches were productively infected with Anhui/1 or Ck/Rizhao and shed virus by the oropharyngeal route for 10–13 dpi (Figure 2, panels A, C; Table 2). No virus was detected in cloacal swab samples. Using AUC analysis, including all animals, we found no statistical difference between cumulative shedding of the 2 viruses from donor finches.

During oropharyngeal sampling of the naive water contacts, we considered the possibility that we were obtaining transient virus that the birds acquired during recent drinking. To differentiate transiently acquired virus from replicated/shed virus, we defined a transmission event as an instance when samples from a naive water contact contained ≥2.5 $\log_{10}$ EID_{50}/mL of virus and/or when the bird shed during ≥2 consecutive time points. Under such criteria, waterborne transmission from finches to chickens was limited. Of 6 water-contact chickens paired with Anhui/1-donor finches, 2 shed <2 logs of virus for a single time point, which did not meet our transmission criteria (Figure 2, panel A; Table 2). Two water-contact

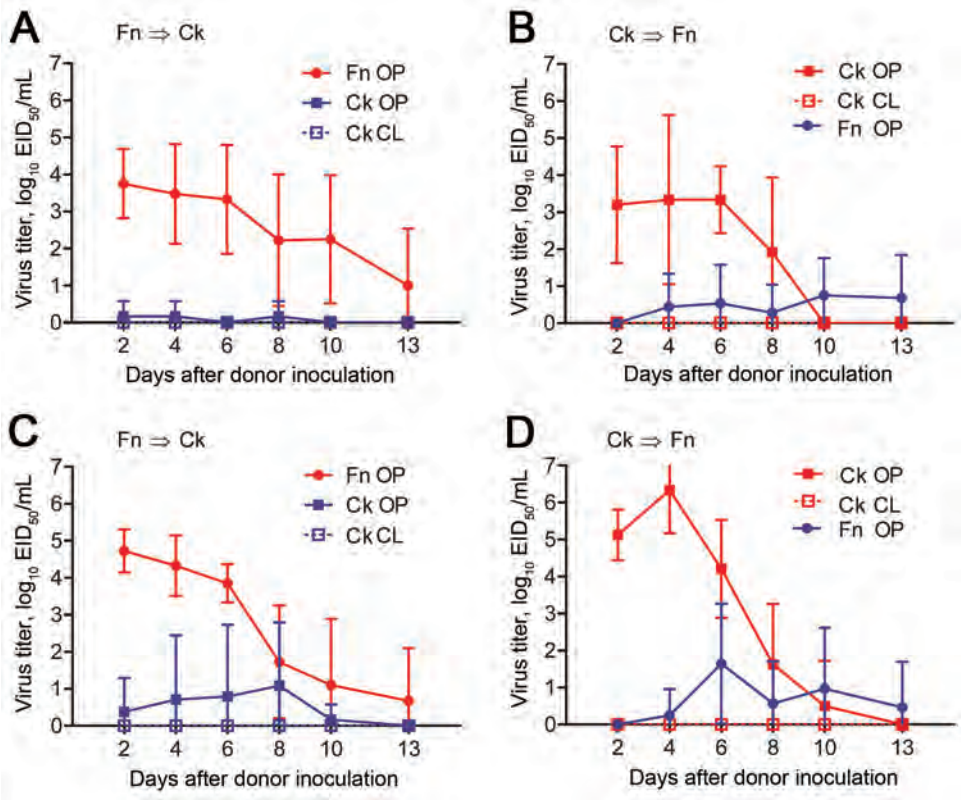

Figure 2. Waterborne transmission of virus between chicken and finches in an interspecies study of influenza A(H7N9) virus transmission. Finches (n = 8 or 10) and chickens (n = 6) were inoculated with strain A/Anhui/1/2013 (H7N9) (A, B) or A/chicken/Rizhao/867/2013 (H7N9) (C, D) and paired with naive birds in an environment in which physical contact was prevented but water was shared (Figure 1, panel A). Swab samples were obtained from birds every 48 h, and virus titers were determined in embryonated chicken eggs. Data are the average titer per time point ± SD. Directionality of transmission (i.e., infected → naive) is indicated in the top left of each panel. Red indicates infected animals; blue indicates naive animals. Ck, chicken; CL, cloacal swab sample; EID_{50}, 50% egg infectious dose; Fn, finch; OP, oropharyngeal swab sample.

Table 2. Virus transmission, shedding, sickness, and death among birds in an interspecies study of influenza A(H7N9) virus transmission*

Virus, donor/naive contact, transmission route	No. with OP virus shedding/no. total (%)		Clinical signs of illness†		No. died/no. total (%) species
	Donors	Naive contacts	Donors	Naive contacts	
A/Anhui/1/2013 (H7N9)					
Finch/chicken					
Waterborne	10/10 (100)	0/6 (0)	–	–	1/10 (10) finches
Airborne	2/2 (100)	0/3 (0)	–	–	0
Finch/quail					
Waterborne	10/10 (100)	6/6 (100)	–	+	1/10 (10) finches; 2/6 (33) quail
Airborne	3/3 (100)	0/3 (0)	–	–	0
Chicken/finch					
Waterborne	6/6 (100)	3/8 (38)	–	–	1/8 (13) finch
Airborne	3/3 (100)	0/2 (0)	–	–	1/2 (50) finch
Quail/finch					
Waterborne	6/6 (100)	2/10 (20)	+	–	0
Airborne	3/3 (100)	0/3 (0)	+	–	1/3 (33) quail
A/chicken/Rizhao/867/2013 (H7N9)					
Finch/chicken					
Waterborne	10/10 (100)	2/6 (33)	–	–	0
Airborne	NA	NA	NA	NA	NA
Finch/quail					
Waterborne	10/10 (100)	6/6 (100)	–	–	1/6 (17) quail
Airborne	NA	NA	NA	NA	NA
Chicken/finch					
Waterborne	6/6 (100)	4/8 (50)	–	–	1/8 (13) finch
Airborne	NA	NA	NA	NA	NA
Quail/finch					
Waterborne	6/6 (100)	2/10 (20)	–	–	0
Airborne	NA	NA	NA	NA	NA

*OP, oropharyngeal route; NA, not applicable; +, symptoms present; –, symptoms not present.
†Clinical signs or symptoms were observed at least once and included ≥1 of the following: hunched posture, ruffled feathers, and lethargy.

chickens paired with Ck/Rizhao-donor finches shed virus over multiple time points (Figure 2, panel C; Table 2). Water-contact chickens shed virus by the oropharyngeal route; virus was not detected in cloacal swab samples. Cumulative shedding was not significantly different for the 2 viruses in the water contacts.

In the converse experiment, all donor chickens were productively infected with both viruses and shed virus an average of 10 days (Figure 2, panels B, D; Table 2). Chickens shed virus by the oropharyngeal route only, and cumulative shedding was statistically higher in birds inoculated with Ck/Rizhao than with Anhui/1 (AUC analysis, $p \leq 0.01$).

Three water-contact finches paired with Anhui/1-donor chickens met our transmission criteria, although average virus titers were low (peak titers 1.0–2.8 $\log_{10}$ EID_{50}/mL) (Figure 2, panel B; Table 2). Four water-contact finches paired with Ck/Rizhao-donor chickens became infected; 3 had low virus titers (peak titer 1.0–3.5 $\log_{10}$ EID_{50}/mL), but the fourth finch had higher virus titers (peak titer 4.3 $\log_{10}$ EID_{50}/mL) and shed virus for 7 days (4 sampling time points) (Figure 2, panel D; Table 2). All water-contact finches shed virus by the oropharyngeal route; no virus was isolated from cloacal swab samples. Cumulative shedding of the 2 viruses did not differ statistically in the water-contact finches. Overall, our data showed that, when water resources are shared, virus transmission between society finches and chickens is sporadic, and contact birds generally shed virus at low titers for short periods.

Waterborne Transmission between Finches and Quail

As in the finch–chicken experiments, society finches or bobwhite quail in this experiment were inoculated with Ck/Rizhao or Anhui/1 (donors) and shared water with naive birds. As in the other experiments, all donor finches in this experiment shed both viruses an average of 10 days (Figure 3, panels A, C; Table 2). Virus was shed by the oropharyngeal route, with the exception of 1 Ck/Rizhao-inoculated finch that shed virus via the cloaca 4 dpi (3.5 $\log_{10}$ EID_{50}/mL). Water-contact quail were quickly infected and shed virus by 2 dpi.

All water-contact quail shed Anhui/1 by the oropharyngeal route (Figure 3, panels A, C; Table 2), and peak virus titers were equal to or exceeded those of the donor finches beginning at 4 dpi (AUC analysis, $p = 0.0005$). All water-contact quail also shed virus via the cloaca (Figure 3, panels A, C). A comparable trend was observed in Ck/Rizhao experiments: by 2 dpi, all water-contact quail shed virus by the oropharyngeal route, and cumulative titers exceeded those of the donor finches (AUC analysis, $p<0.001$). Only half these birds shed virus via the cloaca (Figure 3,

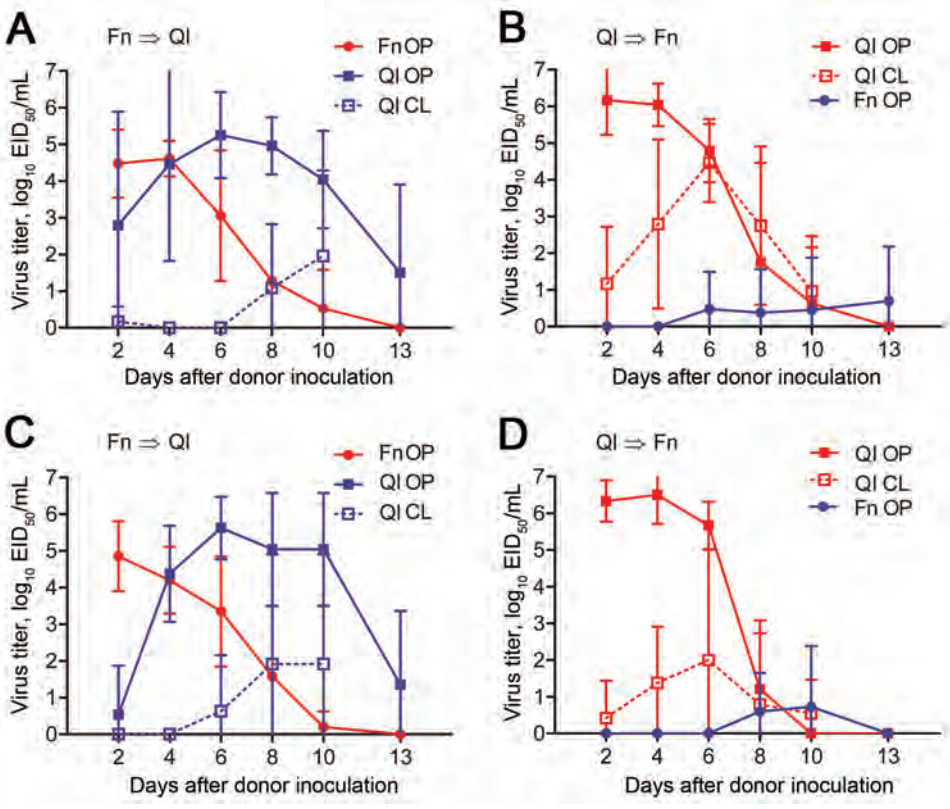

Figure 3. Waterborne transmission of virus between birds in an interspecies study of influenza A(H7N9) virus transmission. Finches (n = 10) and quail (n = 6) were inoculated with influenza virus strain A/Anhui/1/2013 (H7N9) (A, B) or A/chicken/Rizhao/867/2013 (H7N9) (C,D) and paired with naive birds in an environment in which physical contact was prevented but water was shared (Figure 1, panel A). Swab samples were obtained from birds every 48 h, and virus titers were determined in embryonated chicken eggs. Data are the average titer per time point ± SD. Directionality of transmission (i.e., infected→naive) is indicated in the top left of each panel. Red indicates infected animals; blue indicates naive animals. EID_{50}, 50% egg infectious dose; Fn, finch; Ql, quail; OP, oropharyngeal swab sample; CL cloacal swab sample.

panel C). Cumulative oropharyngeal shedding of the 2 viruses did not differ statistically in the water-contact quail.

In the converse experiment, in which quail served as donors, all quail shed Anhui/1 and Ck/Rizhao by the oropharyngeal and cloacal routes for 13 and 10 days, respectively (Figure 3, panels B, D; Table 2). Despite the high titers of virus shed from quail, transmission to the water-contact finches was infrequent. Two of 10 finches in the Anhui/1 experiment shed virus (1 shed for 7 days) (Figure 3, panel B; Table 2). Two of 10 finches in the Ck/Rizhao experiment shed virus, although only for 2 consecutive time points each (Figure 3, panel D; Table 2). Cumulative shedding of the 2 viruses did not differ statistically in the water-contact finches. Thus, when finches and quail share water resources, efficient and sustained interspecies transmission occurs from finches to quail, but only sporadic transmission occurs from quail to finches.

Airborne Transmission between Finches and Poultry

The birds in our experiments shared the same airspace, so we examined whether virus transmission occurred between the species via large droplet particles or smaller, fully aerosolized particles. Droplet transmission was less likely to occur because the animals were separated and droplet sources (e.g., water splashes) were minimized through placement of separate water pans. Society finches (n = 2 or 3) were housed with chickens or bobwhite quail (n = 3) (Figure 1, panel B). Consistent with birds in the previous experiments, donor finches, chickens, and quail shed virus for 6–10, 8, and 13 days, respectively (Figure 4; Table 2). Finches and chickens shed virus by the oropharyngeal route only; quail shed virus by the oropharyngeal and cloacal routes (Figure 4; Table 2). In the chicken–finch pairings, neither naive species shed virus at any time point (Figure 4, panels A, B; Table 2). In the quail–finch pairings, naive finches also shed no virus (Figure 4, panel C; Table 2), but 1 naive quail shed virus via the cloaca on dpi 8. Shedding from the naive quail was the only instance of virus detection in this group and did not meet our transmission criteria, and the shed virus was at the lower limit of detection (Figure 4, panel D). Thus, in our experimental setting, there was no airborne transmission between finches and chickens and very little if any between finches and quail.

Virus Load in Shared Water Pans

In the previously described experiments, we observed interspecies transmission of H7N9 virus when birds shared water, but transmission did not occur when they shared airspace but not water. We hypothesized that this effect was primarily mediated by water contact. Because finches, chickens, and quail shed virus by the oropharyngeal route (often exclusively), transmission via water is possible and may occur during

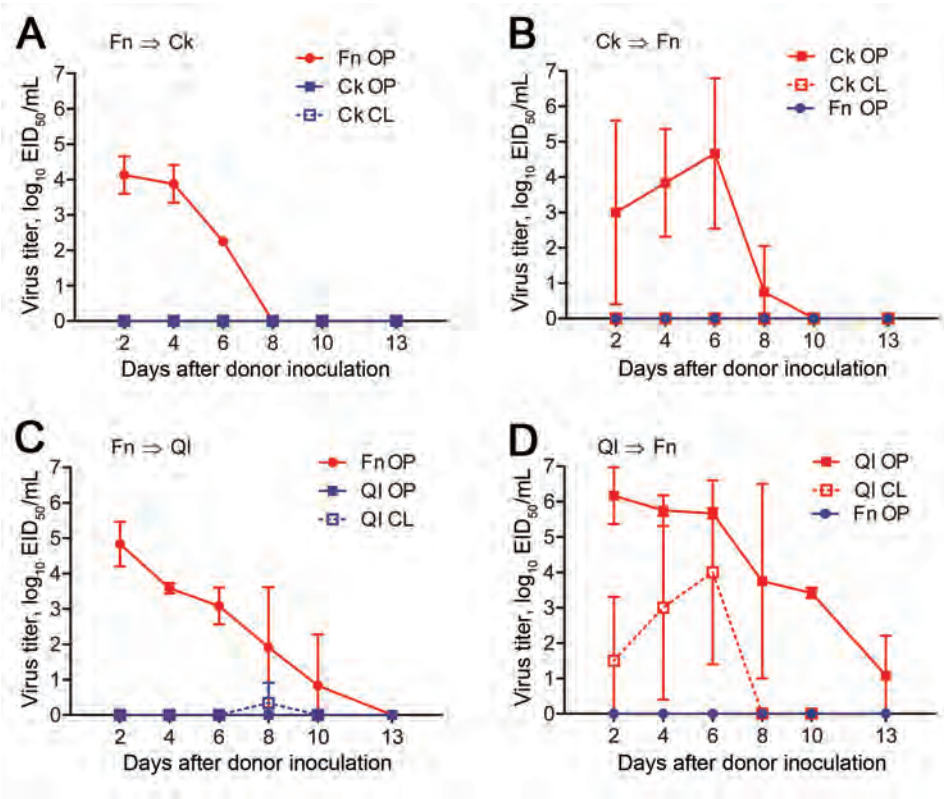

Figure 4. Airborne transmission of virus between birds in an interspecies study of influenza A(H7N9) virus transmission. Finches (n = 2 or 3) and chickens or quail (n = 3) were inoculated with influenza virus strain A/Anhui/1/2013 (H7N9) and paired with naive birds in an environment in which physical contact was prevented but by an air-permeable barrier and food/water resources were not shared (Figure 1, panel B). Swab samples were obtained from birds every 48 h, and virus titers were determined in embryonated chicken eggs. Data are the average titer per time point ± SD. Directionality of transmission (i.e., infected→naive) is indicated in the top left of each panel. Red indicates infected animals; blue indicates naive animals. EID_{50}, 50% egg infectious dose; Fn, finch; Ql, quail; OP, oropharyngeal swab sample; CL cloacal swab sample.

drinking and other events associated with water contact. To test this hypothesis, we sampled 500 mL of the water remaining in each pan on 4 different dpi and at 8 dpi (i.e., representing the water supply 4 days after a full water change).

During ≥4 of 5 sampled time points, virus was detected in all water pans, regardless of the inoculated bird species (Figure 5). Peak virus titers in water among the 4 initial time points were 3.8–6.5 $\log_{10}$ EID_{50}/mL for finch–chicken experiments and 3.8–4.5 $\log_{10}$ EID_{50}/mL for finch–quail experiments (Figure 5). At 8 dpi, 4 days after a full water change, virus was still present (2.8–3.5 $\log_{10}$ EID_{50}/mL) (Figure 5). Therefore, substantial and sustained amounts of infectious virus were shed from infected birds into shared water pans. Shedding patterns in our past (*13*) and present studies suggest that this virus was deposited into the water via oropharyngeal shedding. Because of variations in water levels and consumption by different species, quantitative comparisons were not possible.

Illness among Birds and Virus Isolation from Organs

Overall, 5 of 86 finches, 4 of 30 quail, and 0 of 30 chickens died during the experiments (Tables 1, 2). All chickens remained free of clinical signs of disease, and none died.

Two of 25 finches inoculated with Anhui/1 died at 2 and 6 dpi, respectively; neither bird had clinical signs of disease. Virus titers in trachea and lung samples were 2.3–4.5 $\log_{10}$ EID_{50}/mL (Table 2).

Three contact finches died: 1/18 water contacts and 1/5 airborne contacts paired with Anhui/1-infected chickens, and 1/18 water contacts paired with Ck/Rizhao-inoculated chickens. No clinical signs of disease were observed in these birds. Virus was not isolated from swab samples for water contacts before death but was isolated from trachea and lung samples at necropsy (4.3–6.5 $\log_{10}$ EID_{50}/mL) (Figure 2, panels B, D; Tables 1, 2). An airborne-contact finch that died 4 dpi showed no clinical signs, did not shed virus (Figure 4, panel B; Table 2), and had no virus in its organs (Table 2). This death was likely caused by cage stress, although the definitive cause could not be determined.

One of 9 Anhui/1-donor quail died 15 dpi; virus was isolated from its trachea (3.3 $\log_{10}$ EID_{50}/mL) and lung (4.5 $\log_{10}$ EID_{50}/mL) at necropsy (Table 2). Three days before death, the bird displayed hunched posture, ruffled feathers, and lethargy. All inoculated cage mates of the bird showed less severe degrees of lethargy. All Ck/Rizhao-donor quail remained free of clinical signs (Table 2).

Two of 6 naive water-contact quail in Anhui/1 experiments died at 15 dpi, and 1 of 6 naive water-contact quail in Ck/Rizhao experiments died 10 dpi (Table 1). These quail displayed clinical signs of disease (ruffled feathers, hunched posture, a drop in temperature) 2–4 days before death. Virus was detected in respiratory organs at necropsy. We noted sporadic and less severe clinical signs among contact quail in Anhui/1 experiments; these birds survived

(Table 2). One water-contact quail in an Anhui/1 experiment had conjunctivitis beginning 6 dpi (data not shown), and eye swab samples were positive for H7N9 virus (4.8 $\log_{10}$ EID_{50}/mL).

Seroconversion of Finches and Poultry

Seroconversion was tested by using IDEXX ELISA. Prechallenge serum and swab samples were negative for avian influenza antibodies and virus (data not shown), strongly suggesting the lack of prior exposure to influenza A virus (Table 3).

More than half of donor birds seroconverted: for Anhui/1-inoculated birds, finches 66%–77% (range for donor groups), chickens 50%, quail 100%; for Ck/Rizhao-inoculated birds, finches 50%–100%, chickens 83%, quail 100% (Table 3). Among water-contacts, all quail seroconverted, irrespective of virus. No water-contact chickens paired with Anhui/1-donor finches seroconverted, but 2 of 6 chickens paired with Ck/Rizhao-donor finches seroconverted. Twenty percent of finches in water contact with Ck/Rizhao-donor quail seroconverted, but none of the other contact finches seroconverted. Aerosol-contact birds remained seronegative (Table 3).

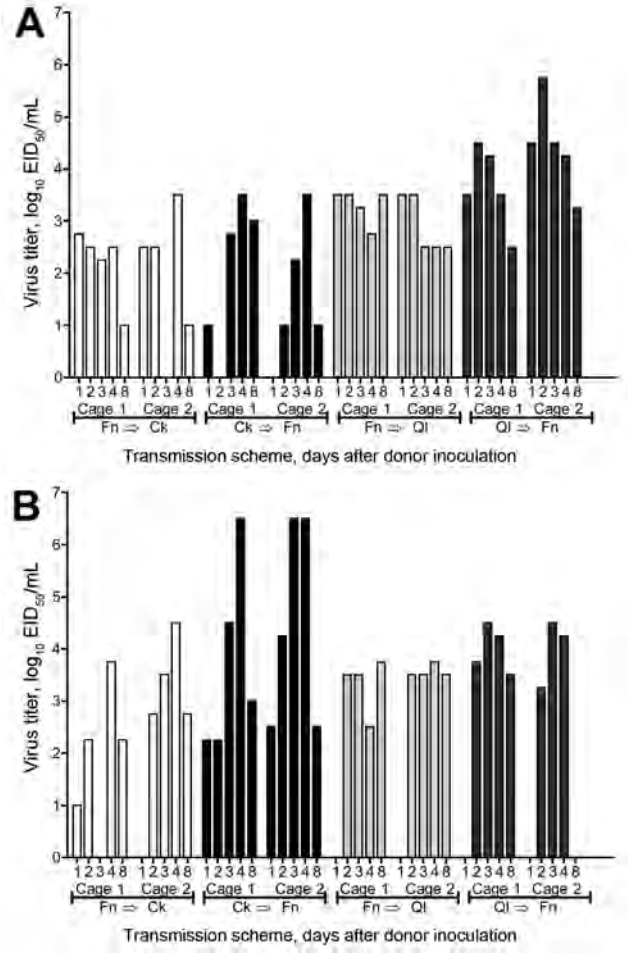

Figure 5. Virus load in shared water pans for birds in an interspecies study of influenza A(H7N9) virus transmission. A shared drinking water sample (500-mL) was collected daily on postinoculation days 1–4 and 8. Virus titers in samples were determined in embryonated chicken eggs. A) Shedding from A/Anhui/1/2013 (H7N9)–infected birds. B) Shedding from A/Chicken/Rizhao/867/2013 (H7N9)–infected birds. Cages 1 and 2 indicate results from duplicate experimental groups. EID_{50}, 50% egg infectious dose; Ck, chicken; Fn, finch; QI, quail.

Discussion

Novel influenza A(H7N9) viruses emerged in China in 2013 and were first detected in humans with severe illness (*7*). The viruses are maintained in Chinese poultry and continue to cause human disease. We previously showed that songbirds and parakeets are susceptible to H7N9 virus and shed virus into drinking water (*13*). Here we examined interspecies transmission of H7N9 virus and demonstrated that waterborne, but not airborne, transmission occurs between society finches and poultry. Virus was more likely to transmit from chickens to naive finches than vice versa, and such transmission occurred more frequently with chicken virus (Ck/Rizhao) than human virus (Anhui/1). In contrast, virus transmitted more easily from finches to naive quail than vice versa.

H7N9 viruses and viruses with genes homologous to those of H7N9 virus have been isolated from 2 passerine birds: bramblings and a tree sparrow (*1*,*8*,*12*). We used a related passerine bird, the society finch, which originates from munias, close relatives to true finches and true sparrows. Japanese quail are prevalent in East Asia markets but were unavailable for use; thus, we used bobwhite quail (same taxonomic order/family). Bobwhite quail support influenza replication, and virus receptors in their respiratory tracts (*19*) and titers and routes of H7N9 virus shedding are similar to those for Japanese quail (*6*). We believe the model species we used can reflect the dynamic interaction and transmission events we tested.

The isolation of LPAIVs from water has been reported (*20*–*23*). Water plays a key role in the transmission of LPAIVs among waterfowl (*22*,*24*–*26*) and has experimentally been implicated in influenza virus transmission among poultry and other bird species (*27*–*29*). Therefore, our finding of waterborne transmission of H7N9 virus between finches and poultry is consistent with previous findings. However, a study by Ku et al. (*30*) demonstrated that contact transmission of H7N9 between infected and naive chickens does not occur. Presumably, the birds in that study shared a water source, but virus titers in the water were not measured, and inoculated chickens in that study shed for a shorter period than those in our study and other studies (*6*).

Airborne transmission of LPAIV among poultry has been demonstrated for multiple influenza subtypes, but Zhong et al. (*31*) reported that airborne transmission of H9N2 likely

Table 3. Seroconversion among birds in an interspecies study of influenza A(H7N9) virus transmission*

	No. seroconverted/no. total, by transmission route†			
	Waterborne		Airborne	
Challenge virus, species	Donor	Naive contact	Donor	Naive contact
A/Anhui/1/2013 (H7N9), donor → naive contact				
Finch → chicken	7/9	0/6	1/2	0/3
Chicken → finch	3/6	0/7	3/3	0/1
Finch → quail	6/9	4/4	2/3	0/3
Quail → finch	6/6	0/6	2/2	0/3
A/chicken/Rizhao/867/2013 (H7N9), donor → naive contact				
Finch → chicken	5/10	2/6	NT	NT
Chicken → finch	5/6	0/7	NT	NT
Finch → quail	10/10	5/5	NT	NT
Quail → finch	6/6	2/10	NT	NT

*Prechallenge serum samples from 7 finches, 5 chickens, and 5 quail were confirmed influenza virus–negative by IIDEXX ELISA. Donor birds were inoculated with the challenge virus; contacts were not inoculated. NT, not tested.
†Determined by using an influenza A virus blocking ELISA (IDEXX AI MultiS-Screen Ab Test; IDEXX Laboratories, Westbrook, ME, USA). A signal-to-noise cutoff of <0.5 was considered seroconversion.

requires mutations that stabilize the HA (363K) protein, alter PA activity (672L), or both. Anhui/1 and Ck/Rizhao possess PA-672L but lack HA-363K, which may explain the lack of airborne transmission in our experiments. Our data are also in line with those of Spekreijse et al. (*32*), who reported that airborne transmission of H5N1 virus occurs at a low rate or not at all in chickens. Adaptation of H7N9 viruses in poultry or passerine species may be required for airborne transmission. For now, this route appears to represent a low risk. However, in our settings, the small number of animals used and the testing of a human-origin virus could have hindered detection of low-level airborne transmission.

We found little difference between the 2 viruses used in our study, except that replication of Ck/Rizhao was significantly better than that of Anhui/1 in donor chickens. Anhui/1 has several mutations conferring mammalian replication and receptor binding (*1*) and would be predisposed to replicate more efficiently than Ck/Rizhao in mammalian tissues. Thus, although Anhui/1 does replicate in chickens, molecular adaptions to mammals may constrain this replication. Bobwhite and Japanese quail are susceptible to a variety of influenza viruses (*33*–*36*) and have avian and mammalian influenza virus receptors in their respiratory and digestive tracts (*19*,*34*,*37*). It is not surprising that human and avian H7N9 viruses replicated to levels similar to those in these birds.

Although the type of finches used in this study would not necessarily be expected to have poultry contact, other finch and sparrow species are peridomestic and susceptible to H7N9 virus; intermingling of these birds in nonsecured poultry operations like farms or live bird markets could facilitate transmission of H7N9 virus to poultry. Chickens and, in particular, quail could then act as an amplifying host, releasing large amounts of H7N9 virus into the environment, thereby posing a health risk to humans with direct contact. Bobwhite quail and Japanese quail have been shown to be highly susceptible to LPAIVs, including subtype H7N9, and to highly pathogenic avian influenza viruses (*6*,*34*,*36*,*38*); those findings are consistent with our observations of quail as the most receptive recipient species.

We did not address interspecies transmission between birds and mammals. Passerine and psittacine birds shed H7N9 virus at levels lower than or nearly equal to those of domesticated poultry (*6, 13*). However, data correlating virus shedding by poultry with infectivity in mammals is absent, so the correlation between levels of shed virus and transmission is not known. Nevertheless, direct transmission of H7N9 virus from passerines to humans is enhanced because of the prevalence of passerine birds as household pets. Modeling such events is difficult because of the husbandry/cohabitation of the laboratory models required (i.e., ferrets with birds). A recent study demonstrated that experimental airborne transmission of H7N9 virus from donor chickens to naive ferrets does not occur (*30*). However, for humans, the handling of contaminated water (containing virus deposited by small birds or infected poultry) should be considered a risk factor for influenza virus transmission in addition to the already identified risk factor of direct poultry contact. Root et al. (*39*) demonstrated that raccoons exposed to influenza virus–spiked water, duck eggs, or duck carcasses became infected and shed virus only when exposed to the water (*39*). In addition, subtype H7 viruses can cause conjunctivitis in mammals (*40*), so human contact with H7N9 virus–contaminated water could lead to virus inoculation by the ocular route. Cases of H7N9 virus conjunctivitis have not been reported, but incidences of conjunctivitis in poultry workers or those in contact with live poultry should be investigated and monitored.

In summary, in this follow-up of our study identifying small bird species as potential vectors of H7N9 virus (*13*), we found that waterborne transmission of human and avian H7N9 viruses occurred between society finches and poultry (chickens and bobwhite quail). Quail shed virus at the highest titers and were the most susceptible species. We conclude that finches, and likely other passerines, can act as vectors for virus transmission to poultry via shared water.

Acknowledgments

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Dr. Jones is a postdoctoral fellow at St. Jude Children's Research Hospital. His research interests include the host response to influenza viruses, emergence of novel influenza virus variants, and zoonotic transmission of influenza between species.

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Highly Pathogenic Avian Influenza A(H5N1) Virus Infection among Workers at Live Bird Markets, Bangladesh, 2009–2010

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The risk for influenza A(H5N1) virus infection is unclear among poultry workers in countries where the virus is endemic. To assess H5N1 seroprevalence and seroconversion among workers at live bird markets (LBMs) in Bangladesh, we followed a cohort of workers from 12 LBMs with existing avian influenza surveillance. Serum samples from workers were tested for H5N1 antibodies at the end of the study or when LBM samples first had H5N1 virus–positive test results. Of 404 workers, 9 (2%) were seropositive at baseline. Of 284 workers who completed the study and were seronegative at baseline, 6 (2%) seroconverted (7 cases/100 poultry worker–years). Workers who frequently fed poultry, cleaned feces from pens, cleaned food/water containers, and did not wash hands after touching sick poultry had a 7.6 times higher risk for infection compared with workers who infrequently performed these behaviors. Despite frequent exposure to H5N1 virus, LBM workers showed evidence of only sporadic infection.

Human infections and deaths caused by highly pathogenic avian influenza A (H5N1) viruses in several countries (*1*); by A(H9N2) virus in Bangladesh (*2*); and by A(H7N2), A(H7N9), A(H9N2), and A(H10N8) viruses in China (*3*–*5*) reflect the persistent public health threat posed by different avian influenza A virus subtypes. Subtype H5N1 virus remains endemic among poultry in Bangladesh, China, Egypt, Indonesia, and Vietnam (*6*). Among these countries the first human cases of H5N1 virus were identified in China and Vietnam during 2003 (*1*). The seroprevalence of antibodies against H5N1 virus among poultry workers was 0%–4% in Bangladesh, China, Indonesia, and Vietnam during 2001–2009 (*7*–*13*); published data on seroprevalence among poultry workers in Egypt are not available. Beyond the countries where H5N1 is endemic, 0%–10% seroprevalence has been reported among poultry workers in Nigeria; South Korea; Thailand; and Hong Kong, China (*14*–*17*). The incidence of H5N1 virus infection among occupationally exposed populations has not been determined in countries where the virus is endemic or nonendemic.

In Bangladesh, a country with a population density of 964/km^2 and 257 million poultry (*18*,*19*), H5N1 virus infection was first detected among poultry in 2007. By the end of 2013, the country had reported 549 outbreaks among poultry to the World Organisation for Animal Health (*20*). The first human case of H5N1 virus infection in Bangladesh was identified during 2008 (*21*). Live bird markets (LBMs) are often associated with poultry-to-human transmission of H5N1 virus (*22*). For example, butchering and exposure to sick poultry were associated with detection of H5 antibody among LBM workers in Hong Kong (*17*). In one study, workers from 16 LBMs in Bangladesh were rarely observed using personal protective equipment (PPE) or washing their hands during the handling of poultry, suggesting a high likelihood of exposures to H5N1 virus (*23*). Data are limited on the risk for avian influenza A virus infections among poultry workers in Bangladesh (*7*).

Seroprevalence studies among humans yield information about how many persons have serologic evidence of infection at a certain point and time, but they do not provide information about when people became infected or the risk for infection with prolonged exposures to contaminated animals or environments. Studies designed to estimate the rate of seroconversion of antibodies to H5N1 virus among poultry workers may also help elucidate the

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risks of poultry-to-human transmission of H5N1 virus in countries, such as Bangladesh, where H5N1 virus is endemic among poultry. Such information may help public health officials develop, prioritize, and reinforce prevention and control strategies. During 2009–2010, a total of 61 H5N1 outbreaks, resulting in the culling of 220,432 birds, were reported among poultry in Bangladesh (*24*); no human cases were identified during this period. We followed a cohort of LBM workers in Bangladesh to determine the seroprevalence of antibodies to H5N1 virus, the incidence of seroconversion, and risk factors for poultry-to-human transmission of H5N1 virus.

Methods

Study Sites

We conducted this study among workers in 12 LBMs in 4 districts of Bangladesh: 8 in Dhaka, 2 in Chittagong, and 1 each in Netrokona and Rajshahi. We selected these LBMs because they served as sentinel sites for existing avian influenza surveillance throughout the study period; surveillance included the monthly collection of poultry and environmental samples (*25*,*26*). The samples were tested for influenza A and subtype H5 by using real-time reverse transcription PCR (*27*). By April 2009, H5N1 virus was detected from farms in 47 of 64 districts in Bangladesh, including the 4 districts where the LBMs in our study were located (*20*).

The LBMs in Dhaka, which were open daily from 6:00 AM to midnight, sold chickens, ducks, geese, and quail. The workers slaughtered, defeathered, eviscerated, and sold the poultry. LBMs outside Dhaka were in rural subdistricts and were open once or twice a week. Backyard poultry farmers and, occasionally, commercial poultry farmers sold poultry at these LBMs.

Poultry Worker Enrollment and Baseline Data Collection

We aimed to recruit ≈400 workers. All workers 18–59 years of age were eligible for enrollment. This age limit maximized the specificity of detection of H5N1 virus antibodies by microneutralization assay with confirmatory Western blot because the specificity of these assays is lower among older adults (*28*). The field team prepared a list of 721 eligible poultry workers present at the LBMs from 8:00 AM to 5:00 PM.

In 2009, we enrolled a convenience sample of consenting workers from rural subdistrict LBMs during May–June and from urban Dhaka LBMs during October–November, when poultry surveillance became operational (Figure). The poultry workers were enrolled as a closed cohort. The field team used a structured questionnaire (online Technical Appendix 1 Figure 1, http://wwwnc.cdc.gov/EID/article/21/4/14-1281-Techapp1.pdf) to collect demographic data and information about any history of chronic medical conditions; habits involving frequent hand-to-mouth contact (i.e., smoking, smokeless tobacco use, and betel leaf/nut use); the location of poultry handling; and practices that may have placed the workers at risk for H5N1 virus infection (i.e., not wearing PPE, eating while working with poultry, holding or carrying poultry, and eating raw or undercooked poultry or eggs). Medical technologists collected a 10-mL blood specimen from each study participant.

Follow-up Data Collection

During January–April 2010, which included the peak period of H5N1 virus circulation among poultry (*26*), we followed up with study participants one time. Follow-up occurred ≥21 days after virus was first detected through poultry surveillance (*25*) or 1 year after enrollment if H5N1 virus was not detected in an LBM where a study participant worked (Figure). At follow-up, the field team collected information about any history of influenza-like illness (i.e., subjective or measured fever and cough or sore throat) and shortness of breath or difficulty breathing within the 21 days before the follow-up visit and about exposure to sick poultry and precautions taken in the 3 days before respiratory symptom onset (if applicable) or 7 days before collection of the H5N1 virus–positive poultry or environmental surveillance sample (online Technical Appendix 1 Figure 2). In LBMs where H5N1 virus was not detected through poultry surveillance within 1 year after baseline data collection, the field team obtained follow-up data during June 2010, using a questionnaire similar to the one used at baseline. Medical technologists collected a 10-mL blood specimen from all participants during follow-up.

Data Collection from Nonpoultry Workers

In 2010, to get a sense of the baseline seroprevalence rate in a seemingly lower-risk population and to optimize the interpretation of the microneutralization assay results, we obtained samples from a group of nonpoultry workers. We enrolled a convenience sample of nonpoultry workers (18–59 years of age) from 3 accommodating nongovernmental organizations; these persons worked in Dhaka, did not own poultry, and had not participated in studies associated with influenza or other animals since the first detection of H5N1 virus among poultry in Bangladesh during 2007. During July and August 2010, using a structured questionnaire (online Technical Appendix 1 Figure 3), the field team collected demographic data and information about any history of chronic medical conditions; habits involving frequent hand-to-mouth contact (e.g., smoking, smokeless tobacco use, and betel leaf/nut use); and lifetime history of ever handling poultry. Medical technologists collected a 10-mL blood specimen from each nonpoultry worker.

Processing of Blood Specimens and Laboratory Analysis

All blood specimens were transported to the icddr,b laboratory in Dhaka on frozen cold packs at 2°C–8°C. Specimens collected outside Dhaka were centrifuged at the end of each day to separate serum and then transported. Specimens collected in Dhaka were transported to and centrifuged at icddr,b the same day. All serum samples were split into 3 aliquots and stored at icddr,b at –70°C. One aliquot was shipped on dry ice to the Influenza Division at the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) for H5N1 serologic testing.

We performed the microneutralization assay as previously described (*28*,*29*), using H5N1 clade 2.2 (A/Bangladesh/3233/2011) virus, the most common strain identified through surveillance in Bangladesh during the study period. Serial 2-fold dilutions of serum (1:10–1:1,280) were tested. Samples that tested positive by microneutralization assay were also tested by a confirmatory Western blot assay against influenza strain recombinant hemagglutinin A/bar-headed goose/Qinghai/1A/2005 (clade 2.2). Samples with positive assay results or that demonstrated evidence of seroconversion against H5N1 virus were also tested by microneutralization and hemagglutination inhibition assays against pandemic influenza A(H1N1)pdm09 virus strain A/Mexico/4108/2009 (H1N1) to exclude potential serum antibody cross-reactivity. Serum samples that had high titers to A(H1N1)pdm09 virus were adsorbed with A(H1N1)pdm09 virus and then retested by microneutralization for reactivity to H5N1 virus. A seropositive result was defined as an H5N1 virus microneutralization titer ≥40 (equivalent to World Health Organization criteria ≥80) and confirmation by an H5-specific Western blot (*28*–*30*). Seroconversion against H5N1 virus was defined as detection of a ≥4-fold rise in microneutralization antibody titer between the initial serum sample and a paired second serum sample, with the second sample achieving a titer ≥40. Serum samples were tested ≥2 times by using the microneutralization assay. Microneutralization titers were expressed as the geometric mean of replicate titers.

Estimating Seroprevalence and Seroconversion

We calculated the proportion of poultry workers and non-poultry workers that were seropositive at baseline, the proportion of poultry workers that seroconverted against H5N1 virus, and 95% CIs of the proportions, assuming binomial distribution. We calculated the incidence of seroconversion against H5N1 virus among workers with

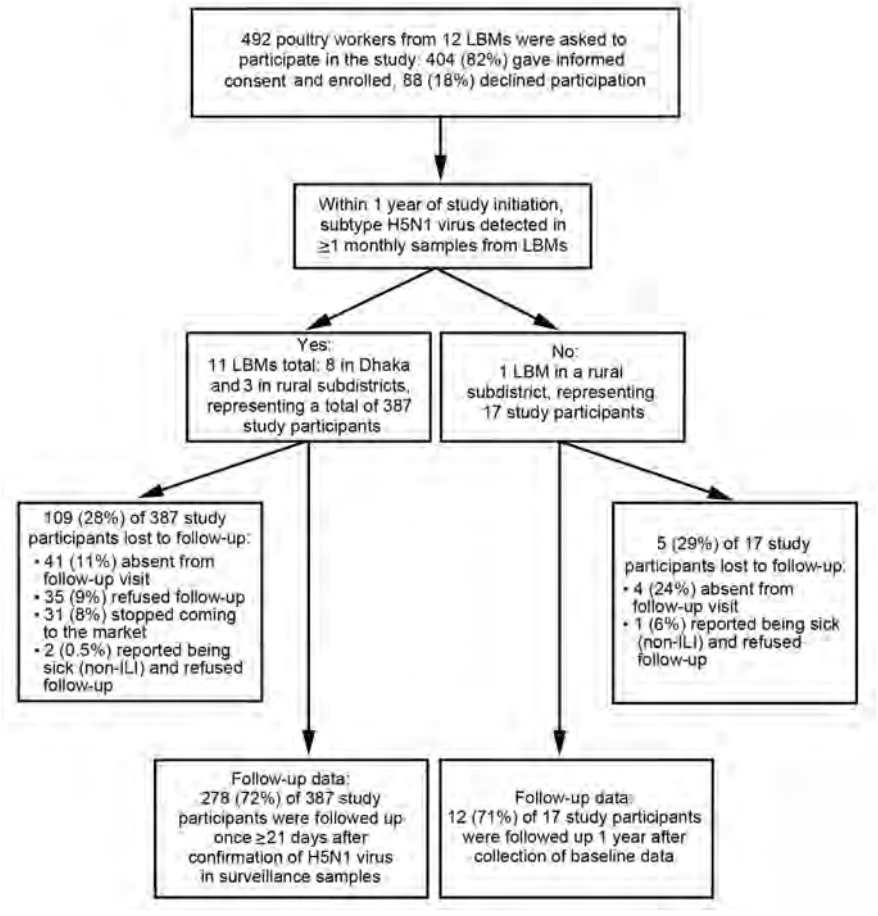

Figure. Enrollment and data for participants in a study of influenza A(H5N1) virus infection among workers at live bird markets (LBMs), Bangladesh, 2009–2010. ILI, influenza-like illness.

paired serum samples who were from LBMs where H5N1 virus was detected through poultry surveillance; workers who were seropositive at baseline were excluded. We calculated the incidence by dividing the number of seroconversions by the person-time each participant contributed to the study between baseline and follow-up data collection and calculated 95% CIs, assuming a Poisson distribution. To be conservative, we assumed that workers were at risk of acquiring H5N1 virus between baseline and follow-up serum collection even though the LBM may have been free of H5N1 virus during some of that period. We extrapolated our calculated incidence of seroconversion among the participating poultry workers to estimate the annual number of poultry workers infected with H5N1 virus among the 721 eligible workers. To compare characteristics between poultry workers and nonpoultry workers, exposure to poultry, and use of PPE between workers who were followed versus those who were lost to follow-up, we performed the 2-sample Wilcoxon rank-sum test and 2-sample test of proportions.

Statistical Analysis of Potential Risk Factors for H5N1 Virus Infection

We assessed risk factors for H5N1 virus infection (seropositivity or seroconversion) only among poultry workers with paired serum samples. Candidate risk factors were collinear, precluding the use of a regression model. Therefore, we performed the Kaiser-Meyer-Olkin test to assess the applicability of factor analysis for this dataset (*31*) and selected sets of common behaviors that explained >90% of variance among the candidate variables. Using the contribution of individual behavior (factor loading) as the basis, we grouped the behaviors into 3 sets and estimated the factor score for each set. Poultry workers with scores above median and those with scores below median were classified, respectively, as frequently and infrequently engaging in these sets of behaviors. We used a log-linear model, adjusted for clustering at the market level, to calculate risk ratio of serologic evidence of H5N1 virus infection for each set of behaviors between workers who were seropositive or seroconverted and those who were not seropositive and did not seroconvert against H5N1 virus (*32*). We applied robust sandwich SE estimation strategy to account for the correlation (*33*).

Protection of Human Subjects

We obtained written informed consent from all participants before enrollment. Institutional review boards at icddr,b and CDC approved the study protocol.

Results

We enrolled 404 LBM poultry workers in the study: 332 from Dhaka and 72 from rural subdistricts. The percentage of refusals was 18% (71/403) in LBMs in Dhaka and 17% (15/89) in those outside Dhaka. Most refusals were due to an unwillingness to provide a serum sample. We collected data from 101 nonpoultry workers, all of whom were from Dhaka. Overall, compared with nonpoultry workers, poultry workers were younger (median age 28.0 years [interquartile range (IQR) 22.5–38.0 y] vs. 36.0 years [IQR 32–40 y]) and more likely to be male (100% vs. 78%) and to smoke (58% vs. 34%) ($p<0.001$) (Table 1).

H5N1 Serologic Testing Results

Of 404 poultry workers, 9 (2%) were seropositive for H5N1 virus antibodies at baseline (95% CI 1%–4%). During November 2009–March 2010, routine icddr,b poultry surveillance identified H5N1 virus at 11 (92%) of the 12 LBMs and in 25 (93%) of 27 monthly samples. We obtained a second blood specimen from 278 (72%) of 387 participating poultry workers from the 11 LBMs (online Technical Appendix 2 Table 1, http://wwwnc.cdc.gov/EID/article/21/4/14-1281-Techapp2.pdf). Because of a delay in the availability of laboratory results for poultry and environmental surveillance samples, the median interval between detection of H5N1 virus at LBMs and collection of a second blood sample from poultry workers at the corresponding LBM was 56 days (IQR 49–61 days).

Of 9 seropositive poultry workers at baseline, 5 remained seropositive and 1 was seronegative for H5N1 virus antibodies at follow-up (online Technical Appendix 2 Figure); the remaining 3 workers were lost to follow-up. Six (2%) of 284 poultry workers seroconverted during the study period (95% CI 1%–5%) (Table 2). Six other workers

Table 1. Characteristics of live bird market workers and nonpoultry workers, Bangladesh, 2009–2010*

Characteristic	Poultry workers, n = 404	Nonpoultry workers, n = 101	p value
Male sex	404 (100)	79 (78)	<0.001†
Median age, y (IQR)	28 (22–38)	36 (32–40)	<0.001‡
Smoke tobacco	236 (58)	34 (34)	<0.001†
Median duration of smoking, y (IQR)	8 (4–16)	15 (9–20)	0.003‡
Use betel leaf or nut	151 (37)	22 (22)	0.003†
Use smokeless tobacco	15 (4)	1 (1)	0.2
Have chronic medical condition§	28 (7)	11 (11)	0.2

*Data are no. (%) persons except as indicated. IQR, interquartile range.
†Value for 2-sample test of proportion.
‡Value for 2-sample Wilcoxon rank-sum test.
§Conditions such as asthma; diabetes; chronic heart, lung, kidney, and liver disease; immune disorders; and cancer.

Table 2. Characteristics of live bird market workers with evidence of seroconversion against avian influenza A(H5N1) virus, Bangladesh, 2009–2010*

Characteristic	PW1	PW2	PW3	PW4	PW5	PW6
Personal characteristic						
Age, y	28	20	22	24	38	19
Smoke tobacco	+	–	+	–	–	+
Have chronic medical condition	–	–	–	–	–	–
Exposure to poultry or virus						
Handle sick poultry	+	+	+	+	+	+
Perform tasks with sick poultry						
Transport poultry	+	+	+	+	–	+
Feed poultry	–	+	+	+	–	+
Clean feeding tray	–	–	+	+	–	+
Clean water container	–	+	+	+	–	+
Medicate sick poultry	–	–	+	–	–	–
Separate sick poultry	–	+	+	+	+	+
Slaughter poultry	–	+	+	+	–	+
Defeather poultry	–	+	–	+	–	+
Eviscerate poultry	–	+	–	+	–	+
Stuff poultry into bags	+	–	–	+	+	+
Clean feces from pen	–	–	+	–	–	+
Hand-carry sick poultry or held poultry on lap	+	+	+	+	+	+
Carry baskets containing sick poultry on head	–	–	–	–	–	–
Eat raw/undercooked poultry or eggs	–	+	+	–	–	–
Precautions taken when handling sick poultry						
Use personal protective equipment†	–	–	–	–	–	–
Wash hands at the market after working with the poultry	+	+	–	+	–	–
Change clothes upon returning home	+	+	+	+	+	+
Presence of influenza-like illness in past 21 d	–	–	–	–	–	–
Interval, d, between detection of virus–positive surveillance sample and follow-up collection of serum sample from worker	49	50	54	56	91	26
Neutralizing antibody titer, geometric mean‡						
Baseline	5	10	5	5	5	5
Follow-up	40	61	49	40	66	67

*PW, poultry worker; +, characteristic present; –, characteristic not present.
†Apron, gloves, dedicated coveralls, cloth mask, and boots.
‡By microneutralization assay, using influenza A/Bangladesh/3233/2011 (H5N1, clade 2.2) virus.

met the criteria for seropositivity in the follow-up serum samples, but they were not considered to have seroconverted because baseline titers were >10 and a >4-fold rise in titer was not achieved.

H5N1 virus was not detected by routine poultry surveillance in 1 subdistrict LBM during the study period. We collected follow-up data from 12 (71%) of 17 participating poultry workers at this LBM 1 year after baseline enrollment, and all 12 were seronegative for H5N1 virus at enrollment and follow-up. The overall seroprevalence of antibodies to H5N1 virus among poultry workers from all LBMs during the study period was 5% (20/404, 95% CI 3%–7%). In comparison, none of the 101 nonpoultry workers was seropositive (95% CI 0%–4%).

Incidence of Seroconversion

In LBMs where H5N1 virus was detected through routine poultry surveillance, we followed 278 poultry workers, of whom 266 were H5N1 virus–seronegative at baseline. These 266 workers contributed 30,043 days (≈82 years) of observation between the collection of paired blood samples, resulting in an incidence of 7 cases/100 poultry worker–years (95% CI 3–16). Using this incidence, we estimate that the annual incidence of H5N1 virus infection after exposure to H5N1 virus at the study LBMs was 50 cases per 721 enlisted poultry workers.

Risk Factors for H5N1 Virus Infection

Seventeen (94%) of the 18 workers who were seropositive or seroconverted against H5N1 virus and 180 (66%) of the 272 seronegative workers reported exposure to poultry through >1 activity. None of the workers who were seropositive or who seroconverted reported exposure to poultry at home, at their farm, or at another place.

Three sets of behaviors explained 95% of the variability among risk behaviors at baseline and follow-up. However, the risk for H5N1 virus infection (risk ratio) was not equal for each set of behaviors (online Technical Appendix 2 Table 2). The set of behaviors with the highest risk ratio consisted of feeding poultry, cleaning feeding trays and water containers, not washing hands after working with sick poultry, and cleaning feces from pens; this set of behaviors was classified as high exposure. The set of behaviors with the second highest risk ratio consisted of slaughtering, defeathering, eviscerating, collecting or transporting feces, and stuffing poultry into bags; this set

Table 3. Risks for testing seropositive or seroconverting against avian influenza A(H5N1) virus among live bird market workers, Bangladesh 2009–2010*

Characteristic/behavior	Poultry workers		Regression model		
	Seronegative, n = 272	Seropositive or seroconverted, n = 18	Simple RR (95% CI)	Multiple RR (95% CI)	p value†
Median age, y (IQR)	27 (23–38)	27 (20–30)	0.9 (0.9–1.0)	0.9 (0.9–1.1)	0.8
Risk behavior					
High exposure			4.8 (0.8–28.2)	7.6 (2.8–20.9)	<0.001
Feed poultry	196 (72)	17 (94)			
Clean feeding tray	156 (57)	15 (83)			
Clean water container	155 (57)	16 (89)			
Clean feces from poultry pen	125 (46)	14 (78)			
Do not wash hands after handling sick poultry	133 (49)	10 (56)			
Medium exposure			3.5 (0.8–14.7)	5.1 (1.8–14.1)	0.002
Slaughter poultry	198 (73)	17 (94)			
Defeather poultry	142 (52)	15 (83)			
Eviscerate poultry	143 (53)	15 (83)			
Collect or transport poultry feces	53 (19)	1 (6)			
Stuff poultry into bags	113 (42)	14 (78)			
Low exposure			1.0 (0.3–3.3)	–	–
Smoke	159 (58)	7 (39)			
Medicate poultry	15 (6)	2 (11)			
Isolate sick poultry	130 (48)	10 (56)			
Eat raw/undercooked poultry or eggs	103 (38)	6 (33)			
Risk of infection from					
Medium-exposure behaviors when frequently performing both medium- and high-exposure behaviors‡	–	–	–	1.4 (0.3–6.2)	0.6
High-exposure behaviors when frequently performing both high- and medium-exposure behaviors‡	–	–	–	2.1 (0.4–12.9)	0.4

*Data are no. (%) except as indicated. IQR, interquartile range; RR, risk ratio; –, not applicable.
†Value for multivariate model.
‡The ratio of RR for interaction between medium- and high-exposure behaviors was 0.3 (1.4/5.1 for medium-exposure behaviors and 2.1/7.6 for high-exposure behaviors (95% CI 0.08–0.88; p = 0.031).

of behaviors was classified as medium exposure. The set of behaviors with the lowest risk ratio included smoking, medicating poultry, isolating sick poultry, and eating raw or undercooked poultry or eggs; this set of behaviors was classified as low exposure.

Poultry workers who frequently performed high-exposure behaviors had a 7.6 times higher risk for H5N1 virus infection compared with poultry workers who infrequently performed high-exposure behaviors when they also infrequently performed medium-exposure behaviors (p<0.001) (Table 3). Poultry workers who frequently performed medium-exposure behaviors had a 5.1 times higher risk of H5N1 virus infection compared with poultry workers who infrequently performed medium-exposure behaviors when they also infrequently performed high-exposure behaviors (p = 0.002).

Discussion

Our study demonstrates that, despite frequent exposure to infected poultry and low PPE use, LBM workers in Bangladesh had low serologic evidence of H5N1 virus infection. These results also suggest that cross-sectional seroprevalence studies may underestimate the risk for H5N1 virus infection if conducted outside the peak time for H5N1 virus circulation or if samples are obtained from infected workers long after exposure to the virus (i.e., when antibody titers have declined below the seropositive threshold).

Two percent of poultry workers were H5N1 virus–seropositive at baseline. This finding suggests that previous infection with H5N1 virus was uncommon despite the frequent exposure of workers to poultry. One of the workers who was seropositive at baseline became seronegative at follow-up, possibly because neutralizing antibodies decreased below the threshold for laboratory detection (*34*). The overall 5% seroprevalence of H5N1 virus antibody among poultry workers in our study is similar to the 4% seroprevalence among LBM workers in Vietnam in 2001 (*13*) but higher than the <1% seroprevalence reported among LBM workers from Bangladesh, Nigeria, Indonesia, and China during 2005–2009 (*7*,*9*,*11*,*14*). This finding suggests that human infection with H5N1 virus among heavily exposed workers at LBMs occurs infrequently but may be more common than previously reported. Routine poultry surveillance that included subdistrict LBMs in our study detected H5N1 virus from a higher proportion of poultry and environmental samples collected in 2011 than in 2009 and 2010 (3.8% vs. 0.4% and 0.5%, respectively) (*26*). Indeed, we would expect an increase in seroprevalence of

H5N1 virus antibodies or seroconversion rates among exposed poultry workers during periods with increased H5N1 virus activity among poultry (*35*). Nevertheless, it is unclear whether the current proportion represents a substantive opportunity for virus reassortment and the generation of a novel virus with pandemic potential.

We identified 2 sets of correlated behaviors that increased the risk of acquiring H5N1 virus infection among poultry workers. Frequently performing high-exposure behaviors was associated with 1.5 times higher risk of acquiring H5N1 virus infection compared with performing medium-exposure behaviors. Only butchering and exposure to ill poultry were associated with H5 seropositivity among LBM workers performing >1 poultry-related task in Hong Kong (*17*). The single seropositive LBM worker in China also reported slaughtering birds for 5 years (*36*). The use of PPE while performing high-exposure behaviors and frequent handwashing may reduce the risk for H5N1 virus infection (*37*). Nevertheless, because poultry workers handle poultry throughout the workday, it may be challenging for them to use PPE every time they have contact with poultry or their feces (*38*). Virus exposure and subsequent infection via mucous membranes and the respiratory tract may also be reduced among workers if they avoid touching their eyes, mouth, and nose while at work. Formative research would be helpful to explore if and how environmental controls (e.g., handwashing stands, improved ventilation flow, scalding pots); improved poultry handling techniques (e.g., slaughtering poultry inside plastic bags); and improved PPE (e.g., more accessible, cost-effective, and better tolerated equipment) could help decrease the risk for virus transmission at LBMs.

In Bangladesh, most identified cases of H5N1 virus infection in humans have been asymptomatic or mildly symptomatic (*2*,*21*). However, in 2013, the potential for severe and fatal illness from H5N1 virus infection in Bangladesh was highlighted by a fatal case in a child who had been exposed to infected backyard poultry (*39*). An increase in H5N1 virus infections among occupationally exposed poultry workers could signal the emergence of a virus with increased transmissibility among humans (*40*).

Our study has several limitations. First, almost 20% of the poultry workers declined to participate, and 28% of those enrolled at baseline were lost to follow-up. The refusals and losses to follow-up may have led to selection bias, resulting in an underestimation of seroprevalence and incidence of seroconversion if some of these workers were infected or in an overestimation if none of them were infected. Second, once H5N1 virus was detected in surveillance samples from an LBM, we conducted a final follow-up with workers at that LBM. Thus, we may have missed seroconversions that occurred after follow-up. Third, because the modified horse erythrocyte hemagglutination assay is insensitive for the detection of antibody to A/Bangladesh/3233/2011 (H5N1, clade 2.2) virus, we could not use it for confirmation of seropositivity and seroconversion in this study. Fourth, poultry workers in Bangladesh were engaged in multiple activities, making it difficult to identify which specific behavior was the predominant risk factor for H5N1 virus infection. Last, we were unlikely to have accurately ascertained clinical illness associated with H5N1 virus infections because of the lag between collection of H5N1 virus–positive poultry and environmental surveillance samples and the collection of follow-up blood samples from workers.

In conclusion, our study suggests that a low but substantive proportion of LBM poultry workers in Bangladesh become infected with H5N1 virus after unprotected, ongoing sporadic exposures to H5N1 virus–infected poultry and virus-contaminated environments of LBMs. The risk behaviors identified in our study may help public health officials explore interventions to interrupt poultry-to-human transmission of H5N1 virus and other avian influenza A viruses among the poultry workers. The cost of any interventions needs to take into account the anticipated potential modest benefit of decreasing an infrequent event with uncertain pandemic potential.

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Increased Risk for Group B *Streptococcus* Sepsis in Young Infants Exposed to HIV, Soweto, South Africa, 2004–2008[1]

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Although group B *Streptococcus* (GBS) is a leading cause of severe invasive disease in young infants worldwide, epidemiologic data and knowledge about risk factors for the disease are lacking from low- to middle-income countries. To determine the epidemiology of invasive GBS disease among young infants in a setting with high maternal HIV infection, we conducted hospital-based surveillance during 2004–2008 in Soweto, South Africa. Overall GBS incidence was 2.72 cases/1,000 live births (1.50 and 1.22, respectively, among infants with early-onset disease [EOD] and late-onset [LOD] disease). Risk for EOD and LOD was higher for HIV-exposed than HIV-unexposed infants. GBS serotypes Ia and III accounted for 84.0% of cases, and 16.9% of infected infants died. We estimate that use of trivalent GBS vaccine (serotypes Ia, Ib, and III) could prevent 2,105 invasive GBS cases and 278 deaths annually among infants in South Africa; therefore, vaccination of all pregnant women in this country should be explored.

In 2013, a total of 41.6% (2.6 million) of deaths worldwide in children <5 years of age occurred in neonates; 76.7% occurred within 6 days after birth (*1*). Furthermore, in 2012, ≈6.9 million probable cases of severe bacterial infections and 680,000 associated deaths occurred among neonates (*2*). Nevertheless, there is a paucity of data from low- and middle-income countries on pathogen-specific causes of neonatal sepsis, particularly during the first day of life, and it is unknown whether in utero HIV exposure increases susceptibility to severe neonatal bacterial infections.

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Group B *Streptococcus* (GBS) is a leading cause of severe invasive disease in young infants. A meta-analysis dominated by studies from high-income countries estimated global incidence of 0.53 cases/1,000 live births during 2000–2011 (*3*). Considerable intra- and interregional variation in the incidence of invasive early-onset disease (EOD; disease in 0- to 6-day-old infants) was observed (*3*–*5*), ranging from 1.21 cases/1,000 live births (95% CI 0.5–1.91) in Africa to 0.02 cases/1,000 live births (95% CI 0–0.07) in Southeast Asia (*3*). This variability is inconsistent with the lesser difference in prevalence of maternal GBS colonization, the major risk factor for EOD, in women from different regions (20.9% in Africa, 13.4% in Southeast Asia) (*6*). Maternal HIV infection has not been associated with a higher prevalence of GBS colonization (*7*,*8*), except among women with CD4+ lymphocyte counts of >500 cells/mm^3 (*8*).

Providing intrapartum antimicrobial drug prophylaxis (IAP) to women identified as rectovaginally colonized by GBS at 35–37 weeks' of pregnancy has been associated with a >80.0% reduction in EOD (*9*); however, this strategy is logistically challenging to implement and maintain in resource-constrained settings. Furthermore, IAP has not decreased the incidence of late-onset disease (LOD; disease in 7- to 90-day-old infants) (*10*).

Progress has been made in the development of a trivalent GBS polysaccharide–protein conjugate vaccine (GBS-CV), which is targeted for use in pregnant women; the goal is to enhance transplacental transfer of capsular antibody to the fetus (*1*,*8*–*10*), which could protect against EOD and LOD. Improved estimates of the incidence of invasive GBS disease are needed from low- and middle-income countries to contextualize the prioritization of GBS vaccination and determine whether temporal changes in invasive serotypes should be considered in the design of serotype-specific GBS vaccine.

We evaluated the clinical and microbiological epidemiology, incidence, and serotype distribution of invasive

[1]Preliminary results from this study were presented at the 8th World Congress of the World Society for Pediatric Infectious Diseases, November 19–22, 2013, Cape Town, South Africa.

GBS disease among young infants in a setting with a high prevalence of maternal HIV infection. A secondary aim was to estimate the potential effect of a trivalent GBS-CV in reducing the number of invasive GBS cases nationally.

Materials and Methods

Study Setting and Design

During 2004–2008, we undertook hospital-based surveillance of culture-confirmed invasive bacterial sepsis in infants 0–90 days of age at Chris Hani Baragwanath Academic Hospital (CHBAH), a public secondary–tertiary health care facility in Soweto, South Africa. CHBAH is the only public hospital in Soweto with neonatal care facilities; ≈90.0% of all hospitalizations from the community occur in this hospital. Soweto is a predominantly black-African community and has 1.4 million inhabitants, including 125,000 children <5 years of age and a birth cohort of ≈28,000/year (*11*); ≈21,000 are delivered at CHBAH and 7,000 are delivered at 1 of 6 community-based midwife obstetric units. Women with potentially complicated deliveries at midwife obstetric units and clinically ill newborns are referred to CHBAH by ambulance.

Health care for pregnant women and children is provided free of charge in South Africa (*12*). Most deliveries in Soweto (95.0%) (E. Buchmann, pers. comm., 2014 Jul 19) and in South Africa as a whole (87.3% in 2010) (*13*) occur in health facilities. Voluntary counseling and testing for HIV is offered at antenatal clinics; >96.0% of pregnant women accept testing (C. Mnyani, pers. comm,, 2014 Jul 28). Single-dose nevirapine, administered as standard of care to women in labor and their newborns to prevent mother-to-child HIV transmission, was supplemented in 2007 with triple antiretroviral therapy to immunocompromised women (<350 CD4+ cells/mm^3) from 34 weeks' gestation onward.

During the surveillance period, HIV prevalence in pregnant women remained stable at 29.9% (*14*), and ≈18.0% of all children were born prematurely (<37 weeks gestational age) or had a low birthweight (<2,500 g) (CHBAH, unpub. data). Gestational age was determined on the basis of the available obstetric or neonatal assessments by attending physicians. Healthy newborns are routinely discharged home 12 h after vaginal or 72 h after cesarian delivery.

At CHBAH, newborns with signs and symptoms of severe illness at birth or before discharge from postnatal wards are admitted to the neonatal unit; infants who are discharged home after delivery and subsequently return for suspected bacterial infections are hospitalized in the general pediatric wards. Investigation and treatment of neonates and young infants with suspected invasive bacterial disease were conducted according to standard of care by attending physicians. Investigations included complete blood cell counts and blood cultures for all infants. Lumbar punctures (to obtain cerebrospinal fluid [CSF] samples for biochemistry, microscopy, and antimicrobial drug sensitivity testing and culture) were limited to infants with GBS-positive cultures of blood samples obtained at birth and to all infants admitted from the community for suspected sepsis. Sterile-site cultures were processed at the National Health Laboratory Service (NHLS). Blood cultures were evaluated by using the BacT/Alert microbial system (Organon Teknika, Durham, NC, USA). GBS isolates were retrieved from NHLS, stored at –70°C, and serotyped by latex agglutination (*15*). During the surveillance period, empiric treatment for suspected sepsis consisted of intravenous penicillin and gentamicin for neonates and ampicillin and gentamicin for infants 1–12 months of age; case-patients with suspected meningitis received ampicillin and cefotaxime empirically.

Maternal screening for rectovaginal GBS colonization during pregnancy is not routinely performed in public health facilities in South Africa. Before 2007, CHBAH IAP guidelines recommended administration of intravenous ampicillin (1 g/6 h) and oral metronidazole (400 mg 3×/d) for suspected chorioamnionitis and prolonged rupture of membranes. In January 2007, targeted risk-based IAP was implemented for possible GBS infection in women with preterm labor, a previous infant infected with GBS, or a GBS-positive culture; treatment consisted of an initial 2-g dose of intravenous ampicillin, followed by intravenous ampicillin (1 g/4 h) until delivery. During the surveillance period, 10.5% of women received IAP during labor (*16*).

Infants 0–90 days of age who were admitted to CHBAH with GBS isolated from a normally sterile site were identified through screening of ward admissions and microbiological records within 24 h of identification of GBS. We also undertook an audit of the NHLS database to identify all invasive pathogens isolated from infants over the study period. Invasive GBS disease was categorized as bacteremia if identified in blood only and as meningitis if identified from CSF or if there was CSF cytologic evidence of purulent meningitis (>5 leukocytes/mm^3; adjusted in traumatic lumbar punctures to allow 1 leukocyte/500 erythrocytes) in an infant with GBS bacteremia.

Demographic, birth, maternal HIV infection status, and other clinical data were abstracted from infants' medical records by study doctors. HIV exposure was determined by abstracting antenatal HIV test results of mothers and supplemented by HIV ELISA results from maternal or infant blood tests conducted by attending physicians. The HIV infection status of HIV-exposed infants was determined by using a qualitative HIV PCR at the discretion of the attending physician.

Statistical Considerations

Incidence was calculated as cases per 1,000 live births. Administrative live birth data from CHBAH and community clinics for Soweto and HIV prevalence survey data for the surveillance period were used to determine population denominators for HIV-infected and -uninfected women. Established patient referral protocols limited the chance that neonates, especially newborns, living outside the hospital catchment area were admitted to CHBAH. A sensitivity analysis of incidence was undertaken to account for GBS case-patients for whom maternal HIV status was unknown. For overall and annual incidence calculations, we attributed the prevalence of HIV exposure among case-patients with known exposure status to case-patients with unknown exposure status. Alternate sensitivity analyses assumed all cases with unknown maternal HIV status were HIV-exposed or, conversely, HIV-unexposed.

On the basis of 2012 population data for number of live births in South Africa (1,168,403) (*17*) and national prevalence of HIV infection in pregnant women (29.5%) (*18*), we used our study data to estimate annual national number of invasive GBS cases and deaths and stratified estimates by HIV exposure. We estimated the number of annual vaccine-preventable invasive GBS cases and deaths in South Africa on the basis of the conservative assumption that administration of GBS-CV to pregnant women (*19*) would not protect against invasive GBS disease in infants born at ≤33 weeks' gestation, and we adjusted for the proportion of serotypes included in the current experimental trivalent GBS-CV (Ia, Ib, and III) and a future pentavalent vaccine (addition of serotypes II and V). These estimates were based on hypothetical vaccine efficacy assumptions of 75.0% for the overall population; 85.0% for HIV-unexposed infants; and 65.0% for HIV-exposed infants, as determined on the basis of the lower immunogenicity of trivalent GBS-CV in HIV-infected pregnant women (*19*).

Proportions were compared by using the χ^2 and Fisher exact tests, as appropriate; Wilcoxon rank sum test (nonparametric) was applied for continuous variables. Univariate analysis was performed to determine factors associated with GBS-related death. Two-sided p values ≤0.05 were considered statistically significant, and 95% CIs were calculated. Analyses were conducted by using STATA/IC 13.0 (StataCorp, College Station, TX, USA).

Ethics Consideration

The study was approved by the Human Research Ethics Committee, University of the Witwatersrand (M03–10–07 and M10–367) and the institutional review board of the Centers for Disease Control and Prevention. Mothers of infants prospectively identified with invasive GBS disease signed informed, written consent. Consent was waived by the ethics committees for retrospective review of records of cases identified after discharge or death of the infant.

Results

During the surveillance period, 389 invasive GBS cases were identified in infants 0–90 days of age; 214 (55.0%) cases were EOD. Complete medical records were unavailable for 17 cases (10 EOD, 7 LOD), which were included in incidence calculations but not in univariable analysis. Overall incidence of invasive GBS was 2.72 cases/1,000 live births (95% CI 2.46–3.01). EOD incidence was 1.50 cases/1,000 live births (95% CI 1.30–1.71), and LOD incidence was 1.22 cases/1,000 live births (95% CI 1.05–1.42); incidences for both were generally similar across years (data not shown). Overall, 26.6% of case-patients were born prematurely, including 29.8% of EOD and 22.3% of LOD case-patients. Most (69.4%) preterm births occurred at ≤33 weeks' gestation, including 63.0% and 80.6% of those for EOD and LOD case-patients, respectively (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/4/14-1562-Techapp1.pdf). Of the 214 EOD case-patients, 138 (64.5%) had positive culture results for blood samples obtained at birth (median age 0 days, interquartile range 0–1; Figure 1, panel A). Forty-four percent of LOD cases were detected during week 2 of life (median age 16 days, interquartile range 11–29; Figure 1, panel B). Infants with LOD were 5.57-fold (95% CI 3.50–8.90) more likely than infants with EOD to have meningitis (61.7% vs. 22.4%; p<0.0001).

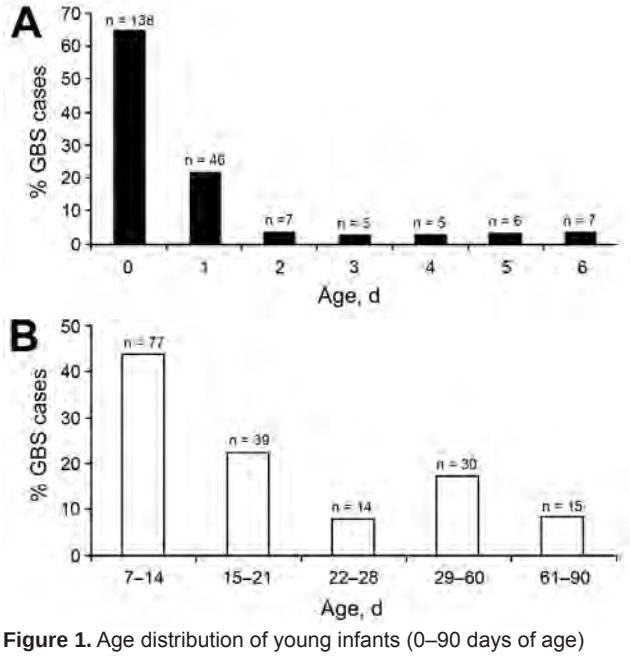

Figure 1. Age distribution of young infants (0–90 days of age) with invasive group B *Streptococcus* (GBS) sepsis, Soweto, South Africa, 2004–2008. A) Distribution for 214 infants with early-onset disease. B) Distribution for 175 infants with late-onset disease.

Invasive GBS Disease and HIV Exposure

Maternal HIV infection status was available for 327 (84.1%) GBS case-patients, of whom 161 (49.2%) were HIV-exposed (online Technical Appendix Table 1). HIV-exposure data were unavailable for 12.3% of EOD and 11.9% of LOD case-patients. Regimens to prevent mother-to-child HIV transmission were documented in only 41.6% of the infants' medical records and were therefore not analyzed in this study. HIV PCR results were available for 46 (28.6%) of 161 HIV-exposed infants: 6 had EOD (all nonreactive results) and 40 had LOD (8 [20.0%] had reactive results).

Infants with LOD were more likely than those with EOD to be HIV-exposed (58.1% vs. 41.9%; p = 0.004) (online Technical Appendix Table 1). HIV-exposed and -unexposed case-patients overall or when stratified by EOD and LOD did not differ substantially with regard to mode of delivery, preterm birth, and low birthweight (online Technical Appendix Table 1) or to exposure to meconium-stained liquor, prolonged rupture of membranes, or IAP during labor (data not shown).

The incidence of invasive GBS disease was 2.25-fold (95% CI 1.84–2.76) greater in HIV-exposed than HIV-unexposed infants (4.46 cases/1,000 live births [95% CI 3.85–5.13] vs. 1.98 cases/1,000 live births [95% CI 1.71–2.28]) (Table 1). The higher incidence of GBS disease in HIV-exposed compared with HIV-unexposed infants was evident for EOD case-patients (2.10 vs. 1.24 cases/1,000 live births, respectively; risk ratio 1.69, 95% CI 1.28–2.24) and more so for LOD case-patients (2.36 vs. 0.74 cases/1,000 live births, respectively; risk ratio 3.18, 95% CI 2.34–4.36). Bacteremia and meningitis incidence was also higher in HIV-exposed than HIV-unexposed infants (Table 1). These differences in incidence of invasive GBS disease remained significant in all sensitivity analyses in which missing maternal HIV infection status were extrapolated (Table 1), except for EOD when infants with unknown HIV-exposure status were assumed to be HIV-unexposed (Table 1).

Table 1. Incidence of invasive group B *Streptococcus* sepsis in 0- to 90-day-old infants, by in utero exposure to HIV, Soweto, South Africa, 2004–2008*

	Overall		Bacteremia		Meningitis	
HIV exposure status	No. cases, incidence (95% CI)†	RR (95 % CI)	No. cases, incidence (95% CI) †	RR (95%CI)	No. cases, incidence (95% CI) †	RR (95% CI)
Early-onset disease						
Proration of unknown exposure‡						
Unexposed	124, 1.24 (1.03–1.48)	1.69 (1.28–2.24)	103, 1.03 (0.84–1.25)	1.43 (1.03–1.97)	21, 0.21 (0.13–0.32)	3.00 (1.63–5.58)
Exposed	90, 2.10 (1.69–2.58)		63, 1.47 (1.13–1.88)		27, 0.63 (0.41–0.92)	
Unknown, assume exposed						
Unexposed	104, 1.04 (0.85–1.26)	2.47 (1.87–3.26)	ND		ND	
Exposed	110, 2.57 (2.11–3.09)		ND		ND	
Unknown, assume unexposed						
Unexposed	139, 1.39 (1.17–1.64)	1.26 (0.94–1.68)	ND		ND	
Exposed	75, 1.75 (1.38–2.19)		ND		ND	
Late-onset disease						
Proration of unknown exposure‡						
Unexposed	74, 0.74 (0.58–0.93)	3.18 (2.34–4.36)	27, 0.27 (0.18–0.39)	3.37 (2.01–5.73)	47, 0.47 (0.35–0.62)	3.08 (2.07–4.60)
Exposed	101, 2.36 (1.92–2.86)		39, 0.91 (0.65–1.24)		62, 1.45 (1.11–1.85)	
Unknown, assume exposed						
Unexposed	62, 0.62 (0.48–0.79)	4.25 (3.09–5.89)	ND		ND	
Exposed	113, 2.64 (2.17–3.17)		ND		ND	
Unknown, assume unexposed						
Unexposed	89, 0.89 (0.71–1.09)	2.25 (1.66–3.07)	ND		ND	
Exposed	86, 2.01 (1.61–2.48)		ND		ND	
Early-onset plus late-onset disease, exposed vs. unexposed	2.25 (1.84–2.76)		1.83 (1.40–2.39)		3.05 (2.20–4.25)	

*RR, relative risk; ND, not done.
†Incidence = no. cases/1,000 live births.
‡Based on prevalence of HIV exposure among those tested.

Factors Associated with Death among Infants with Invasive GBS

The overall case-fatality rate (CFR) was 16.9%; the CFR for meningitis (24.3%) was 2.4-fold greater than that for bacteremia (11.8%; odds ratio 2.24, 95% CI 1.33–4.35; p = 0.0015). The median duration of hospitalization was 1 day for infants who died and 15 days for those who survived (online Technical Appendix Table 1). In univariate analysis, meningitis and very low birthweight (<1,500 g) were associated with a higher overall CFR (p = 0.002 and p = 0.003, respectively). Prematurity (≤33 weeks' gestation) was associated with a higher CFR in EOD but not LOD case-patients (p = 0.008 and p = 0.68, respectively) (online Technical Appendix Table 2).

Antimicrobial Drug Susceptibility and Serotyping of GBS

Antimicrobial drug sensitivity profiles were available for 385 (98.9%) of 389 isolates; all were penicillin sensitive. Macrolide resistance was prevalent in 15 (5.5%) of 273 of isolates.

Of 389 isolates, 213 (54.8%) were available for serotyping, including 125 (58.6%) from EOD case-patients and 88 (41.3%) from LOD case-patients. The proportion of isolates available for serotyping increased each year: 2004, 15.6%; 2005, 45.1%; 2006, 65.8%; 2007, 63.3%; 2008, 75.0%. Overall, serotypes Ia and III accounted for 84.0% of all serotypes (19.2% [41/213] and 64.8% [138/213], respectively) and for 74.4% of EOD and 97.7% of LOD cases (p<0.001; Figure 2). Serotype distribution remained similar throughout the study and did not differ by HIV-exposure status (data not shown). Overall, a greater proportion of meningitis than bacteremia cases were caused by serotype III (77.8% [63/81] vs. 56.8% [75/132]; p = 0.002), and serotype V was more commonly identified in bacteremia than meningitis cases (7.6% [10/132] vs. 3.7% [3/81]; p = 0.25; online Technical Appendix Figure). Serotype distribution between survivors and nonsurvivors did not differ (p = 0.51; data not shown).

Nationwide Number and Potential Vaccine-Preventable Fraction of Invasive GBS Disease

We estimated ≈3,178 invasive GBS cases and 549 GBS-associated deaths among South Africa's 2012 birth cohort (Table 2). For HIV-unexposed and -exposed infants, these estimates represent ≈1,639 and 1,544 cases and 283 and 266 deaths, respectively. On the basis of the predefined estimated efficacy of trivalent GBS vaccine to protect infants born at >33 weeks gestation, we estimated that, each year, 1,230 cases of invasive GBS disease and 163 deaths could be prevented in HIV-unexposed infants and 886 cases and 117 deaths could be prevented in HIV-exposed infants (Table 2).

Discussion

We report a high overall incidence of invasive GBS disease in Soweto (2.72 cases/1,000 live births), which is greater than global (0.53 cases/1,000) and African (1.21 cases/1,000) incidence estimates reported in a meta-analysis of studies conducted during 2000–2011 (*3*). That overall estimate included an incidence of 1.98 cases/1,000 live births (95% CI 1.71– 2.28) among HIV-unexposed infants, which is similar to or greater than incidences reported in many resource-rich countries before the widespread use of IAP (*20*). Furthermore, the observed overall incidence was similar to that for the same population a decade earlier (3.0 cases/1,000 live births) (*21*) and to that for women of South Asian descent in South Africa during the 1980s (2.65 cases/1,000 live births) (*22*). Despite the 2007 implementation of a risk-based IAP strategy at CHBAH, the high incidence of GBS disease has persisted, indicating the limited effect of the strategy in a resource-restricted setting with high maternal HIV infection prevalence. The limited effect could indicate poor strategy adherence or that the strategy missed most women whose newborns were at risk for EOD.

The high incidence of invasive GBS disease in South Africa contrasts with the lower incidence reported in South

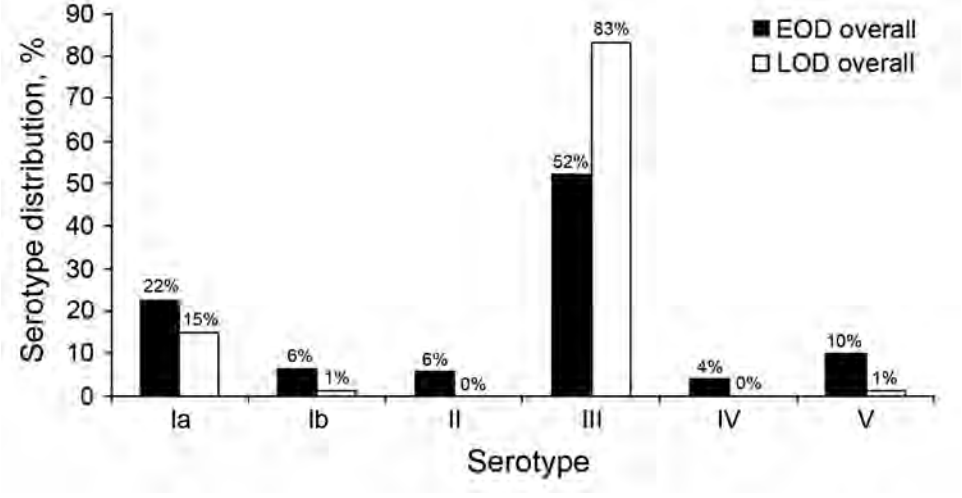

Figure 2. Group B *Streptococcus* serotype distribution among 125 patients with early-onset disease (EOD) and 88 patients with late-onset disease (LOD), Soweto, South Africa, 2004–2008.

Table 2. Estimated annual number of invasive GBS disease cases and associated deaths and potential annual vaccine-preventable fraction, South Africa*

	Overall		HIV unexposed†		HIV exposed†	
National estimates	No.	Incidence (95% CI)	No.	Incidence (95% CI)	No.	Incidence (95% CI)
Births	1,168,403‡		823,724		34,4679	
Invasive GBS cases	3,178§	2.72 (2.62–2.81)	1,639¶	1.99 (1.89–2.09)	1,544#	4.48 (4.26–4.71)
Invasive GBS-associated deaths						
No total**	549	0.47 (0.43–0.51)	283	0.34 (0.30–0.39)	266	0.77 (0.68–0.87)
No. in infants born at >33 weeks' gestation††	420	0.36 (0.33–0.40)	217	0.26 (0.23–0.30)	204	0.59 (0.51–0.68)
Vaccine-preventable cases and deaths						
Trivalent GBS-CV‡‡						
Cases	2,105§§	1.80 (1.73–1.88)	1,230¶¶	1.49 (1.14–1.58)	886##	2.57 (2.40–2.75)
Deaths	278§§	0.24 (0.21–0.27)	163¶¶	0.20 (0.17–2.31)	117##	0.34 (0.28–0.41)
Pentavalent GBS-CV***						
Cases	2,317§§	1.99 (1.90–2.07)	1,354¶¶	1.64 (1.56–1.73)	976##	2.83 (2.66–3.01)
Deaths	306§§	0.26 (0.23–0.29)	179¶¶	0.22 (0.19–0.25)	129##	0.37 (0.31–0.44)

*GBS, group B *Streptococcus;* GBS-CV, GBS polysaccharide–protein conjugate vaccine.
†HIV-exposed and -unexposed values were calculated on the basis of national HIV prevalence in pregnant women (29.5%). Incidence values represent cases/1,000 live births.
‡2012 live births.
§Overall GBS incidence is 2.72/1,000 live births.
¶GBS incidence for HIV-unexposed infants is 1.99/1,000 live births.
#GBS incidence in HIV-exposed infants is 4.48/1,000 live births.
**Total deaths in infants ≤90 days old, assuming 15.2% were born at ≤33 weeks of gestation and have a case-fatality rate (CFR) of 26.5% and assuming 84.8% were born at >33 weeks of gestation and have a CFR of 15.6%.
††Deaths in infants <90 days old who were born at >33 weeks of gestation (84.8% of infants); CFR 15.6%.
‡‡Trivalent GBS-CV contains serotypes Ia, Ib, and III, which account for 88.3% of cases.
§§Assuming vaccine efficacy of 75%.
¶¶Assuming vaccine efficacy of 85%.
##Assuming vaccine efficacy of 65%.
***Pentavalent GBS-CV contains serotypes Ia, Ib, II, III, and V, which account for 97.2% of cases.

Asia and the Western Pacific, despite similarity in prevalence of maternal vaginal GBS colonization at delivery (*6*). Possible reasons for this discrepancy include differences in delivery location, presence of trained birth-care attendants, and access to health facilities with adequate capability to diagnose and treat invasive GBS disease. Possible reasons for differences are exemplified by the findings in a study conducted in Bangladesh (*23*), which reported that only 1 of 30 culture-confirmed neonatal bacteremia cases was caused by GBS; however, >50.0% of the 259 reported neonatal deaths (many among infants born outside health care facilities) occurred within 24 h of birth, and 62.0% of those cases were not investigated for bacteremia. Our finding that 64.5% of EOD cases were diagnosed within 24 h of birth highlights the effect that births outside of health care facilities or where there is limited capacity for investigating invasive disease in newborns could have on measuring the incidence of EOD. Also, even though case-patients were treated in a secondary–tertiary hospital, death caused by invasive GBS disease was rapid (median 1 d from hospitalization).

In our study, the overall incidence of GBS disease in HIV-exposed infants was 4.46 cases/1,000 live births. The high prevalence of maternal HIV infection is contributing to the high incidence of GBS disease in South Africa, which corroborates data from a Belgium study that reported an incidence of 15.5 cases/1,000 live births (i.e., 5 cases/322 infants, predominantly LOD) in HIV-exposed infants, compared with 0.8 cases/1,000 live births (i.e., 16 cases/20,158 infants) in HIV-unexposed infants (*24*). Our study was not designed to evaluate whether the lower threshold used for investigating for sepsis in HIV-exposed than for HIV-unexposed infants may have contributed to ascertainment bias. However, such bias is unlikely because the threshold for investigating for sepsis among neonates is low in general at CHBAH; an investigation is done only when clinically indicated.

In addition to the higher incidence of LOD observed in HIV-exposed compared with HIV-unexposed infants, we observed that risk for EOD was 1.69-fold (95% CI 1.28–2.24) greater in HIV-exposed infants. This increased risk was present despite our previous observation that the prevalence of vaginal GBS colonization at delivery was lower in HIV-infected than HIV-uninfected women (17.0% vs. 23.0%; p = 0.002), even though rates of vertical colonization were similar for their newborns (52.0%–58.0%) (*25*). Although our study did not identify other differences in prevalence of risk factors for invasive GBS disease between HIV-exposed and -unexposed infants (*26*), we did not have population-level data on prevalence rates of these maternal risk factors for HIV-infected and -uninfected women. However, the observation of a greater difference in risk for LOD than EOD in HIV-exposed newborns compared with HIV-unexposed newborns suggests that risk factors other than peripartum

EOD–associated risk factors likely contribute to the heightened susceptibility of invasive disease in HIV-exposed infants. We were unable to determine whether HIV infection in the neonates contributed to an enhanced susceptibility to invasive GBS disease. None of the newborns with EOD for whom HIV testing was done were HIV-positive, whereas 20.0% (8/40) of tested LOD case-patients were HIV-positive. The population-based vertical transmission rate of HIV during the course of the study was 9.6% (*27*). A further limitation of our study was the lack of data on the clinical, immunologic, and HIV-virologic characteristics of the HIV-infected women and analysis of whether these characteristics could have contributed to a heightened susceptibility of invasive disease in their neonates.

Multiple studies have reported an inverse association between maternal GBS serotype–specific antibody levels and an infant's risk for EOD and LOD (*28*). Lower levels of maternally derived antibodies to several childhood vaccine epitopes have been reported in HIV-exposed but uninfected infants at birth to at least 6 weeks of age (*29*,*30*). Thus, it is plausible that lower naturally acquired capsular antibody in HIV-infected women may contribute to increased susceptibility to invasive GBS disease in HIV-exposed infants; this possibility warrants further investigation. The increased incidence of invasive GBS disease in HIV-exposed but uninfected infants could also be due to observed perturbations of their immune systems (*31*,*32*).

The serotype distribution of GBS isolates from EOD and LOD cases in this study was similar to that observed previously (*21*) and did not differ by HIV-exposure status. Serotypes Ia, Ib, and III, which are included in the current investigational trivalent GBS-CV, covered 78% of EOD and 100% of LOD invasive isolates in this study. However, the overall potential disease reduction of a vaccine against invasive GBS disease may be lower than this potential coverage because the vaccine is unlikely to confer protection through antibody acquisition in neonates born at <34 weeks' gestation (*33*). The protection of premature newborns against EOD would require prevention of GBS acquisition from the mother. This strategy is pertinent to settings like ours, in which 29.8% of all EOD-associated and 31.4% of all GBS-associated deaths occurred in premature infants despite the rate of premature birth in the community being only 18.0%.

Using a conservative approach of assuming no efficacy against invasive GBS disease in infants born at ≤33 weeks' gestation and vaccine efficacy of 85% and 65% in HIV-unexposed and HIV-exposed infants, respectively, we estimate that vaccination of pregnant women with the current investigational trivalent conjugate vaccine could potentially prevent 3,178 GBS cases and 549 GBS-associated deaths annually in South Africa. An effective GBS vaccine could also be used to probe the possible role of GBS in causing sickness and death in countries with limited epidemiologic laboratory capacity (*34*) and in causing stillbirths (*35*).

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La Crosse Virus in *Aedes japonicus japonicus* Mosquitoes in the Appalachian Region, United States

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La Crosse virus (LACV), a leading cause of arboviral encephalitis in children in the United States, is emerging in Appalachia. For local arboviral surveillance, mosquitoes were tested. LACV RNA was detected and isolated from *Aedes japonicus* mosquitoes. These invasive mosquitoes may significantly affect LACV range expansion and dynamics.

La Crosse virus (LACV; family *Bunyaviridae,* genus *Orthobunyavirus*), in the California serogroup, is the major cause of arboviral encephalitis among children in the United States (*1*). Since its 1963 discovery in Wisconsin, LACV has been identified in 30 other US states (*2*). These include states within the Appalachian Mountain region (West Virginia, Virginia, Ohio, Tennessee, and North Carolina), which is an emerging focus of LACV (*3*).

The primary vectors of LACV, *Aedes triseriatus* mosquitoes, are present in southwestern Virginia and West Virginia, but 2 invasive congeners—*Ae. albopictus* and *Ae. japonicus*—have recently emerged (*3*). Both species have been shown to be competent experimental LACV vectors (*4*,*5*). Although LACV has been isolated from *Ae. albopictus* mosquitoes (*6*), previously it had only been detected in the Asian bush mosquito (*Ae. japonicus japonicus*) in Tennessee (*7*). *Ae. japonicus* mosquitoes are mammalophilic container breeders that co-occur with the primary LACV vector (*Ae. triseriatus* mosquitoes). Known to feed on humans (*8*), *Ae. japonicus* mosquitoes are found in woodlands (where this "rural encephalitis" virus is endemic) and urban areas (*9*).

To ascertain the public health risk that *Ae. japonicus* mosquito vectors represent for LACV transmission, we examined mosquitoes from West Virginia and Virginia for presence of this arbovirus. We report 2 independent isolations of LACV from adult *Ae. japonicus* mosquitoes in southwestern Virginia and 7 field detections of LACV RNA from adults (Virginia and West Virginia) and adults reared from eggs (Virginia). Our findings suggest a potential role of this invasive vector in the ecology of LACV in Appalachia (Figure 1).

The Study

In 2005, mosquito eggs were collected weekly in Wise County, Virginia, by using ovitraps. The resultant larvae were reared to adults in a Biosafety Level 2 insectary at 24°C, 75% relative humidity, and a photoperiod of 16 hours of light and 8 hours of dark. In 2008 and 2009, adult mosquitoes were collected weekly from infusion-baited gravid traps in Montgomery and Craig Counties, Virginia. Mosquitoes were identified to the species level according to morphology and grouped in pools of ≤50 individuals according to species, collection location, and collection date. Adults were stored at –80°C until testing. Reverse transcription PCR (RT-PCR) was used for LACV detection in mosquitoes collected in 2005 and 2008. In 2009, mosquito pools were homogenized by using previously described methods for LACV isolation (*6*). Homogenate supernatant (150 μL) was inoculated onto Vero cells, incubated at 37°C, and monitored daily for cytopathic effect. Isolates that showed marked cytopathic effect were harvested and submitted to the Centers for Disease Control and Prevention (CDC) in Fort Collins, Colorado, USA, and the Wadsworth Center in Slingerlands, New York, USA, for quantitative RT-PCR (qRT-PCR).

In 2013, mosquito surveillance was conducted as part of the West Virginia Department of Health and Human Resources Mosquito Surveillance Program. Gravid traps, carbon dioxide–emitting light traps, and BG Sentinel (Biogents AG, Regensburg, Germany) traps baited with octenol lures were used to collect adult mosquitoes weekly from counties with high (Nicholas, Fayette, Raleigh) and low (Kanawha, Jackson, Wood) incidence of LACV among humans as defined (*10*) and on a somewhat regular basis in additional counties. Specimens were pooled by species, county, and collection date for qRT-PCR at the West Virginia Office of Laboratory Services, by use of previously described methods (*11*).

LACV RNA was detected in a pool of *Ae. japonicus* mosquitoes collected as eggs in August 2005 from Wise

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County, Virginia. LACV was also detected in a pool of *Ae. japonicus* mosquitoes from Montgomery County, Virginia, in July 2008. LACV RNA was detected in 5 separate pools of *Ae. japonicus* mosquitoes collected in West Virginia in 2013, representing 3 counties over a 4-month period. Of 3,529 *Ae. japonicus* mosquitoes collected from Montgomery County in 2009, we isolated LACV from 1 pool (n = 3). In that same year, of 796 *Ae. japonicus* mosquitoes from Craig County tested, LACV was isolated from 1 pool (n = 50) (Table). These isolations were verified by qRT-PCR at CDC (Montgomery County isolate) and the Wadsworth Center (Craig County isolate).

Nucleotide sequencing and a BLAST (http://blast.ncbi.nlm.nih.gov//Blast.cgi) query were performed on the amplified cDNA from both isolates. The LACV medium segment was used to infer phylogeny (Figure 2). Coding sequences were aligned in Mesquite version 2.75 with Opalescent version 2.10 (*12*,*13*). Phylogenetic trees for the polyprotein genes were estimated by using a maximum likelihood–based method and assuming a general time-reversible (GTR) model with gamma-distributed rate heterogeneity of nucleotide substitution GTR + Γ in RAxML version 8.0.0 (*14*). Support values for each clade were generated in RAxML by using 1,000 rapid bootstrap replicates. The Virginia 2009 isolates were within the previously described lineage I, which contains the reference LACV strain isolated from the brain tissue of a child in Wisconsin (National Center for Biotechnology Information accession no. U18979) (*15*). The 2 isolates from mosquitoes collected in Virginia in 2009 differed by only 1 bp.

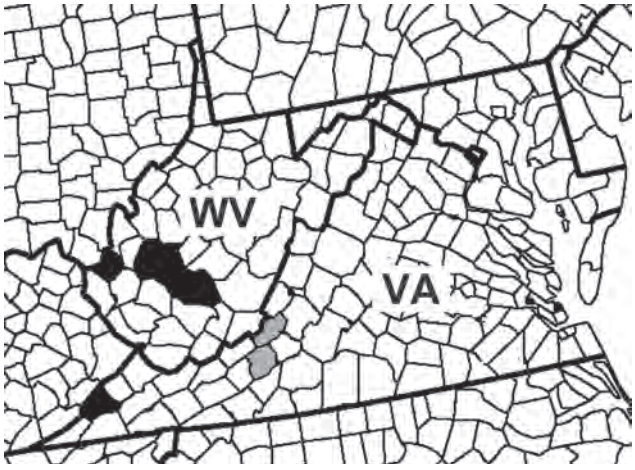

Figure 1. Locations of detection of La Crosse virus (LACV) RNA and virus isolation from *Aedes japonicus* mosquito pools. Gray represents counties of the *Ae. japonicus* LACV isolates and black represents counties of *Ae. japonicus* LACV RNA detection.

Conclusions

The isolation of LACV from field-collected *Ae. japonicus* mosquitoes, and particularly from mosquitoes collected as eggs, is highly significant because of the pervasiveness of this species in the United States. The large number of LACV detections in this invasive species highlights the need for LACV mosquito surveillance and control efforts to include *Ae. japonicus* in addition to *Ae. triseriatus* mosquito populations. Most (3/5) detections of LACV in West Virginia were in mosquitoes from Fayette County, where incidence of LACV among humans is high, suggesting that *Ae. japonicus* mosquitoes may play a major role in transmission of LACV to humans. Our detection of LACV in *Ae. japonicus* mosquitoes from field-collected eggs in 2005 and the ability of LACV to be transmitted transovarially in *Ae. triseriatus* mosquitoes suggests that future research should examine the possibility of vertical transmission of LACV in invasive *Ae. japonicus* mosquitoes. In states east of the Mississippi River, where *Ae. japonicus* mosquitoes (*9*) and LACV (*2*) co-exist, this mosquito may play a major role in the maintenance, transmission, and range expansion of LACV.

Acknowledgments

We thank Amy Lambert for conducting RT-PCR and sequencing of the 2009 isolate from Montgomery County, Virginia; Dee Petit and Andrew Luna for technical assistance; and Nate

Table. Detection of LACV RNA and virus isolations in *Aedes japonicus* mosquito pools from Virginia and West Virginia, USA*

Collection date	County, state	Trap type†	Pool size‡	LACV detection method	C_t value	MLE (95% CI)
2005 Aug	Wise, VA	Ovitrap	9	RT-PCR	38.04	8.59 (0.54 –41.00)
2008 Jul	Montgomery, VA	Gravid	22	RT-PCR	37.57	4.51 (0.26–22.00)
2009 Jul	Montgomery, VA	Gravid	3	Isolation, RT-PCR	14.00	0.23 (0.01–1.11)
2009 Jul	Craig, VA	Gravid	50	Isolation, RT-PCR	23.00	1.28 (0.07–6.29)
2013 Jun	Fayette, WV	Multiple adult	36	RT-PCR	37.66	13.41 (5.18–29.14)
2013 Jul	Cabell, WV	Multiple adult	1	RT-PCR	34.72	13.41 (5.18–29.14)
2013 Aug	Fayette, WV	Multiple adult	15	RT-PCR	37.35	13.41 (5.18–29.14)
2013 Aug	Fayette, WV	Multiple adult	2	RT-PCR	34.64	13.41 (5.18–29.14)
2013 Sep	Kanawha, WV	Multiple adult	1	RT-PCR	37.43	13.41 (5.18–29.14)

*C_t, cycle threshold; LACV, La Crosse virus; MLE, maximum-likelihood estimate of the proportion of infected mosquitoes; RT-PCR, reverse transcription PCR.
†All mosquitoes were collected from the field as adults except when ovitraps were used to collect eggs. Adults were reared from the field-collected eggs before testing for arboviruses. Multiple adult traps were gravid traps, carbon dioxide–emitting light traps, and BG Sentinel (Biogents AG, Regensburg, Germany) traps baited with octenol lures.
‡Pool size indicates no. adult mosquitoes tested for LACV.

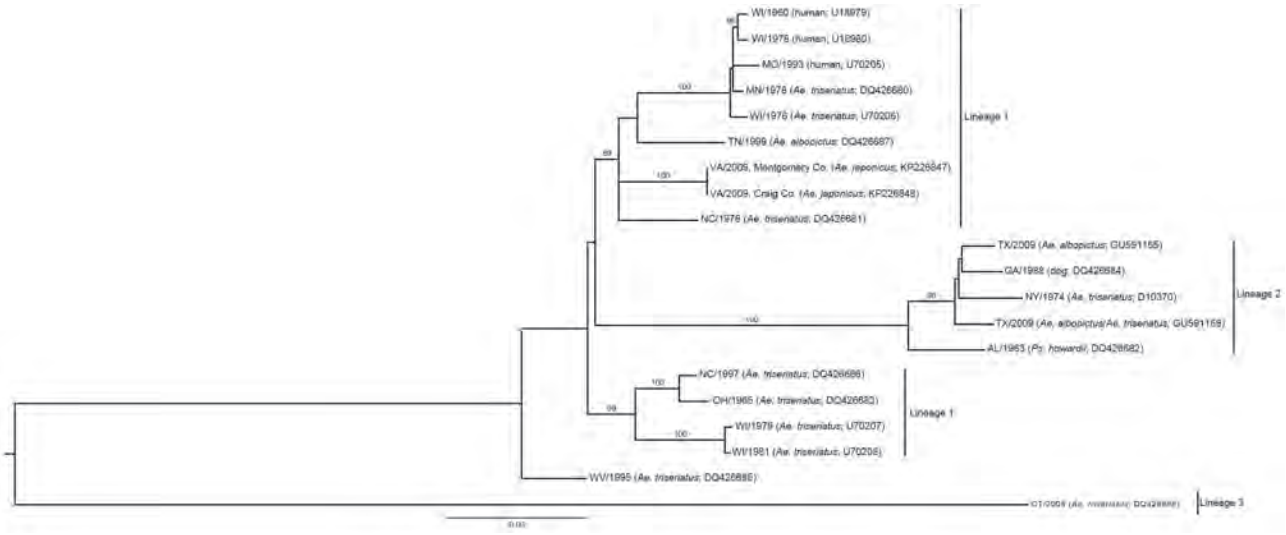

Figure 2. Phylogeny of La Crosse virus (LACV) based on the medium (M) segment of the viral polyprotein gene. State of isolate origin, isolation year, mosquito, or vertebrate isolate source and the National Center for Biotechnology Information (NCBI) accession numbers are listed for each isolate within the tree. The scale bar represents the number of nucleotide substitutions per site. LACV historical lineages are identified by vertical bars. The 2009 isolates from Virginia (NCBI accession nos. KP226847, KP226848) group with lineage 1 viruses. *Ae*., *Aedes*; *Ps*., *Psorophora*.

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Pathogenicity of 2 Porcine Deltacoronavirus Strains in Gnotobiotic Pigs

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To verify whether porcine deltacoronavirus infection induces disease, we inoculated gnotobiotic pigs with 2 virus strains (OH-FD22 and OH-FD100) identified by 2 specific reverse transcription PCRs. At 21–120 h postinoculation, pigs exhibited severe diarrhea, vomiting, fecal shedding of virus, and severe atrophic enteritis. These findings confirm that these 2 strains are enteropathogenic in pigs.

Porcine epidemic diarrhea virus (PEDV) (family *Coronaviridae*, genus *Alphacoronavirus*) was discovered in the United States in May 2013. The virus has now spread nationwide and caused a high number of deaths among suckling pigs (*1*,*2*). In regions of the United States to which PEDV is epidemic, a new coronavirus, genetically distinct from PEDV, porcine deltacoronavirus (PDCoV) (genus *Deltacoronavirus*), has been simultaneously and frequently detected in diarrheic fecal samples from pigs (*3*–*5*).

The clinical role and disease severity of PDCoV in the field is reportedly less than that of PEDV (*6*). To confirm the role of PDCoV as an enteric viral pathogen and understand disease progression, we studied the pathogenicity of 2 strains of PDCoV (OH-FD22 and OH-FD100) in gnotobiotic pigs. We developed in situ hybridization and immunofluorescence staining methods to verify the tissue sites of PDCoV replication in infected pigs.

The Study

In February and July 2014, intestinal contents were obtained from young nursing piglets with diarrhea on farms in Ohio, USA. PDCoV strains OH-FD22 and OH-FD100 were detected in samples by using a TaqMan quantitative reverse transcription RT-PCR (qRT-PCR) specific for the membrane gene (nt 23395–23466) as reported (*7*), or an RT-PCR specific for the PDCoV membrane gene (nt 23111–23651) based on the sequence of PDCoV strain (USA/Illinois121/2014; GenBank accession no. KJ481931), as described in the in situ hybridization method. The partial membrane gene sequences of OH-FD22 and OH-FD100 were identical to that of USA/Illinois121/2014. Samples were negative for PEDV, rotavirus groups A–C, transmissible gastroenteritis virus (TGEV)/porcine respiratory coronavirus (PRCV), and caliciviruses (noroviruses, sapoviruses, and St. Valerien–like viruses) by RT-PCR as reported (*8*). The samples were bacteriologically sterilized by using 0.22-μm syringe filters and then prepared as inoculum.

Near-term gnotobiotic pigs were delivered aseptically by hysterectomy from 2 specific pathogen–free sows (*9*). Seven 11- to 14-day-old pigs were randomly assigned to a PDCoV-inoculated group (pigs 1–5) or as negative controls (pigs 6 and 7). Pigs 1–3 and pigs 4 and 5 were inoculated orally with 8.8 $\log_{10}$ genomic equivalents (GEs) of PDCoV strain OH-FD22 and 11.0 $\log_{10}$ GEs of OH-FD100, respectively. Clinical signs were monitored hourly. Pig 2 was monitored for long-term clinical signs and virus shedding until day postinoculation (dpi) 23. Pigs were euthanized for pathologic examination at 24–48 h or >48 h after onset of clinical signs. All animal-related experimental protocols were approved by the Ohio State University Institutional Animal Care and Use Committee.

Fecal or rectal swab samples were prepared as described (*8*,*9*). Virus RNA was extracted by using the Mag-MAX Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Titers of virus shed in feces were determined by using the qRT-PCR and the OneStep RT-PCR Kit (QIAGEN, Valencia, CA, USA) as reported (*7*). A standard curve was generated by using the PCR amplicon (nt 23111–23651) of strain OH-FD22. The detection limit of the qRT-PCR was 10 GEs/reaction, which corresponded to 4.6 $\log_{10}$ and 3.6 $\log_{10}$ GEs/mL of PDCoV in fecal and serum samples, respectively.

Small and large intestinal tissues and other major organs (lung, liver, heart, kidney, spleen, and mesenteric lymph nodes) were examined. Mean jejunal ratios of villous height to crypt depth were measured as reported (*8*). For PDCoV RNA detection in formalin-fixed, paraffin-embedded tissues, a nonradioactive digoxigenin-labeled cDNA probe specific for the 541-bp virus membrane gene sequence (nt 23111–23651), amplified with the primers forward 5′-CGCGTAATCGTGTGATCTATGT-3′ and reverse 5′-CCGGCCTTTGAAGTGGTTAT-3′, was used for in situ hybridization as described (*10*). Reverse transcription was conducted at 50°C for 30 min, followed by denaturation at 94°C for 5 min; 35 cycles at 94°C for 40 s, 55°C for 40 s, and 72°C for 1 min; and final extension at 72°C for 7 min.

OH-FD22–infected pig 2 was immunized intramuscularly with OH-FD22 from the gnotobiotic pig–passaged

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Table 1. Fecal shedding of virus and clinical signs after inoculation of gnotobiotic pigs with PDCoV strains OH-FD22 and OH-FD100*

Pig status, no. (age at inoculation, d)	Virus strain	Oral inoculum, log_{10} GEs	Fecal shedding, log_{10} GE/mL by hpi†						Clinical signs (onset hpi)
			0	24	48	72	96	120	
PDCoV-inoculated									
1 (14)	OH-FD22	8.8	<4.6	<4.6	8.1	7.3	7.4	6.7‡	Diarrhea/vomiting (21–24)§
2 (14)	OH-FD22	8.8	<4.6	6.2	8.5	8.4	6.1	7.6	Diarrhea/vomiting (21–24)
3 (14)	OH-FD22	8.8	<4.6	8.4	8.2	8.8‡	–	–	Diarrhea/vomiting (21–24)
4 (11)	OH-FD100	11.0	<4.6	7.1	6.0	ND	ND‡	–	Diarrhea/vomiting (22–24)
5 (11)	OH-FD100	11.0	<4.6	8.2	8.5	ND‡	–	–	Diarrhea/vomiting (22–24)
Negative control									
6 (16)¶	None	None	<4.6	<4.6	<4.6	<4.6‡	–	–	None
7 (17)¶	None	None	<4.6	<4.6	<4.6	<4.6‡	–	–	None

*PDCoV, porcine deltacoronavirus; GE, genome equivalent; hpi, hour postinoculation; –, no result (pig euthanized); ND, not determined.
†Detected by real-time quantitative reverse transcription PCR. The detection limit of the PCR was <4.6 log_{10} GE/mL for a fecal sample and <3.6 log_{10} GE/mL for a serum sample.
‡Pig was euthanized.
§Pig 1 vomited at days postinoculation 21–24 and then had diarrhea.
¶At euthanasia.

intestinal contents that were semipurifed by sucrose gradient ultracentrifugation (*11*) and mixed with complete and incomplete Freund's adjuvants at dpi 30 and dpi 44 (*11*). Immunofluorescence staining was performed on frozen or formalin-fixed, paraffin-embedded tissues as described (*8*,*9*) by using hyperimmune gnotobiotic pig antiserum against OH-FD22. Tissues from control pigs 6 and 7 and PEDV-infected gnotobiotic pigs (*8*) were used negative controls for in situ hybridization/immunofluorescence staining.

Acute, severe, watery diarrhea, vomiting, or both developed in all inoculated pigs. Clinical signs developed at hour postinoculation (hpi) 21–24, regardless of the inoculum strain or dose (Table 1). At hpi 96–120, pig 1 exhibited severe dehydration, loss of bodyweight, and lethargy. Pig 2, which was followed up longer, showed diarrhea until dpi 7. All inoculated pigs exhibited onset of clinical disease similar to that of infection with PEDV strain PC21A (6.3–9.0 log_{10} GEs/pig) in gnotobiotic pigs (*8*). Immune electron microscopy with hyperimmune serum to PDCoV from a gnotobiotic pig showed only PDCoV particles in the intestinal contents (Figure 1). For pig-passaged OH-FD22 and OH-FD100 samples, RT-PCR results were negative for PEDV, rotavirus groups A–C, TGEV/PRCV, and caliciviruses. Detection of fecal virus shedding at hpi 24 coincided with onset of clinical signs (Table 1) in pigs 2–5. In pig 1, which showed only vomiting at hpi 24, fecal shedding occurred at hpi 48 at the onset of diarrhea.

Macroscopic examination showed that all infected pigs had PEDV-like lesions characterized by thin and transparent intestinal walls (proximal jejunum to colon) and accumulation of large amounts of yellow fluid in the intestinal lumen (Figure 2, panel A). The stomach was filled with curdled milk. Other internal organs appeared normal. Histologic lesions included acute diffuse, severe atrophic enteritis (Figure 2, panels B, D) and mild vacuolation of superficial epithelial cells in cecum and colon (Figure 2, panel E). The mean jejunal ratios of villous height to crypt depth of infected pigs 3–5 at hpi 72–120 ranged from 1.4 to 3.6 (Table 2), which were similar to those in gnotobiotic pigs experimentally infected with PEDV strain PC21A (*8*). Clinical signs or lesions did not develop in negative control pigs during the experiment (Figure 2, panels C, F).

In situ hybridization–positive or immunofluorescence-stained cells were observed mainly in the villous epithelium of small (duodenum to ileum) and large intestines (Table 2; Figure 2, panels G, I–K). Immunofluorescence was confined to the cytoplasm of villous epithelial cells (Figure 2, panel J) and was infrequently observed in crypt epithelial cells. No other internal organs of infected pigs showed in situ hybridization–positive or immunofluorescence-positive staining.

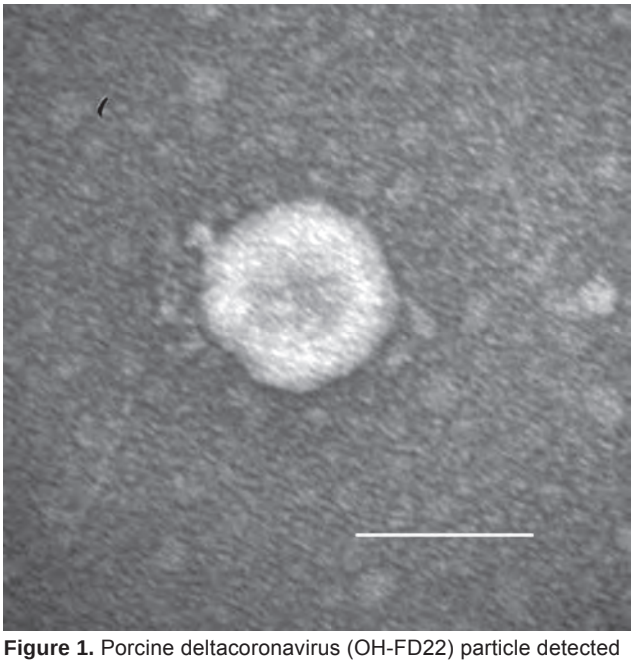

Figure 1. Porcine deltacoronavirus (OH-FD22) particle detected in intestinal contents from a gnotobiotic pig. The sample was negatively stained with 3% phosphotungstic acid. Scale bar indicates 100 nm.

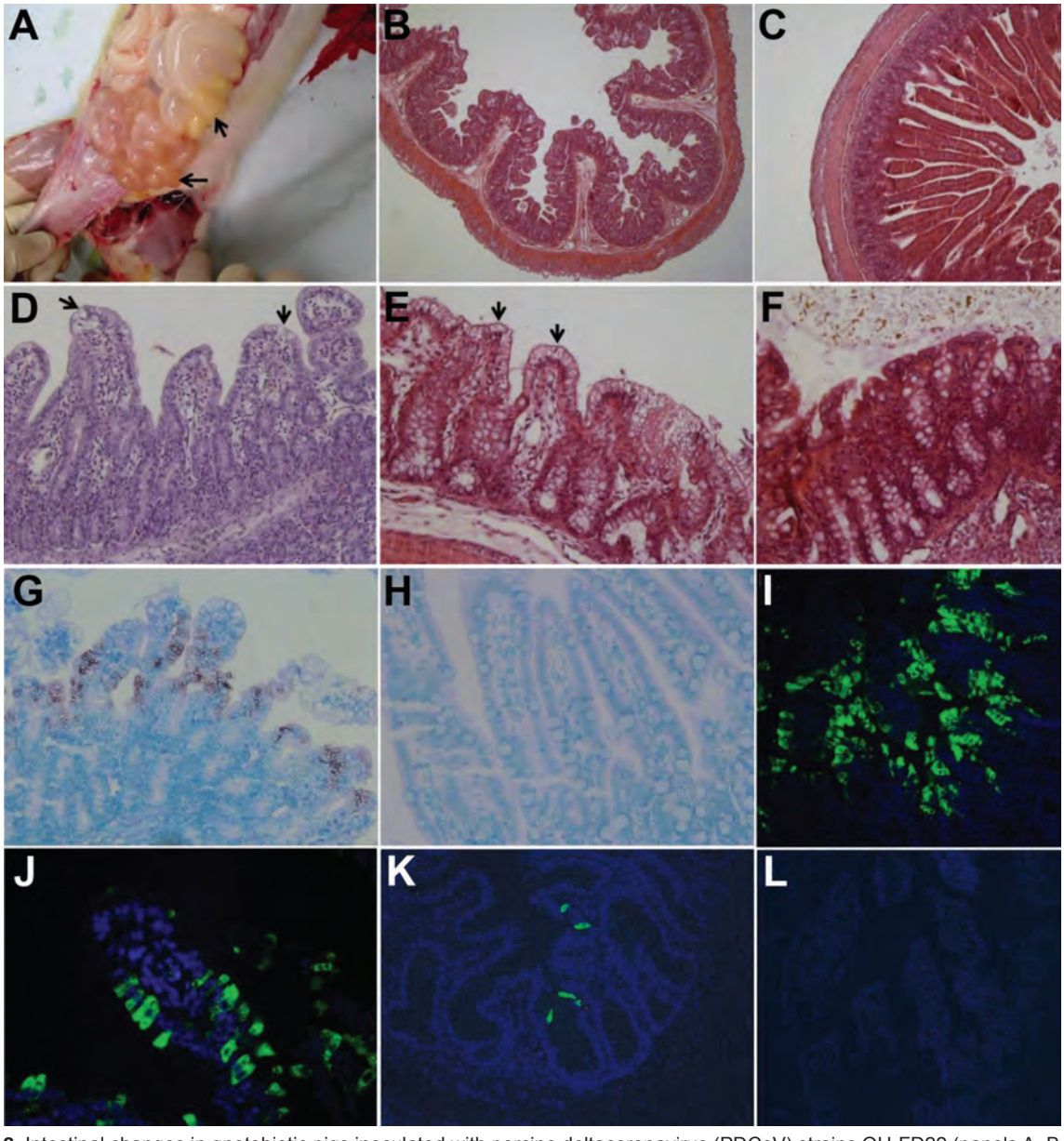

Figure 2. Intestinal changes in gnotobiotic pigs inoculated with porcine deltacoronavirus (PDCoV) strains OH-FD22 (panels A, B, E, G, I, and J) and OH-FD100 (panels D and K). A) Intestine of pig 3 at hour postinoculation (hpi) 72 (48–51 h after onset of clinical signs), showing thin and transparent intestinal walls (duodenum to colon) and accumulation of large amounts of yellow fluid in the intestinal lumen (arrows). B) Jejunum of pig 3 at hpi 72 (48–51 h after onset of clinical signs), showing acute diffuse, severe atrophic jejunitis (original magnification ×40). C) Jejunum of noninoculated pig 7, showing normal villous epithelium (original magnification ×80). D) Jejunum of pig 4 at hpi 96 (72–74 h after onset of clinical signs), showing acute diffuse, severe atrophic jejunitis with mild cytoplasmic vacuolation at the tips of villi (arrows) (original magnification ×200). E) Colon of pig 3 at hpi 72 (48–51 h after onset of clinical signs), showing mild cytoplasmic vacuolation of superficial epithelial cells (arrows) (original magnification ×200). F) Colon of noninoculated pig 7, showing normal colonic epithelium (original magnification ×200). G) Jejunum of pig 3 at hpi 72 (48–51 h after onset of clinical signs), showing that epithelial cells lining the atrophied villi are positive for PDCoV RNA (original magnification ×200). H) Jejunum of noninoculated pig 6, showing absence of PDCoV RNA-positive cells and background staining (original magnification ×200). I) Jejunum of pig 3 at hpi 72 (48–51 h after onset of clinical signs), showing large numbers of PDCoV antigen–positive cells in the epithelium of atrophied villi (original magnification ×200). J) Jejunum of pig 3 at hpi 72 (48–51 h after onset of clinical signs), showing localization of PDCoV antigens in cytoplasm of columnar epithelial cells (original magnification ×400). K) Cecum of pig 4 at hpi 96 (72–74 h after onset of clinical signs), showing a few PDCoV antigen–positive cells in the epithelium (original magnification ×200. L) Jejunum of noninoculated pig 6, showing absence of immunofluorescence-stained cells and background staining (original magnification ×200). Nuclei were stained with blue-fluorescent 4′, 6-diamidino-2-phenylindole dihydrochloride. Hematoxylin and eosin staining in panels B–F; in situ hybridization staining in panels G and H; immunofluorescence staining in panels I–L.

Table 2. Histopathologic findings after inoculation of gnotobiotic pigs with PDCoV strains OH-FD22 and OH-FD100*

Pig status, no.	hpi at euthanasia	Mean VH:CD	RNA detection in formalin-fixed, paraffin-embedded tissues/antigen detection in frozen tissues (ISH/IF results)†			
			Duodenum	Jejunum	Ileum	Cecum/colon
PDCoV-inoculated						
1	120	ND	–/–	+/+++	+/+++	–/±
2‡	NA	NA	NA	NA	NA	NA
3	72	3.6 (1.7)	–/±	++/+++	++/+++	±/±
4	96	1.4 (0.1)	–/–	++/+++	++/+++	–/±
5	72	1.6 (0.5)	–/+	++/+++	++/+++	–/±
Negative control						
6	NA	5.8 (0.9)	–/–	–/–	–/–	–/–
7	NA	5.6 (0.5)	–/–	–/–	–/–	–/–

*PDCoV, porcine deltacoronavirus; hpi, hour postinoculation; VH:CD, ratio of villous height to crypt depth; ISH, in situ hybridization; IF, immunofluorescence staining; ND, not determined because of autolysis at tips of jejunal villi; NA, not applicable.
†Detected by ISH or IF staining. –, no cells showed staining; +, 1%–29% of epithelial cells showed staining; +++, 60%–100% of epithelial cells showed staining as described (*12*); ±, <1% of epithelial cells showed staining; ++, 30%–59% of epithelial cells showed staining. Other internal organs showed no ISH or IF staining.
‡Pig 2 was used for production of hyperimmune gnotobiotic antiserum against PDCoV strain OH-FD22.

In situ hybridization–positive or immunofluorescence-stained cells were not detected in negative control pigs (Figure 2, panels H, L) and PEDV-infected gnotobiotic pigs.

Under the experimental conditions used, no PDCoV-inoculated pigs at hpi 72–168 had detectable virus RNA (<3.6 $\log_{10}$ GEs/mL) in serum. However, viremia was detected frequently in symptomatic PEDV-infected pigs (*8*,*13*).

Conclusions

Since 2013–2014, newly emerged PEDV and PDCoV have spread throughout the United States and caused a high number of pig deaths (*1*,*2*,*6*), but no studies of the pathogenicity of PDCoV have been reported. Our data confirm that PDCoV strains OH-FD22 and OH-FD100 are enteropathogenic and acutely infect the entire intestine. However, the jejunum and ileum are the primary sites of infection. PDCoV infection caused severe atrophic enteritis accompanied by severe diarrhea, vomiting, or both. These clinical and pathologic features in PDCoV-infected pigs resemble those of PEDV and TGEV infections. Differential diagnosis of PDCoV, PEDV, and TGEV is critical to control virus epidemic diarrhea on swine farms in the United States.

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Multidrug-Resistant *Salmonella enterica* Serotype Typhi, Gulf of Guinea Region, Africa

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We identified 3 lineages among multidrug-resistant (MDR) *Salmonella enterica* serotype Typhi isolates in the Gulf of Guinea region in Africa during the 2000s. However, the MDR H58 haplotype, which predominates in southern Asia and Kenya, was not identified. MDR quinolone-susceptible isolates contained a 190-kb incHI1 pST2 plasmid or a 50-kb incN pST3 plasmid.

Typhoid fever, which is caused by *Salmonella enterica* serotype Typhi, is endemic to the developing world; there were an estimated 26.7 million cases in 2010 (*1*). The incidence of typhoid fever in sub-Saharan Africa was an estimated 725 cases/100,000 persons in 2010, despite a lack of incidence studies conducted in West and central Africa (*1*). Antimicrobial susceptibility data are also scarce for this part of Africa. This issue is problematic because treatment with appropriate antimicrobial drugs is essential for recovery in the context of the global emergence of multidrug resistance.

In the Indian subcontinent and Southeast Asia, the multidrug-resistant (MDR) *Salmonella* Typhi H58 clone, which was named after its haplotype (a combination of defined chromosomal single-nucleotide polymorphisms [SNPs]) (*2*,*3*), has spread rapidly and become endemic and predominant. During the 1990s, this clone acquired a large conjugative incHI1 pST6 plasmid encoding resistance to ampicillin, chloramphenicol, and co-trimoxazole (*4*,*5*); also in the 1990s, this MDR clone became resistant to quinolones and showed decreased susceptibility to ciprofloxacin because of point mutations in the chromosomal *gyrA* gene (*2*). The H58 clone has also spread to eastern Africa, where it has been the most prevalent haplotype (87%) in Kenya since the early 2000s (*6*).

During 1997–2011, high incidence of MDR *Salmonella* Typhi was reported in some countries near the Gulf of Guinea in Africa, including Nigeria (*7*), Ghana (*8*,*9*), Togo (*10*), and the Democratic Republic of the Congo (*11*). During 1999–2003, a British surveillance system reported a prevalence of 19% (49/421) for MDR *Salmonella* Typhi isolates among imported cases of typhoid fever acquired in Africa, particularly in Ghana (*12*). However, nothing is known about the genotypes of these isolates, including whether they belong to the spreading MDR H58 clone.

We report data for the occurrence, genotypes, and characterization of the resistance mechanisms of MDR *Salmonella* Typhi isolates. These isolates were obtained from the French National Reference Center for *Salmonella* (FNRC-Salm), Institut Pasteur (Paris, France), and Centre Pasteur du Cameroun (Yaoundé, Cameroon).

The Study

Almost all *Salmonella* Typhi strains isolated in France are referred to the FNRC-Salm. Most isolates were obtained from travelers or immigrants, most of whom were infected in Africa and Asia. In Cameroon, the Centre Pasteur du Cameroun collects *Salmonella* Typhi isolates from several hospitals in.

Antimicrobial susceptibility testing was performed according to the guidelines of the antibiogram committee of the French Society for Microbiology (http://www.sfm.asso.fr/nouv/general.php?pa = 2). Isolates were considered to be MDR if they were resistant to ≥2 of the following antimicrobial drugs: amoxicillin, co-trimoxazole (trimethoprim/sulfamethoxazole), chloramphenicol, or tetracyclines.

During 1996–2013, a total of 1,746 *Salmonella* Typhi isolates were collected through the French national surveillance system and subjected to antimicrobial susceptibility testing; 408 were acquired in sub-Saharan Africa (n = 237) and northern Africa (n = 171), and 55 (13.5%) of those acquired in Africa were MDR (Table). All but 1 of the MDR isolates were acquired in sub-Saharan Africa (Table). The

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Table. Characteristics of *Salmonella enterica* serotype Typhi isolates, France and Cameroon, 1996–2013*

Location	1996–1999	2000–2004	2005–2009	2010–2013
France				
No. isolates studied	345	266	627	508
No. isolates acquired in Africa	86	64	155	103
No. (%) MDR†	0	7 (10.9)	25 (16.1)	23 (22.3)
Country of infection for MDR isolates (no.)		Benin (3), Togo (2), Burkina-Faso (1), Cameroon (1)	Cameroon (7), Côte d'Ivoire (4), Burkina-Faso (3), Angola (2), Congo (1), Mali (1), Benin (1), Nigeria (1), Mauritania (1), Togo (1), Central African Republic (1), Guinea (1), not specified (1)	Côte d'Ivoire (7), Guinea (3), Burkina-Faso (3), Cameroon (2), Congo (1), Central African Republic (1), Niger (1), Mali (1), Nigeria (1), Chad (1), Togo (1), Algeria (1)
Yaounde, Cameroon				
No. isolates studied	ND	61	75	49
No. (%) MDR	ND	29 (47.5)	50 (66.6)	37 (75.5)

*MDR, multidrug resistant; ND, not determined.
†For isolates acquired in Africa.

proportion of MDR isolates increased from 0% during 1996–1999 to 22.3% (n = 23) during 2010–2013. Only 4 isolates from Africa were resistant to nalidixic acid, including 1 isolate resistant to ciprofloxacin. Because these isolates acquired after 2010 were not MDR isolates, they were not studied further.

In Yaoundé, the proportion of MDR isolates was high (45.5 %, 29/61) in the first survey during 2000–2004. However, this proportion increased to 75.5% (37/49) during 2010–2013.

We studied 61 isolates (online Technical Appendix, http://wwwnc.cd.gov/EID/article/21/3/14-1355-Techapp1.xlsx). Of these, 46 were MDR: 29 acquired in Africa and detected at FNRC-Salm before 2010; 2 acquired in France during 2009 at an African festive meal (*13*); 12 randomly selected acquired in Yaoundé during 2002–2007; 2 acquired in the Central African Republic, and 1 acquired in Morocco (*2*). The remaining 15 comparison strains (MDR or drug susceptible) that belonged to various haplotypes and were acquired in Africa and Asia during 1958–2009.

Mechanisms of antimicrobial drug resistance were determined as described (*14*). Genetic diversity and phylogenetic relationships were studied by using standardized *Xba*I–pulsed-field gel electrophoresis (PFGE) (http://www.cdc.gov/pulsenet/pathogens/index.html), haplotyping (*5*), and clustered regularly interspaced short palindromic repeats (CRISPR) typing (*15*). Haplotyping was based on identification of SNPs at 1,487 defined chromosomal loci, and CRISPR typing was based on detection of 32-bp sequences (spacers) within 1 or both CRISPR regions.

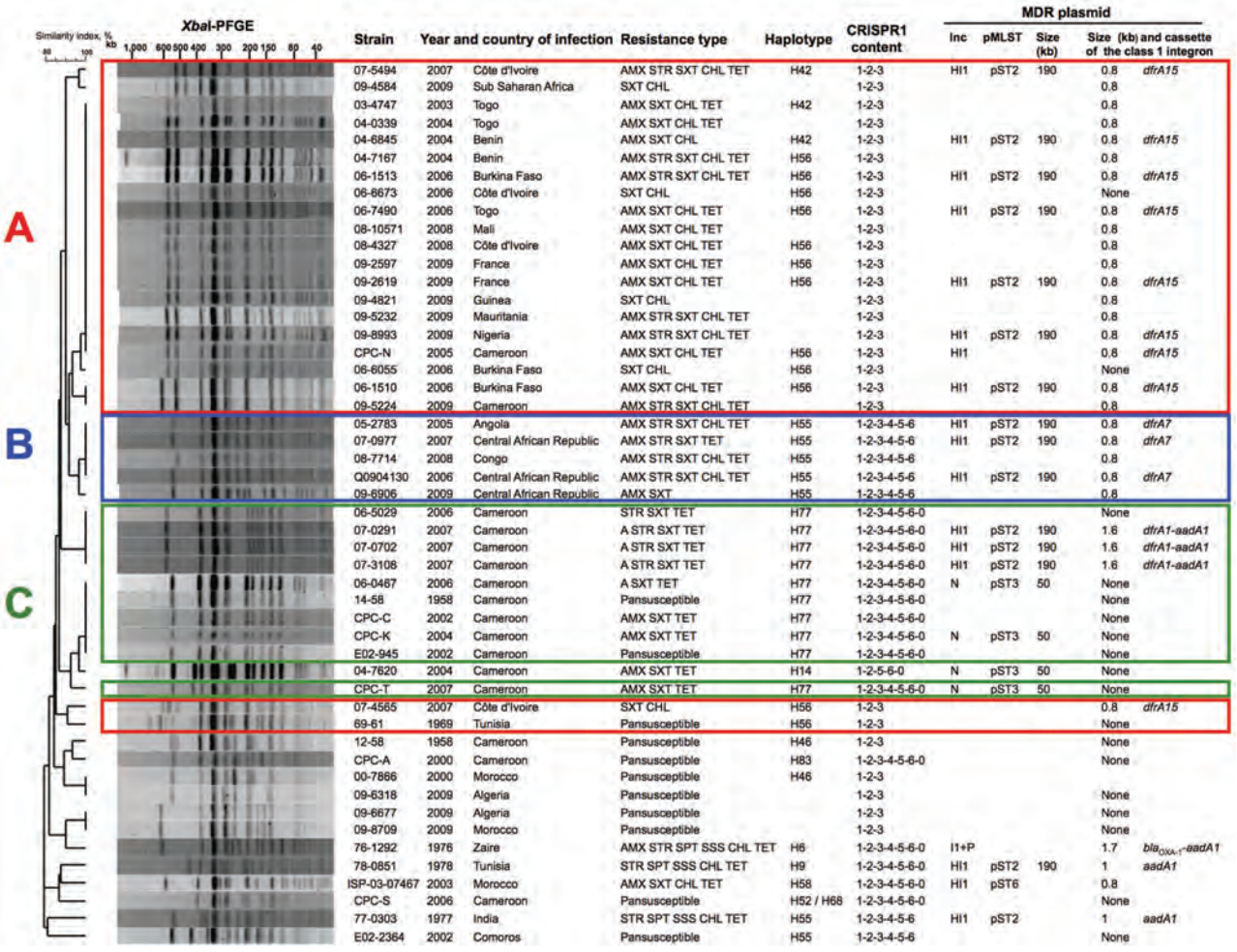
With the exception of the isolate from Morocco (ISP-03-07467) (*2*), none of the isolates had the H58 haplotype or contained the associated incHI1 pST6 MDR plasmid. We found 3 other lineages with different geographic distributions and MDR plasmids (Figures 1, 2).

Lineage A consisted mostly of haplotype H56 isolates and more rarely H42 (which differs from H56 by 2 SNPs) and was found only in the western part of the Gulf of Guinea region. Lineage B consisted of haplotype H55 isolates and was found in the eastern and southern parts of the Gulf of Guinea region. Lineage C consisted of haplotype H77 isolates and was found only in Cameroon. All 3 lineages had distinctive CRISPR1 spacer contents. *Xba*I-PFGE, which used a similarity value of ≥90% as a cutoff, correctly grouped (i.e., concordant with haplotyping and CRISPR results) all but 2 of the MDR isolates from Africa (Figure 1; online Technical Appendix).

The 3 lineages contained a large (≈190 kb) conjugative MDR incHI1 pST2 plasmid that differed among lineages. Resistance to trimethoprim was encoded by different class 1 integron gene cassettes: *dfrA15*, *dfrA7*, and *dfrA1* for incHI1 plasmids of lineages A, B, and C, respectively. All incHI1 plasmids from lineage A encoded resistance to chloramphenicol, and none of those from lineage C encoded such resistance. A second smaller (50-kb) MDR plasmid belonging to the incN incompatibility group (pST3 by plasmid multilocus sequence typing), was present mostly in lineage C isolates, but was also found in 1 lineage A isolate (02-1739) (online Technical Appendix).

Conclusions

Analysis of older isolates and previously published data (*2*) showed that susceptible *Salmonella* Typhi strains of haplotypes H42, H56, and H77 had already been identified in Senegal in 1962, Tunisia in 1969, and Cameroon in 1958, respectively. This finding suggests that the MDR isolates from lineages A and C are derived from local *Salmonella* Typhi populations in Africa, rather than being recently imported from other regions to which this bacterium is endemic. Haplotype H55 was previously restricted largely to the Indian subcontinent and eastern Africa (*2*); it was



Strain	Year	Country of infection	Resistance type	Haplotype	CRISPR1 content	MDR plasmid: Inc	pMLST	Size (kb)	Size (kb) of the class 1 integron	and cassette
07-5494	2007	Côte d'Ivoire	AMX STR SXT CHL TET	H42	1-2-3	HI1	pST2	190	0.8	*dfrA15*
09-4584	2009	Sub Saharan Africa	SXT CHL		1-2-3				0.8	
03-4747	2003	Togo	AMX SXT CHL TET	H42	1-2-3				0.8	
04-0339	2004	Togo	AMX SXT CHL TET		1-2-3				0.8	
04-6845	2004	Benin	AMX SXT CHL	H42	1-2-3	HI1	pST2	190	0.8	*dfrA15*
04-7167	2004	Benin	AMX STR SXT CHL TET	H56	1-2-3				0.8	
06-1513	2006	Burkina Faso	AMX STR SXT CHL TET	H56	1-2-3	HI1	pST2	190	0.8	*dfrA15*
06-6673	2006	Côte d'Ivoire	SXT CHL	H56	1-2-3				None	
06-7490	2006	Togo	AMX SXT CHL TET	H56	1-2-3	HI1	pST2	190	0.8	*dfrA15*
08-10571	2008	Mali	AMX SXT CHL TET		1-2-3				0.8	
08-4327	2008	Côte d'Ivoire	AMX SXT CHL TET	H56	1-2-3				0.8	
09-2597	2009	France	AMX SXT CHL TET	H56	1-2-3				0.8	
09-2619	2009	France	AMX SXT CHL TET	H56	1-2-3	HI1	pST2	190	0.8	*dfrA15*
09-4821	2009	Guinea	SXT CHL		1-2-3				0.8	
09-5232	2009	Mauritania	AMX STR SXT CHL TET		1-2-3				0.8	
09-8993	2009	Nigeria	AMX STR SXT CHL TET		1-2-3	HI1	pST2	190	0.8	*dfrA15*
CPC-N	2005	Cameroon	AMX SXT CHL TET	H56	1-2-3	HI1			0.8	*dfrA15*
06-6055	2006	Burkina Faso	SXT CHL	H56	1-2-3				None	
06-1510	2006	Burkina Faso	AMX SXT CHL TET	H56	1-2-3	HI1	pST2	190	0.8	*dfrA15*
09-5224	2009	Cameroon	AMX STR SXT CHL TET		1-2-3				0.8	
05-2783	2005	Angola	AMX STR SXT CHL TET	H55	1-2-3-4-5-6	HI1	pST2	190	0.8	*dfrA7*
07-0977	2007	Central African Republic	AMX STR SXT TET	H55	1-2-3-4-5-6	HI1	pST2	190	0.8	*dfrA7*
08-7714	2008	Congo	AMX STR SXT CHL TET	H55	1-2-3-4-5-6				0.8	
Q0904130	2006	Central African Republic	AMX STR SXT CHL TET	H55	1-2-3-4-5-6	HI1	pST2	190	0.8	*dfrA7*
09-6906	2009	Central African Republic	AMX SXT	H55	1-2-3-4-5-6				0.8	
06-5029	2006	Cameroon	STR SXT TET	H77	1-2-3-4-5-6-0				None	
07-0291	2007	Cameroon	A STR SXT TET	H77	1-2-3-4-5-6-0	HI1	pST2	190	1.6	*dfrA1-aadA1*
07-0702	2007	Cameroon	A STR SXT TET	H77	1-2-3-4-5-6-0	HI1	pST2	190	1.6	*dfrA1-aadA1*
07-3108	2007	Cameroon	A STR SXT TET	H77	1-2-3-4-5-6-0	HI1	pST2	190	1.6	*dfrA1-aadA1*
06-0487	2006	Cameroon	A SXT TET	H77	1-2-3-4-5-6-0	N	pST3	50	None	
14-58	1958	Cameroon	Pansusceptible	H77	1-2-3-4-5-6-0				None	
CPC-C	2002	Cameroon	AMX SXT TET	H77	1-2-3-4-5-6-0				None	
CPC-K	2004	Cameroon	AMX SXT TET	H77	1-2-3-4-5-6-0	N	pST3	50	None	
E02-945	2002	Cameroon	Pansusceptible	H77	1-2-3-4-5-6-0				None	
04-7620	2004	Cameroon	AMX SXT TET	H14	1-2-5-6-0	N	pST3	50	None	
CPC-T	2007	Cameroon	AMX SXT TET	H77	1-2-3-4-5-6-0	N	pST3	50	None	
07-4565	2007	Côte d'Ivoire	SXT CHL	H56	1-2-3				0.8	*dfrA15*
69-61	1969	Tunisia	Pansusceptible	H56	1-2-3				None	
12-58	1958	Cameroon	Pansusceptible	H46	1-2-3				None	
CPC-A	2000	Cameroon	Pansusceptible	H83	1-2-3-4-5-6-0				None	
00-7866	2000	Morocco	Pansusceptible	H46	1-2-3					
09-6318	2009	Algeria	Pansusceptible		1-2-3				None	
09-6677	2009	Algeria	Pansusceptible		1-2-3				None	
09-8709	2009	Morocco	Pansusceptible		1-2-3				None	
76-1292	1976	Zaire	AMX STR SPT SSS CHL TET	H6	1-2-3-4-5-6-0	I1+P			1.7	bla_{OXA-1}-*aadA1*
78-0851	1978	Tunisia	STR SPT SSS CHL TET	H9	1-2-3-4-5-6-0	HI1	pST2	190	1	*aadA1*
ISP-03-07467	2003	Morocco	AMX SXT CHL TET	H58	1-2-3-4-5-6-0	HI1	pST6		0.8	
CPC-S	2006	Cameroon	Pansusceptible	H52 / H68	1-2-3-4-5-6-0				None	
77-0303	1977	India	STR SPT SSS CHL TET	H55	1-2-3-4-5-6	HI1	pST2		1	*aadA1*
E02-2364	2002	Comoros	Pansusceptible	H55	1-2-3-4-5-6				None	

Figure 1. Characteristics of 50 *Salmonella enterica* serotype Typhi isolates. The dendrogram was generated by using BioNumerics version 6.6 software (Applied Maths, Sint-Martens-Latem, Belgium) and shows results of cluster analysis on the basis of *Xba*I–pulsed-field gel electrophoresis (PFGE) fingerprinting. Similarity analysis was performed by using the Dice coefficient, and clustering analysis was performed by using UPGMA. CRISPR1, clustered regularly interspaced short palindromic repeats 1; MDR, multidrug resistant; AMX, amoxicillin; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole; CHL, chloramphenicol; TET, tetracycline; SPT, spectinomycin; SSS, sulfamethoxazole. Zaire is the former name of the Democratic Republic of the Congo. Plasmid multilocus sequence typing (pMLST) was performed as described for incN http://pubmlst.org/plasmid/) or incHI1 (*4*). Plasmid size was estimated by using S1 nuclease PFGE (*14*). For isolates from Africa, red indicates linage A, blue indicates lineage B, and green indicates lineage C.

detected in association with an incHI1 pST2 plasmid in India during the mid-1970s (*5*). Therefore, lineage B might have been was imported into central Africa from eastern Africa/southern Asia.

A previous study also reported isolation of an MDR clone in the Democratic Republic of the Congo in 2004 that was resistant to quinolones, showed decreased susceptibility to ciprofloxacin, and belonged to the Asian H58 lineage (*2*). Because only a limited number of isolates from central Africa were tested in our study, studies of a larger collection of isolates might provide more information about bacterial genotypes/MDR plasmids circulating in central Africa.

Despite intrinsic limitations of a laboratory surveillance system for typhoid fever that is used mostly for travelers and immigrants and has the bias of preferential links caused by colonial history and choices of tourist destinations, we documented emergence of 3 MDR *Salmonella* Typhi lineages in the Gulf of Guinea area. Two lineages found in Guinea and Cameroon were local lineages that acquired MDR conjugative plasmids, either a large incHI1 pST2 plasmid or a smaller incN pST3 plasmid. The H58 lineage, which is currently predominant in Asia and eastern Africa, was not detected among MDR isolates from West and central Africa.

Acknowledgments

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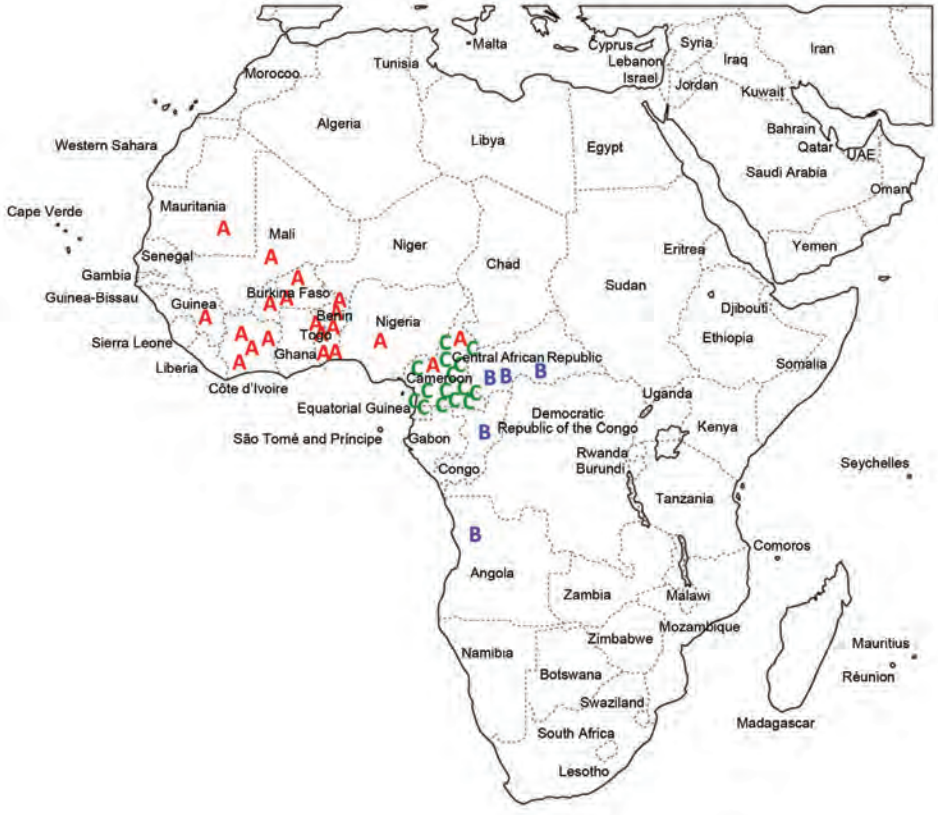

Figure 2. Distribution of multidrug-resistant *Salmonella enterica* serotype Typhi isolates by genetic lineage (A, B, or C), Gulf of Guinea region, Africa. Location within the country of infection/isolation was assigned at random. UAE, United Arab Emirates.

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Candidate New Rotavirus Species in Sheltered Dogs, Hungary

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We identified unusual rotavirus strains in fecal specimens from sheltered dogs in Hungary by viral metagenomics. The novel rotavirus species displayed limited genome sequence homology to representatives of the 8 rotavirus species, A–H, and qualifies as a candidate new rotavirus species that we tentatively named *Rotavirus I*.

Rotaviruses (family *Reoviridae*, genus *Rotavirus*) are major causes of acute dehydrating gastroenteritis in birds and mammals (*1*). Rotaviruses have an 11-segmented dsRNA genome encoding 6 structural proteins (viral protein [VP] 1–4, VP6, and VP7) and at least 5 functional nonstructural proteins (NSPs; NSP1–NSP5) (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/4/14-1370-Techapp1.pdf). Traditionally, rotaviruses have been classified into (sero)groups on the basis of major antigenic differences that predominantly reside in the VP6 and of the genomic RNA profile obtained by polyacrylamide gel electrophoresis and silver staining (*1*). Recently, a VP6 gene sequence–based classification scheme has been proposed to replace the conventional methods. An empirical 53% aa identity was demonstrated to reliably distinguish strains of various rotavirus groups (*2*). Also, reclassification of the 8 rotavirus groups as distinct species within the *Rotavirus* genus, designated *Rotavirus A–H*, has been proposed.

Rotavirus A has been detected in a wide variety of mammals and birds. In mammals, both endemic and epidemic forms of rotavirus B, C, E, and H infections have been described, whereas rotavirus D, F, and G have been identified only in birds (*1*–*3*). Genetically diverse rotaviruses have been found in some viral metagenomics studies (*4*,*5*). Using the metagenomic approach and the VP6-based molecular classification scheme, we found evidence for a novel rotavirus species that we tentatively called *Rotavirus I*.

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The Study

During 2012, we collected fecal specimens from sheltered dogs in northern Hungary to detect enteric viruses. Of 63 samples obtained from 50 animals, 37 randomly selected samples (from 33 animals) were subjected to random primed reverse transcription PCR and semiconductor sequencing by using the Ion Torrent PGM platform (New England Biolabs, Ipswich, MA, USA) (online Technical Appendix). Bioinformatics analysis consisted of the mapping of reads >40 bases against ≈1.7 million viral sequences downloaded from GenBank by applying moderately rigorous mapping parameters (length fraction 0.6; similarity fraction 0.8) within the CLC Genomics Workbench (http://www.clcbio.com/).

One sample (KE135/2012) obtained from a suckling dog in May 2012 was positive for several enteric viruses. When analyzing the initially obtained ≈60.5-K sequence reads, in addition to canine rotavirus A (141 reads), astrovirus (2,399 reads), and parvovirus (3,623 reads), we identified a single 53-nt sequence read that mapped to the VP1 gene of rotavirus B. Another sample, KE528/2012, collected during August 2012 from an adult dog with diarrhea, was positive for coronavirus (30 reads), vesivirus (17 reads), picodicistrovirus (3 reads), and astrovirus (1 read); in addition, 7 and 5 sequence reads, respectively, mapped to the VP1 and VP3 genes of rotavirus H and/or B.

Subsequently, we enriched genomic dsRNA of KE135/2012 by differential LiCl precipitation; however, the enriched dsRNA remained invisible by polyacrylamide gel electrophoresis and silver staining. Because of the apparent low titer of the novel rotavirus, we tried to obtain more sequence data by drastically increasing the output in parallel sequencing runs. De novo assembly of the resulting ≈1.59 million sequence reads readily identified homologs of the structural and some nonstructural genes, which were divergent from rotavirus A–H reference sequences (Table; online Technical Appendix Table 1). Determination of the coding regions in most cases was successful by extension of the termini of consensus sequences using the Ion Torrent sequence reads. However, we found no evidence for NSP3 and NSP4 with this approach, probably because of the great sequence divergence of these genes across members of the genus (*6*,*7*). Because the genomic RNA of each rotavirus species is characterized by low GC (guanine:cytosine) content (29%–40%), we expected that contigs with low GC content and with no GenBank homologs might be good candidates for detecting the missing genes. Indeed, further assembly and subsequent analysis

Table. Sequencing depth for the putative rotavirus I strains obtained by massively parallel sequencing*

	KE135/2012		KE528/2012	
Gene	Mapped read count	Average coverage (X)	Mapped read count	Average coverage (X)
VP1	9632	478	1286	59
VP2	7762	455	860	46
VP3	6361	510	657	49
VP4	5887	436	716	47
VP6	4762	700	582	72
VP7	2841	594	258	45
NSP1	3677	450	561	62
NSP2	2980	529	401	64
NSP3	2528	523	176	32
NSP4	2272	586	229	51
NSP5	1098	387	249	72

*Total sequence reads to obtain genomic RNA sequence for KE135/2012 and KE 528/2012 were 1,591,803, and 144,747, respectively. The minimum overlap with the consensus sequences (i.e., the de novo assembled rotavirus I–specific consensus sequences) was 20 nt, the minimum identity was 80%. To improve the mapping results, the following gap penalties were applied for the dataset: mismatch cost = 2, insertion cost = 3, deletion cost = 3. After visual inspection of the sequence alignments and remapping onto the obtained gene sequence, a single consensus sequence was finalized for each genome segment.

of selected sequence stretches helped to identify the NSP3 by similarity search through the blastx engine (http://blast.ncbi.nlm.nih.gov/Blast.cgi) after an 800-bp long fragment was obtained, and analysis of the structural features of the deduced protein sequence supported detection of the putative NSP4. The obtained consensus sequence was used as reference to map other viral metagenomics data generated from the sheltered dog population; however, except for the aforementioned sample, KE528/2012, we found no additional specimens by this method to contain homologous viruses. The 2 related unusual rotaviruses, KE135/2012 and KE528/2012, had conserved genome segment termini (5′ end, GGC/TA; 3′ end, AACCC) and shared high sequence identities in most genes (e.g., VP2: 88% nt, 95% aa; NSP4: 99% nt, 99% aa) and very low sequence similarity in the VP7 gene (53% nt, 38% aa) (GenBank accession nos. KM369887–KM369908; online Technical Appendix).

The deduced VP6 amino acid sequences served as the basis to classify these 2 unusual rotavirus strains (*2*). The greatest amino acid sequence identity of the VP6 proteins was found when compared to the novel rotavirus H strains (≤46%); lower sequence similarities were found in comparison to randomly selected representatives of other rotvirus species (e.g., rotavirus G and B, ≤37%; rotavirus A, C, D, and F, ≤18%).

To extend the analysis and assess whether the obtained VP6 gene might be functionally integral, we conducted molecular modeling of the amino acid sequence. In brief, amino acid sequence similarity values created a reliable protein model (*8*,*9*) showing similar protein folding of the VP6 monomer and comparable electrostatic charge pattern around the 3-fold axis of the VP6 homotrimer to that experimentally determined for rotavirus A (Figure 1). Subsequent phylogenetic analysis of the VP6 protein identified 2 major clades of rotaviruses (*6*). The novel rotavirus strains

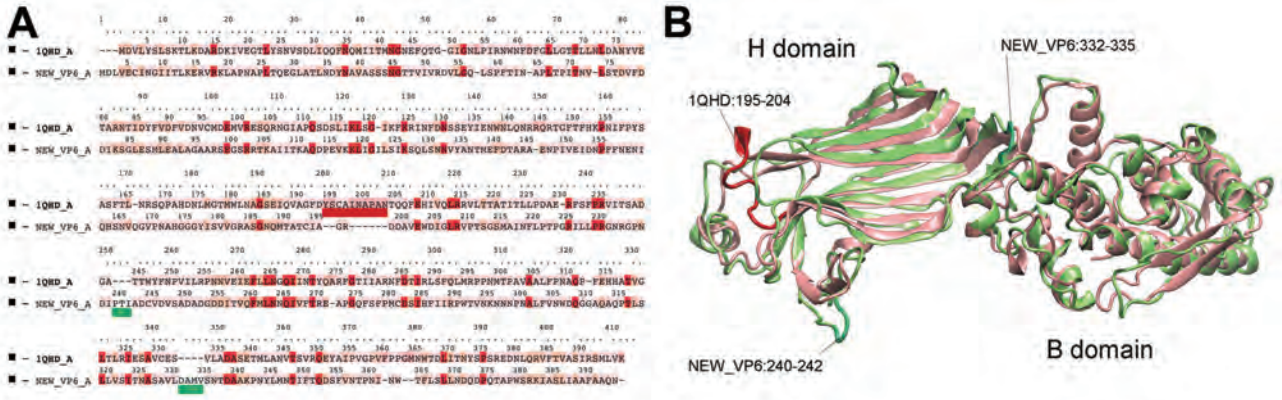

Figure 1. Structure comparison of rotavirus viral protein (VP) 6 proteins. A) Structure-based amino acid sequence alignment of the novel canine rotavirus VP6 protein and the template bovine rotavirus A VP6 protein. The background of the sequence alignments reflects the homology levels of the 2 VP6 sequences. Red, identical amino acid; orange, similar amino acid; pink, different amino acid. The main structural differences are indicated by dark red and menthol green on the sequence alignment and on the superimposed VP6 structures. B) Cartoon presentation of the homologous VP6 proteins: pink, rotavirus A; green, rotavirus I. Further information is available in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/21/4/14-1370-Techapp1.pdf).

clustered with species H, G, and B within clade 2, whereas clade 1 included representative strains of species A, C, D, and F (Figure 2). This pattern of clustering was also evident when we analyzed the remaining genes. Collectively, sequence and phylogenetic analysis demonstrated moderate genetic relatedness of the unusual canine rotaviruses to representative strains of species A–H, suggesting that they belong to a novel species, tentatively called *Rotavirus I*. The prototype strains were named RVI/Dog-wt/HUN/KE135/2012/G1P1 and RVI/Dog-wt/HUN/KE528/2012/G2P1 according to recent guidelines (*10*) (online Technical Appendix).

Short rotavirus sequences detected recently in the fecal viral flora of cats and California sea lions (*4*,*5*) showed closer relatedness to our strains in the amplified VP6- and VP2-specific stretches, respectively, than to the corresponding genomic regions of reference rotavirus species (VP6, ≈70 aa, 67% vs. <55%; VP2, ≈160 aa, 78%–86% vs. <44%) (online Technical Appendix). These published data (*4*,*5*) together with our results suggest that genetically related non–rotavirus A–H strains occur in various carnivore host species and geographic settings.

Conclusions

We identified 2 representative strains of a novel rotavirus species, *Rotavirus I*. Many questions remain, including those related to the epidemiology, host range, and evolution of this species. One intriguing finding was the distantly related VP7 genes expressed on a fairly conserved genetic backbone. Typically, very low sequence identity values within the VP7 gene (e.g., rotavirus A, as low as 60% nt and 55% aa; rotavirus B, 54% nt, 46% aa; rotavirus H, 63% nt, 56% aa) can be seen when strains from different host species are compared (*11*–*13*). Whether the VP7 gene(s) of rotavirus I strains could have been acquired in the past from another rotavirus species by reassortment remains uncertain, given that reassortment among various rotavirus

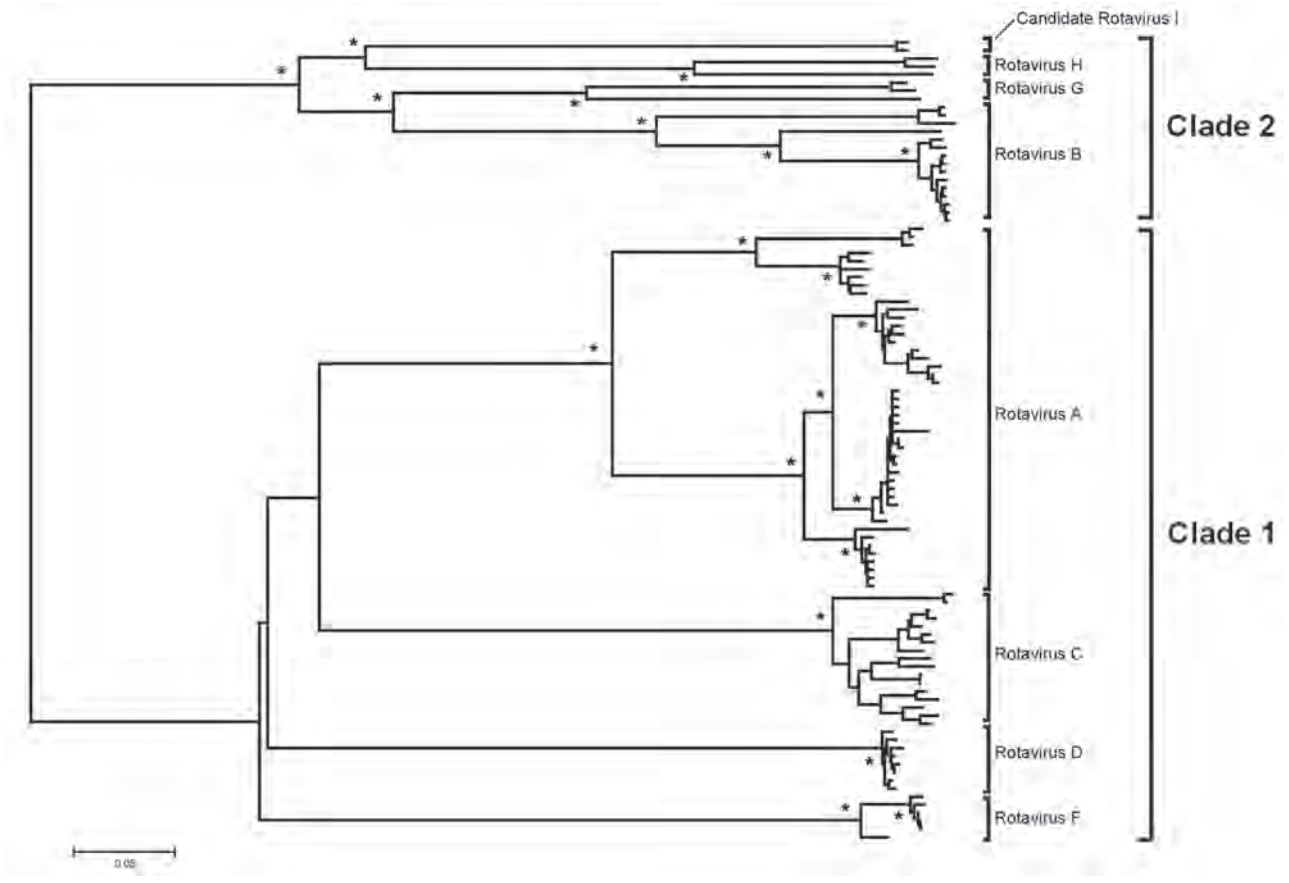

Figure 2. Protein sequence–based phylogenetic tree of the rotavirus viral protein 6 gene obtained by the neighbor-joining algorithm. Asterisks indicate >90% bootstrap values. The 2 canine rotavirus strains from Hungary that belong to the proposed novel *Rotavirus I* cluster with rotavirus H, G, and B within a major clade referred to as clade 2. Rotavirus A, C, D, and F strains belong to clade 1 (*6*). Scale bar indicates nucleotide substitutions per site.

species is thought to occur only rarely (*7*,*14*). Further information is needed to better understand this genetic diversity within rotavirus I.

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Severity of Influenza A(H1N1) Illness and Emergence of D225G Variant, 2013–14 Influenza Season, Florida, USA

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Despite a regional decline in influenza A(H1N1)pdm09 virus infections during 2013–14, cases at a Florida hospital were more severe than those during 2009–10. Examined strains had a hemagglutinin polymorphism associated with enhanced binding to lower respiratory tract receptors. Genetic changes in this virus must be monitored to predict the effect of future pandemic viruses.

In 2009, a novel influenza virus, influenza A(H1N1) pdm09, emerged. The resulting pandemic disproportionately affected persons <65 years of age (*1*), but illness caused by the virus was similar in severity to that caused by seasonal influenza (*2*). As the 2013–14 influenza season progressed, physicians at a Florida hospital noted that patients <65 years of age were affected in numbers similar to those seen in 2009–10, but with increased severity. To investigate these observations, we obtained the number of influenza admissions during the 2013–14 season, characterized pathologic findings in deceased patients, sequenced subtype H1N1 viruses, and assessed receptor-specific characteristics.

The Study

Studies were approved by the University of Florida Institutional Review Board and the Florida Department of Health. Data on influenza-like illness (ILI) were obtained from the 13-county Florida Department of Health Region 3 (2010 population 2.2 million) of Florida's Electronic Surveillance System for the Early Notification of Community-based Epidemics (*3*). ILI-associated emergency department visits were defined as those having a chief complaint containing the words influenza or flu or the word fever plus cough or sore throat. During August 2009–March 2010, a total of 12,022 (2.18%) of 549,101 illnesses among all patients visiting a hospital emergency department met the ILI case definition. During the same period in 2013–14, a total of 12,496 (Figure 1) (1.67%) of 746,560 illnesses met the case definition ($p<0.0001$ by χ^2 test) (Figure 1).

During September 1, 2013–March 21, 2014, we tracked test results for influenza-positive inpatients and outpatients at a major tertiary referral center for the north Florida region. Rapid point-of-care and respiratory virus panel tests were performed: 808 (>97%) of 826 positive samples were influenza A virus, and of 181 samples subtyped, 163 (90%) were H1N1 virus. During this period, 387 patients with laboratory-confirmed influenza were admitted to the hospital; 15 died, yielding an influenza-associated death rate of 3.9%. Each deceased patient had ≥1 risk factors for severe influenza, placing them in a high-risk group as defined by the Centers for Disease Control and Prevention (*4*) (Table). Documentation of influenza vaccination was found for only 3 of the 15 deceased patients. Similar data for the 2009–10 influenza season were not available.

To identify patients admitted to the hospital's medical intensive care unit (MICU) for influenza during August 1, 2013–March 31, 2014, we searched the University of Florida's Health Integrated Data Repository for patients with diagnosis codes from the International Classification of Diseases, 9th Revision (ICD-9), for influenza (487) or novel influenza (488) listed among the first 10 diagnoses. We used the number of patients admitted with these codes as a marker of disease severity. The mean age of these patients was 50.2 years, which did not differ greatly from the mean age of 43.4 years in 2009–10 (Mann-Whitney U test). Of 1,024 patients admitted to the MICU in 2013–14, a total of 49 had influenza, yielding an admission rate of 4.8%. During the same period in 2009–10, a total of 10 of 821 MICU patients were admitted with influenza, yielding an admission rate of 1.2%. This difference was statistically significant ($p<0.0001$ by 2-tailed Fisher exact test). The risk ratio for MICU admission for influenza in 2013–14 versus 2009–10 was 3.7 (95% CI 1.9–7.3). Therefore, although the overall number of ILIs in the community was lower in 2013–14 versus 2009–10 (Figure 1), illness in 2013–14 was more severe, as reflected by a higher MICU admission rate.

Of the 15 deaths in 2013–14, twelve occurred in the MICU, all among the 49 patients admitted for influenza (death rate 24.5%). The mean age of deceased patients was 48.2 (range 25–89) years; 40% were <40 years of

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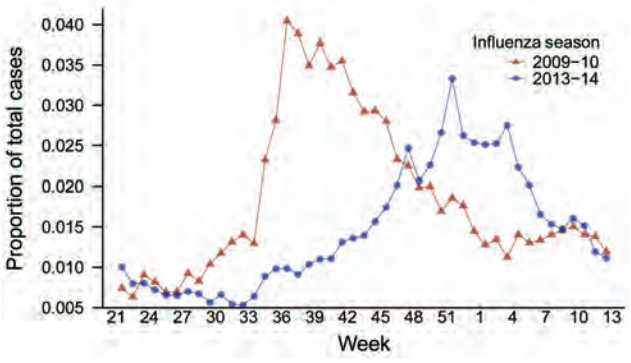

Figure 1. Proportion of all emergency department visits attributable to influenza-like illness, 2009–10 versus 2013–14 influenza seasons, Florida Department of Health Region 3, Florida, USA. Emergency department visits for influenza-like illness are shown as a proportion of total emergency department visits. Week 21 corresponds to the end of May for both influenza seasons, and week 12 corresponds to the end of March.

Table. Characteristics of 15 patients who died from influenza virus infection, Florida, USA, 2013–14*

Characteristic	No. (%)
Sex	
M	8 (53.3)
F	7 (46.7)
Age, y	
18-29	1 (6.7)
30-49	7 (46.6)
50-65	3 (20.0)
>65	4 (26.7)
Influenza type	
A	4 (26.7)
H1N1	11 (73.3)
BMI	
30-40	5 (13.3)
>40	2 (20.0)
Co-occurring conditions	
Asthma/COPD	5 (33.3)
Diabetes	3 (20.0)
Heart disease	3 (20.0)
Immunosuppression	5 (33.3)
Liver disease	1 (6.7)
Neurologic disorder	1 (6.7)
Renal disease	4 (26.7)
Smoking	2 (13.3)
Influenza vaccination record	3 (20.0)

*COPD, chronic obstructive pulmonary disease.

age. Acute respiratory distress syndrome and influenza A virus infection were diagnosed in all 12; none had bacterial infection before testing positive for influenza. Ten of the 12 viruses were subtyped by using the respiratory virus panel: all 10 were H1N1 virus. We reviewed autopsy findings for 5 of the H1N1 virus–infected patients. Although most influenza viruses infect trachea and upper respiratory tract cells (*5*), histopathologic examination of respiratory samples from all 5 patients revealed marked intraalveolar hemorrhage and diffuse alveolar damage (Figure 2, panels A, D), indicating lower respiratory tract disease that clinically manifested as acute respiratory distress syndrome. In 4 patients, neutrophilic alveolar infiltrates consistent with pneumonia were seen (Figure 2, panel B). Changes consistent with the chronic stage of diffuse alveolar damage were noted in 2 patients (Figure 2, panel C). Two others showed necrotizing tracheitis (Figure 2, panel D); swab samples from these tissues were positive for H1N1 virus.

Hemagglutinin of human influenza viruses typically binds to cells bearing sialylglycan receptors configured in an α2-6 orientation. However, some A(H1N1)pdm09 virus isolates also bind to α2-3 sialylglycan receptors (*6*). In humans, α2-6 sialylglycans are found throughout the respiratory tract, but α2-3-sialylglycans are mainly expressed on lower respiratory tract cells (*7*). The permissiveness of wild-type MDCK cells and those overexpressing α2-6-sialylglycan receptors (MDCK-SIAT2,6-UF) and α2-3-sialylglycan receptors (MDCK-SIAT2,3-UF) was assessed by monitoring cytopathic effects of virus strains derived from patients (*8*,*9*). Cytopathic effects were first apparent and were extensive in MDCK-SIAT2,6-UF cells, followed by MDCK-SIAT2,3-UF, then wild-type MDCK cells. These results suggest that the H1N1 virus isolates in our study use both α2-6- and α2-3-sialylglycan receptors, providing a potential mechanism by which lower respiratory tract disease can occur.

The molecular basis for the ability of the H1N1 viruses to cause severe lower respiratory tract disease was first noted with the 1918 pandemic H1N1 virus (*10*). A single amino acid change of aspartic acid at position 225 to glycine (D225G) enabled binding to α2-3- and α2-6-sialylglycans (*10*,*11*). The association of this polymorphism with severe and lower respiratory tract disease was also noted with the A(H1N1)pdm09 virus from small subsets of patients during the 2009–10 influenza season (*12*–*14*). To determine if this polymorphism existed in the H1N1 viruses isolated in our study, we sequenced viral RNA corresponding to the coding regions of all 8 influenza virus genomic segments from viruses isolated from or detected in 7 patient samples (GenBank sequences KJ645758–KJ645765 and KJ645774–KJ645791) (*8*). The consensus sequences were similar to those of key American A(H1N1)pdm09 virus isolates, and like a subset of those isolates, our 7 H1N1 isolates harbored the D225G polymorphism.

None of the 7 isolates harbored the H275Y neuraminidase polymorphism that confers oseltamivir resistance (*15*), and in vitro test results confirmed oseltamivir susceptibility (data not shown). To determine whether polymorphisms in the 3′ and 5′ untranslated regions were associated with the 2013 H1N1 virus, we sequenced 1 isolate by using rapid amplification of cDNA ends (FirstChoice RLM-RACE; Ambion, Bleiswijk, the Netherlands). No substantial differences were detected between this virus and the original A(H1N1) pdm09 virus or circulating contemporary H1N1 viruses.

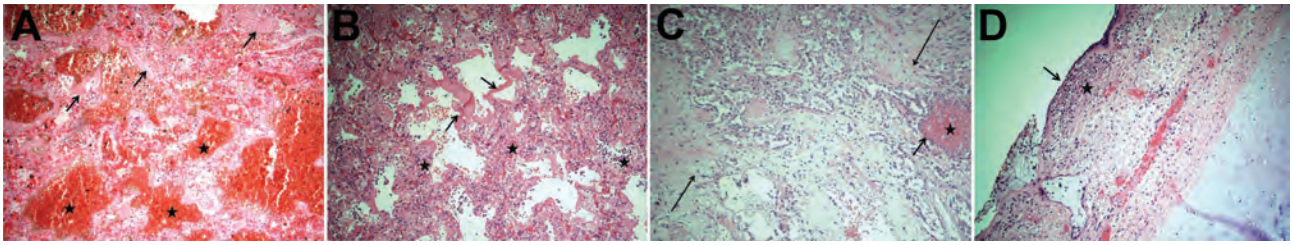

Figure 2. Results of autopsies for patients who died from influenza A(H1N1) virus infection during the 2013–14 influenza season in Florida Department of Health Region 3, Florida, USA. A) Marked intraalveolar hemorrhage (stars) and hyaline membranes (arrows) were seen during the early phase of diffuse alveolar damage and were the most common findings in the 5 autopsy cases reviewed. B) Hyaline membranes, a proteinaceous exudate replacing the alveolar walls (arrows), are prominent in this lung section, as is interstitial edema. Also, a lymphocytic infiltrating inflammation, characteristic of viral pneumonia, is shown (stars). These histologic findings correspond clinically to changes that occur during acute respiratory distress syndrome, and they occur 3–7 days after lung injury. C) The alveolar parenchyma has been replaced predominately by spindled, proliferative fibroblasts (long arrows) and hyperplastic type II pneumocytes (short arrow), indicative of the organizing phase of diffuse alveolar damage seen ≈1 week or more after lung injury. A small focus of intraalveolar hemorrhage is also present (star). D) Necrotizing tracheitis, characterized by desquamation of the tracheal columnar epithelium (arrow) and submucosal acute inflammation (star), is shown. Original magnification ×100 for all panels.

Our work was subject to certain limitations. First, the use of ICD-9 codes for diagnostic information is imprecise. To mitigate this, we considered only 2 ICD-9 codes for influenza. Second, it is not known whether our findings extend beyond our region. Last, changes in health-seeking behavior in 2009–10 versus 2013–14 were not addressed.

Conclusions

We hypothesize that the emergence of an influenza virus variant bearing the D225G polymorphism enabled the 2013 H1N1 virus to infect lower and upper respiratory tract cells, thereby contributing to the increased severity of the 2013–14 influenza season in our region. Our findings highlight the importance of monitoring genetic changes in the 2013 H1N1 virus to predict the effect of future influenza viruses.

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Close Relationship of Ruminant Pestiviruses and Classical Swine Fever Virus

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To determine why serum from small ruminants infected with ruminant pestiviruses reacted positively to classical swine fever virus (CSFV)–specific diagnostic tests, we analyzed 2 pestiviruses from Turkey. They differed genetically and antigenically from known *Pestivirus* species and were closely related to CSFV. Cross-reactions would interfere with classical swine fever diagnosis in pigs.

Pestiviruses are enveloped viruses within the family *Flaviviridae* that have a highly variable single-stranded positive-sense RNA genome of ≈12.3 kb (*1*). The genus *Pestivirus* comprises the established species bovine viral diarrhea virus (BVDV)–1, BVDV-2, border disease virus (BDV), and classical swine fever virus (CSFV), as well as a growing number of additional tentative *Pestivirus* species. CSFV is the causative agent for classical swine fever, which is notifiable to the World Organisation of Animal Health because it is highly contagious and can cause great loss of pigs (*2*–*4*). For a given country, CSFV-positive status severely diminishes international trade of pigs and pig products. Accordingly, because of cross-reacting antibodies, infections of pigs (nonruminants) with ruminant pestiviruses, which occasionally occur under natural conditions, can cause serious problems with regard to serologic diagnosis of classical swine fever (*5*).

In Turkey, 2 pestiviruses, Aydin/04 and Burdur/05, have been isolated from a sheep and a goat with clinical signs of border disease (*6*). A detailed genetic and antigenic characterization revealed that these 2 isolates must be regarded as representatives of a new *Pestivirus* species that is closely related to CSFV and can cause serious diagnostic problems in established CSFV serology.

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The Study

During 2004–2007, serum samples from 1,036 sheep and goats in Turkey were serologically screened for infection with pestiviruses of small ruminants. Of these, 11 serum samples from 7 sheep herds gave positive or doubtful reactions in the CSFV antibody–specific ELISA (HerdChek, IDEXX) and were subjected to commonly used virus neutralization testing (VNT) (*7*). VNT against the 2 established CSFV strains Alfort187 (genotype 1.1) and Diepholz (genotype 2.3) and against the BDV strains Moredun (genotype 1) and Gifhorn (genotype 3) revealed higher BDV titers in only 3 serum samples (Table 1). Equal or slightly higher titers against the CSFV reference strains became evident in 8 of the 11 serum samples, which came from 5 regions of Turkey. Further VNT analyses with the 2 previously obtained isolates, Aydin/04 and Burdur/05, demonstrated neutralizing antibody titers that were equal or higher than those against BDV and CSFV test strains. To elucidate the reason for strong serologic reactivity in CSFV assays, we genetically and antigenically characterized pestiviruses Aydin/04 and Burdur/05.

The complete genome sequence of Aydin/04 was determined as reported previously (*8*). The genome sequence of Burdur/05 was determined by next-generation sequencing on an Illumina MiSeq platform (2 × 250-bp paired end run, 593,328 reads) as recently described (*9*). Template total cellular RNA was extracted from supernatant of sheep fetal thymus cells. Of all reads, 73.9% were found to be of host origin. Of the nonhost reads, 89.9% assembled into a single sequence contig encompassing the entire pestivirus Burdur/05 genome (coverage 196-fold).

Sequence and phylogenetic analyses were performed with complete genome sequences and deduced amino acid sequences of new pestiviruses Aydin/04 (GenBank accession no. JX428945) and Burdur/05 (KM408491). For further analyses, reference sequences were obtained from GenBank (Figure 1). Genetic distances were calculated by using the Kimura 2-parameter substitution model, and phylogenetic analyses were conducted by applying the neighbor-joining method as commonly used for CSFV phylogeny (*11*). With the same set of sequences, a grouping scan was performed by using the SSE platform (*12*). Comparison of the complete coding sequences of Aydin/04 and Burdur/05 revealed a genetic distance of 16.5%. Phylogenetic analyses based on deduced polyprotein sequences showed that isolates Aydin/04 and Burdur/05 form a distinct group located between CSFV and BDV (Figure 1, panel A).

Table 1. Serologic pestivirus testing results for sheep from different districts in Turkey, 2004–2007*

Sample	Province of origin	CSFV ELISA†	Neutralizing antibody titer, ND_{50}					
			BVDV	CSFV‡		BDV§		Pestiviruses from Turkey
			NADL	Alfort187	Diepholz	Moredun	Gifhorn	Aydin	Burdur
1	Antalya¶	+	<5	320	226	28	57	905	905
2	Antalya¶	+	<5	113	113	28	40	226	160
3	Burdur	+	<5	40	640	113	226	226	226
4	Burdur	?	<5	226	226	160	226	640	453
5	Konya	?	<5	<80	<40	160	113	113	57
6	Konya¶	+	<5	160	905	320	160	320	452
7	Konya¶	?	<5	≤28	14	20	10	40	40
8	Kütahya¶	+	<5	≤15	≤15	160	640	57	113
9	Kütahya¶	+	<5	≤15	453	640	640	113	57
10	Kütahya¶	+	<5	28	160	160	160	226	80
11	Sanliurfa	+	<5	226	<1,280	<320	160	452	452

*BDV, border disease virus; BVDV, bovine viral diarrhea virus; CSFV, classical swine fever virus; ND50, 50% neutralizing dose; ?, doubtful result.
†HerdChek (IDEXX).
‡CSFV strains: Alfort187 (genotype 1.1), Diepholz (genotype 2.3).
§BDV strains: Moredun (genotype 1), Gifhorn (genotype 3).
¶Serum obtained from the same herd (samples 1 and 2, 6 and 7, 8–10).

Systematic antigenic characterization was performed by using cross-neutralization assays (Table 2). For this purpose, CSFV and BDV reference strains for which homologous serum was available were tested by VNT as described (*7*). In general, neutralization of both isolates was more efficient when performed with different CSFV antiserum than with BDV antiserum. In addition, the Aydin-specific antiserum obtained from animal experiments neutralized

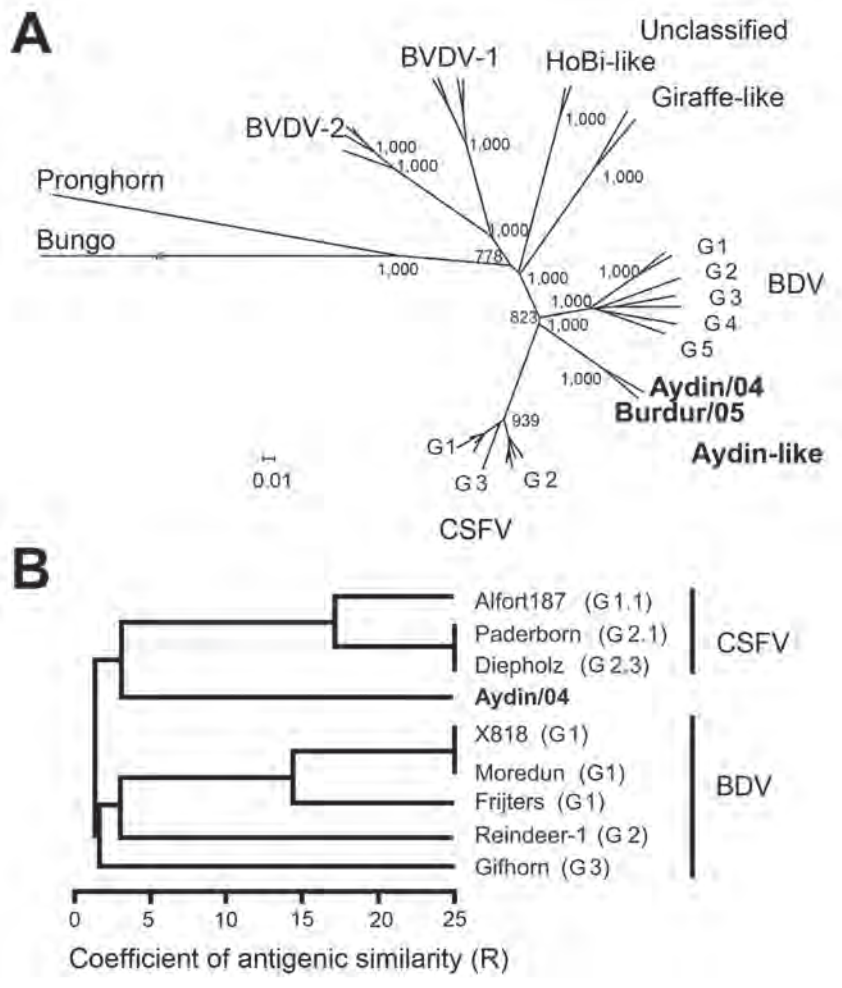

Figure 1. Phylogenetic and antigenic tree displaying relatedness of pestiviruses Aydin/04 and Burdur/05 to other *Pestivirus* species. A) For phylogenetic analysis, deduced polyprotein sequences from GenBank were used (CSFV: J04358, GU233734, JX218094, AY568569, GQ902941, KJ619377, AY382481, AF326963, X87939, AF099102, AY578687, AY646427; BDV: AF037405, U70263, KC963426, AF144618, GQ902940, KF918753, GU270877; BVDV-1: EF101530, AF220247, M96751, AF091605; BVDV-2: AB567658, GQ888686, AF502399, U18059; HoBi-like: AB871953, NC012812; giraffe-like: NC003678, KJ660072; Pronghorn and Bungowannah: NC024018, NC023176). Bootstrap values were calculated for 1,000 iterations. Only significant bootstrap values (≥700) of major nodes are given in the tree. Trees were displayed by using Dendroscope (*10*). Scale bar indicates base substitutions per site. B) Antigenic tree based on coefficients of antigenic similarity (R values) displaying antigenic relatedness of pestiviruses Aydin/04 and Burdur/05 to representative CSFV and BDV strains. R values <25 indicate significant antigenic differences as representing >4-fold differences in titers. R values >25 are considered not significant and are therefore not drawn to scale. Boldface indicates pestiviruses circulating among sheep and goat herds in Turkey. BDV, border disease virus; BVDV, bovine viral diarrhea virus; CSFV, classical swine fever virus; G, genotype.

Table 2. Antigenic relationships determined by cross-neutralization of serum raised against different CSFV and BDV reference strains*

	CSFV†					BDV‡				
Strain	Alfort187	Diepholz	Paderborn	Aydin	Burdur	Moredun	Frijters	X818	Reindeer	Gifhorn
Alfort187	1,810	452	113	**20**	**80**	5	10	≤3.5	10	12
Diepholz	381	1,280	452	**80**	**135**	57	28	10	48	28
Paderborn	34	160	452	**≤3.5**	**14**	28	5	8.4	14	7,1
Aydin	**40**	**40**	**20**	**761**	**135**	**8.4**	**14**	**8.4**	**34**	**24**
Moredun	≤3.5	4.2	≤3.5	**≤3.5**	**≤3.5**	190	4.2	95	≤3.5	≤3.5
Frijters	14	20	17	**14**	**67**	113	761	269	134	12
X818	14	160	95	**24**	**30**	905	381	2,560	381	226
Reindeer	8.4	17	8.4	**4.2**	**17**	80	8.4	48	2,560	40
Gifhorn	14	80	67	**28**	**80**	30	40	67	134	5,120

*Boxes indicate virus neutralization test titers for homologous serum; boldface indicates results for Aydin/04 and Burdur/05. BDV, border disease virus; CSFV, classical swine fever virus.
†CSFV strains: Alfort187 (genotype 1.1), Diepholz (genotype 2.3), Paderborn (genotype 2.1).
‡BDV strains: Moredun (genotype 1), Frijters (genotype 1), X818 (genotype 1), Reindeer (genotype 2), Gifhorn (genotype 3).

the CSFV reference strains with titers higher than those for the BDV strains (Table 2). Because no experimental infection with Burdur/05 has been performed, Burdur/05-specific antiserum was not available; however, close antigenic relatedness of both isolates was demonstrated by the high neutralization titers of the Aydin-specific antiserum for isolate Burdur/05 (Table 2). To quantify and to depict the antigenic relatedness, we calculated coefficients of antigenic similarity (R values) as described previously (*13*). An antigenic tree graphically displaying the R values clearly shows 2 distinct clades, one representing CSFV and the other comprising BDV strains (Figure 1, panel B). Furthermore, Aydin/04 is antigenically more closely related to CSFV than to BDV, but it also clearly differs from these 2 pestivirus species.

Because of their close relationship to CSFV, it was of particular interest to determine the ability of these ruminant pestiviruses to infect pigs and induce clinical disease. Therefore, 3 clinically healthy and pestivirus uninfected weaner (6 weeks of age) piglets were inoculated with 1×10^6 50% tissue culture infectious doses of isolate Aydin/04 and given a booster of 3×10^7 50% tissue culture infectious doses 2 weeks later. Pigs showed no clinical signs of disease, no fever, no platelet or leukocyte depletion, and no viremia (data not shown). For all 3 animals, strong seroconversion was found (50% neutralizing titer of serum for homologus virus was 240–640 on postinoculation day 77).

Conclusions

Several new genetically diverse groups of pestiviruses have emerged in domestic livestock and wild animals, adding to the continuously growing list of approved and tentative pestivirus species (*1*). According to phylogenetic analyses of short partial genome sequences, 2 pestivirus isolates, Aydin/04 and Burdur/05, recently circulating in sheep and goat herds in different regions of Turkey, were classified as novel members of the BDV species (*6*). However, the data from this study demonstrate that these novel Aydin-like pestiviruses are representatives of a new pestivirus species, genetically and antigenically located between CSFV and BDV (Figure 1). The genetic distance of 16.5% between these isolates indicates that distinct ruminant Aydin-like pestiviruses circulate in different regions of Turkey. For some genomic regions, both ruminant pestivirus isolates display an even higher similarity to CSFV than to BDV (Figure 2). The close genetic relatedness to CSFV is in line with the antigenic characterization by cross-neutralization assays as depicted in the antigenic tree (Figure 1, panel B). This finding is in contrast with findings for pestivirus isolates from Tunisia, another group of ruminant pestiviruses genetically closely related to CSFV but antigenically more closely related to BDV (*14*). The close antigenic relationship to CSFV explains the observed strong cross-reactivity of serum from sheep and goat in CSFV-specific ELISAs, even when the variable E2 protein is used as diagnostic antigen (Table 1). In routine diagnosis, questionable ELISA results are further investigated by VNT against CSFV and other pestiviruses (e.g., BVDV and BDV). Usually, VNT titers are highest for the homologous pestivirus species. Remarkably, even if representatives of the Aydin-like pestiviruses were included as test strains in the VNT, CSFV infection still could not be ruled out.

Although these novel pestiviruses are the closest known relatives of CSFV, experimental infection of pigs with Aydin/04 did not result in detectable viremia and clinical signs. Nevertheless, these ruminant pestiviruses are candidates for a switch to porcine hosts after ongoing virus evolution, which would have severe consequences for serologic diagnosis of classical swine fever, affecting control and monitoring programs performed in many parts of the world.

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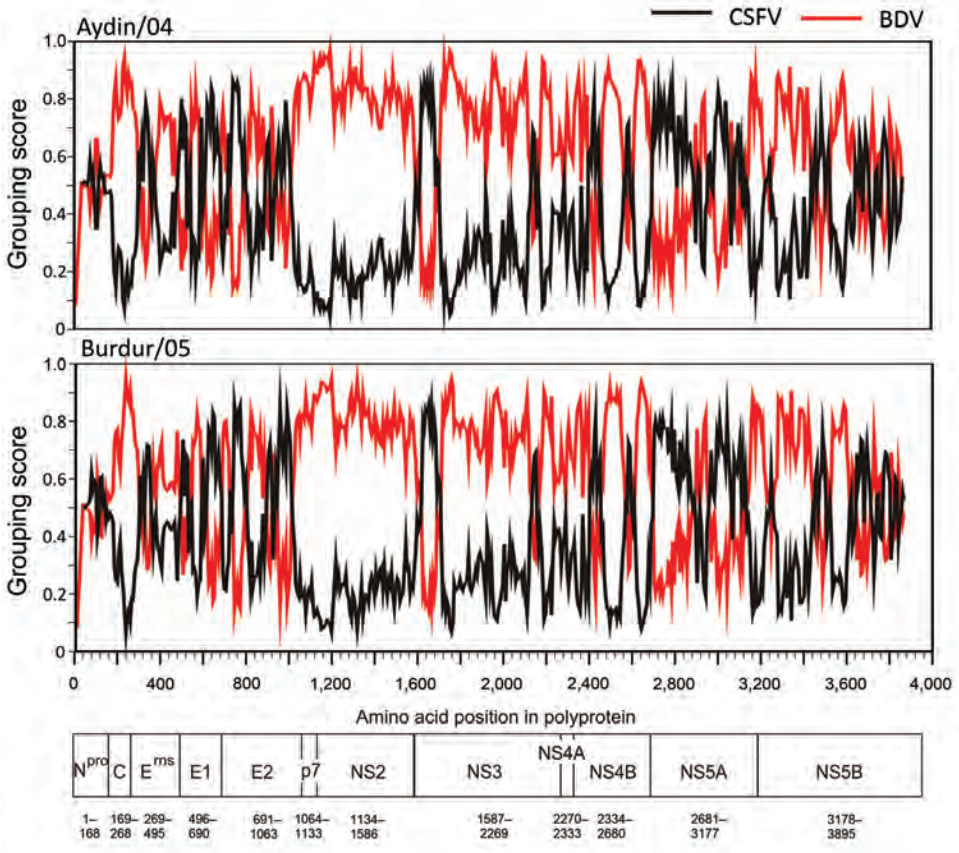

Figure 2. Amino acid similarity of pestiviruses Aydin/04 and Burdur/05 to representative CSFV and BDV polyprotein sequences. The same CSFV and BDV polyprotein sequences as in Figure 1 were used for analysis. Grouping scan was performed with the SSE software platform as described previously, by using a window of 200 aa with 20-aa increments (*12*). For calculation of genetic distances, the Kimura 2-parameter model was applied. Borders of the mature viral proteins in the polyprotein of Aydin/04 are given below. BDV, border disease virus; CSFV, classical swine fever virus; C, core protein; E, envelope protein; rns, ribonuclease secreted; N^{pro}, N-terminal autoprotease; NS, nonstructural protein; p7, protein p7.

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Reassortant Avian Influenza A(H9N2) Viruses in Chickens in Retail Poultry Shops, Pakistan, 2009–2010

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Phylogenetic analysis of influenza viruses collected during December 2009–February 2010 from chickens in live poultry retail shops in Lahore, Pakistan, showed influenza A(H9N2) lineage polymerase and nonstructural genes generate through inter- and intrasubtypic reassortments. Many amino acid signatures observed were characteristic of human isolates; hence, their circulation could enhance inter- or intrasubtypic reassortment.

The first outbreak of illness caused by avian influenza A(H9N2) virus in Pakistan was reported in 1998; isolates showed a close relationship to subtype H9N2 avian influenza viruses (AIVs) circulating in Hong Kong, China during 1997 that were grouped within the G1 lineage (*1*). In recent years, H9N2 genes have reassorted extensively, generating novel genotypes on the Indian subcontinent. Widespread co-circulation of H9N2 with other AIVs (e.g., highly pathogenic AIVs H5N1 and H7N3) could instigate the generation of novel variant and reassorted viruses, possibly with increased zoonotic potential (*2*).

No information was available about the genetic makeup of AIVs circulating in live poultry retail shops (LPRSs) in Pakistan. This study was conducted to genetically characterize AIVs in LPRSs in Lahore District, Pakistan.

The Study

We conducted a cross-sectional survey of LPRSs in Lahore (Figure), which is the capital of the Punjab Province in Pakistan. In each of 280 LPRSs, we collected tracheal swab samples from 5 randomly selected chickens and pooled them into 1 composite sample, totaling 280 pooled samples. The samples were characterized at the World Organisation for Animal Health/Food and Agriculture Organization of the United Nations National Reference Laboratory for Avian Influenza in Padua, Italy.

Of the 280 samples, 10 tested positive for H9N2 subtype by real-time reverse transcription PCR (rRT-PCR) (*3*) and virus isolation test (United Nations www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf) (Table 1). Results were negative for H5 and H7 subtypes. Two of the 280 samples were positive for Newcastle disease virus. Each of the 10 H9N2 isolates was characterized by sequencing for 6 gene segments (hemagglutinin [HA]: neuraminidase [NA], nonstructural [NS], matrix [M], and polymerase basic 1 and 2 [PB1, PB2]); 2 isolates (A/chicken/Pakistan/10RS3039-283-11/2010 and A/chicken/Pakistan/10RS3039-289-186/2010) were sequenced for all 8 gene segments (HA, NA, NS, M, PB1, PB2, polymerase [PA], and nucleoprotein [NP]), as described (*4*). Sequences for these viruses were deposited into GenBank (accession nos. KF975457–KF975503, and KP223678–KP223693). The NS gene sequence of A/chicken/Pakistan/ 10RS3039-284-11/2010 could not be deposited due to poor data quality.

We generated neighbor-joining phylogenetic trees for all gene segments using the distance-based method in MEGA version 5.2.2 (http://www.megasoftware.net/). We calculated bootstrap values based on 1,000 replicates of alignment (*5*). HA and NA genes of viruses in this study tightly clustered within the G1 lineage along with H9N2 viruses from Pakistan, India, Iran, Israel, Saudi Arabia, and Bangladesh, suggesting derivation from a common ancestor: A/quail/Hong Kong/G1/97 (online Technical Appendix Figure panels A, B http://wwwnc.cdc.gov/EID/article/21/4/14-1570-Techapp1.pdf). M and NP genes clustered within the G1 lineage (online Technical Appendix Figure panels C, D) along with an influenza (H7N3) isolate from Pakistan (A/chicken/Karachi/NARC-100/2004; nucleotide identity >96% for M gene and 95% for NP gene). Two polymerase complex genes, PB1 and PA, and the NS gene did not cluster within G1 lineage or any other established Eurasian lineages: these gene sequences made a separate well-supported cluster with highly similar H9N2 viruses that circulated in Pakistan (2005–2008), Iran, India, and Bangladesh; these sequences had a high sequence

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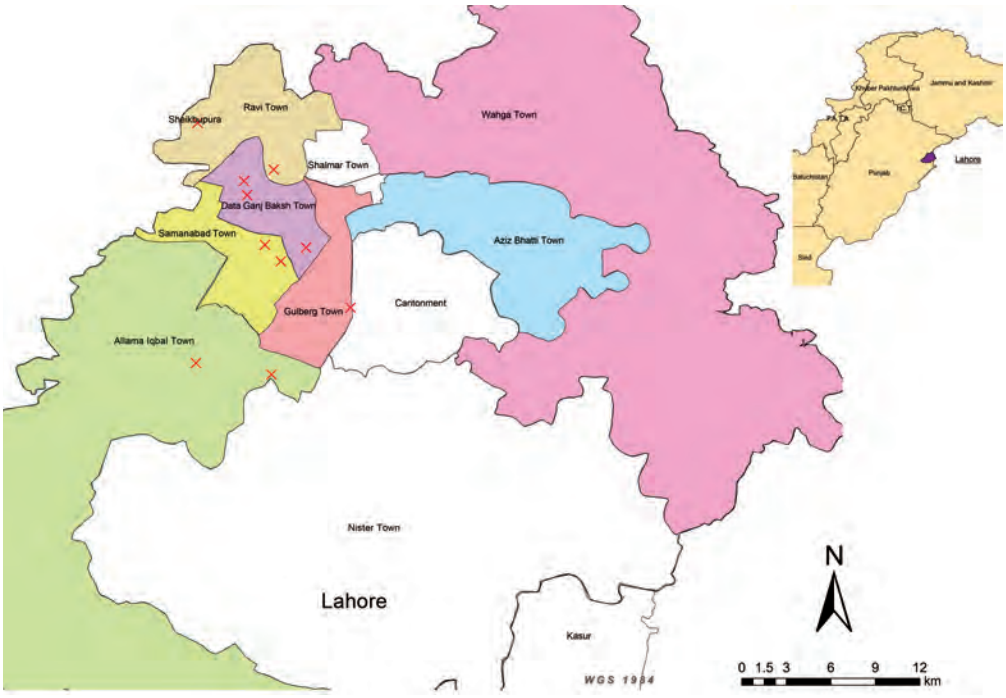

Figure. Location of live poultry retail shops (X) in 5 towns in Lahore, Pakistan, where avian influenza A(H9N2) virus isolates were identified in chickens, 2009–2010. Inset shows location of Lahore in Punjab Province.

identity (>95%) with A/chicken/Karachi/NARC-100/04 (H7N3) (*2*,*6*,*7*) (online Technical Appendix Figure panels E, F, G). The PB2 segment clustered separately with H9N2 viruses from the subcontinent and Middle East (online Technical Appendix Figure panel H), except A/chicken/Karachi/NARC-100/04. These results indicate a separate Indian subcontinental lineage of H9N2 viruses has emerged (*2*).

On the basis of these analyses, we could conclude that internal genes PB1, PA, and NS of these viruses originated by intersubtypic (between different HA subtypes) reassortment events from local H7N3 viruses circulating in Pakistan (nucleotide identity >95%). This suggests that intersubtypic reassortment events continuously result from mixing of AIV subtypes in domestic poultry and wild birds in Pakistan (*8*), and that PB2, M, and NP genes were acquired by intrasubtypic reassortment between H9N2 viruses of G1 lineage circulating within Pakistan. These results support speculation that the currently circulating H9N2 lineage is a reassortment of G1 lineage from Hong Kong and the highly pathogenic H7N3 virus that circulated in Pakistan and can be assigned to genetic group B (*8*).

We aligned amino acid sequences of current viruses and compared them to representative H9N2 lineages. When compared to the prototype G1 viruses, H9N2 viruses isolated from LPRSs showed that they have evolved to acquire mammalian host-specific mutations throughout the genome (online Technical Appendix Table 1). Of these mutations, certain amino acid substitutions throughout the viral genome have become fixed (*9*). All LPRS isolates possessed the K-S-S-R motif at the cleavage site. The presence of lysine at position 4 was observed in H9 isolates from the Indian subcontinent (*2*,*9*), but it is uncommon elsewhere. Amino acid signature changes of human influenza viruses were also observed in internal gene segments (Table 2).

Table 1. Influenza A(H9N2) viruses isolated from oropharyngeal swab samples from chickens in live poultry retail shops of Lahore, Pakistan, 2010

Isolates	Collection date	Towns of Lahore District*
A/chicken/Pakistan/10RS3039-283-11/2010	Jan 23	Gulberg Town
A/chicken/Pakistan/10RS3039-284-48/2010	Jan 25	Data Gunj Bakhsh Town
A/chicken/Pakistan/10RS3039-285-63/2010	Jan 26	Data Gunj Bakhsh Town
A/chicken/Pakistan/10RS3039-286-65/2010	Jan 26	Data Gunj Bakhsh Town
A/chicken/Pakistan/10RS3039-287-98/2010	Jan 27	Ravi Town
A/chicken/Pakistan/10RS3039-288-102/2010	Jan 27	Ravi Town
A/chicken/Pakistan/10RS3039-289-186/2010	Feb 11	Samanabad Town
A/chicken/Pakistan/10RS3039-199-199/2010	Feb 11	Samanabad Town
A/chicken/Pakistan/10RS3039-290-230/2010	Feb 13	Allama Iqbal Town
A/chicken/Pakistan/10RS3039-291-266/2010	Feb 14	Allama Iqbal Town

*Lahore District contains 9 towns and 1 cantonment area.

Table 2. Amino acid signature changes observed in M1, NP, PB1 and NS1 proteins of influenza A(H9N2) viruses isolated from chickens in live poultry retail shops, Lahore, Pakistan, 2009–2010*

Protein	Amino acid position	Predicted aa		Viruses with detected mutation	Mutation
		Avian	Human		
M1	15	V	I	All 10 isolates analyzed in this study	V15I
NP	372	E	D	Only 2 viruses were analyzed (A/chicken/ Pakistan/10RS3039-283-11/2010 A/chicken/ Pakistan/10RS3039-289-186/2010)	E372D
PB1	13	L	P	All 10 isolates analyzed in this study	L13P
NS1	149	V	A	All 10 isolates analyzed in this study	V149A
	227	E	R or K (H1N1 1918)		E227K

*M, matrix; NP, nucleoprotein; PB, basic polymerase; NS, nonstructured.

Each of the 10 isolates had the $Q^{226}L$ substitution (H3 numbering) in the receptor-binding site of HA, correlating to a shift in affinity from avian-type sialic acid receptor to human-type (*10*). We identified 3 representative substitutions: E/T^{190} A in 9 viruses and $E/T^{190}V$ in 1 virus, and $Q^{227}I$ in all 10 viruses (H3 numbering). The outcomes of these substitutions have not been investigated; further study is needed (*2*). Glycosylation sites at positions 551, 218, and 206 were absent in the study viruses, suggesting a frequent alteration in sequences from this region (*2*,*6*,*9*) and possibly signifying the selected adaptation of H9N2 to poultry (*10*). We did not find an $R^{292}K$ substitution, which is associated with resistance to the sialidase inhibitors oseltamivir and zanamivir, in the NA proteins of any of the 10 LPRS virus isolates. HB sites were also well conserved with few substitutions ($K^{367}E$ in 5 viruses, $K^{367}G$ in 1 virus, and $S^{372}A$ and $W^{403}R$ in all 10 viruses). An additional glycosylation site was present at position 44, which is believed to enhance virulence caused by altered antigenicity or sialidase activity (*11*) (online Technical Appendix Table 1).

Many residues in nucleoprotein and polymerase are considered determinants of the host range of AIVs and increase virulence or replication in the mammalian host (*2*,*10*,*12*). The analyses of internal genes showed that these viruses also contained mammalian host-specific markers (*2*,*8*) that have become permanent in M protein (M1, $V^{15}I$, $T^{37}A$; M2, $E^{16}G$, $L^{55}F$); in PB1 protein ($L^{13}P$); and in NS1 protein ($V^{149}A$) of all LPRS isolates and in NP ($E^{372}D$) of the 2 isolates we sequenced (online Technical Appendix Table 2). In M2 protein, no substitutions linked to resistant amantadine were seen. All viruses contained an uncommon K-S-E-I sequence as a PDZ ligand motif in the NS protein. Residue isoleucine at the C-terminus of PDZ ligand motif has been reported as a rare substitution (*12*). NS also harbored $E^{227}K$ mutation in the C-terminal, which has been demonstrated to modulate pathogenicity of AIVs (*13*) and appeared to be a rare amino acid signature, although in this study it was observed in all 10 virus isolates from chickens in LPRSs.

Conclusions

Our analysis confirmed that continuous gene reassortment has occurred among influenza A(H9N2) viruses since their emergence in poultry in Pakistan. Because H9N2 viruses infect multiple species, they may donate genes to emerging human pathogens; it has been observed that H7N9 acquired internal genes from the avian H9N2 virus (*14*). In wet markets, availability of freshly slaughtered poultry, live poultry transportation, and mixed trading of domestic animals provide a favorable environment for gene reassortment, mutation, and interspecies transmission of AIVs (*15*). Continuous circulation of these viruses in LPRSs increases the chances of their evolution into new genotypes. Close contact of humans and poultry in LPRSs with no biosecurity barriers increases the risk for emergence of novel influenza viruses with zoonotic or human pandemic potential. Continued surveillance in LPRSs is essential to better understand the public health risk posed by H9N2 AIVs.

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Peste des Petits Ruminants Virus in Heilongjiang Province, China, 2014

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During March 25–May 5, 2014, we investigated 11 outbreaks of peste des petits ruminants in Heilongjiang Province, China. We found that the most likely source of the outbreaks was animals from livestock markets in Shandong. Peste des petits ruminants viruses belonging to lineages II and IV were detected in sick animals.

Peste des petits ruminants (PPR) is a contagious disease that infects goats and sheep and has a case-mortality rate of ≈80% for acute cases. PPR virus (PPRV) is a member of the family *Paramyxoviridae*, genus *Morbillivirus* (*1*). The disease is present mainly in Africa, the Middle East, and the Indian subcontinent (*2*–*8*).

In July 2007, a PPR outbreak was reported in the Ngari region of southwestern Tibet, China. The outbreak was eliminated by using strict control measures. These measures included culling of all animals suspected to be infected; using a PPR vaccine (75/1 strain) (Tecon Animal Husbandry Bio-Technology Co. Ltd., Xinjiang, China) throughout Tibet and neighboring areas; and restriction of transport of animals (*9*).

On December 5, 2013, a new outbreak of PPR was reported in Huocheng County in Xinjiang Province. PPR was also detected in Gansu, Inner Mongolia, Ningxia, Jiangxi, and Hunan Provinces by mid-March 2014.

Heilongjiang Province is the northernmost province of China (Figure 1, panel A). The total population of small ruminants in this province was ≈9 million in 2012 (http://www.stats.gov.cn), and nearly all ruminants were raised for meat or wool production. Sheep and goats were always kept separately, and registered flocks contained 120–6,000 animals (mean 434 animals). Before 2014, no PPR outbreaks had been documented in this region. We report outbreaks of PPR in 11 counties in Heilongjiang Province during 2014.

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The Study

On March 25, 2014, health authorities reported that 43 of 84 goats on 5 breeding farms had died in Huanan County in Heilongjiang Province after being transported from Jiaxiang County in Shandong Province on March 13. Animals showed clinical signs and symptoms of PPRV infection, including fever (temperature >39.5°C), ocular and nasal discharge, pneumonia, and severe diarrhea. On March 26, health authorities reported that 13 of 87 imported breeding goats died of suspected PPR in Huachuan County, which also borders Huanan County. These animals had also been transported from Jiaxiang County on March 13. Because of this animal deaths, a systematic investigation of suspected PPR outbreaks was launched by the Center for Animal Disease Control and Prevention of Heilongjiang Province.

After confirmation of the first case of PPR, active clinical surveillance was conducted by the local veterinary authorities, and farmers were urged to report suspected cases. Suspected flocks were defined as those that 1) during the previous month had ≥1 sheep or goats with ≥1 signs of illness: fever (temperature >39.5°C), depression, anorexia, ocular and nasal discharge, pneumonia, necrosis and ulceration of mucous membranes, and severe diarrhea; 2) had sheep or goats transported from other provinces, especially Shandong Province; or 3) had neighboring farms on which animals had suspected PPRV infections.

During March 25–May 5, a total of 141 suspected flocks (of which 41 contained imported animals [defined as imported flocks]; other flocks were defined as local flocks) from 13 counties were investigated. Sources of all introduced animals were livestock markets in Shandong Province, which were located mainly in 3 counties (Jiaxiang, Heze, and Liangshan). Because spring is traditionally the time when farmers increase numbers of animals on their farms, breeding sheep or goats are bought from livestock markets for this purpose. Shandong Province has the largest number of small ruminant markets in China. It takes 3–5 days for animals to be transported by truck from these markets to most destinations.

From the 141 suspected flocks, 1,887 serum samples were randomly collected from clinically healthy animals, and 285 nasal swab and 28 tissue samples (lymph node, spleen, lung, and intestine) were collected from all ill or dead animals. Competitive ELISAs were performed to detect antibodies against PPRV in the serum samples as described (*10*,*11*).

Viral genomic RNA was extracted from nasal swab and tissue samples by using the QIAamp Viral RNA Mini

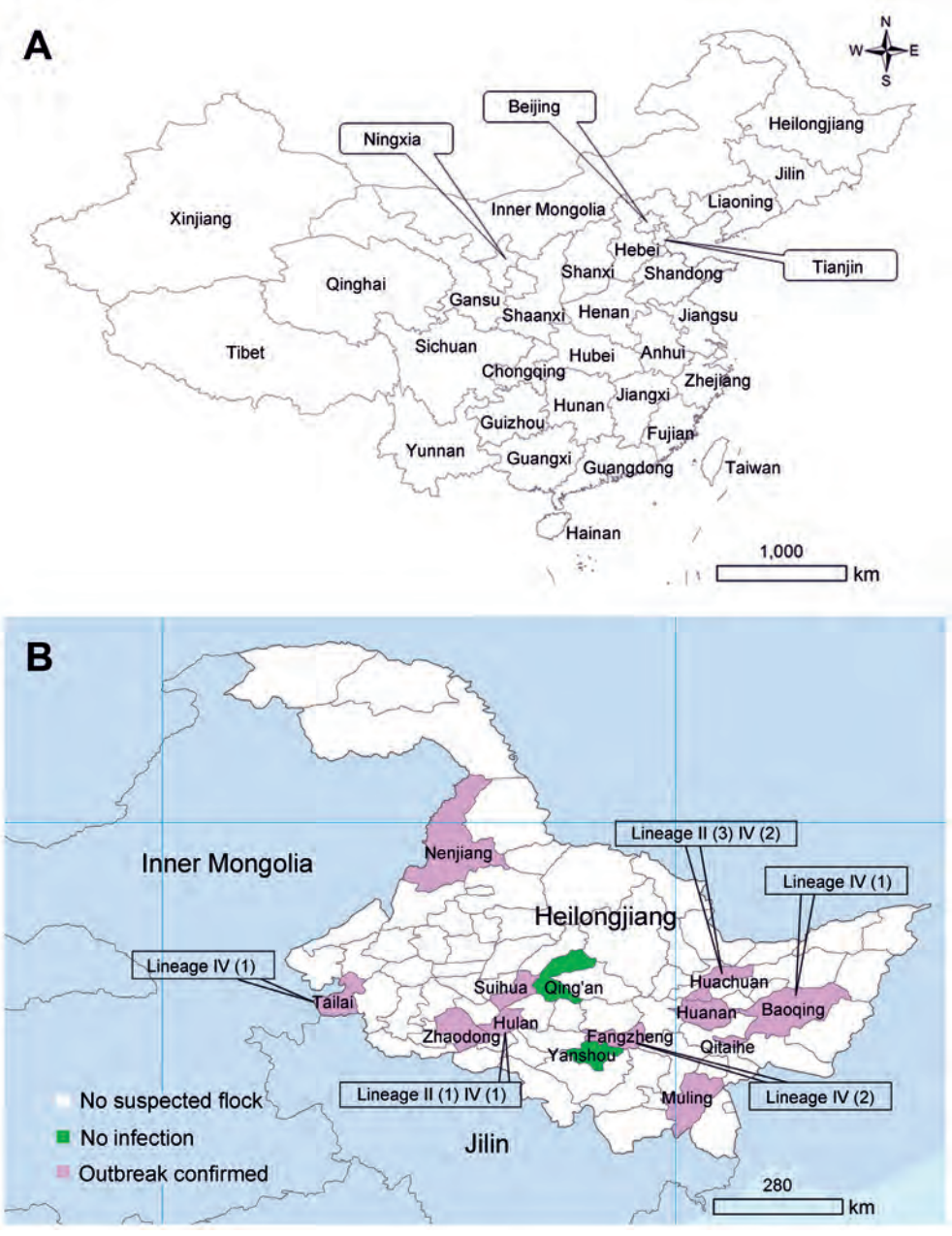

Figure 1. A) Provinces of China and B) distribution of confirmed outbreaks of peste des petits ruminants and peste des petits ruminants virus detected in Heilongjiang Province, March 25–May 5, 2014. Values in parentheses are number of virus isolates.

Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. PPRV was detected by using a reverse transcription PCR (RT-PCR) specific for the 3′ end of the nucleoprotein gene of PPRV; this RT-PCR yields an amplification product of 351 bp (*12*). PCR products were purified by using the QIA Quick Gel Extraction Kit (QIAGEN) and sequenced.

Multiple sequence alignment was performed by using ClustalX2.0 (*13*), and a phylogenetic tree was constructed by using the neighbor-joining method with MEGA6 (*14*). A bootstrap analysis of 1,000 replicates was performed to test the degree of branching.

Serologic analysis indicated that 17% (312/1887) of sampled animals and 76% (31/41) of imported flocks had antibodies against PPRV; all animals from Suihua, Yanshou, and Qing'an Counties were antibody negative (Table). A total of 29% (29/100) of local flocks in 5 counties (Zhaodong, Hulan, Huachuan, Nenjiang, and Baoqing) had antibody-positive animals, which suggests that transmission of PPRV between local and imported flocks had occurred in these areas.

RT-PCRs showed that 14% (39/285) of nasal swab samples and 89% (25/28) of tissue samples were positive for the nucleoprotein gene of PPRV; overall PPRV positive

Table. Serologic and molecular diagnosis for peste des petits ruminants virus in 13 counties in Heilongjiang Province, China, March 25–May 5, 2014*

	No. flocks/no. tested (%)				No. samples/no. tested (%)			Lineage
	Antibody positive		PCR positive		Antibody-positive	PCR-positive		(no. viruses
County	Imported	Local	Imported	Local	Serum	Nasal swab	Tissue	detected)
Baoqing	10/10 (100)	11/70 (16)	10/10 (100)	8/70 (11)	146/533 (27)	11/146 (8)	10/10 (100)	IV (1)
Hulan	1/2 (50)	8/14 (57)	1/2 (50)	2/14 (11)	38/315 (12)	4/24 (17)	1/2 (50)	II (1), IV (1)
Zhaodong	1/1 (100)	4/5 (80)	1/1 (100)	2/5 (40)	30/222 (14)	6/25 (24)	1/1 (100)	0
Huanan	5/5 (100)	0/3 (0)	1/5 (20)	0/3 (0)	3/157 (2)	0/13 (0)	2/2 (100)	0
Huachuan	6/6 (100)	1/1 (100)	4/6 (67)	0/1 (0)	29/130 (22)	11/20 (55)	2/2 (100)	II (3), IV (2)
Tailai	1/3 (33)	0/2 (0)	1/3 (33)	0/2 (0)	24/111 (22)	0/12 (0)	1/1 (100)	IV (1)
Nenjiang	2/2 (100)	5/5 (100)	2/2 (100)	3/5 (60)	16/99 (16)	5/15 (33)	1/1 (100)	0
Qitaihe	3/3 (100)	0	2/3 (67)	0	14/79 (18)	1/15 (7)	1/1 (100)	0
Fangzheng	1/1 (100)	0	1/1 (100)	0	11/70 (16)	1/12 (8)	2/2 (100)	IV (2)
Suihua	0/1 (0)	0	1/1 (100)	0	0/52 (0)	0/3 (0)	3/3 (100)	0
Muling	1/1 (100)	0	1/1 (100)	0	1/2 (50)	0	1/3 (33)	0
Yanshou	0/3 (0)	0	0	0	0/67 (0)	0	0	0
Qing'an	0/3 (0)	0	0	0	0/50 (0)	0	0	0
Total	31/41 (76)	29/29 (100)	25/35 (71)	15/15 (100)	312/1,887 (16)	39/285 (14)	25/28 (89)	II (4), IV (7)

*An antibody-positive flock contained ≥1 animal in positive for antibody against peste des petits ruminants virus. A PCR-positive flock contained ≥1 animal positive for peste des petits ruminants virus by PCR.

rate was 20% (64/313) (Table). Combined results of serologic and the molecular diagnostic tests indicated that 43% (60/141) of suspected flocks were positive for PPRV. Eleven outbreaks were confirmed in Heilongjiang Province; the geographic distribution of these outbreaks is shown in Figure 1, panel B.

To identify genetic characteristics of PPRVs in Heilongjiang Province, a phylogenetic tree was generated by using the 3′ ends of nucleoprotein genes of 49 viruses: 11 were obtained from RT-PCR–positive samples in this study and 38 were obtained from GenBank. Seven of the 11 viruses belonged to lineage IV and were closely related to the strains Tajikistan/04 (*12*) and Tibet/07 (*9*). The remaining 4 viruses from this study belonged to lineage II and were closely related to vaccine strain Nigeria/75/1 (Figure 2). All 11 viruses were obtained from sick animals. These 11 animals had not been vaccinated in Shandong or Heilongjiang Provinces; previous vaccination information was not available.

To control and prevent further spread of PPR, several measures were initiated by the animal health authorities in Heilongjiang Province. These measures included 1) culling of all animals in PPRV-infected flocks and on all farms within a radius of 5 km; 2) restriction of transport of sheep and goats throughout the province; 3) disinfection of PPRV-contaminated areas and disposal of dead animal carcasses; and 4) enhancement of the animal disease–reporting mechanisms. Before the PPR outbreak, sheep and goats with signs and symptoms of disease were not required to be reported to the local veterinary authorities, except for animals with foot-and-mouth disease. After the outbreak, farmers were required to report all sick animals and vaccination of all susceptible animals. These control measures eventually contained the outbreaks.

Conclusions

The emergence of PPR in Heilongjiang Province poses a great threat to the livestock industry in this province. Our investigation showed that the most likely source of the outbreaks was animals from livestock markets in Shandong Province. This finding suggests that transportation of unidentified PPRV-infected sheep and goats is a major risk factor for spread of PPR in China.

Lineage II and lineage IV PPRVs were detected in sick animals. Although the partial nucleoprotein gene of the lineage II viruses had similar sequence identity (>99%) to that of vaccine strain 75/1, we could not determine the vaccination status of these sick animals. Future studies should focus on determining the virulence of these viruses.

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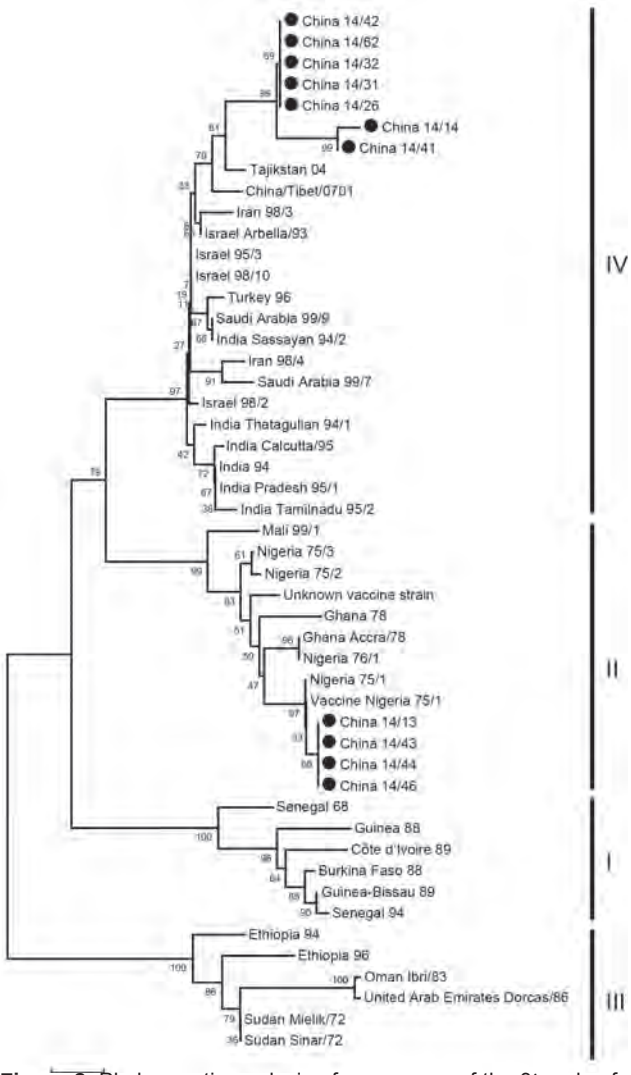

Figure 2. Phylogenetic analysis of sequences of the 3′ ends of nucleoprotein genes of peste des petits ruminant virus (PPRV), Heilongjiang Province, China, March 25–May 5, 2014. The tree was constructed by using the neighbor-joining method in MEGA6 (*14*). Values along branches indicate bootstrap values of 1,000 replicates, and numbers on the right indicate lineages. Black dots indicate PPRV-positive samples isolated in this study. Scale bar indicates estimated number of substitutions per 20 nt.

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West Nile Virus Infection Incidence Based on Donated Blood Samples and Neuroinvasive Disease Reports, Northern Texas, USA, 2012

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During the 2012 outbreak of West Nile virus in the United States, approximately one third of the cases were in Texas. Of those, about half occurred in northern Texas. Models based on infected blood donors and persons with neuroinvasive disease showed, respectively, that ≈0.72% and 1.98% of persons in northern Texas became infected.

From the first reported cases of West Nile Virus (WNV) in North America in August of 1999 through 2013, more than 39,000 cases of West Nile virus (WNV) were reported in the United States (*1*). In 2003, identification of transfusion-transmitted WNV infections (*2*) led to screening of the blood supply for WNV by using nucleic acid amplification technology (NAT) assays in mini-pools (MP-NAT) (*3*). Despite the success of MP-NAT screening of samples from blood donors, WNV transmission from infected donors continued. During 2004, screening algorithms expanded, including triggered individual donation NAT (ID-NAT) (*3*). Approximately 25% of viremic blood donors can be detected by ID-NAT (*4*).

Estimates of WNV infections in 2003 were derived from viremic blood donor rates detected by MP-NAT throughout the United States. West Nile neuroinvasive disease (WNND) reports were then used to approximate the number of infections relative to WNND cases (*5*). With the introduction of targeted ID-NAT, estimates of WNV infections from viremic blood donors must account for differential ID-NAT and MP-NAT screening during epidemic seasons.

Nationwide, the largest WNV epidemic since 2003 occurred in 2012, and approximately one third of cases were reported from Texas. Approximately 48% of cases in Texas were in 4 counties: Collin, Dallas, Denton, and Tarrant, located in the northern area of the state. The aim of this study was to estimate the number of WNV infections in this area during the 2012 arboviral season using 2 models: blood donor NAT yield and WNND-based models (*5*,*6*).

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The Study

Counts of screened blood donations and confirmed WNV viremic donations detected by MP-NAT or ID-NAT from northern Texas residents during the WNV season (April 1, 2012, through November 30, 2012, the WNV surveillance period used by AABB for triggering ID-NAT screening [*4*]) were obtained from Carter BloodCare and Creative Testing Solutions, the area blood collection organization and donor screening laboratory, respectively. Carter BloodCare accounts for ≥95% of blood donation centers in the North Texas Region. These data were used to derive the WNV seasonal incidence rate in 2012.

Calculations were performed separately for ID-NAT– and MP-NAT–screened donations. The length of time WNV RNA is detectable by MP-NAT has been previously reported (*7*). For this analysis, we derived new estimates for the duration of the MP-NAT and ID-NAT detection periods (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/4/14-1178-Techapp1.pdf).

WNV seasonal incidence rates were obtained using a previously derived formula (*5*) by using rates of detection and durations of ID-NAT and MP-NAT WNV RNA detection periods. CIs were obtained assuming a Poisson distribution for ID-NAT and MP-NAT yields. The WNV seasonal incidence rate in blood donors and days screened per method were then applied to the estimated 2011 population of the 4 counties who were age-eligible for blood donation (≥16 years of age) (*8*) to estimate the number of WNV infections in that area during the 2012 WNV season.

To estimate the number of WNV infections by age and gender, we used confirmed and probable WNND cases in persons ≥16 years of age reported during the WNV season to the Texas Department of State Health Services and included in ArboNET, a national surveillance system which monitors WNV activity (*6*). CIs were obtained by applying Taylor series expansion (*9*), based on a Poisson distribution for the WNND cases and the estimated variance of the ratio of WNV cases to WNND cases as reported (*6*).

Results

Fifty-four WNV viremic donations were detected: 30 by MP-NAT and 24 by ID-NAT (Table 1). Dividing the

Table 1. Blood donor NAT yield model derived West Nile Virus infection estimates, northern Texas, 2012*

Model type	No. samples tested	Person-months†	No. WNV RNA+ donations	Viremic donations/10,000 donations	Incidence/10,000 donor months	% Population infected (95% CI)	Estimated no. infections (95% CI)
MP-NAT	118,593	41,604.76	30	2.5	7.2	0.72 (0.44–1.00)	31,013 (19,133–42,893)
ID-NAT	15,134	9,725.46	24	15.9	24.7		

*NAT, Nucleic acid amplification technology.
†Person-months = number of donations tested multiplied by WNV RNA detection period/30.5.

number of viremic donations detected by donations screened by each method, 2.5 WNV-confirmed RNA-positive donations (MP-NAT screening periods) and 15.9 WNV-confirmed RNA-positive donations (ID-NAT screening periods) were detected per 10,000 donations, reflecting higher sensitivity of ID-NAT than MP-NAT screening.

The time at risk for donors differed; detection period is estimated as 19.6 days for ID-NAT and 10.7 days for MP-NAT (online Technical Appendix). The incidence rates also differed, estimated as 7.2 WNV infections (95% CI 3.5–10.9) per 10,000 donor-months (MP-NAT screening periods) and 24.7 WNV infections (95% CI 13.3–36.0) per 10,000 donor-months (ID-NAT screening periods). During the 239-day WNV season, the ratio of blood donations screened by each method was assumed to be equal to the ratio of days screened by each method (because donations per day are roughly constant throughout the season). Incidence was presumed to be 0 outside the WNV season. Applying the 4-county area's 2011 population estimates and the number of days screened by each method to the NAT yield-derived incidence rates resulted in an estimated 31,013 WNV infections (95% CI 19,133–42,893) or 0.72% (95% CI 0.44%–1.00%) infection proportion during the 2012 epidemic season.

Of 356 probable and confirmed WNND case-patients, 7 were ≤15 years of age. Therefore, based on 349 probable and confirmed WNND cases, we estimated 85,156 WNV infections (95% CI 68,302–103,866) or 1.98% (95% CI 1.59%–2.41%) infection proportion during the 2012 epidemic season (Table 2). Age- and sex-based point estimates are shown in Table 2; however, these infection proportions are not statistically significant (p = 0.54), as evident by 95% CIs.

Conclusions

Our findings reflect low incidence of WNV in this area; ≤2% of the population was infected during a large WNV epidemic, with potential incidence differences by age and sex. Low incidence was found regardless of method (NAT yield vs. WNND-based). The donor NAT yield model resulted in lower numbers of projected WNV infections in northern Texas during the 2012 arboviral season compared with the WNND-based model. These estimation differences may be caused by issues affecting internal validity in the model, resulting in overestimation or underestimation of WNV infections.

Because the donor NAT yield model used blood donors who tested WNV RNA-positive and the WNND-based model used ratios derived from blood donors, we emphasize that persons who donate blood may not reflect the total population sampling frame. Blood donors differ from the general population in age, sex, and racial and ethnic descriptions to (*10*). WNV infection rates and WNND rates also differ by age, sex, and possibly race and ethnicity (*6*). In addition, 25% of WNV-infected persons may have signs and symptoms that result in self-exclusion or deferral from blood donation (*11*). Also, the RNA detection periods on which the NAT yield model relies continues to be refined.

For the WNND-based model, although WNND cases may be more reflective of the total population sampling frame because of reporting requirements, issues with case

Table 2. West Nile virus infection estimates derived from WNND case-based model, by sex and age, northern Texas, 2012*

Sex and age groups†	Total population	No. WNND case-patients (A)	Inverse ratio (B)	Estimated no. infections‡ (95% CI)	% Population infected§ (95% CI)
M	2,096,657	203	220	44,660 (33,841–56,977)	2.13 (1.61–2.72)
16–24 y	361,910	11	719	7,909 (2,357–16,723)	2.19 (0.65–4.62)
25–44 y	858,076	41	356	14,596 (7,260–24,468)	1.70 (0.85–2.85)
45–64 y	667,136	75	248	18,600 (11,210–27,851)	2.79 (1.68–4.17)
≥65 y	209,535	75	50	3,750 (1,953–6,129)	1.79 (0.93–2.92)
F	2,211,115	146	291	42,486 (28,943–58,621)	1.92 (1.31–2.65)
16–24 y	350,330	5	1,231	6,155 (626–17,397)	1.76 (0.18–4.97)
25–44 y	879,401	36	330	11,880 (5,306–21,068)	1.35 (0.60–2.40)
45–64 y	699,480	52	387	20,124 (9,949–33,847)	2.88 (1.42–4.84)
≥65 y	281,904	54	61	3,294 (1,017–6,873)	1.17 (0.36–2.44)
Total	4,307,772	349	244	85,156 (68,302–103,866)	1.98 (1.59–2.41)

*WNND, West Nile neuroinvasive disease.
†Age and sex information presented for confirmed and probable case-patients ≥16 y of age (n = 349).
‡A × B.
§No. estimated infections/population.

determination and completeness of WNND reporting exist, likely resulting in underreporting. In addition, ratios used in the model were determined from North Dakota (2002–2008). This population may differ regarding exposure, disease, and reporting from that of the study population. Although interval estimation did not support differences by age and sex, possibly because of small counts, potential differences in point estimates are consistent with other observations (2,*6*,*11*).

Seroprevalence studies conducted in the United States have described varying WNV infection proportions in the population after an epidemic, ranging from 2.6% to 19.7% in different geographic areas (*12*). This estimation of WNV infections in the southern United States contributes to defining the incidence of WNV infection. Despite limitations in the models, data on viremic blood donors and persons with WNND should continue to be used to determine the external validity of the models in conjunction with seroprevalence studies during outbreaks. Valid estimations of WNV infections may give insight into the overall effects of infection and could guide public health interventions in the future.

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Influenza A(H10N7) Virus in Dead Harbor Seals, Denmark

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Since April 2014, an outbreak of influenza in harbor seals has been ongoing in northern Europe. In Denmark during June–August, 152 harbor seals on the island of Anholt were found dead from severe pneumonia. We detected influenza A(H10N7) virus in 2 of 4 seals examined.

Influenza A virus is widespread and affects a wide range of species, including humans (*1*). Waterfowl are considered the natural reservoir for most subtypes of influenza A virus, and most mammalian-adapted viruses initially originated in interspecies transmission from aquatic birds (*2*). Avian influenza A virus (AIV) replicates primarily in the intestinal tract of birds and is transmitted mainly through the fecal–oral route (*1*). Pinnipeds (e.g., seals) share the same shoreline habitats as many waterfowl species and therefore can be exposed to AIV. Several instances of interspecies transmission between birds and harbor seals (*Phoca vitulina*) with AIV subtypes H7N7, H4N5, H4N6, H3N8, and H3N3 have been reported in the United States, and antibodies against a wide range of subtypes have been identified in Europe, Asia, and South America (reviewed by White [*3*]). Human infections with seal influenza A virus have occasionally been reported (*3*). More recently, A(H1N1) pdm09 virus was isolated from elephant seals (*Mirounga angustirostris*) off the central coast of California, USA (*4*). To our knowledge, AIV in harbor seals off the coast of northern Europe was first reported in April 2014 (*5*).

The Study

During June 16–August 13, 2014, a total of 152 harbor seals were found dead on the shore of the small island of Anholt in Denmark. A few carcasses were reported in late June, and deaths peaked in mid-July (Figure 1). Four freshly or recently dead harbor seals were submitted to the National Veterinary Institute, Technical University of Denmark, for necropsy and laboratory examination. The seals were juveniles; 3 were males; and the animals' body conditions were normal or slightly below normal (2 seals each). All had intense reddening of the lungs, with multifocal condensation of lung tissue, moderate to massive amounts of blood in thorax, and an intensely hyperemic trachea. One seal also had blood in the mouth. No lesions were apparent in other organs, but all 4 animals had empty stomachs. In all seals, histopathologic examination of the lungs showed massive suppurative and necrotizing bronchopneumonia and many bacteria in the alveoli. In the interstitial tissues, edema, fibrin, and neutrophils were seen. Samples from relevant organs were tested for bacteria on selective and nonselective culture media. Samples from the lungs revealed massive growth of *Pseudomonas aeruginosa* in all animals, indicating severe bacterial pneumonia (*6*). In addition, variable growth of *Streptococcus equi* subsp. *zooepidemicus* was found.

We tested samples of lung and spleen for influenza A virus by a slightly modified version of a previously described real-time reverse transcription PCR (RT-PCR) that targeted the matrix gene (*7*) and for phocine and canine distemper virus by 1-step RT-PCR using previously described primers that targeted the P (phosphoprotein) gene (*8*). All 4 samples were negative for distemper virus. Samples from lungs and spleen from 2 seals were positive for influenza A virus (Table). RNA from the influenza A virus–positive samples subsequently tested negative for H5 and H7 subtypes by subtype-specific real-time RT-PCR.

Full length hemagglutinin (HA) and neuraminidase (NA) genes were amplified using RT-PCR on the RNA extracted from the spleen and lung. Sequences of HA and NA were only obtained from lung samples and used in the following phylogenetic analysis. We could not

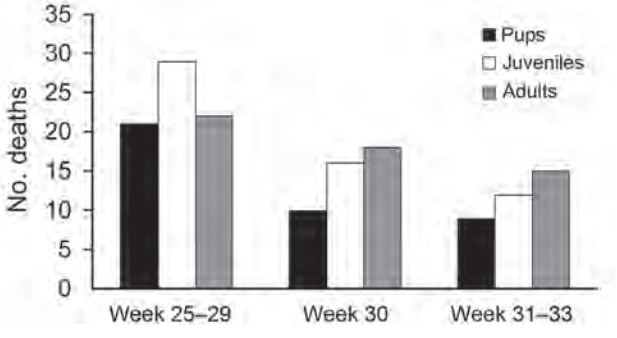

Figure 1. Deaths of harbor seals (*Phoca vitulina*) on the small island of Anholt, Denmark, summer 2014.

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Table. Results of PCR and sequence analysis of the 2 influenza A virus–positive harbor seals, Denmark, 2014*

Virus	Tissue	Real-time RT-PCR, M (C_t)	Sequence	
			Hemagglutinin	Neuraminidase
A/harbor seal/Denmark/14–5060–1lu/2014	Lung	Positive (32.9)	Negative	Positive
A/harbor seal/Denmark/14–5060–1sp/2014	Spleen	Positive (37.3)	Negative	Negative
A/harbor seal/Denmark/14–5061–1lu/2014	Lung	Positive (27.8)	Positive	Positive
A/harbor seal/Denmark/14–5061–1sp/2014	Spleen	Positive (38.2)	Negative	Negative

*C_t, cycle threshold; M, matrix; RT-PCR, reverse transcription PCR.

sequence the spleen samples because of low viral loads (Table). Using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and phylogenetic analysis, we identified the subtype as H10N7. The highest nucleotide similarity of HA gene from the seal to AIVs was to A/mallard/Sweden/133546/2011(H10N4) (98.4%) and a wild bird AIV from Denmark A/mallard/Denmark/16109-4/2011(H10N6) (98.3%). The NA segments from the harbor seals were 99.5% identical to each other and 99.3% identical to an AIV isolated from a commercial flock of game birds in Denmark in 2013 (A/mallard/Denmark/303878-1s/2013(H7N7)) and 97.6% identical to A/domestic duck/Republic of Georgia/1/2010(H10N7). In general, both the HA (Figure 2, panel A) and NA (Figure 2, panel B) segments showed high-level nucleotide sequence identity to AIVs from birds sampled in Scandinavia and the Republic of Georgia. Detection of the H10N7 virus in seals has been reported from Denmark,

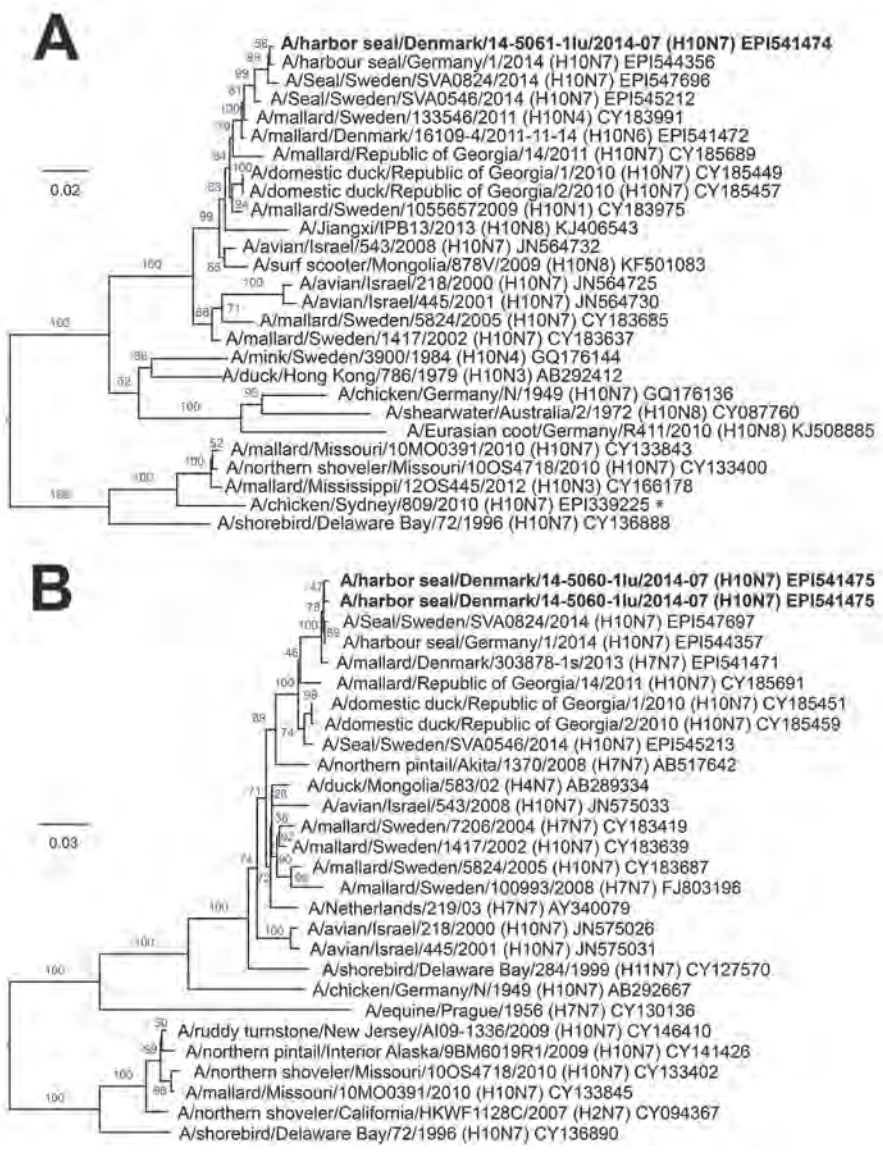

Figure 2. Phylogenetic trees of selected avian influenza A virus sequences. Boldface indicates sequences identified from harbor seals in Denmark during 2014. A) H10 avian influenza A virus sequences. The asterisk denotes the H10N7 subtype that also caused disease in humans (*9*). B) N7 avian influenza A virus sequences. Sequences were aligned with CLC Main Workbench version 7.02 (CLC bio, Aarhus, Denmark) by using the MUSCLE algorithm, and phylogenetic trees were constructed by using the neighbor-joining method with 1,000 bootstrap replicates. Only bootstrap values >75% are shown. Scale bars indicate mutations per site.

Germany, the Netherlands, and Sweden. The seal influenza virus was initially collected in Sweden in April (*5*); this isolate is 98.7% nt (HA) and 97.0% nt (NA) identical to the strain from Denmark. The samples collected from Germany and Sweden in late August are more similar to the samples from Denmark, with identities of 99.2%–99.7% nt; the strains from Denmark and Germany are most similar. At the amino acid level, the HA of the viruses from Denmark and Germany was 99.3% identical, whereas the early strain from Sweden was only ≈97.5% identical to all the other strains. The most recent reported strain from Sweden was 98.5% aa identical to the Denmark and Germany strains. The lower amino acid identity of HA reflects the large proportion of nucleotide mutations, which results in amino acid changes (dN/dS = 0.7) indicating that adaptation to seals is in progress. Further analysis of the HA sequence showed that the amino acids in the receptor binding site were conserved compared to other H10 sequences (*10*). The predicted amino acid sequence of the HA cleavage site of all the A (H10N7) seal viruses were unique (PELVQGR*GLF) and contained only 1 basic amino acid, which indicated low pathogenicity.

Conclusions

Outbreaks of influenza A virus in marine mammals have been repeatedly described in North America and Asia and have often been linked to increased deaths and severe lung lesions (*11*–*14*), as in the outbreak reported here (*5*). The pathologic findings are consistent with findings described in previous outbreaks in seals, but the histopathologic findings indicate severe secondary bacterial pneumonia, which was also confirmed by cultures of several different bacteria. Thus, the severity of infection probably was caused by AIV infection combined with secondary bacterial infections, which also explain why experimentally reproducing severe disease in seals has been difficult (*14*).

The influenza A (H10N7) virus that we detected probably was of avian origin, as indicated by its high similarity to contemporary AIV isolates. The predicted amino acid sequence of the HA cleavage site indicated low pathogenic AIV because it did not contain multiple basic amino acids. However, because the amino acid sequence of the cleavage site is unique and because the virus also was detected in the spleens, systemic spread and a highly pathogenic phenotype of the virus cannot be ruled out. Indeed, influenza A virus of the H10 subtype has been shown to have characteristics of highly pathogenic AIV in chickens, despite the lack of multiple basic amino acids at the cleavage site (*15*). Furthermore, some strains of H10 have proved to be highly pathogenic in mink (*10*).

During the current outbreak, we found no epidemiologic evidence of human exposure, but human infection has been reported in connection with previous outbreaks of influenza A virus in harbor seals (*3*). Furthermore, another A(H10N7) virus, A/chicken/Sydney/809/2010(H10N7), from chicken infected workers in a poultry abattoir in Australia was of US lineage and thus differed significantly (81.0% identity) from the harbor seal HA in our current study (Figure 2, panel A) (*9*). Thus, whether humans are at risk for infection from contact with seals infected with A(H10N7) virus is unclear.

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High Seroprevalence of Antibodies against Spotted Fever and Scrub Typhus Bacteria in Patients with Febrile Illness, Kenya

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Serum samples from patients in Kenya with febrile illnesses were screened for antibodies against bacteria that cause spotted fever, typhus, and scrub typhus. Seroprevalence was 10% for spotted fever group, <1% for typhus group, and 5% for scrub typhus group. Results should help clinicians expand their list of differential diagnoses for undifferentiated fevers.

Rickettsioses are zoonoses that are increasingly being recognized as noteworthy infectious diseases (*1*). They are caused by bacteria of the genera *Rickettsia* and *Orientia*, which are small, gram-negative, obligate intracellular bacteria that are transmitted to humans through bites of infected arthropod vectors, such as fleas, mites, ticks, and lice. These bacteria are able to invade various host cells, including vascular endothelium, causing characteristic symptoms such as rash and petecchial hemorrhages. The genus *Rickettsia* is divided into 2 main biogroups: spotted fever group (SFG) and typhus group (TG). The scrub typhus group (STG) previously belonged to the genus *Rickettsia* but now belongs to the genus *Orientia* (*2*), which consists of 2 species: *O. tsutsugamushi* and *O. chuto* (*3*).

The past 5 years have seen concerted efforts to understand the etiology of undifferentiated febrile illnesses, a group of diseases that includes rickettsioses. These efforts have confirmed the occurrence of infections with *R. felis*, transmitted mainly by the cat flea (*Ctenocephalides felis felis*), as a common cause of fever in rural areas (*4*–*6*). Studies have also shown the preponderance of *R. africae*, the causative agent of African tick-bite fever, as well as *R. conorii* and *R. aeschlimannii*, in different ecoregions of Kenya (*7*). The study reported here is part of a broader study aimed at identifying pathogens or their surrogates, such as immunoglobulins, in patients with febrile illnesses. It is hoped that these types of reports will help local clinicians expand their list of differential diagnoses for undifferentiated fevers.

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The Study

The study protocol was approved by the Ethical Review Committee of the Kenya Medical Research Institute (SSC #1282) and the Walter Reed Army Research Institute's Human Subject Protection Board (WRAIR HSPB #1402). All patients provided informed consent.

Serum samples were collected from patients with fever (≥38°C) at 8 hospitals in 6 ecoregions of Kenya (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/20/4/14-1387-Techapp1.pdf). The samples were screened for IgG against whole-cell antigens of *R. conorii* for SFG, *R. typhi* for TG, and Karp and Gilliam strains of *O. tsutsugumishi* for STG as previously described (*8*,*9*). Serum samples that were reactive at 1:100 dilutions were further titrated by using 4-fold dilutions to 1:6,400.

Because scrub typhus has not been reported in Kenya, Western blot was performed to confirm specificity of the reactive serum samples, essentially as described before (*5*). For Western blot, 0.06 μg/well of the Otr47b antigen was applied to a 10% sodium dodecyl sulfate–polyacrylamide gel and separated by electrophoresis. The proteins were transferred to a nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA). After blocking nonspecific binding at 4°C in 10% skim milk (Difco Becton, Dickinson, Franklin Lakes, NJ, USA), lanes of migrated Otr47b antigens were probed with serum samples that had titers ≥1:1,600. IgG against Otr47b antigen was detected by a horseradish peroxidase–conjugated anti-human IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) at a 1:25,000 dilution, signal visualized by chemiluminescence, and acquired on Kodak X-Ray Film (Carestream Health Inc., Toronto, Ontario, Canada).

A total of 2,225 patients 1–72 years of age (mean age 5 years) were enrolled in the study. There was no difference in the male:female ratio across age groups. Overall, 212 (10%) febrile patients were seropositive for SFG. A substantially higher prevalence rate was seen in Garissa (57/226, 25%) than in Alupe (27/176, 15%), Marigat (37/320, 12%), Malindi (9/102, 9%), Kisii (39/656, 6%), or Kisumu (43/745, 6%) ($p<0.05$) (online Technical Appendix Figure 2).

In all regions, most SFG-seropositive patients had titers ≥1:1,600 (38%), with the highest numbers coming from Garissa (29%, n = 23), followed by Kisumu (18%, n = 16), and Malindi (5%, n = 4) (Figure 1, panel A). Only 4/1,611

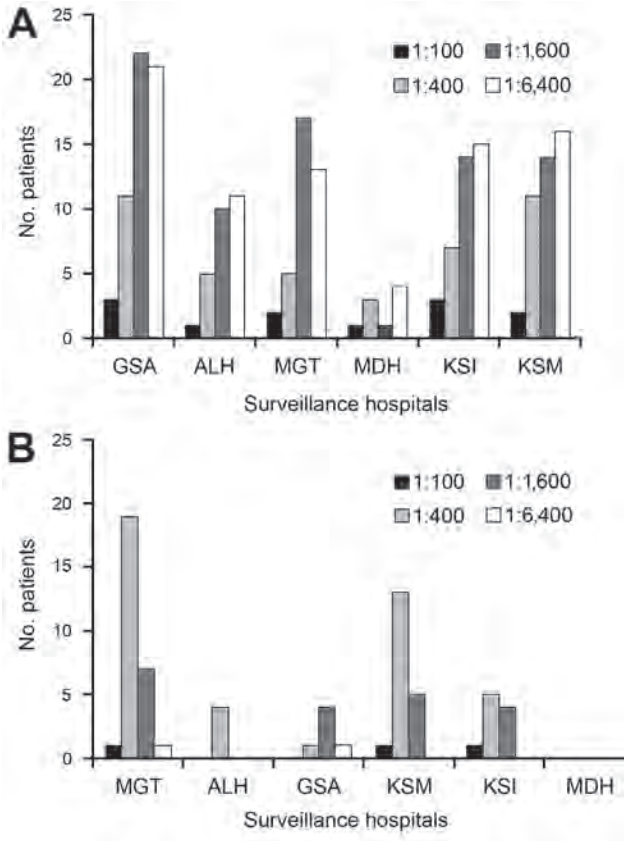

Figure 1. Distribution of titers to spotted fever (SFG) and scrub typhus (STG) groups in patients recruited in various surveillance hospitals. A) For SFG, Garissa District Hospital (GSA), in semiarid northeastern Kenya, had more patients with higher titers compared with Alupe District Hospital (ALH), on the Kenya-Uganda border; Marigat District Hospital (MGT), on the floor of the Rift Valley; Malindi District Hospital (MDH), on the Indian Ocean coast; Kisii District Hospital (KSI), in the Kisii highlands; and Kisumu District Hospital and Obama Children Hospital (KSM), on the Lake Victoria basin. B) For STG, MGT had the most patients with titers of 1:400 and 1:1,600 compared with ALH, MDH, KSI, and KSM.

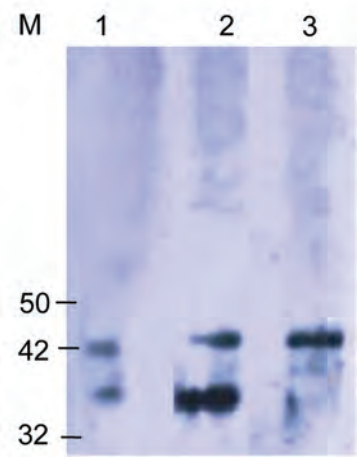

Figure 2. Western blot analysis using the *Orientia* spp.–specific antigen (Otr47b). Twenty scrub typhus reactive serum samples at a titer ≥1:6,000 were used. Negative controls were serum samples that were reactive to spotted fever and typhus group antigens. The scrub typhus reactive serum samples recognized the Otr47b antigen (lanes 2 and 3), but the spotted fever group and typhus group reactive serum samples did not (data not shown). Lane 1 was probed with a positive control serum sample from an earlier scrub typhus outbreak study (*5*). M, molecular mass standard, kDa.

(<1%) febrile patients were seropositive for TG: 3 patients in Malindi and 1 in Kisumu. Antibodies against STG rickettsiae were detected in 67/1401 (5%) febrile patients. The highest prevalence was seen in Marigat District Hospital (28/238, 12%), followed by Alupe Sub-District Hospital (4/68, 6%), Garissa (6/134, 5%), Kisumu (19/464, 4%), and Kisii (10/458, 2%) ($p<0.05$) (online Technical Appendix Figure 3). Most STG patients had titers of 1:400 (62%), with the highest coming from Marigat (107/238, 45%) and Kisumu (142/458, 31%) (Figure 1, panel B). Western blot analysis confirmed reactivity of STG serum samples to *O. tsutsugumishi* antigen (Figure 2).

Table 1 shows the prevalence of SFG and STG antibodies by patient age, sex, and animal contact. Female patients were 1.88 times more likely to be exposed to STG than male patients ($p = 0.0169$), unlike with SFG. Seroprevalence for SFG and STG increased with patients' age ($p<0.05$). Having camels and dogs was positively associated with SFG ($p<0.05$) and having goats with STG ($p<0.05$).

Conclusions

Seventy-eight percent of the study population was ≥12 years of age; >50% were <5 years of age. This age weighting may have led to underreporting of seroprevalence, because seroprevalence increased with age for SFG and STG (Table). The overall seroprevalence of SFG was 10% (212/2,225), similar to the percentage reported among febrile patients in northern Tanzania (8%) (*10*). Substantial differences in seroprevalence were observed among patients in the surveillance hospitals in different ecoregions of Kenya (online Technical Appendix Figure 2). Patients' land use influenced seroprevalence; the highest rates of seroprevalence were recorded among the pastoralists of Garissa and Marigat, who keep large herds of cattle, sheep, goats, and camels. In other locales with high seroprevalence rates (Alupe, Malindi, Kisii, and Kisumu), farmers practice small-scale animal husbandry. IgG titers in most seropositive patients were high (1,600–6,400), perhaps indicating patients' repeated exposure to other homologous or heterologous SFG organisms (Figure 1, Panel A). In contrast to seroprevalence of SFG, seroprevalence of TG rickettsioses was low (4/1,611, <1%) and comparable to that reported among febrile patients in northern Tanzania (*10*).

Table. Demographic characteristics of febrile patients tested for seropositivity for SFG and STG rickettsioses, Kenya*

Characteristic	SFG, no. positive/no. tested (%)	OR (95% CI)	STG, no. positive/no. tested (%)	OR (95% CI)
Sex				
F	96/1,094 (9)	1.0	43/694 (6)	1.0
M	116/1,131 (10)	1.2 (0.9–1.6)	24/707 (3)	0.5 (0.3–0.9)†*
Age, y				
<5	41/1,107 (4)	1.0	17/687 (3)	1.0
5–12	62/622 (10)	2.9 (1.9–4.4)†	29/423 (6)	2.9 (1.5–5.7)
13–26	63/290 (22)	7.2 (4.7–11.2)†	10/ 166 (6)	2.5 (1.0–6.0)†
>26	46/206 (22)	7.5 (4.6–12.1)†	11/125 (9)	3.8 (1.6–8.8)†
Animal contact				
Goats				
No contact	205/2,188 (9)	1.0	60/1,372 (4)	1.0
Contact	7/37 (19)	2.3 (0.8– 5.3)	7/29 (24)	7.0 (2.4–17.7)†
Cows				
No contact	207/2,187 (10)	1.0	65/1,377 (5)	1.0
Contact	5/38 (13)	1.4 (0.4–3.8)	2/24 (8)	1.8 (0.2–7.7)
Donkeys				
No contact	211/2,223 (10)	1.0	67/1,399 (5)	1.0
Contact	1/2 (50)	9.5 (0.1–748.8)	0/2 (0)	0 (0–38.7)
Cats				
No contact	203/2,106 (10)	1.0	66/1,315 (5)	1.0
Contact	9/119 (8)	0.8 (0.3–1.5)	1/86 (1)	0.2 (0.05–1.3)
Sheep				
No contact	212/2,218 (10)	1.0	67/1,387 (5)	1.0
Contact	0/7 (0)	0 (0–5.2)	0/14 (0)	0.0 (0–5.5)
Dogs				
No contact	210/2,146 (10)	1.0	67/1,398 (5)	1.0
Contact	2/79 (3)	0.2 (0.03–0.9)†	0/3 (0)	0.0 (0–11)
Camels				
No contact	196/2,173 (9)	1.0	67/1,365 (5)	1.0
Contact	16/52 (31)	4.5 (2.3–8.5)†	0/36 (0)	0.0 (0.0–2.1)

*SFG, spotted fever group; STG, scrub typhus group; OR, odds ratio.
†Seroprevalence for both spotted fever and scrub typhus increased with age; there were significant differences ($p<0.05$) between those <5 years of age and those in older age groups for SFG and those >12 years of age for STG. Exposure to SFG and STG were more likely in patients who had contact with dogs and camels for SFG and goats for STG ($p<0.05$).

Considering that STG had not been reported in Kenya, its seroprevalence was surprisingly high (67/1,401, 5%) and was highest in Marigat (28/238, 12%) (online Technical Appendix Figure 3). Marigat also had the highest number of persons with titers of 1:400 and 1:1,600 (Figure 1, Panel B). The reported determinants for STG are presence of rodents and vectors (chigger mites, especially *Leptotrombidium deliense*) that infest areas of heavy scrub vegetation.

Exposure to scrub typhus increased with age; persons >26 years of age were more likely to be seropositive than younger persons. Similar results were reported in a study conducted in Sri Lanka (*11*). Scrub typhus seropositivity was associated with contact with goats, perhaps because the short dense shrubs that are forage for goats are also the habitat for Trombiculid mites. As in South Korea but not Japan (*12*), more girls and women were exposed to tsutsugamushi disease than boys and men, possibly because women's culturally sanctioned activities expose them to plant tissues inhabited by chiggers.

This study had several limitations. First, the serum samples used were from a 1-time encounter with the patient (acute-phase sample only). Convalescent-phase serum samples would have better defined the cases. Second, to demonstrate the disease unequivocally, the Trombiculid mites and the infectious *Orientia* spp. will need to be identified. Last, it remains to be determined whether the findings of STG in Kenya represents spread of *Orientia* species outside the tsutsugamushi triangle (an area that includes Pakistan, Australia, Japan, South Korea, and Thailand), as reported recently (*3*,*13*), or identifies a hitherto unknown disease-endemic focus.

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At the time of this study, Ms. Thiga was a master of science student at the Jomo Kenyatta University of Agriculture and Technology. Her research interests include rickettsial diagnosis and epidemiology.

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Prevalence of Hepatitis E Virus Antibodies, Israel, 2009–2010

Orna Mor, Ravit Bassal, Michal Michaeli, Marina Wax, Daniela Ram, Oranit Cohen-Ezra, Dani Cohen, Ella Mendelson, Ziv Ben-Ari, Tamy Shohat

We investigated prevalence of hepatitis E virus in a sample of the population of Israel. The overall seroprevalence of antibodies to the virus was 10.6% (95% CI 8.4%–13.0%); age-adjusted prevalence was 7.6%. Seropositivity was associated with age, Arab ethnicity, low socioeconomic status, and birth in Africa, Asia, or the former Soviet Union.

Acute viral hepatitis is caused by hepatitis E virus (HEV), which can be divided into 4 genotypes (*1*–*4*). Genotype 1, transmitted mainly through the fecal-oral route, is most common in developing countries in Asia, Africa, and Central America, where the disease is highly endemic. In industrialized countries, hepatitis E is infrequent, although in recent years, sporadic cases have been reported, resulting mainly from zoonotic transmission of HEV genotype 3 (*1*–*3*).

Prevalence rates of HEV IgG, a marker of previous exposure to HEV, range from 1% to 20% in industrialized countries (*1*). This marked variability in reported prevalence has been attributed to the use of different IgG assays with significantly different sensitivities (*4*).

The population of Israel comprises 2 major population groups, Jews (80%) and Arabs (including Muslims, Christians, and Druze); each has distinct cultural and socioeconomic features (*5*). In a study published in 1995, a low seroprevalence of anti-HEV antibodies in Israel was found among Jews (2.81%) and Arabs (1.81%) (*6*). These data were obtained by using an immunoassay developed 20 years ago; newer assays are considered more sensitive and specific to HEV IgG (*4*).

The Study

We used an age-stratified sampling design to systematically select 729 serum samples from those deposited during 2009–2010 at the National Serum Bank of the Israeli Center of Disease Control. Included were anonymous residual samples from diagnostic laboratories (403 samples) and from healthy blood donors (326 samples). Demographic information (place of birth, population group, and place of residence) was available for all donors. Socioeconomic status (SES) was assessed by the socioeconomic rank as defined by the Israel Central Bureau of Statistics; this system categorizes each place of residence on a 10-point scale based on parameters such as financial resources, housing density, motorization, education, and employment profile (*7*). Levels of HEV IgG were determined by using the DS-Anti-HEV-IgG kit (Diagnostic Systems Italy, Saronno, Italy), which is capable of detecting antibodies against all 4 HEV genotypes. Statistical analyses included χ^2, *t* test, and univariate and multivariate logistic regression models to evaluate factors associated with hepatitis E infection. Odds ratios (OR) and 95% CI were calculated. A p value of <0.05 was considered statistically significant. Data were analyzed by using SAS version 9.1.3 (SAS Institute, Cary, NC, USA). This study was approved by the Institutional Review Board of Sheba Medical Center (approval no. 9927–12-SMC).

Of 729 samples, 77 (10.6%, 95% CI 8.4%–13.0%) tested positive for HEV IgG. The calculated age-adjusted prevalence rate for the population of Israel was 7.6%. Seropositivity increased significantly with age (Table 1); seroprevalence among persons ≥60 years of age was 37.5%, compared with 0.5% among those <20 years of age. HEV seropositivity, which was mainly observed in older persons, was significantly higher among Arabs (22.5%) compared with non-Arabs (10.3%). Among Jews, a significant association was found between samples testing positive for anti-HEV IgG and having been born in Africa (50%), Asia (53.8%), or the former Soviet Union (17.9%) compared with Israel (OR 10.4, 95% CI 6.1–17.9; p<0.0001) and also with an earlier year of immigration to Israel (OR 2.5, 95% CI 1.2–5.4; p = 0.02). The odds for testing HEV IgG–positive were highest among those of low SES (OR 2.9, 95% CI 1.4–5.9; p = 0.003). The multivariate logistic regression model also showed significant association between HEV seropositivity and advanced age, low SES, Arab ethnicity, and having been born in Asia, Africa, or the former Soviet Union (Table 2).

The prevalence of HEV antibodies we found is higher than previously reported in Israel and is consistent with other studies that have reported higher prevalence rates

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Table 1. Prevalence of antibodies to hepatitis E virus categorized by study population demographics, Israel, 2009–2010*

Characteristics	No. samples tested	HEV-seropositive samples		Odds ratio (95% CI)	p value
		No.	% (95% CI)		
Age, y					
<20	212	1	0.5 (0.01–2.6)	Reference	
20–39	189	2	1.1 (0.1–3.8)	2.3 (0.2–25.1)	0.51
40–59	168	14	8.3 (4.6–14.0)	19.2 (2.5–147.4)	0.005
≥60	160	60	37.5 (30.0–45.5)	126.6 (17.3–926.6)	<0.0001
Sex					
M	394	38	9.6 (6.9–13.0)	Reference	0.38
F	335	39	11.6 (8.4–15.6)	1.2 (0.8–2.0)	
Birthplace					
Israel	518	26	5 (3.3–7.3)	Reference	<0.0001
Africa, FSU, Asia	121	43	35.5 (27.-44.8)	10.4 (6.1–17.9)	
Population group					
Jews	562	58	10.3 (7.9–13.1)	Reference	0.002
Arabs	80	18	22.5 (13.9–33.2)	2.5 (1.4–4.6)	
Year of immigration					
<1970	89	37	41.6 (31.2–52.5)	2.5 (1.2–5.4)	0.02
1970–1989	31	2	6.4 (0.8–21.4)	0.2 (0.1–1.2)	0.08
1990–2000	54	12	22.2 (12.0–35.6)	Reference	
>2000	13	1	1 (0.2–36.0)	0.3 (0.03–2.5)	0.26
Socioeconomic status					
High, ranks 7–10	203	12	5.9 (3.1–10.1)	Reference	
Intermediate, ranks 4–6	211	25	11.8 (7.8–17.0)	2.1 (1.0–4.4)	0.03
Low, ranks 1–3	193	30	15.5 (10.7–21.4)	2.9 (1.4–5.9)	0.003
Total	729	77	10.6 (8.4–13.0)	NA	NA

*FSU, former Soviet Union; NA, not applicable.

with the use of new, more sensitive immunoassays (*4*). Although it was argued that high prevalence of anti-HEV antibodies could be attributed to nonspecific or false-positive serum reactions (*8*), the low prevalence we found among those <40 years of age and the significant association between the prevalence of HEV antibodies and older age suggest that it is unlikely to be a result of nonspecific serum reactions.

Association of seropositivity with age was also reported in Denmark and the United Kingdom (*9*,*10*). Such associations could represent a cohort effect related to infection in the past or could be a result of ongoing low incidence of HEV infection resulting in cumulative exposure to infection over time. In Denmark, a statistically significant difference was detected in the overall HEV prevalence among samples from blood donors collected in 1983 versus those collected in 2003, suggesting that past exposure contributed to the anti-HEV response and that the prevalence of HEV seropositivity had decreased over the years (*9*). In contrast, in HEV-endemic countries, transmission might be ongoing; in India, the age-specific prevalence of anti-HEV did not change during 1982–1992 (*11*). Similarly, overall, 3.2% of blood donors in France were HEV-positive, but 52.5% of blood donors in southwest France, which includes the Midi-Pyrénées region, where HEV is endemic, were HEV positive (*12*).

No HEV outbreak has been documented in Israel; the total number of autochthonous HEV infections is unknown. Acute infections were reported only among travelers returning to Israel from HEV-endemic countries (*13*), further suggesting that ongoing transmission of HEV in Israel is unlikely.

Our findings that low SES is associated with HEV seropositivity is supported by others who have suggested low SES and poor environmental conditions are risk factors for HEV infection (*14*). The higher seropositivity observed among persons born in Africa, Asia, or the former Soviet Union corroborates with HEV endemicity and documented large outbreaks in these regions (*3*).

Although we found the seropositivity rate in the population of Israel to be higher than previously reported, and associated with specific population subgroups, this study has several limitations. Being a cross-sectional study, it is impossible to rule out recent or ongoing infections among older

Table 2. Multivariate logistic regression analysis for factors associated with anti HEV seropositivity, Israel, 2009–2010*

Characteristics	Odds ratio (95% CI)	p value
Age, y		
<20	Reference	
20–39	1.5 (0.1–24.3)	0.79
40–59	17.6 (2.2–143.8)	0.008
≥60	100.1 (12.1–830.3)	<0.0001
Socioeconomic status		
High, ranks 7–10	Reference	
Intermediate, ranks 4–6	2.1 (0.7–5.7)	0.16
Low, ranks 1–3	3.4 (1.1–10.1)	0.03
Population group		
Jew	Reference	
Arab	7.1 (2.1–24.0)	0.002
Country of birth		
Israel	Reference	
Africa, FSU, Asia	3.8 (1.4–10.8)	0.1

*FSU, former Soviet Union.

persons. To better address this issue, the presence of HEV RNA and anti HEV IgM antibodies, which together provide the most sensitive measure for acute infection, should be assessed in a much larger representative sample of older persons of all populations. Moreover, HEV genotype which was not addressed in this study should be determined in persons positive for HEV RNA. Because the prevalence of HEV infection in animals in Israel has not been documented, a study to better understand the HEV transmission root would be useful, especially because the consumption of pork, a common source of HEV infection, is religiously prohibited for both Arab Muslims and Jews (*15*).

Conclusions

The findings of this study indicate high numbers of past HEV infections among immigrants to Israel, which seemingly occurred in countries in which HEV is endemic. The higher HEV prevalence found among older persons in the Arab population, which was also associated with low SES, compared with the non-Arab population could be attributed to previous local exposure. The current overall HEV infection rate in Israel is low, as suggested both by the low prevalence in the younger population and the absence of any documented HEV outbreaks in Israel. However, the nature of this study cannot rule out ongoing infections among older persons. Surveillance of HEV through mandatory reporting of communicable diseases should continue to enable detection of emerging and reemerging infections.

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Outbreak of Severe Zoonotic Vaccinia Virus Infection, Southeastern Brazil

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In 2010, a vaccinia virus isolate caused an atypically severe outbreak that affected humans and cattle in Brazil. Of 26 rural workers affected, 12 were hospitalized. Our data raise questions about the risk factors related to the increasing number and severity of vaccinia virus infections.

After the World Health Organization declared in 1980 that smallpox had been eradicated, smallpox vaccination was suspended (*1*). This fact led to the emergence of a generation of humans that is susceptible to infection by zoonotic viruses of the genus *Orthopoxvirus*, which includes cowpox virus in Europe; monkeypox virus, which occurs naturally in Africa and of which 1 introduction was event reported in the United States; and vaccinia virus (VACV) in Asia and South America (*2*–*5*).

Especially during the past decade, orthopoxvirus (OPV) infections have increased worldwide, and the immunologic status of the population against OPV is a major risk factor for its reemergence (*6*). We describe an outbreak of atypically severe VACV infection in which 12 rural workers in Brazil, who were not vaccinated against smallpox, were hospitalized because of systemic clinical manifestations.

The Study

In June 2010, an outbreak of exanthematic VACV infection was reported in the rural region of Doresópolis County (20°17′13 ″S, 45°54′10 ″ W), Minas Gerais State, Brazil. This region is characterized by small rural properties, where cattle are kept for milk production. Outbreaks of VACV infection had been reported in the neighboring counties in previous dry seasons. In dairy cattle, typical lesions had developed on teats and udders that caused a decrease in milk production; however, the source (index case) was not identified. The reported virulence of the disease in cattle was not atypical and was similar to previously described cases (*4*).

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During our collection of epidemiologic data, we were directed to the local health facility, where 12 rural workers were hospitalized because of high fever; lymphadenopathy; prostration; and painful vesicular–pustular lesions on the hands, arms, faces, and/or knees. All patients were occupationally infected (after milking cows that had lesions on teats). Patients reported that in case of multiple lesions, autoinoculation probably occurred from lesions on hands, the first site of infection; therefore, we have no clinical evidence of "generalized vaccinia." Three patients also had convulsion, vomiting, diarrhea, and mental confusion.

The patients received clinical support and remained hospitalized for 3–18 days. They had no history of immunologic disorders and took no medications that could cause this severe clinical condition. The patients were 15–26 years of age, and none had a history of smallpox vaccination; 1 patient reported having similar clinical illness in 2009. Our investigations also identified 14 additional rural workers who were occupationally infected but not hospitalized; 7 were >40 years old and probably vaccinated against smallpox.

To characterize the etiologic agent of this outbreak, we collected serum from 4 infected cows, scabs from 3 cows, and swab samples from the lesions of 4 hospitalized patients and 1 nonhospitalized patient. The serum samples were submitted to plaque reduction–neutralizing tests as previously described (*7*). Neutralizing antibodies against OPV were detected in 3 (75%) cows; titers ranged from 1:40 to 1:160 neutralizing units/mL. Scabs and swab samples were macerated, and the supernatant, which was diluted 1:100 in phosphate-buffered saline (PBS), was used in a nested PCR for the C11R (viral growth factor) gene, as described previously (*8*,*9*). OPV-specific fragments from all samples were amplified. The samples were also submitted to viral isolation in BSC-40 cells. We isolated the virus from 3 of the nested PCR viral growth factor–positive samples (1each from a cow, a hospitalized patient, and a nonhospitalized patient). All isolates induced the formation of small plaques, similar to group 1 VACV isolates previously identified in Brazil (*4*). After we observed typical poxvirus cytopathic effect, the viruses were plaque-purified and used to reinoculate a Vero cell monolayer for viral amplification.

The viral DNA from the A56R (hemagglutinin) gene was amplified and sequenced from all isolated viruses (*10*). The A56R gene is traditionally used for phylogenetic analysis and usually clusters VACV from Brazil (VACV-BR) into 2 groups (group 1: mice, nonvirulent; group 2: mice, virulent) (*4*). In addition, we sequenced DNA from the A26L viral gene (A-type inclusion body) (*11*). The obtained

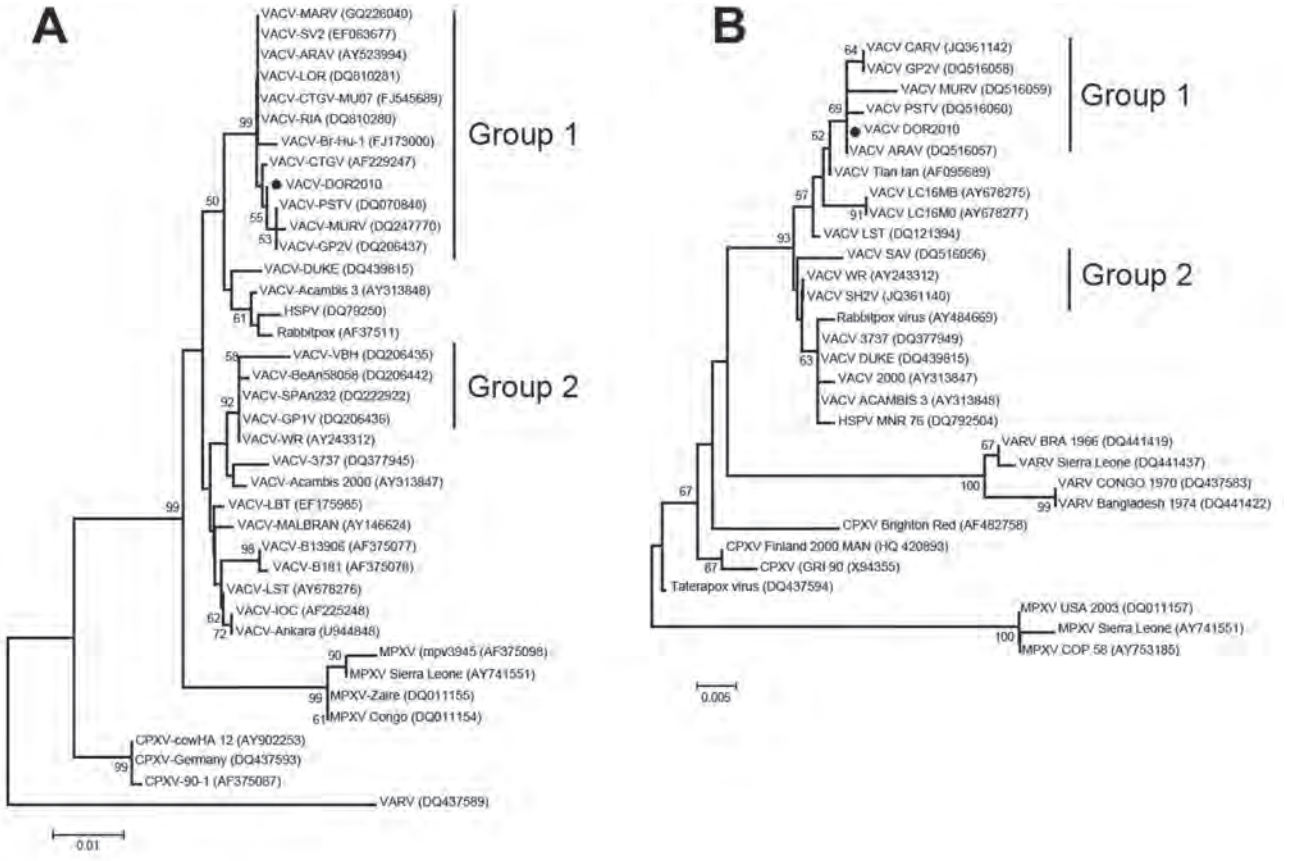

Figure. Phylogenetic trees based on the nucleotide sequences of the A56R (A) and A26L (B) genes of orthopoxvirus showing that DOR clusters with Brazilian vaccinia virus(VACV) genogroup 1.The trees were constructed by using the neighbor-joining method and the Tamura-Nei model of nucleotide substitutions with a bootstrap of 1,000 replicates using MEGA 4.0 (http://www.megasoftware.net). Dots highlight VACV DOR2010 among group 1 VACV isolates. GenBank accession numbers appear in parentheses. Scale bars indicate nucleotide substitutions per site.

PCR fragments were directly sequenced in both orientations in triplicate (Mega-BACE 1,000 sequencer; GE Healthcare, Buckinghamshire, UK). The sequences were aligned with previously published OPV sequences from GenBank by using ClustalW (*12*), and the alignments were manually verified by using MEGA 4.0 software (http://www.megasoftware.net). We named these isolates VACV DOR2010 (provisional GenBank accession no. 1606198).

Optimal alignment of the nucleotides from the A56R and A26L genes using ClustalW showed that all amplified DNA sequences from DOR2010 were identical and were highly identical to several group 1 VACV-BR isolates (99.7% identity [A56R] and 99.8% identity [A26L] average) (Figure). DOR2010 also showed a signature deletion of 18 nt in the A56R sequences of other group 1 VACV-BR isolates. Therefore, no special genetic feature was identified in the DOR2010 isolates in regard to A56R and A26L. Phylogenetic trees based on the nucleotide sequences of the A56R and A26L genes of OPV showed that DOR clustered with VACV-BR group 1 (Figure).

Given the severity of the outbreak, we investigated the virulence of this isolate in mice (following the rules of the Committee of Ethics for Animal Experimentation, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil). Sixteen BALB/c mice were divided into 4 groups of 4 mice each. We intranasally inoculated 4 mice with 10-μL doses of viral suspensions containing 10^6 plaque-forming units, as described previously (*4*). Two groups were inoculated with VACV-GuaraniP1 or VACV-GuaraniP2 (*4*), which served as virulent or nonvirulent controls, respectively. Another group was inoculated with PBS. The animals infected with VAVC-GuaraniP1 exhibited ruffled fur, arched backs, and weight loss. No clinical signs were observed in mice inoculated with DOR2010, VACV-GuaraniP2, or PBS, which supported the grouping of the DOR2010 sample into the nonvirulent cluster.

Conclusions

Our biologic, epidemiologic, and molecular data indicate that the VACV isolate DOR2010 was associated with an

Table. Clinical data of vaccinia virus–infected patients, Brazil, 2010*

Patient no.	Age, y	Signs/symptoms	Reported similar clinical features in previous years	Hospitalization, d	Case definition	Smallpox vaccinated
1	17	F, L, Ls hands, P	No	4	CC, H	No
2	25	F, L, Ls hands/arms, P	No	3	CC, H	No
3	24	F, L, Ls hands/knees, P	No	3	CC, H	No
4	20	F, L, Ls hands, P	No	3	CC, H, LC	No
5	21	F, L, Ls hands/face, P	No	4	CC, H, LC	No
6	21	F, L, Ls hands, P, C, V, D, M	NA	5	C, H	No
7	23	F, L, Ls hands, P	No	7	CC, H, LC	No
8	21	F, L, Ls hands, P, C, V, D, M	No	3	C, H	No
9	18	F, L, Ls hands, P	Yes	10	C, H	No
10	15	F, L, Ls hands/arms, P, C, V, D, M	No	18	CC, H, LC	No
11	26	F, L, Ls hands, P	NA	15	CC, H	No
12	18	F, L, Ls hands, P	NA	6	CC, H	No
13	46	F, L, Ls hands, P	No	No	CC, H	No
14	17	F, L, Ls hands/arms, P	No	No	CC, H	No
15	25	F, L, Ls hands, P	No	No	CC, H	NA
16	28	F, L, Ls hands, P	No	No	CC, H	NA
17	42	F, L, Ls hands/arm, P	No	No	CC, H, LC	NA
18	56	F, L, Ls hands, P	NA	No	CC, H	Yes
19	51	F, L, Ls hands, P	No	No	CC, H	NA
20	62	F, L, Ls hands, P	No	No	CC, H	Yes
21	55	F, L, Ls hands/knee, P	No	No	CC, H	Yes
22	43	F, L, Ls hands, P	NA	No	CC, H	NA
23	19	F, L, Ls hands/arm, P	NA	No	CC, H	No
24	18	F, L, Ls hands, P	NA	No	CC, H	No
25	25	F, L, Ls hands/arms, P	NA	No	CC, H	No
26	26	F, L, Ls hands, P	No	No	CC, H	No

*Data were obtained from the health center and based on observations and patient reports. C, convulsion; CC, clinical confirmation; D, diarrhea; F, fever; H, history of exposure; L, lymphadenopathy; LC, laboratory confirmation; Ls, exhanthematous lesion(s); M, mental confusion; NA, information not available; P, prostration; V, vomiting.

outbreak of severe, exanthematous vaccinia virus infection that resulted in the hospitalization of 12 workers in a rural area in Brazil. During the past decade, VACV has spread to all regions of Brazil, and no specific official national programs are in place to prevent the disease (*4*,*13*–*15*). Notifications and scientific efforts are needed to clarify the circulation, virulence, and diversity of VACV. Our data showed that DOR2010 does not present any special feature in A56R or A26L that justifies this unprecedented severity and infectivity among humans. Other studies suggested that group 2 VACV might be associated with severe illness, but such is not the case in the outbreak studied; however, very limited information is available about the relation between viral genotype and virulence in humans. Although useful for pathogenesis studies, our data indicate that the mouse model might not be considered a precise approach to indicate virulence potential of a viral isolate in humans (*4*). Exposure of OPV-nonvaccinated workers to VACV might, in part, explain those clinical features. Although previously infected—and vaccinated—patients were among the patients studied (Table), vaccination history (based on age, vaccine scar, and patient report) was strongly associated with severity of disease. The increased number of VACV outbreaks in recent years should be analyzed in the context of a worldwide phenomenon involving other zoonotic OPVs (*1*–*6*). We believe that the increased number of notifications will be followed by a concomitant increase in reports of atypically severe cases. A worldwide scientific and governmental debate is essential for zoonotic OPV control and prevention on the different continents affected by these viruses.

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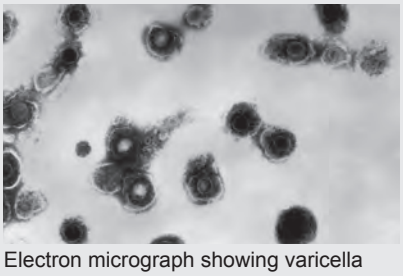
etymologia

Varicella Zoster Virus [var″i-sel′ə zos′ tər vi′rəs]

A member of the family *Herpesviridae*, varicella zoster virus (VZV) is named for the 2 main diseases (chickenpox and herpes zoster [shingles]) it causes. Varicella may be a diminutive of "variola" because it was considered a mild form of smallpox. "Variola" was coined by Rudolph Augustin Vogel in 1764 and is possibly derived from the Latin *varus* ("pimple") or *varius* ("speckled"). Herpes zoster derives from the Greek terms *herpein* ("to creep") and *zoster* ("belt"). Not until the twentieth century was VZV recognized as the cause of both these diseases.



Electron micrograph showing varicella zoster virus.

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Lack of Middle East Respiratory Syndrome Coronavirus Transmission from Infected Camels

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To determine risk for Middle East respiratory syndrome coronavirus transmission from camels to humans, we tested serum from 191 persons with various levels of exposure to an infected dromedary herd. We found no serologic evidence of human infection, suggesting that zoonotic transmission of this virus from dromedaries is rare.

Cases of Middle East respiratory syndrome (MERS) in humans continue to be reported from the Arabian Peninsula and the Middle East. The largest number of cases has been reported by Saudia Arabia: 818 cases leading to 351 deaths as of December 5, 2014 (*1*). The causative agent is MERS coronavirus (MERS-CoV), which is endemic to and ubiquitous among dromedary camels in the Arabian Peninsula and East and North Africa; seroprevalence among adult animals is typically >90% (*2*). MERS-CoV infection causes mild upper respiratory illness in dromedaries and remains detectable in nasal swab specimens for ≈1 week (*3*).

To look for serologic evidence of MERS-CoV infection in humans extensively exposed to a herd of infected dromedaries, we assessed seroprevalence among persons in close contact with an infected herd of ≈70 animals in Al Hasa, Saudi Arabia, during peak calving season, December 2013–February 2014 (*4*). The study was approved by the King Faisal University Research Ethics Committee.

The Study

The dromedaries were maintained in a fenced enclosure with barns. MERS-CoV was first detected in this herd on November 30, 2013; by December 30, of 11 sampled dromedaries, 9 were positive for viral RNA by reverse transcription PCR. The viruses isolated in November and December were genetically identical, suggesting ongoing transmission arising from introduction of a single virus (*4*).

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In February 2014, serum samples were obtained from persons with various levels of exposure to camels. Persons were divided into 5 groups.

Group 1 comprised 4 herdsmen who were in daily contact with the infected herd (feeding, grooming, administering treatment when needed). They frequently consumed fresh unboiled milk from the camels, of which at least 1 dam and 7 calves were retrospectively confirmed to have been MERS-CoV infected (*4*).

Group 2 comprised 8 persons who had intermittent but regular (several times/week) direct contact with the infected herd (animal management, feeding, manure removal) and included veterinary staff and attendants. Because this herd was also used for veterinary teaching and research, animals were frequently handled for clinical examination and specimen collection. With the exception of disposable gloves, which were worn by the veterinarians during examinations, personal protective equipment (masks, gloves, eye protection) was not used.

Group 3 comprised 30 veterinary surgeons and clinical support staff working at the Clinical Research Center at the Faculty of Veterinary Medicine and Animal Resources, King Faisal University, Saudi Arabia, who were not exposed to the infected herd. This largest clinical veterinary center in southeastern Saudi Arabia also serves the adjacent countries of the United Arab Emirates, Qatar, Bahrain, Kuwait, and Oman. Camels from across Saudi Arabia and the Gulf states are brought to this research center. Although we did not conduct MERS-CoV testing on camels brought for routine clinical care, some animals may have been shedding MERS-CoV. Staff members came into daily contact with domestic livestock of all species, including dromedaries, of which at least 20 arrived daily for treatment. Disposable gloves were used for examinations, but no respiratory or eye protection was used.

Group 4 comprised 3 workers in a camel abattoir in Al Hasa, where 25–35 camels were slaughtered daily. These workers did not wear personal protective equipment.

Group 5 comprised 146 persons in the same (Al Hasa) region who were not exposed to camels in their professional work. This group served as negative controls.

Serum from these 191 persons was tested for MERS-CoV antibodies by using a pseudoparticle neutralization assay that has been previously described, validated, and demonstrated to be at least as sensitive as microneutralization

[1]These authors contributed equally to this article.

assays (*5*,*6*). Evidence of a ≥50% reduction of signal was also sought as a more sensitive signal of neutralization, which would have been further confirmed in virus plaque neutralization assays.

Of the 191 human serum samples tested, none had serologic evidence of infection, even when a 50% reduction in luciferase signal was used as the threshold for suspected antibody (including those in groups 1 and 2 who had repeated close contact with the infected herd through December 2013). The virus infecting the herd was genetically similar to viruses that had previously infected humans, including in the amino acid sequence of the virus spike protein (*4*,*7*–*10*). We previously demonstrated that MERS-CoV from this infected herd had the capacity to efficiently infect ex vivo cultures from the human respiratory tract (*7*). These findings imply that group 1 and 2 members were repeatedly exposed over at least 1 month to dromedaries shedding a virus that was potentially competent to infect humans.

Conclusions

We conclude that MERS-CoV was not highly transmissible from dromedaries to humans with various levels of exposure to this infected dromedary herd. Two other studies have reported lack of serologic evidence of MERS-CoV infection among persons with various levels of exposure to dromedaries (*11*,*12*), but neither study documented MERS-CoV infection in the relevant animals. Studies of MERS-CoV RNA prevalence in nasal swab specimens from dromedaries in the field or abattoirs have found rates of 0%–15% (by PCR) among adults and 35% among calves (1 study) (*4*,*8*–*10*).

A study limitation is that precise events of individual-level human exposure to infected animals cannot be ascertained because different animals were infected at different times over the 1-month period. The timing of serum collection (4–6 weeks after putative exposure) from persons exposed to the infected herd was optimal in terms of detecting a serologic response. However, data on antibody titers after asymptomatic or mild MERS-CoV infections are limited, and our conclusion about lack of human infection must be subject to that caveat.

Our findings do not imply that dromedaries are not a source of infection for humans. Spillover infection of humans may be more common in other settings in which humans are exposed over sustained periods to animals among which virus prevalence is higher. For example, animals from diverse origins with varying immune status that are housed together in abattoirs before slaughter may provide opportunity for virus amplification and persistence, analogous to that seen with avian influenza virus in live poultry markets (*13*). Our study period (December–February) was timed to coincide with the calving season. However, human cases seem to peak during April–June, although this peak may be skewed by clusters of human-to-human transmission. Future longitudinal studies of dromedaries should be of longer duration.

Our finding that MERS-CoV is poorly transmissible from dromedaries to humans is compatible with the observed epidemiology of MERS-CoV infection in humans, in which human disease is not directly proportional to potential exposure to a virus that seems to be common in dromedary camels. Infections in dromedaries in settings such as abattoirs are regularly documented; thus, the numbers of humans exposed to virus-infected animals must greatly exceed the number of humans with diagnosed MERS-CoV infection. Conversely, some persons seem to acquire infection with apparently minimal or no apparent exposure to camels, even when secondary transmission from other infected humans is excluded. This setting is analogous to that observed for avian influenza (H5N1) virus, in which the virus can be ubiquitous in live poultry markets in some settings but human infection and disease remain stochastic and rare (i.e., not directly proportional to exposure) (*13*). The biological basis for such an epidemiologic pattern remains obscure for both viruses, avian influenza (H5N1) and MERS-CoV, but the heterogeneity of host susceptibility is a hypothesis to be explored (*7*,*14*). Further studies on the mechanisms by which MERS-CoV is transmitted from dromedaries to humans, whether by direct or indirect routes, and the heterogeneity of human susceptibility to this virus are needed.

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Safety of Recombinant VSV–Ebola Virus Vaccine Vector in Pigs

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The ongoing Ebola outbreak in West Africa has resulted in fast-track development of vaccine candidates. We tested a vesicular stomatitis virus vector expressing Ebola virus glycoprotein for safety in pigs. Inoculation did not cause disease and vaccine virus shedding was minimal, which indicated that the vaccine virus does not pose a risk of dissemination in pigs.

The current Ebola virus (EBOV) outbreak in West Africa has shown the need for an effective vaccine against this virus. As a result, clinical trials to test several vaccine candidates have been expedited (*1*) in hopes of contributing to containment of the outbreak. One of these vaccine candidates is based on a recombinant vesiculovirus vector, species vesicular stomatitis Indiana virus (here designated and more commonly known as VSV) expressing the EBOV strain Mayinga glycoprotein (here designated rVSVΔG/EBOVGP; formerly designated VSVΔG/ZEBOVGP) (*2*–*4*). This vaccine was highly efficacious in preexposure and postexposure studies in nonhuman primates after a single injection (*5*). In addition, the vaccine has been shown to be safe in simian HIV–infected rhesus macaques (*6*) and was not neurovirulent after intrathalamic inoculation into macaques (*7*).

However, because VSV is a World Organisation for Animal Health–listed pathogen (*8*), concerns might arise with regard to spillover of the vaccine vector to livestock when this vaccine is used on a larger scale in humans. To evaluate the safety of rVSVΔG/EBOVGP in a relevant livestock species, we inoculated pigs with this vaccine and compared clinical signs and virus replication with those of a recombinant wild-type VSV vector (rVSVwt) described previously (*3*).

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The Study

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Rocky Mountain Laboratories and performed following the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care, International. Experiments were performed by certified staff in an Association for Assessment and Accreditation of Laboratory Animal Care AAALAC–approved facility, following the guidelines and basic principles in the US Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for Care and Use of Laboratory Animals.

Four-week old pigs (Yorkshire cross) were obtained from the Washington State University College of Veterinary Medicine (Pullman, WA, USA). One group of 5 pigs and 1 group of 6 pigs were inoculated with rVSVwt and rVSVΔG/EBOVGP, respectively, as controls; 2 animals were mock inoculated with culture medium (Dulbecco modified Eagle medium). Animals were inoculated with 10^6 PFUs of either virus in a 100-μL volume, or an equal volume of Dulbecco modified Eagle medium by intradermal injection in the apex of the snout (*9*).

At regular intervals after inoculation, clinical examinations were performed to determine the health status of the animals and to collect nasal, throat, and rectal swab samples for virologic analysis; blood was collected to determine the humoral immune response. Three animals inoculated with rVSVwt and rVSVΔG/EBOVGP were euthanized at 3 days postinoculation (dpi) as per protocol; the remaining animals were euthanized at 21 dpi.

Inoculation of pigs with rVSVwt and rVSVΔG/EBOVGP did not result in obvious signs of disease (Table), changes in body temperature, or a decrease in weight gain compared with mock-inoculated controls. A nose lesion developed at 4 dpi at the injection site in 1 animal inoculated with rVSVwt, but this lesion healed by 9 dpi. Swab specimens collected from the lesion site on 5, 6, 7, 8, and 10 dpi were negative by virus titration. Nose, throat, and rectal swab specimens were collected at 1, 3, 6, 10, 14, and 21 dpi; a nose swab specimen collected at 3 dpi from a pig inoculated with rVSVΔG/EBOVGP was the only specimen in which virus could be detected (virus titer $10^{0.83}$ 50% tissue culture infectious dose [$TCID_{50}$]/mL) (Table).

Three animals in each group were euthanized at 3 dpi. Tissue samples from lip, tongue, snout, footpad, coronary

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Table. Findings for pigs inoculated with rVSVwt and rVSVΔG/EBOVGP*

Inoculum	Clinical signs	VSV lesions	Virus shedding from			Viremia	Virus replication in tissues	Seroconversion at 21 dpi	
			Nose	Throat	Rectum			VSV-G	EBOVGP
Mock (control)	ND	ND	ND	ND	ND	ND	ND	ND	ND
rVSVwt	ND	1/5†	ND	ND	ND	ND	2/5‡	2/2	ND
rVSVΔG/EBOVGP	ND	ND	ND	1/6§	ND	ND	2/6¶	ND	3/3

*rVSVwt, recombinant wild-type vesicular stomatitis virus; rVSVΔG/EBOVGP, recombinant VSV expressing Ebola virus strain Mayinga glycoprotein; VSV-G, VSV glycoprotein; dpi, day postinoculation; ND, not detected.
†Lesion at inoculation site.
‡Snout positive in virus titration in 2 of 3 animals at 3 dpi.
§One swab collected from 1 animal positive at 3 dpi.
¶Snout positive in 1 of 3 animals at 3 dpi; inguinal lymph node positive in 1 of 3 animals at 3 dpi.

band, interdigital skin, tonsil, oronasopharynx, inguinal lymph node, axillary lymph node, cervical lymph node, mesenteric lymph node, bronchial lymph node, nasal mucosa, trachea, bronchus, lungs, heart, liver, spleen, kidney, adrenal gland, pancreas, jejunum, colon, urinary bladder, cervical spinal cord, frontal brain, cerebellum, and brain stem of these animals were collected for virus titration and histologic analysis.

In 2 of 3 animals inoculated with rVSVwt, virus was detected in the snout (virus titers $10^{2.3}$ and $10^{2.6}$ $TCID_{50}$/g), but virus was not detected in any of the other tissues collected at 3 dpi. In 1 of the pigs inoculated with rVSVΔG/EBOVGP, virus was detected in the snout (virus titer $10^{2.5}$ $TCID_{50}$/g) and in another pig in an inguinal lymph node (virus titer $10^{5.1}$ $TCID_{50}$/g). This high titer in the inguinal lymph node might have been the result of a change in cell tropism caused by use of EBOVGP rather than VSV glycoprotein (VSV-G). Histologic analysis did not identify lesions consistent with VSV infection in the rVSVwt– or rVSVΔG/EBOVGP–inoculated animals. At the end of the experiment at 21 dpi, the same tissues were collected for virologic and histologic analysis; virus could no longer be detected in any of the tissues derived from the inoculated animals, and no histologic lesions were present.

Serum samples collected at 21 dpi were analyzed for IgG against VSV-G or EBOVGP by using an IgG ELISA and secreted forms of these glycoproteins as antigens (*10*,*11*), respectively. In the VSV-G ELISA, pigs inoculated with rVSVwt showed an antibody response to VSV-G at 21 dpi (titers 1:400 and 1:1600); animals inoculated with rVSVΔG/EBOVGP did not show seroconversion to VSV-G. In an ELISA specific for the EBOVGP, pigs inoculated with rVSVΔG/EBOVGP showed a robust antibody response (titers 1:800, 1:1,600, and 1:3,200 by 21 dpi), but animals inoculated with rVSVwt did not show seroconversion to EBOVGP.

Conclusions

Our data indicate that, although rVSVΔG/EBOVGP can replicate in pigs, this vaccine virus does not result in overt clinical disease, and virus shedding is minimal. Because a high dose of the vaccine was directly injected intradermally into the snouts of the animals in this study and yet did not cause disease, it is unlikely that vaccination of humans with the rVSVΔG/EBOVGP vector would result in a productive infection with clinical disease in domestic pigs during a spillover event. Moreover, even if this spillover were to occur, the near absence of virus shedding in the rVSVΔG/EBOVGP–infected animals suggests that spillover would not result in maintenance of rVSVΔG/EBOVGP within a pig herd. This study provides data to support the safety of the live-attenuated VSVΔG/EBOVGP vaccine in a relevant livestock species. Should exposure/infection of pigs occur during a vaccination trial in humans, it is highly unlikely that signs of disease would develop in pigs or that the vaccine virus would be disseminated by interspecies or intraspecies transmission.

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Lives of a Cell: 40 Years Later, A Third Interpretation

Virginia M. Dato[1]

In 1974, Lewis Thomas (1913–1993), physician, professor, and dean, published The Lives of A Cell (*1*), the first of 2 books subtitled Notes of a Biology Watcher (*1*,*2*). The phrase "lives of a cell" refers to the independent yet interrelated parts of a human cell—including mitochondria, centrioles, and basal bodies—that once led independent lives. Without these previously independent lives working together, we would not have the capacity for thought, communication, and movement. Dr. Thomas wrote, "Our membranes hold against equilibrium, maintain imbalance, bank against entropy…. We are shared, rented and occupied."

Our human lives do not depend just on the lives in our individual cells. Our lives depend fully on the earth, including the atmosphere, and the many other human and nonhuman lives that occupy it. In explaining this complex interdependence, Dr. Thomas observed that the earth is "most like a cell." This second interpretation of lives of a cell refers to the many interrelated earthly entities, such as plants, whales, humans, and even viruses, that "dart rather like bees from organism to organism, from plant to insect to mammal to me and back again," all protected by the sky—a membrane that "works, and for what it is designed to accomplish it is as infallible as anything in nature."

PulseNet identifies disease outbreaks by connecting DNA "fingerprints." PulseNet, established by the Centers for Disease Control and Prevention (Atlanta, GA, USA) in 1996, comprises 87 laboratories in all 50 states (*3*,*4*). The labs develop and compare DNA patterns from bacterial pathogens submitted by state, Food and Drug Administration, and US Department of Agriculture laboratories from across the nation (*5*). The work of PulseNet provides insight into the lives of a bacterial cell through DNA pattern matches. For some matches, the connection is clear; for other matches, no connection is known. Yet these bacteria are not just the same strain or type; they have identical or nearly identical patterns of DNA.

In his essay "The Wonderful Mistake" (*2*), Dr. Thomas wrote, "The capacity to blunder slightly is the real marvel of DNA. Without this special attribute, we would still be anaerobic bacteria and there would be no music." Some DNA differences result from blunders during the DNA replication necessary for cell division. Others result from mobile genetic elements—snippets of DNA that are able to move between bacteria (*6*). Suddenly a cell exists that creates a brand new pattern. As each of the cell progeny divides, the new pattern lives on in each new clone. These progeny are the lives of a cell.

Some patterns are stable and spread so far and wide so long ago that they exist throughout nature. The PulseNet specimens have no connection other than symbiotic flora, colonization, contamination, or infection by bacteria that at some point—years ago—were progeny of the same cell. Then one of those cells mutates through a DNA insertion, deletion, or point mutation, and suddenly a new pattern appears. If the lives of this cell appear in 2 specimens sent to PulseNet, connections are revealed across time and space.

A factory produces a contaminated product, and the lives of a cell connect a retired person on a limited income to a young adult consuming the same product in a different county. A contamination in dog food can sicken a dog's human owners, while the dogs remain healthy. Contamination on a farm can send lives of a cell across the country to rich and poor.

Dr. Thomas described a "half way technology" (*1*); "at the same time highly sophisticated and profoundly primitive." It represents things "one must continue to do until there is a genuine understanding of the mechanisms involved in disease." Examples included organ transplants and iron lungs. Like these, connecting lives of a cell can be labor- and resource-intensive.

In the case of human infections, unavoidable delays occur between the time a person is exposed to bacteria, becomes sick, and provides a sample for culture; the culture grows; the bacterium is identified in the laboratory, grown on media for transportation, and sent to a second laboratory; a pattern is determined; and matches are identified. After a match is determined, investigations often require repeated phone calls, followed by intensive testing of products and finally massive recalls. If the pattern disappears, investigations may be suspended before a common source is found.

All 3 interpretations of lives of a cell—the once independent lives of a single cell, the many lives (human, animal, bacterium, fungus, virus) of the earth, and the lives of a bacterial cell that travels throughout the earth—strongly suggest a need for multidisciplinary and interdisciplinary collaborations, i.e., "One Health." The One Health Initiative

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—One World, One Medicine, One Health—has as its foundation the work of Dr. Thomas and many other great scientists. The One Health vision is to improve (and help save) "the lives of all species—human and animal—through the integration of human medicine, veterinary medicine and environmental science" (http://www.onehealthinitiative.com/mission.php).

Publicity about recalls may leave the perception that our food supply is riskier than before PulseNet. On the contrary, following the lives of a cell leads to a new understanding of disease mechanisms. As many diverse professionals work together and pool knowledge to develop economical solutions, our food supply becomes safer. This is the "real high technology" that Lewis Thomas described in The Technology of Medicine (*7*). "When it becomes available it is relatively inexpensive, simple, efficacious, expeditious and easy to deliver"—and is thus One Health in action (*8*).

Acknowledgment

I thank Bruce Kaplan and many anonymous reviewers for thoughtful suggestions and editing necessary to honor a great man—Lewis Thomas—and advance his observations as a biology watcher.

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Enterovirus A71 Subgenotype B5, France, 2013

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To the Editor: We report the detection of human enterovirus A71 (EV-A71) subgenotype B5 in France, 6 years after it was first detected in Europe. EV-A71 belongs to the *Enterovirus A* species (genus *Enterovirus*, family *Picornaviridae*) and is a major cause of hand, foot and mouth disease (HFMD), sometimes associated with severe neurologic complications (*1*). EV-A71 strains are classified in 6 genotypes, A–F, (*2*) but most of the circulating strains belong to genotypes B and C and to 11 subgenotypes (B0–B5, C1–C5) (*1*). Genotypes B and C have been reported in HFMD epidemics in the Asia-Pacific region; different subgenotypes cause nationwide epidemics that usually occur every 2–3 years (*1*). During 1963-1986 in Europe, Australia, and the United States, enterovirus infections were caused by viruses from subgenotypes B0, B1, and B2, but since 2000, infections with C1 and C2 viruses have begun to predominate (*3*,*4*). The other subgenotypes have been reported rarely in Europe; the C4b and C4a strains were identified in France, Germany, Austria, and Denmark in 2004 and 2012, respectively (*4*–*6*), and the B5 subgenotype was reported in Denmark in 2007 (*7*).

In November 2013, a 3-week-old boy was admitted to the emergency unit of a hospital in Compiègne, France, with a 48-hour history of fever and irritability. He was born at term after an uneventful pregnancy and delivery. On admission, he had normal vital signs. He had had contact with a cousin with oral ulcerations, but no information was available about the source of the cousin's infection.

Laboratory testing revealed moderate cytolytic hepatitis. Complete blood count results were within reference values. Cerebrospinal fluid showed pleiocytosis (38 leukocytes, 81% polymorphonuclear cells) with protein and glucose levels with reference ranges. Bacterial cultures of blood and cerebrospinal fluid were negative. Enterovirus genome was detected in serum samples and cerebrospinal fluid by reverse transcription PCR. The infant made a steady recovery and was discharged 10 days after admission, with no apparent adverse outcome. Final diagnosis was neonatal enterovirus infection with meningitis.

Genotyping was performed on the serum specimen by using seminested reverse transcription PCR amplification and sequencing of the viral protein 1 gene. Phylogenetic investigation with sequences of reference strains representing all subgenotypes indicated that the isolate from the patient, designated PAR024102_FRA13, belonged to the EV-A71 B5 subgenotype. We investigated the putative origin of the strain by comparing 248 nonredundant complete 1D sequences of EV-A71 B5 strains following a Bayesian phylogenetic approach. PAR024103_FRA13 shared a most recent common ancestor (posterior probability = 1) with virus strains sampled in China in 2009 and in Taiwan during the 2011–2012 outbreak, (*8*) but was only distantly related to them (data not shown).

Further analyses with 274 partial 1D gene sequences from GenBank (on March 19, 2014) indicated close genetic relationships (posterior probability = 1) with strains isolated in Thailand in 2012 (*9*) (Figure). The complete genome of PAR024103_FRA13 was determined by nucleotide sequencing of 4 overlapping segments obtained by gene amplification (GenBank accession no. LK985324). Sequence comparisons were performed with 13 available EV-A71 B5 complete genomes; the virus strain isolated in France exhibited 92%–99.5% nt similarity (98.9%–99.4% aa similarity) throughout the genome.

The EV-A71 B5 subgenotype was first detected in 1999 in Malaysia and spread to several other countries in Asia during the 2000s. Outbreaks causing severe illness and deaths were reported in Japan (2003), Brunei (2006), and Taiwan (2008 and 2012) (*1*,*8*). The first detection of subgenotype B5 in Europe was associated with a recrudescence of EV-A71 infections associated with meningitis and HFMD in Denmark in 2007 (*7*). The overall phylogenetic data are consistent with an introduction of EV-A71 B5 in France by importation of a strain from Asia, possibly from Thailand. Transmission of EV-A71 strains has been shown to occur in Europe as discrete and temporally defined virus introductions, occasionally followed by sustained dissemination (C. Hassel, unpub. data).

The emergence of the Asiatic lineage EV-A71 C4a in Denmark in 2012 is a recent event (*6*). The reemergence of EV-A71 subgenotype B5 in 2008 in Taiwan resulted in the largest outbreak of EV-A71 infection in the past 11 years (*8*). To our knowledge, the B5 subgenotype has not previously been detected in Europe. Global herd immunity produced by circulation of the C2 genotype may protect the European population from the spread of other subgenotypes (*3*). However, given that most countries in Europe do not perform specific surveillance for HFMD and most enterovirus infections are asymptomatic, this particular subgenotype could be circulating more widely without detection.

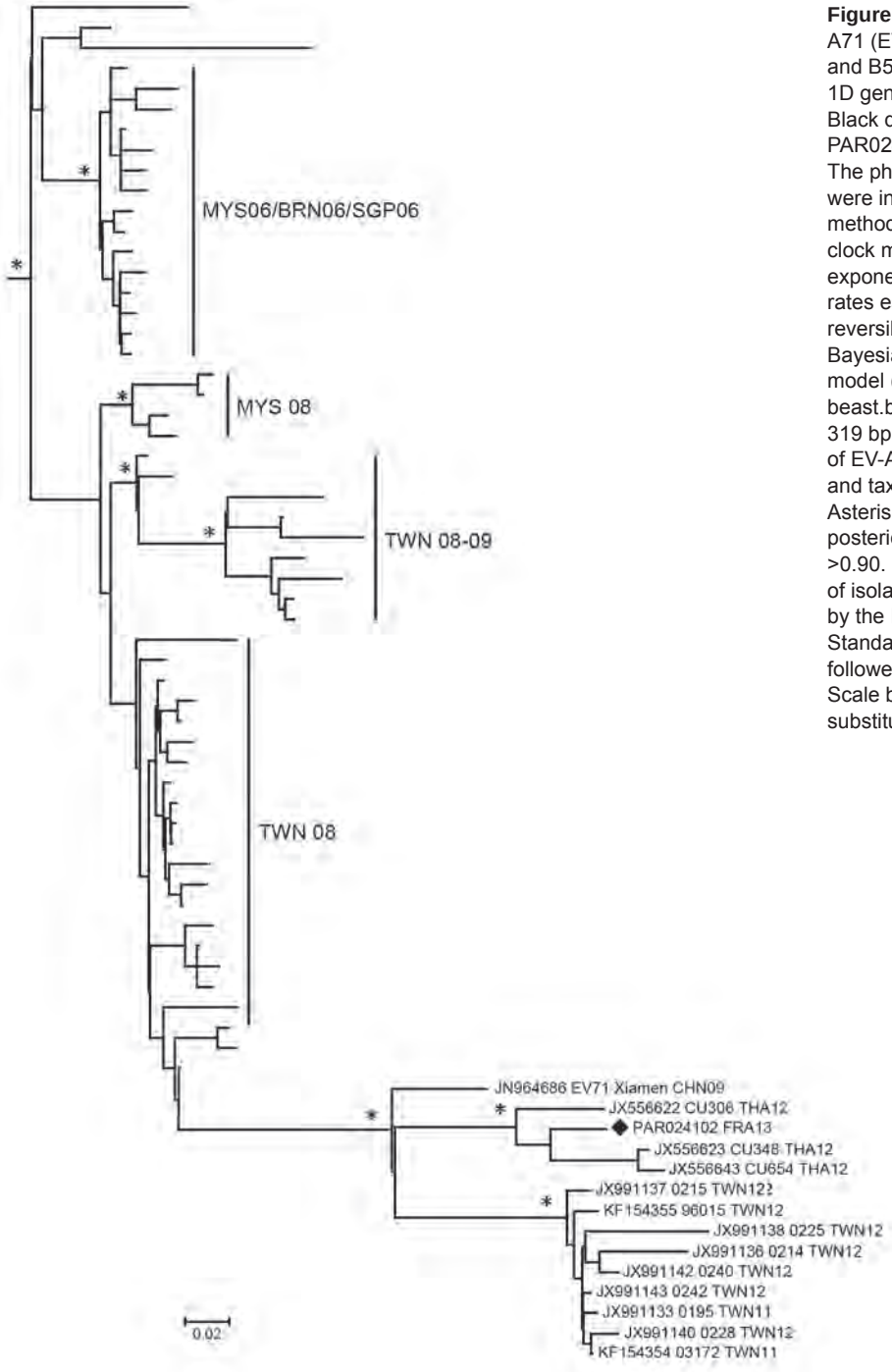

Figure. Phylogeny of enterovirus A71 (EV-A71) subgenogroups B4 and B5 inferred with 274 partial 1D gene sequences, France. Black diamond indicates strain PAR024103_FRA13 from this study. The phylogenetic relationships were inferred following a Bayesian method by using a relaxed molecular clock model with an uncorrelated exponential distribution of evolution rates estimated with a general time reversible substitution model and a Bayesian skyline plot as a population model (BEAST version 1.7.5; http://beast.bio.ed.ac.uk/). Sequences were 319 bp. For clarity, only a subtree of EV-A71 B5 sequences is shown, and taxon names are not included. Asterisks indicate nodes with posterior probability density values >0.90. Geographic origins and time of isolation of strains are indicated by the International Organization of Standardization code abbreviation followed by the year of isolation. Scale bar indicates nucleotide substitutions per site.

Enterovirus infections in neonates and infants are a frequent cause of hospitalization, which may contribute to EV-A71 detection (*5*). However, the development of a national syndromic surveillance targeting HFMD would enable early detection of HFMD outbreaks and any new EV-A71 subgenotype. Attention should also be paid to the potential risks of epidemic spread of EV-A71 outside Asia posed by international travelers.

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Avian Influenza A(H7N9) Virus Antibodies in Close Contacts of Infected Persons, China, 2013–2014

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To the Editor: From early 2013 (*1*) through November 2014, >460 human cases of laboratory-confirmed avian influenza A(H7N9) virus infection occurred in China. Although human-to-human transmission of subtype H7N9 virus is not common, evidence has been reported of probable transmission among several family clusters (*2*), between 2 household contacts (*3*), and between a doctor and an infected patient (*4*). Taken together, these observations suggest that family members, health care providers, and other close contacts (hereafter called contacts) of H7N9-infected persons may be at risk for infection.

In China, national guidelines regarding H7N9-infected patients call for observation of contacts for 7 days after exposure for signs and symptoms of infection and, if any occur, collection of throat swab specimens for testing by molecular assays (*5*). The guidelines do not call for serologic testing. Because human avian influenza infections may be mild or asymptomatic, we sought to determine whether serologic testing would show evidence of H7N9 virus infection among contacts of infected persons during the 2013–2014 epidemic in China. Contacts were defined in accordance with China's guidelines for prevention and control of human H7N9 virus infection (*5*,*6*). The institutional review board of Wuxi Center for Disease Control and Prevention, Wuxi, Jiangsu Province, China, reviewed and approved this study.

During the epidemic, we recruited contacts of patients in Wuxi and collected throat swab specimens when signs or symptoms of infection developed; serum samples were collected 2–3 weeks later. Swab specimens were tested for H7N9 virus by using real-time reverse transcription PCR (*7*). Serum samples were tested for antibodies against hemagglutinin antigens of 3 avian influenza viruses (A/Anhui/1/2013 [H7N9], A/Anhui/1/2005 [H5N1]-RG5, and A/chicken/Jiangsu/1/00 [H9N2]) (*8*) by using a horse erythrocyte hemagglutination inhibition (HI) assay and against the hemagglutinin antigens of 2 seasonal influenza viruses (A/California/07/2009 [H1N1] and A/Victoria/210/2009 [H3N2]) by using a turkey erythrocyte HI assay. Serum samples with HI titers ≥1:40 against H7N9 virus were confirmed positive by microneutralization assay.

Ten laboratory-confirmed human infections with H7N9 virus occurred in Wuxi during March 29, 2013–May 15, 2014. In total, 225 contacts of 7 H7N9-infected patients

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[2]These senior authors contributed equally to this article.

were enrolled in the study (Table); contacts included 30 family members; 177 health care workers (54 physicians, 119 nurses who provided patient care with standard precautions, 2 hospital attendants, and 2 nurse assistants who provided services related to patient care, safety, and comfort, including anxiety relief, and medical observation); and 18 other contacts (8 friends who visited the patient in the hospital, 2 patients who shared the same room, and 8 patients who shared the same hospital area). The contacts of 3 other H7N9-infected patients declined to participate in the study.

Serologic assay results showed that, 14–28 days after their earliest exposure to an H7N9-infected patient, 22 (9.8%) contacts had elevated HI antibody titers (≥1:40) against H7N9 virus; titers were 1:40 for 17 contacts and 1:80 for 5 contacts. Positive results for all 22 serum samples were validated by microneutralization assay; 15 (68.2%) samples had microneutralization antibody titers of ≥1:10 against H7N9 virus antigen (Table). Of the contacts with an HI titer of ≥1:80 and microneutralization titer of ≥1:40, 3 were nurses, 1 was a nurse assistant, and 1 was a family member (a patient's daughter). All 5 of these contacts had antibody titers of <1:40 to influenza subtype H1N1, H5N1, and H9N2 viruses, and 2 of the nurses had HI antibody titers of 1:80 against subtype H3N2 virus. All contacts denied having influenza-like respiratory symptoms during the 28 days of follow-up and also denied recent exposure to poultry or pigs or their environments. Of contacts with an HI titer of ≥1:80 to seasonal H1N1 virus, 3 had titer of 1:80, and 1 each had titer of 1:160 or 1:640. Of the 225 contacts, 108 had HI titers ≥1:80 against seasonal H3N2 virus (1:80 for 63 contacts, 1:160 for 27 contacts, 1:320 for 9 contacts, and ≥1:640 for 8 contacts). All contacts had influenza subtype H5N1 and H9N2 antibody titers of <1:80.

A previous epidemiologic study (*2*) reported the medical monitoring of 2,657 contacts of H7N9-infected patients in mainland China and found that, for 28 of the contacts, respiratory symptoms developed within 7 days after monitoring began. Results of molecular assay testing of throat swab specimens for H7N9 virus were negative for all 28 contacts; the study did not include serologic testing. However, small serologic survey studies in Taiwan (*9*) and household contacts in mainland China (*10*) showed no evidence of human-to-human transmission among contacts.

A limitation of our study is that we did not collect serum samples from all contacts of infected persons or from controls; therefore, we could not assess the possibility of

Table. Demographic characteristics and HI antibody titers against influenza subtype H7N9, H5N1, H9N2, H1N1, and H3N2 viruses among close contacts of avian influenza A(H7N9)–infected persons, China, 2013–2014*

Characteristics	Close contacts, N = 225		
	Family members, n = 30	Health care workers, n = 177	Others, n = 18
Mean age, y ± SD	48.03 ± 17.79	33.71 ± 7.97	68.50 ± 14.89
Sex			
F	18 (60.0)	135 (76.3)	4 (22.2)
M	12 (40.0)	42 (23.7)	14 (77.8)
Exposure duration, mean days ± SD	7.38 ± 4.70	4.42 ± 3.67	3 ± 1.48
Virus subtype and HI titer†			
H7N9			
<1:80	29 (96.7)	173 (97.7)	18 (100.0)
≥1:80	1 (3.3)	4 (2.3)	0
H5N1			
<1:80	30 (100.0)	177 (100.0)	20 (100.0)
≥1:80	0	0	0
H9N2			
<1:80	30 (100.0)	177 (100.0)	20 (100.0)
≥1:80	0	0	0
H1N1			
<1:80	30 (100.0)	172 (97.1)	18 (100.0)
≥1:80	0	5 (2.9)	0
H3N2			
<1:80	20 (66.7)	89 (50.3)	9 (50.0)
≥1:80	10 (33.3)	88 (49.7)	9 (50.0)
MN titer, H7N9‡			
<1:10	0	6 (35.3)	1 (100.0)
1:10	0	4 (23.5)	0
1:20	3 (75.0)	3 (17.6)	0
1:40	0	3 (17.6)	0
1:80	1 (25.0)	1 (5.9)	0

*Data are no. (%) unless otherwise indicated. A comparison of HI titers for control serum samples against reference influenza virus strains used in this study is shown in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/21/4/14-1442-Techapp1.pdf). HI, hemagglutination inhibition; MN, microneutralization assay.
†HI titer cut points were selected conservatively at ≥1:80 on the basis of World Health Organization recommendations for human infection with influenza A(H5N1) virus (http://www.who.int/influenza/resources/documents/RecAllabtestsAug07.pdf).
‡Results for 22 close contacts (17 health care workers, 4 family members, and 1 other close contact) with an HI titer of ≥1:40.

false-positive results or asymptomatic infections. However, our findings of elevated levels of subtype H7N9 antibody among 6.7% of contacts during this epidemic in China offer evidence that human-to-human transmission of H7N9 virus may occur among contacts of infected persons.

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Hepatitis E Epidemic, Biratnagar, Nepal, 2014

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To the Editor: We report a recent epidemic of hepatitis E in Biratnagar, Nepal. During the third week of April 2014, a total of 11 patients with acute jaundice came to hospitals in Biratnagar. IgM against hepatitis E virus (HEV) was detected in serum samples from all 11 patients. During the next 7 weeks, 1,861 patients with acute jaundice came to the outpatient departments of 2 of 5 large hospitals in Biratnagar; 123 patients were admitted to these 2 hospitals.

Registries at these 2 hospitals indicated that 2 patients with acute jaundice came to these hospitals on April 14. On April 28; May 5, 12, 19, and 26; and June 2, 9, 16, 23, and 30, the number of patients with acute jaundice who came to these 2 hospitals were 42, 67, 58, 69, 48, 21, 5, 3, 1, and 0, respectively. Registries showed that this increased frequency of acute jaundice lasted until the end of May 2014, when it began to decrease and reached near zero by the first week of July. In addition, unusually large numbers of patients with acute jaundice came to 25 smaller private health care facilities in Biratnagar during April–May 2014.

The Private and Boarding Schools' Organization of Nepal closed 80 schools in Biratnagar and surrounding areas during the second week of May 2014 because of risk for disease transmission (*1*). The Biratnagar Zonal Health Authority and National Health Authority of Nepal issued special alerts by mass media regarding jaundice after the third week of April and advised using boiled water for consumption (*2*).

Registries of major hospitals, smaller health clinics, and private physicians indicated that ≈7,000 patients were

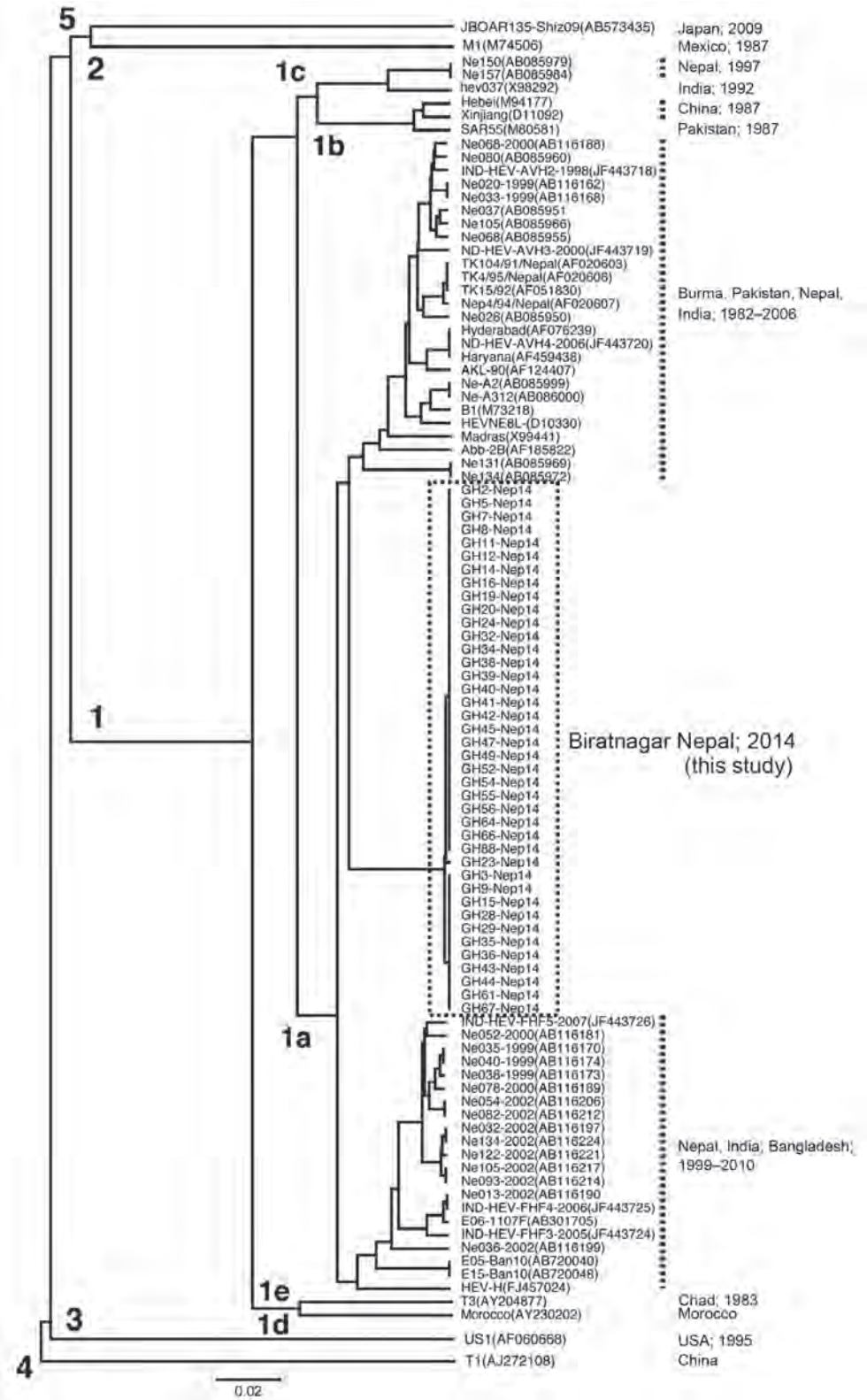

Figure. Phylogenetic analysis (UPGMA) of hepatitis E virus (HEV) sequences from the epidemic in Biratnagar, Nepal, 2014, on the basis of 412 nt within open reading frame 2 (DDBJ/EMBL/GenBank accession nos. AB98608–AB986107). All 40 HEV isolates from epidemic in Biratnagar segregated into a single, new cluster within genotype 1a. Values along branches indicate genotypes. Scale bar indicates nucleotide substitutions per site.

affected during this epidemic. Fourteen patients died; these deaths occurred in Kathmandu, the capital of Nepal, or in different cities in India after these patients were transferred there for better treatment. Fifty pregnant women had acute jaundice, but none of these women died.

The epidemic was presumed to be caused by consumption of contaminated water (*3*). In February and March 2014, water and sewerage pipelines were damaged in different areas of Biratnagar during construction and repair of roads. A survey conducted by the Department of Community Medicine, B.P. Koirala Institute of Health (Dharan, Nepal), found high levels of coliform bacteria in water supplies from different areas in Biratnagar during the epidemic. Tap water also looked cloudy and visibly contaminated (*3*).

To obtain more information about the epidemic, the incidence of acute jaundice was determined for 656 prisoners and 75 security personnel at the Biratnagar Jail. The study protocol was approved by the Liver Foundation Nepal. Informed consent was not obtained because identity of patient samples remained anonymous. Acute jaundice was detected among 30 (4.6%) prisoners and 4 (5.3%) security personnel. The same source of consumable water was used by the general population, inmates, and security personnel in Biratnagar.

To identify the causative agent of this epidemic, serum samples from 48 patients were obtained at Koshi Zonal Hospital, the largest government hospital in this zone. Hepatitis A virus RNA and IgM against hepatitis B virus core antigen was not detected in the 48 serum samples. Conversely, IgG, IgM, and IgA against HEV were detected in 47 (97.9%), 45 (94%), and 45 (94%) serum samples, respectively, and HEV RNA was detected in 42 (87.5%) of 48 serum samples, which indicated that the epidemic was caused by HEV.

A partial 412-nt sequence from open reading frame 2 corresponding to nt 5944–6355 of the HEV B1 genome (*4*) was obtained as reported (*5*). We obtained 40 HEV isolates from the 42 samples and sequenced partial 412-nt segments. All 40 HEV sequences from the epidemic in Biratnagar segregated into a cluster within genotype 1a (Figure). These sequences showed 99.8% nt identity with each other but only 90.8%–95.4% nt identity with other HEV isolates from Nepal and those from India, Bangladesh, Pakistan, and China.

Compared with previous HEV epidemics in Nepal (*6*) and other parts of the Indian subcontinent, the local government of Biratnagar and central government of Nepal took steps to contain the reported epidemic. Activities of public and private sectors in Biratnagar ended the epidemic in ≈12 weeks, and no new cases of acute jaundice have been reported in Biratnagar.

Persons in Biratnagar were given information regarding epidemics and ways to contain them. They were instructed by electronic media to use boiled water for consumption. It became clear that additional information regarding about maintaining water and sewerage systems during road construction and repair should also be provided. Because 14 patients died of HEV infection during this epidemic, more preparedness for epidemics of waterborne diseases is required to minimize unnecessary illnesses and deaths.

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Human Parvovirus 4 Infection among Mothers and Children in South Africa

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To the Editor: Human parvovirus 4 (PARV4) is a single-stranded DNA virus in the family *Parvoviridae* (*1*).

In Western countries, IgG against PARV4 is largely found only in persons with risk factors for parenteral infection and is strongly associated with co-infection with bloodborne viruses (*2*–*4*). In Africa, transmission seems to be more complicated; reported PARV4 seroprevalence is 4%–37%, even among persons at low risk and with no evidence of HIV or hepatitis C virus (HCV) co-infection (*1*,*5*,*6*).

The clinical significance of PARV4 infection remains uncertain. Infections may be asymptomatic, but a variety of clinical associations have been reported (*1*,*7*), including an increased rate of progression to AIDS in persons co-infected with HIV (*8*). This association raises particular concerns for many African populations in which these viruses are co-endemic.

To characterize the epidemiology of PARV4 infection in South Africa, we studied adults and children from pediatric outpatient clinics in Kimberley, South Africa, during May 2009–August 2013. Of the 157 participants, 90 were HIV-1–infected children, 24 their HIV-negative siblings, and 43 HIV-1–infected mothers (of whom 4 had >1 child enrolled). Approval was given by the Ethics Committee of the Faculty of Health Science, University of Free State, South Africa. Written consent was given by all adults and parents/guardians on behalf of their children.

Blood samples were collected from participants, and serum was tested for evidence of PARV4 infection by using ELISA (in duplicate) to detect IgG against PARV4 viral protein 2 (*3*,*6*) and by using PCR to detect PARV4 DNA (*9*). For 92 patients, HIV RNA loads were available; testing was performed by using the Abbott Laboratories m2000 platform (Abbott Park, IL, USA). For 118 of the HIV-infected patients, CD4+ T-cell counts were ascertained by flow cytometry. Statistical analyses were undertaken by using Prism version 6.0f and online software (http://graphpad.com/quickcalcs/). Confidence intervals were calculated by using the adjusted Wald method (http://www.measuringusability.com/wald.htm).

We detected IgG against PARV4 in 58 (37%) of 157 patients; this proportion is broadly comparable with that reported from other settings in sub-Saharan Africa, including Burkina Faso, the Democratic Republic of the Congo, and a previous cohort of HIV-infected persons in South Africa (*5*). Although routes of transmission in Africa remain to be characterized, these high seroprevalence rates support the possibility that some PARV4 transmission may be occurring by nonparenteral routes, as suggested by others (*5*,*10*).

PARV4 IgG seroprevalence was higher among adults (49%) than children (33%), although this difference did not reach statistical significance (p = 0.07, Fisher exact test; Figure, panel A). We found a significant relationship between increasing age and PARV4 IgG serostatus ($R^2 = 0.59$ by linear regression, p = 0.025; Figure, panel B). The numbers in each group are small, and further work is needed to define this association with more confidence. We did not detect any cases of PARV4 viremia, suggesting that chronic viremia or reactivation are probably uncommon, even among HIV-infected patients.

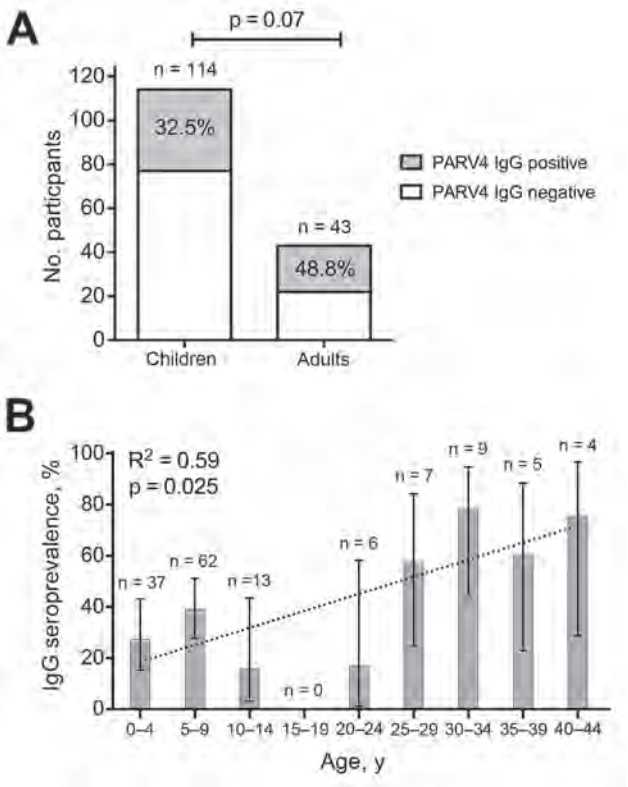

Figure. Relationship between age and seroprevalence of IgG against human parvovirus 4 (PARV4) among 157 mothers and children in Kimberley, South Africa, 2009–2013. A) Number and proportion of children and adults seropositive for IgG against PARV4; the number in each group is shown above the bar. p value calculated by using the Fisher exact test. B) Proportion of population seropositive for IgG against PARV4 according to age; the number in each group is shown above the bar. Data are shown for 143 persons because no date of birth was recorded for 2 children and 12 adults. Error bars show 95% CIs calculated by the adjusted Wald method. R^2 was calculated by linear regression (dotted line). We considered whether maternal antibodies might be contributing to PARV4 IgG seroprevalence among those 0–4 years of age. However, from 11 children in this group who were ≤12 months of age (in whom detection of maternal antibody might still be expected), 2 were PARV4 IgG seropositive, and only 1 of these had an IgG-positive mother, suggesting that maternal antibodies did not contribute significantly to PARV4 seropositivity in this cohort.

On the basis of previously reported data demonstrating PARV4 viremia in neonates (*7*), we hypothesized that vertical transmission is possible. To investigate further, we sought evidence of concordance between IgG serostatus of mothers and their children. Maternal PARV4 IgG status did not differ between IgG-positive and IgG-negative children (p = 1.00, Fisher exact test; online Technical Appendix Table 1,

http://wwwnc.cdc.gov/EID/article/21/4/14-1545-Techapp1.pdf). The absence of correlation between the IgG statuses of mothers and children suggests that vertical transmission is probably not a major contributor to new infections, although it remains plausible that it may sometimes occur.

Data from Europe that suggest an association between PARV4 infection and progression to Centers for Disease Control and Prevention B-syndromes in HIV-positive persons are problematic because of confounding high rates of HCV infection and injection drug use in the PARV4-positive group (*8*). We sought evidence for this effect in our cohort, in which rates of HCV infection and injection drug use were likely to be negligible. We found no evidence of a PARV4 serostatus effect on HIV RNA load or CD4+ T cells in children ($p = 0.13$, $p = 0.68$, respectively; online Technical Appendix Table 1) or adults ($p = 0.15$, $p = 0.77$, respectively; online Technical Appendix Table 2).

We found an unexpected negative correlation between PARV4 IgG and HIV status in children ($p = 0.05$, Fisher exact test; online Technical Appendix Table 1). One possible explanation is that a detectable PARV4 IgG response is not mounted or maintained in the context of HIV infection; however, this theory is not supported by previous studies in which PARV4 IgG seems to be more prevalent in HIV-infected populations (*5*,*8*).

Our analysis was limited by small numbers tested and the retrospective approach to sample testing. Demographic data were not recorded for this cohort, so we are unable to explore further possible social or demographic risk factors that might correlate with PARV4 infection.

This study contributes to an evolving body of data suggesting that PARV4 is highly endemic to different settings across Africa. The unknown clinical effects and transmission routes of this virus remain pressing questions for future research.

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Co-infection with Avian (H7N9) and Pandemic (H1N1) 2009 Influenza Viruses, China

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To the Editor: Since the first case of avian-origin influenza (H7N9) virus infection was reported in March 2013 in Shanghai, China, this virus has caused ≈400 confirmed cases (http://www.who.int/influenza/human_animal_interface/influenza_h7n9/Data_Reports/en/). According to the Chinese Influenza Weekly Report (http://www.cnic.org.cn/eng/show.php?contentid=699), during the winter of 2013–14, multiple influenza viruses that infect humans co-circulated in southern China; these viruses were influenza A(H1N1) pdm09, seasonal influenza A(H3N2), and influenza B viruses, together with avian influenza (H7N9) virus,. Co-infection of humans with avian virus subtype H7N9 and human virus subtypes were reported in Jiangsu Province in June 2013 (*1*) and in Zhejiang Province in January 2014 (*2*), respectively. We report a fatal case of co-infection with influenza (H7N9) and A(H1N1)pdm09 viruses, which was further confirmed by virus isolation and neutralization antibody detection.

In a retrospective study, we identified co-infection with A(H1N1)pdm09 and novel avian-origin H7N9 virus in a 61-year-old female patient from the urban area of Shanghai, China. She had a 3-year history of thrombocythemia. In October 2013, she received an annual vaccine that included an A/California/7/2009(H1N1)pdm09–like virus, an A/Texas/50/2012(H3N2)–like virus, and an influenza B virus, as recommended by the World Health Organization (http://www.who.int/influenza/vaccines/virus/recommendations/2013_14_north/en/). On December 26, 2013, the patient experienced influenza-like symptoms; the next day, she was hospitalized. Despite oral administration of oseltamivir, a rapidly progressive pneumonia developed, and the patient died of multiple organ failure 9 days after admission. On December 31, 2013 (day 5), 1 serum and 2 throat swab samples were collected; both throat swab samples were positive for influenza A and A(H1N1)pdm09 virus, according to the universal and subtype-specific primers provided by the Chinese Center for Disease Control and Prevention (http://www.who.int/influenza/resources/documents/diagnostic_recommendations/en/). On January 3, 2014 (day 8), 1 bronchial secretion and 1 serum sample were also collected.

Recent DNA sequencing analysis of the stored bronchial secretion specimen, performed on a Roche 454 platform (*3*) (Roche, Basel, Switzerland) showed co-existence of H1, H7, N1, and N9 genomic segments, which were further confirmed by real-time reverse transcription PCR (RT-PCR). The cycle threshold values of H7 and H1 were 35.65 and 29.94, respectively.

We then determined antibody levels of the 2 serum samples collected on days 5 and 8 by using a microneutralization assay (*4*) with MDCK cells infected with 2 representative strains, A/Shanghai/4664T/2013(H7N9) and A/Shanghai/37T/2009(H1N1), respectively. The samples were serially diluted 2-fold (1:10 to 1:1,280) at a starting dilution of 1:10; titers were expressed as the reciprocal of the highest dilution of 50% neutralization. The microneutralization titers against novel subtype H7N9 virus were 160 and 320 for the samples collected on days 5 and 8, respectively. This finding indicated that the patient could have been infected by novel subtype H7N9 viruses (*5*); microneutralization titers against A/Shanghai/37T/2009(H1N1) were 320 for both samples.

To determine whether the reassortment had occurred between the 2 subtypes of influenza A viruses during the co-infection, we isolated the virus strains from the bronchial secretion on either MDCK cells (A[H1N1]pdm09) (http://www.who.int/influenza/gisrs_laboratory/manual_diagnosis_surveillance_influenza/en/) or embryonated eggs (H7N9) through several passages. The hemagglutination titers of the isolated H7N9 (A/Shanghai/Mix1/2014[H7N9]) and A(H1N1)pdm09 viruses (A/Shanghai/Mix1/2014[H1N1]) in allantoic fluid were 32 and 8, respectively. Cultures of the 2 isolates were subjected to RT-PCR for detection of 8 genomic segments. The RT-PCR products were sequenced on an ABI 3730XL Automatic DNA Analyzer by using an ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (both from Applied Biosystems, Foster City, CA, USA). The full-genome sequences were submitted to the National Center for Biotechnology Information Influenza Virus Resource (GenBank accession nos. KJ946415, KJ946416 and KP058484–KP058489 for A/Shanghai/Mix1/201[H7N9] and KM607860, KM607861 and KP058490–KP058495 for A/Shanghai/Mix1/2014[H1N1]).

Phylogenetic trees were constructed by using the neighbor-joining method in MEGA 5.1 (http://www.megasoftware.net) to estimate relationships with selected influenza A virus reference sequences obtained from GenBank and the Global Initiative on Sharing Avian Influenza Data database. DNA sequence analysis showed that no genetic reassortment had occurred between the 2 subtypes because only H7N9 or A(H1N1)pdm09 genomic fragments were found in the culture of the respective isolate (Figure; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/4/14-1560-Techapp1.pdf). Phylogenetic analyses of the hemagglutinin and neuraminidase genes revealed that the A/Shanghai/Mix1/2014(H7N9) and A/Shanghai/Mix1/2014(H1N1) viruses were clustered into the clade of A/Shanghai/01/2014(H7N9)-like viruses (*6*) (Figure, panels A and B) and A/Shanghai/6109/2014(H1N1)-like viruses (Figure, panels C and D), respectively. In addition, the phylogenetic trees of the 6 internal genes were close to those of H7N9 viruses or A(H1N1)pdm09-lineage viruses that had recently circulated in China (online Technical Appendix).

Co-infections with H7N9 and other influenza subtypes have been reported from Jiangsu (*1*) and Zhejiang (*2*) Provinces as well as in our study, indicating the potential risk for co-infection during the influenza season

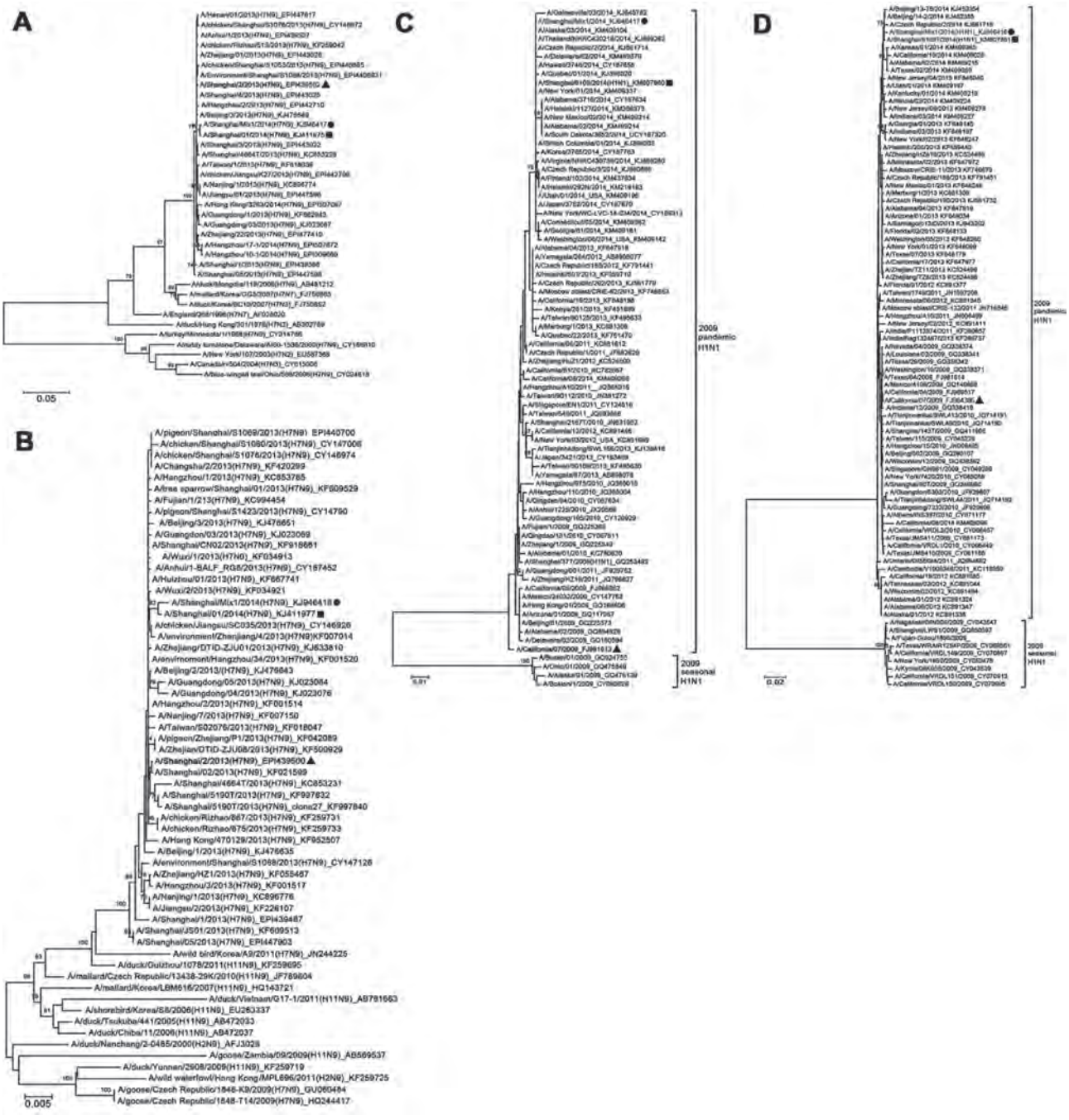

Figure. Phylogenetic analyses of A/Shanghai/Mix1/2014(H7N9) and A/Shanghai/Mix1/2014(H1N1) viruses from Shanghai, China, January 2014. A) Hemagglutinin and B) neuraminidase of A/Shanghai/Mix1/2014(H7N9); C) hemagglutinin and D) neuraminidase of A/Shanghai/Mix1/2014(H1N1). Multiple alignments were constructed by using the ClustalW (http://www.clustal.org/) algorithm based on the sequences of the hemagglutinin and neuraminidase genes. Genetic distances were calculated by using the Kimura 2-parameter method, and phylogenetic trees were constructed by using the neighbor-joining method with bootstrap analysis of 1,000 replicates in MEGA 5.1 (http://www.megasoftware.net). Horizontal distances are proportional to genetic distances. Numbers next to nodes indicate bootstrap value percentages (>70%). Circles indicate the A/Shanghai/Mix1/2014(H7N9) and A/Shanghai/Mix1/2014(H1N1) virus strains isolated from the co-infection patient from Shanghai, China. Triangles indicate the representative strains of the Shanghai area during the same period as the influenza (H7N9) outbreak in China. Squares indicate the representative strains of the Shanghai area during the same wave of the H7N9 outbreak in China. Scale bars indicate nucleotide substitutions per site. The Global Initiative on Sharing Avian Influenza Data database or GenBank accession numbers of the influenza viruses used in the construction of the phylogenetic analyses are displayed in the phylogenetic trees, which follow the taxon names.

in China. Although reassortment was not detected in this co-infection, a potential risk for emergence of a new pandemic strain by reassortment between these 2 viruses (with humans as mixing vessels) should not be ignored. To reduce the risk for emergence of new viral subtypes, the public health and scientific communities should enhance surveillance for co-infection with influenza (H7N9) virus and other influenza virus subtypes.

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Nairobi Sheep Disease Virus RNA in Ixodid Ticks, China, 2013

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To the Editor: Nairobi sheep disease virus (NSDV; genus *Nairovirus*, family *Bunyaviridae*) causes acute hemorrhagic gastroenteritis in sheep and goats (*1*,*2*). First identified in Nairobi, Kenya, in 1910, it is considered endemic in East Africa (*1*,*3*). Ganjam virus, a variant of NSDV, is found in India and Sri Lanka (*2*). NSDV has a limited effect on animals bred in areas to which the virus is endemic but can cause large losses of animals during introduction of new livestock or transport of animals through these areas (*4*). In humans, NSDV infection can cause febrile illness, headache, nausea, and vomiting (*5*).

Ticks are the main transmission vectors of NSDV and many other viral pathogens and therefore pose a major threat to public health (*6*,*7*). Here, we describe a newly discovered NSDV, named NSDV (China), identified by viral metagenomic analysis of ticks collected from the northeast region of the People's Republic of China (Liaoning, Jilin, and Heilongjiang provinces) during May–July, 2013, and divided into 9 groups according to tick species and sampling sites. Four tick species were morphologically identified: *Haemaphysalis longicornis* (84.8%); *Dermacentor silvarum* (7.2%); *D. nuttalli* (5.5%); and *Ixodes persulcatus* (2.5%) (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/4/14-1602-Techapp1.pdf).

Of the 6,427 ticks collected, 3,410 were divided into 9 groups (average 379 ticks/group, range 163–512); each group was homogenized in SM buffer (50 mmol/L Tris, 10 mmol/L $MgSO_4$, 0.1 mol/L NaCl, pH 7.5). Viral RNA extraction, Solexa sequencing, and analysis are described in the online Technical Appendix. Among the sequences annotated to mammalian viruses, 65 contigs were found to target the small (n = 15), medium (n = 27), and large (n = 23) segments of the NSDV genome (online Technical Appendix Tables 2–4).

[1]These authors contributed equally to this article.

To confirm the Solexa results, a 376-nt fragment of the NSDV small gene segment was amplified by reverse transcription PCR (RT-PCR) by using primers P1 (5′-AGCAAAGAGCACATTGACTGGGC-3′) and P2 (5′-CTGTCACACCTGCCTTCCAA-3′). Ticks in 3 *H. longicornis* groups were positive for NSDV: group 1 from sheep in Jian, Jilin Province (125°34′E, 40°52′N); group 2 from cattle in Jinxing, Jilin Province (130°38′E, 42°25′N); and group 5 from sheep in Dandong, Liaoning Province (124°23′E, 40°07′N). Ticks in the other groups were negative. The obtained sequences shared 92% identity with NSDV from *H. intermedia* in India.

The full-length sequence of NSDV was then obtained from group 2 by RT-PCR by using primers based on the Solexa sequences or the conserved sequences of nairoviruses (online Technical Appendix Table 5). The complete sequences of the small, medium, and large segments of NSDV (China) (GenBank accession nos. KM464724–KM464726) contained 1,590, 5,077, and 12,081 nt, respectively; that is, they were similar to other NSDVs. Sequence comparisons showed 75.1%–89.6% identity with other NSDVs at the nucleotide level and 81.3%–96.7% at the deduced amino acid level (online Technical Appendix Table 6). Compared with other member species within the genus *Nairovirus* (Dugbe, Kupe, Hazara, and Crimean Congo hemorrhagic fever viruses), low identities (37.5%–68.6%) were observed at both nucleotide and amino acid levels (online Technical Appendix Table 6). Phylogenetic analysis based on the amino acid sequences grouped the virus together with NSDVs from Africa and South Asia (Figure).

The remaining tick samples of the NSDV-positive groups were used to determine the infection frequency by using RT-PCR to analyze primers P1 and P2. We assayed 104 tick pools (average 15 ticks/pool, range 8–40), 13 pools of 416 ticks in Jian Province and 91 pools of 1,095 ticks in Jinxing Province; 12.5% (13/104) tested positive, 38.5% (5/13) in Jian and 8.8% (8/91) in Jinxing. The higher prevalence in Jian Province may result from more ticks in the pools. Attempts to isolate virus from the positive samples in cell lines (Vero and BHK-21) and suckling mice were unsuccessful; thus, its pathogenicity could not be determined.

In Africa, NSDV is primarily transmitted by *R. appendiculatus* ticks (*5*). In South Asia (India and Sri Lanka), NSDV has been isolated from ticks (*H. intermedia*, *H. wellingtoni*, and *R. haemaphysaloides*), mosquitoes, sheep and humans; *H. intermedia* ticks are considered the main vector for the virus (*5*,*8*,*9*). NSDV had not previously been reported from East Asia. The isolate we identified, NSDV (China), is genetically divergent from the NSDVs of South Asia and Africa and is therefore a novel strain, with *H. longicornis* likely the main vector. Nairobi sheep disease has not been reported in China and East Asia, but our results

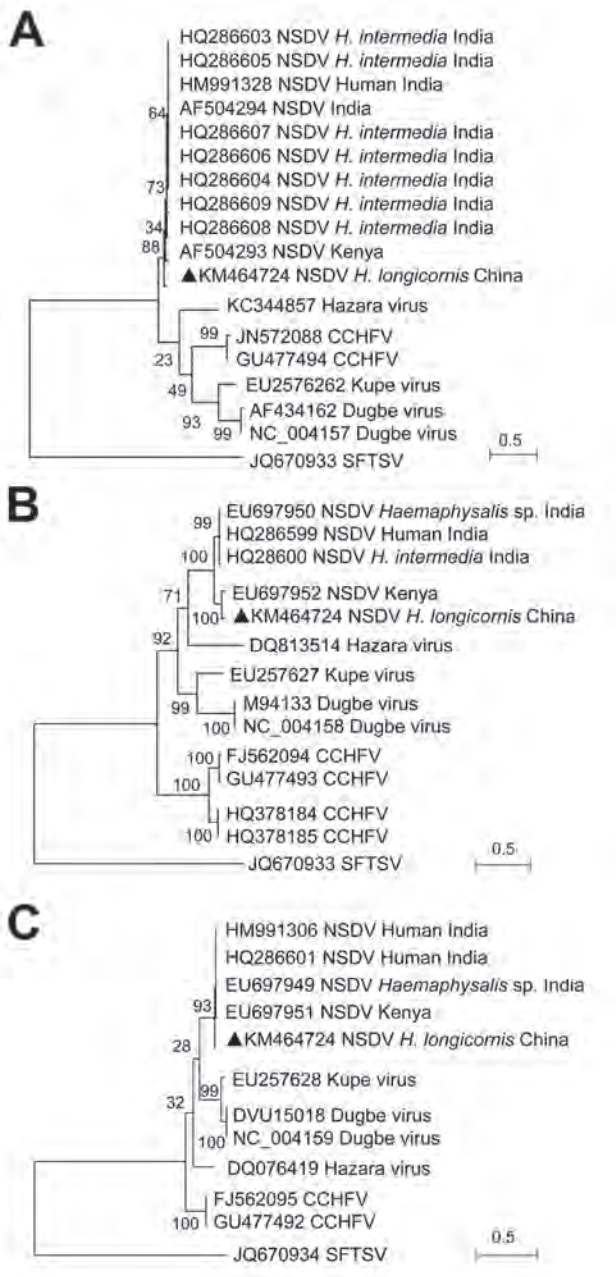

Figure. Phylogenetic analysis of Nairobi sheep disease virus (China) and other nairoviruses. The phylogenetic trees were generated in MEGA5.2 software (http://www.megasoftware.net). The complete coding regions for nucleocapsid protein in the small segment (A), glycoprotein precursor in the medium segment (B), and RNA dependent RNA polymerase in the large segment (C) were analyzed by the maximum-likelihood method. An emergent severe fever thrombocytopenia syndrome virus (SFTSV; family *Bunyaviridae*, genus *Phlebovirus*) was used as the outgroup. Bootstrap testing (1,000 replicates) was performed, and the bootstrap values are indicated. Sequences are identified by their GenBank accession numbers, followed by the virus name, host, and country. Black triangles indicate novel strain NSDV (China). Scale bars indicate substitutions per site. CCHFV, Crimean-Congo hemorrhagic fever virus.

indicate the risk of its occurrence in these regions, where *H. longicornis* is widely distributed (*10*). More extensive investigation to clarify the natural circulation of NSDV among ticks should be conducted and surveillance of sheep improved to prevent outbreaks of Nairobi sheep disease in China and East Asia.

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Avian Influenza A(H10N7) Virus–Associated Mass Deaths among Harbor Seals

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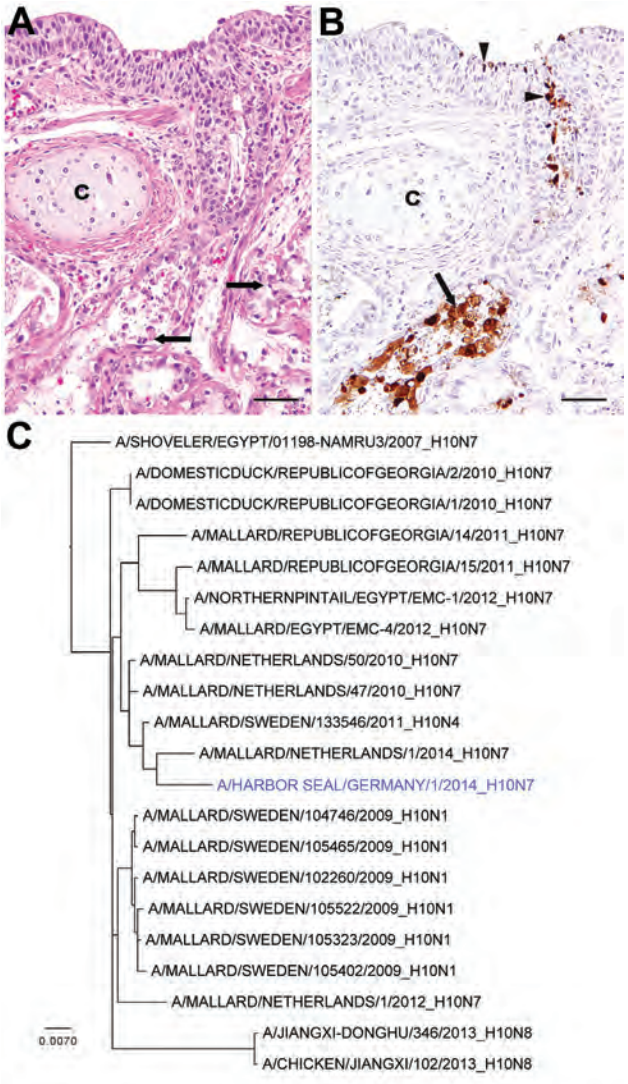
To the Editor: Avian influenza A viruses occasionally cross the species barrier; influenza A(H5N1) virus and the recently emerged influenza A(H7N9) virus are prime examples of bird-to-human transmission (*1*,*2*). In addition, avian influenza A viruses can cross to various other mammalian species, including pinnipeds (e.g., seals) (*3*,*4*).

Recently, mass deaths have occurred among harbor seals (*Phoca vitulina*); hundreds of carcasses washed up the shores of Sweden (March 2014), Denmark (July 2014), and Germany (October 2014). Approximately 1,400 dead harbor seals were seen in the coastal waters of Schleswig-Holstein in Germany alone, where the population is ≈12,000 animals.

We report the investigation of the deaths of 17 seals from different age groups that were found dead on the islands of Helgoland and Sylt, Germany, during the second week of October 2014. Complete necropsies were performed on the carcasses, which were in variable nutritional conditions, ranging from very poor to good. Necropsy results showed consistently poorly retracted lungs with severe congestion, occasional diffuse consolidation, and multifocal firm nodular areas of gray-yellow discoloration with varying numbers of metazoic parasites. Histologic examinations showed acute necrotizing bronchitis and adenitis of bronchial glands with sloughing of epithelial cells (Figure, panel A). Occasionally, mild interstitial pneumonia was found. Multifocal pyogranulomatous to necrotizing pneumonia was associated with parasite infestation. A few animals had suppurative to necrotizing or nonsuppurative rhinitis and tracheitis.

Because mass deaths among seals were caused by phocine distemper virus in the same area in 1988 and 2002,

we tested lung and throat swab samples for morbillivirus using reverse transcription PCR (RT-PCR) and immunohistochemical analysis (*5*). In addition, real-time RT-PCR targeting the influenza A virus matrix gene was performed (*6*). No indications for the morbillivirus were detected by RT-PCR and immunohistochemistry; however, in lung lesions and throat swab samples of 11 animals, a positive signal was observed by the influenza A matrix gene real-time RT-PCR (cycle threshold values 15.0–33.9). Influenza A virus (A/harbor seal/Germany/1/2014) was subsequently isolated from lung and throat swab samples; the virus replicated to high titers in 11-day-old embryonated chicken eggs and on MDCK cells. By PCR using specific primers and subsequent Sanger sequencing of the hemagglutinin and neuraminidase genes, this virus was characterized as an influenza A virus of the H10N7 subtype, commonly found in migratory waterfowl (*6*). In addition, genetic analyses of all other gene segments indicated that the influenza virus A/harbor seal/Germany/1/2014 was most closely related to various influenza A viruses detected in wild birds. Specifically, the hemagglutinin and neuraminidase genes were genetically most closely related to subtype H10N7 viruses recently found in migratory ducks in Georgia, Egypt, and the Netherlands (Figure, panel C) (*7*). Genetic analyses were based on BLAST analyses using public databases available as of October 17, 2014 (http://www.ncbi.nlm.nih.gov, http://www.gisaid.com) and supplemented with H10 and N7 sequences from the international wild bird surveillance program of Erasmus Medical Center (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/4/14-1675-Techapp1.pdf). A maximum-likelihood phylogenetic tree of the hemagglutinin gene was generated by using PhyML version 3.1 (*8*) with the general time reversible +I+G model of nucleotide substitution; a full heuristic search and subtree pruning and regrafting searches were performed. The tree was visualized by using Figtree version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree).



Histopathologic and phylogenetic analyses of necropsy samples from harbor seals infected with avian influenza A(H10N7) virus, Germany, 2014. A) Lung of harbor seal showing marked necrosis and sloughing of epithelial cells in bronchial glands (arrows); c = bronchial cartilage; hematoxylin and eosin stain. Scale bar indicates 50 μm B) Immunohistochemical labeling of influenza A nucleoprotein in bronchial epithelial cells (arrowheads) and glandular epithelial cells (arrows); c = bronchial cartilage; avidin-biotin-peroxidase complex method. Scale bar indicates 50 μm. C) Maximum-likelihood phylogenetic tree of the partial hemagglutinin gene (1,577 nt) of the influenza A/harbor seal/Germany/1/2014 (H10N7) isolate and various other closely related viruses. GenBank accession numbers are provided in online Technical Appendix Table 1 (http://wwwnc.cdc.gov/EID/article/21/4/14-1675-Techapp1.pdf). Scale bar indicates nucleotide substitutions per site.

To further elucidate the role of influenza A(H10N7) virus in the pathogenesis of the disease causing deaths among the seals, we conducted immunohistochemical analysis on the lungs using an influenza A virus nucleoprotein-specific monoclonal antibody (*9*). Evaluation of the lung tissues of the dead seals showed viral antigen in cytoplasm and nuclei of epithelial cells of bronchi and bronchial glands of affected lung areas (Figure, panel B), which suggests that this virus played a major role in the deaths. Immunohistochemical analysis performed on various organs (including brain and olfactory bulb) indicated that viral antigen was restricted to the respiratory tract.

Although avian influenza A virus infections previously have caused mass deaths in seals (*3*,*4*,*10*), subtype H10N7 has not been associated with such events. We can speculate that the ongoing deaths could eventually affect all harbor seal populations of northwestern Europe and have consequences for wildlife management and seal rehabilitation activities. In addition, preliminary analysis of the hemagglutinin sequence of the influenza A(H10N7) virus suggests the presence of molecular determinants that indicate

mammalian adaptation. Various analyses are ongoing to answer questions about the route of transmission among seals and possible transmissibility to humans.

Note added in proof: Zohari et al. also recently reported the involvement of avian influenza A(H10N7) virus in mass deaths of harbor seals in Sweden (Euro Surveill. 2014;19:pii: 20967).

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Zika Virus Infection, Philippines, 2012

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To the Editor: Zika virus (ZIKV), a mosquitoborne flavivirus, was first isolated from a rhesus monkey in Uganda in 1947 (*1*). This positive-sense, single-stranded RNA virus (family *Flaviviridae*, genus *Flavivirus*) has a 10,794-nt genome and is most closely related to Spondweni virus (*2*,*3*). Phylogenetic analyses have revealed 2 major lineages: Asian and African (*2*–*4*).

The first human infection with ZIKV was reported in Nigeria in 1954 (*5*). The virus caused only sporadic infections until 2007, when a large outbreak occurred on Yap, an island in the Federated States of Micronesia (*6*). In October 2013, ZIKV was detected in French Polynesia; since then, >400 laboratory-confirmed cases have been reported (*7*). ZIKV has spread across the South Pacific, and autochthonous cases have been reported in New Caledonia, Easter Island, and the Cook Islands. Several cases of ZIKV infections have been reported in travelers to Southeast Asia (*4*,*8*) and French Polynesia (*3*,*7*).

In March 2012, a prospective longitudinal cohort study, which included active surveillance for acute febrile illness, was initiated in Cebu City, Philippines (I. Yoon, unpub. data). Participants contacted study staff to report fever and were also contacted weekly by staff to determine if they had fever during the previous 7 days. Fever episodes triggered an acute-illness visit by a study nurse, who performed a clinical assessment of the patient and collected an acute-phase blood sample. During the first year of surveillance, 270 acute febrile

illnesses were detected; 267 of the patients had samples available for serologic testing for evidence of influenza, dengue, chikungunya, Japanese encephalitis, and Zika virus infections.

In May 2012, a 15-year-old boy in Cebu City reported a subjective fever; an acute-illness investigation followed. Other symptoms included headache, conjunctivitis, sore throat, myalgias, stomach pain, anorexia, nausea, and vomiting, but no rash. The boy did not seek medical care or require hospitalization; his only treatment was acetaminophen. He had no recent travel history, and no other members of his household were ill. The boy recovered fully by the 3-week study follow-up visit. An acute-phase blood sample, collected 2 days after symptom onset, was negative for dengue and chikungunya viruses by reverse transcription PCR. An in-house dengue/Japanese encephalitis IgM/IgG capture ELISA and chikungunya ELISA were used to test paired acute- and convalescent-phase blood samples; all results were negative. ZIKV ELISA was not available at the testing laboratory. However, by using real-time reverse transcription PCR targeting the gene that encodes the precursor of membrane protein, we detected ZIKV RNA in the patient's serum sample (*6*).

Virus was then isolated by intrathoracically inoculating *Toxorhynchites splendens* mosquitoes and by inoculating C6/36 cells with patient serum. The MiSeq platform (Illumina, Hayward, CA, USA) was used to obtain sequence reads by next-generation genomic sequencing, which identified a 789-bp contig as a partial sequence of the ZIKV gene that encodes the nonstructural 5 protein (GenBank accession no. KM851038).

Maximum-likelihood phylogenetic analysis of the gene encoding the nonstructural 5 protein sequence showed that the isolate belonged to the ZIKV Asian lineage (Figure). Pair-wise genetic distance calculation indicated that the isolate was most closely related to the 2007 strain from Micronesia (p-distance = 0.013), with which it shared ≈99% nt (779/789) similarity.

During the past decade, ZIKV has caused 2 large epidemics in Micronesia and French Polynesia. The virus has a high potential for ongoing geographic expansion into countries where *Aedes* spp. mosquitoes are present and are known to transmit ZIKV; most notable among these vectors are *A. aegypti* mosquitoes, which are widespread throughout the Philippines (*9*). ZIKV infections have been reported in travelers to areas in the South Pacific with known ZIKV transmission and to areas such as Thailand (*4*) and Indonesia (*8*), where no recent endemic cases have been described. However, a case of endemic ZIKV infection has been reported in a child in Cambodia, and serologic evidence of ZIKV infection has been reported in Thailand, Vietnam, Malaysia, Indonesia, and the Philippines (*2*).

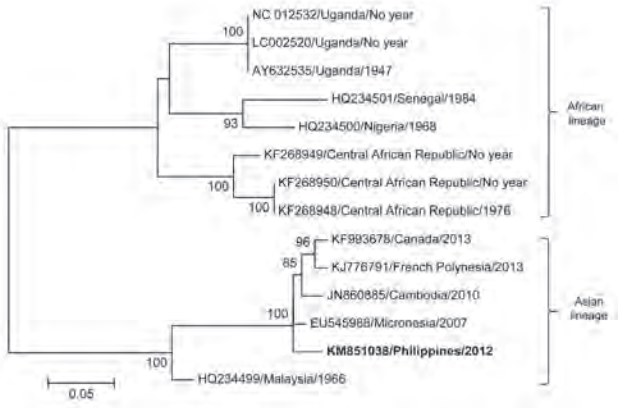

Figure. Maximum-likelihood phylogenetic tree of fragments of Zika virus was determined using the general time-reversible plus gamma distribution plus invariable site model with 13 reference Zika virus strains from GenBank. The contig sequence, obtained from de novo assembly and blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), of the Philippines isolate from 2012 (GenBank accession no. KM851038; bold font) was analyzed against 8 reference strains from Africa (GenBank accession nos. KF268948, KF268949, KF268950, LC002520, AY632535, NC012532, HQ234500, HQ234501) and 5 reference strains from Asia (GenBank accession nos. KJ776791, JN860885, EU545988, HQ234499, KF993678). The year of collection is unknown for several strains from Africa. Bootstrap values ≥70 are indicated at nodes. Scale bar indicates nucleotide substitutions per site. The drawing is not to scale.

Phylogenetic analysis of the isolate from our study indicated that it is more closely related to the strain from Micronesia that was responsible for the 2007 Yap outbreak than to strains identified in Cambodia (2010), Thailand (2013), or French Polynesia (2013). It is possible that the ZIKV infection in our study was an isolated case (the virus was confirmed in only 1/270 episodes of acute febrile illness). However, because the symptoms of disease are similar to those for other known endemic arboviruses, it is also possible that the strain was introduced into the Philippines before 2012 and remained undetected.

The spectrum of ZIKV-associated clinical disease remains uncertain. Although reports indicate most cases of infection are mild, infections may be associated with more severe disease outcomes, such as Guillain-Barre syndrome (*10*). Increased surveillance for ZIKV disease, using pan-flavivirus or ZIKV-specific molecular testing, may lead to more frequent identification of cases, which could give a clearer indication of the true number of ZIKV infections. Additional surveillance and research studies are needed to improve our understanding of this disease, including the potential epidemiologic and clinical effects of ZIKV co-circulation with other flaviviruses.

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Chikungunya Outbreak, French Polynesia, 2014

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To the Editor: Chikungunya virus (CHIKV), an arthropod-borne virus (arbovirus) of the family *Togaviridae*, genus *Alphavirus*, is transmitted by mosquitoes of the *Aedes* genus, especially *Ae. aegypti* and *Ae. albopictus* (*1*). The main clinical manifestations of CHIKV infections are sudden high fever, headache, back pain, myalgia, arthralgia affecting mainly the extremities, and rash.

CHIKV emerged in the Pacific region in New Caledonia in March 2011. Additional outbreaks occurred in Papua New Guinea in June 2012; Yap State (Federated States of Micronesia) in August 2013; Tonga in April 2014; and American Samoa, Samoa, and Tokelau in July 2014 (*2*). Phylogenetic analysis of CHIKV strains showed the existence of 3 lineages: West African, Asian, and East/Central/South African (*1*).

French Polynesia is a French territory in the South Pacific, with 270,000 inhabitants living on 5 archipelagoes. Arboviruses are a common cause of outbreaks in French Polynesia: the last dengue virus (DENV) outbreaks caused by DENV-1 and DENV-3 occurred in 2013 (*3*), and DENV-1 still circulates. French Polynesia also experienced the largest Zika virus (ZIKV) outbreak ever reported during October 2013–April 2014 (*4*). In May 2014, CHIKV infection was detected for the first time in French Polynesia in a traveler returning from Guadeloupe, (*5*) where a chikungunya outbreak was ongoing (*6*).

In late September 2014, an increasing number of patients with fever and rash who tested negative for DENV and ZIKV by real-time reverse transcription PCR (RT-PCR) were recorded by the French Polynesia Department of Health on the south coast of Tahiti, French Polynesia's main island. Serum samples collected from 19 of these patients were tested for CHIKV by RT-PCR using previously reported primers and a probe (*7*). Seven of the 19 (37%) were positive; all 7 were autochthonous. The first specimen that tested positive for CHIKV had been collected from a patient on September 25, and by October

25, a total of 318 patients were confirmed by RT-PCR to be infected by CHIKV. Nearly all districts of Tahiti were affected, and cases were reported on 4 of French Polynesia's 5 archipelagoes.

Partial sequencing of the CHIKV E1 gene of a strain isolated from a patient and collected on September 29 (strain PF14-290914-16, GenBank accession no. KM985619) was performed as previously reported (*8*). Phylogenetic analysis showed that French Polynesia's CHIKV strain belongs to the Asian lineage and is more closely related to a strain collected in the British Virgin Islands in 2014 (VG14/99659) and to the French Polynesian strain imported from Guadeloupe in May 2014 (PF14-270514-51impGP), with 99.9% homology, than to the strains that recently circulated in Yap State (FM13/3807), Tonga (TO14-080414-3007 and TO14-080414-3042), and New Caledonia (NC11-568) (Figure).

No cases of CHIKV infection were reported in French Polynesia within the 4 months after the imported case detected on May 25, 2014. Because of the active, ongoing circulation of CHIKV in the Pacific, introduction of this virus in French Polynesia was expected from other Pacific islands, especially from New Caledonia, because of extensive travel between the 2 French territories.

The fact that the CHIKV strain circulating in French Polynesia is closely related to the strains currently circulating in the Caribbean suggests that the French Polynesia outbreak is a result of the introduction of CHIKV from the Caribbean rather than from another Pacific island. The delay between the current outbreak and the first infected patient detected in 2014 also suggests a new introduction rather than a circulation of the strain introduced in May. However, an undetected low-level circulation of CHIKV during the cooler and drier low transmission season, simultaneously with DENV-1 circulation, cannot be excluded.

The introduction of arboviruses into French Polynesia from other French overseas territories rather than from other Pacific islands was previously reported for DENV. In 2013, DENV-3 reappeared in French Polynesia 3 months after the Solomon Islands had declared a DENV-3 outbreak. However, epidemiologic and phylogenetic investigations revealed that the DENV-3 strain that caused the outbreak in French Polynesia had been introduced by a traveler returning from French Guiana and belonged to a different genotype than the one that was circulating in the Solomon Islands (*3*).

Several conditions are favorable to a large chikungunya outbreak in French Polynesia. First, because CHIKV has never been previously reported in French Polynesia, the entire population is thought to be immunologically naive for CHIKV infection. Second, 2 potential vectors for CHIKV are present in French Polynesia: *Ae. aegypti* (*1*) and *Ae. polynesiensis* mosquitoes (*9*). Third, in French Polynesia the hot and rainy season that lasts from October through March is conducive to the proliferation of mosquitoes. We have the experience of the French Polynesian ZIKV outbreak that started with the same favorable conditions in October 2013 and was responsible for 28,000 estimated symptomatic cases from October 2013 through April 2014 (*10*). This new outbreak corroborates the recent observation that the expansion of arboviruses in the Pacific is ongoing and inevitable (*2*).

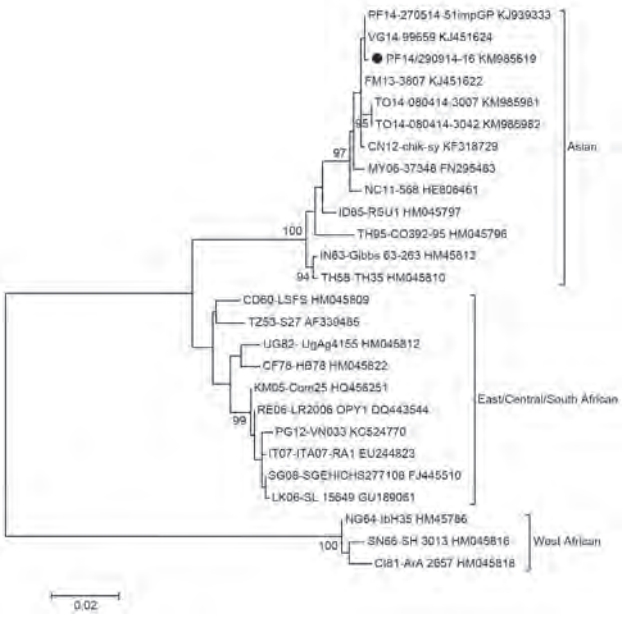

Figure. Phylogenetic analysis of chikungunya virus strain isolated in French Polynesia on September 29, 2014. The evolutionary history was inferred by using the maximum-likelihood method based on the Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown for values >90 next to the branches (1,000 replicates). Evolutionary analyses were conducted in MEGA6 (http://www.megasoftware.net/mega.php). Each strain is labeled by country (iso country code, 2-letter) and date of origin/strain name/GenBank accession number. The chikungunya virus strain isolated in French Polynesia on September 2014 is marked with a black circle. Scale bar indicates nucleotide substitutions per site.

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Influenza A and B Viruses but Not MERS-CoV in Hajj Pilgrims, Austria, 2014

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To the Editor: The World Health Organization recommends that persons who return from pilgrimages to the Middle East with acute severe respiratory infections be tested to determine the cause of infection; the aim is to identify infections with the Middle East respiratory syndrome coronavirus (MERS-CoV), which have been occurring in Saudi Arabia since 2012. Each year, >2.5 million persons from >180 countries, including 240,000 pilgrims from Europe, participate in Hajj, the Muslim pilgrimage to Mecca, Saudi Arabia. The gathering of mass numbers of persons during the Hajj increases the risk for the spread of respiratory infections among participants, and this risk has raised global concern that travelers returning from this pilgrimage could contribute to the international spread of MERS-CoV. During the 2012 and 2013 Hajj and Umrah (a minor pilgrimage) pilgrimages, no MERS cases in pilgrims were reported (*1*,*2*). However, in 2014, cases of MERS-CoV infection were confirmed in 2 returning pilgrims in the Netherlands (*3*). The International Health Regulations Emergency Committee advised all countries to improve awareness about MERS-CoV among pilgrims and to conduct surveillance for MERS-CoV among pilgrims during and after Hajj (*4*).

According to data from the International Air Transport Association, Austria received an estimated 68,000 air travelers from Saudi Arabia, Jordan, Qatar, and the United Arab Emirates during June–November 2012 (a period encompassing 1 month before Ramadan and 1 month after the Hajj) (*5*); of these travelers, 1,000 were pilgrims performing the Hajj. Relatively constant travel volumes to Austria on commercial flights out of these countries during 2010–July 2014 have been confirmed by an analysis of air traffic statistics for Austria (A. Herndler, M. Rudolf, pers. comm.). We report on the investigation of illness among Austrian residents just after their return home from the 2014 Hajj pilgrimage, which ended in early October.

As of October 27, 2014, a total of 7 Hajj pilgrims from Austria had sought medical care in different Austrian hospitals/medical centers just after returning from Saudi Arabia. The patients had fever and/or respiratory symptoms. A summary of the patients' characteristics is presented in the Table. Of the 7 patients, 4 had cough, 1 had dyspnea, and 4 had fever. Patients 1, 2, and 7 had an acute febrile illness and clinical and/or radiologic evidence of pulmonary parenchymal disease. Patient 1 had sought medical care in Saudi Arabia, and patient 2 had been hospitalized for 10 days in Saudi Arabia.

For the diagnosis of viral infection, a serum sample and a sputum, throat swab, or bronchoalveolar lavage sample were collected and sent to the Department of Virology, Medical University of Vienna, Austria, for analysis. All samples were tested for MERS-CoV by using reverse transcription PCR targeting regions upstream of the envelope gene (*6*). Respiratory and serum samples from all 7 patients were negative for MERS-CoV. The respiratory samples were also tested for influenza A and B viruses and for rhinoviruses, as previously described (*7*–*9*). Of the 7 patients, 3 were positive for influenza B virus, 2 for influenza A(H3N2) virus, and 2 for rhinoviruses (Table). Subsequent phylogenetic analysis showed that the influenza A(H3N2) strains belonged to the A/Hong Kong/146/2013-like viruses and the influenza B strains belonged to the B/Phuket/3073/2013-like viruses of the Yamagata lineage, both of which are subtype H3N2 and B strains included in the 2014–15 seasonal influenza vaccine for the Northern Hemisphere.

Our results showed that MERS-CoV was not detected in any of these patients, and our findings support those from reports investigating illness among 2013 Hajj

Table. Characteristics of pilgrims who returned from the Hajj with acute respiratory illness and detectable virus in respiratory specimens, Austria, 2014*

Patient no.	Age, y/sex	Other condition(s)	Signs/symptoms	Date of return from Hajj, Oct 2014	Date of sample collection, Oct 2014	Detected respiratory virus
1	50/M	Diabetes	Fever of 38°C, pneumonia, bronchitis	22	23	Influenza B†
2	49/M	Hypothyroidism	Fever of 39°C, pneumonia, bronchitis	21	22	Influenza A(H3N2)‡
3	47/F	None	Fever, cough	19	21	Influenza B†
4	57/M	None	Cough, bronchitis	19	21	Influenza B†
5	66/M	Hypertension, cardiomyopathy	Cough, lung infiltrates	13	20	Rhinovirus
6	54/M	Diabetes, hypertension	Cough, bronchitis	10	13	Rhinovirus
7	52/F	Diabetes, hypertension, bronchial asthma	Fever of 40°C, dyspnea	12	16	Influenza A(H3N2)‡

*All patients were negative for Middle East respiratory syndrome coronavirus.
†B/Phuket/3073/2013-like virus, Yamagata lineage.
‡A/Hong Kong/146/2013-like virus.

pilgrims (*2*). Our data indicate that all patients had a respiratory virus infection, and 5 of the 7 patients were infected with influenza virus. Six of the patients had not received seasonal influenza vaccine before traveling; for the seventh patient, influenza vaccine uptake data were not available. In 2014 in Austria, the incidence of influenza-like illnesses was below the epidemic threshold during weeks 40–43: no cases of influenza were reported by the National Influenza Surveillance network, Austria (http://www.influenza.at). Therefore, it is likely that these 7 patients became infected while at the Hajj, as reported for patients during previous Hajj seasons (*10*), or while traveling through international airports.

Our preliminary data indicate that influenza virus infection was the cause of severe respiratory illness in 5 of 7 Austrian pilgrims returning from the 2014 Hajj. Because influenza is a serious and potentially life-threatening illness, seasonal influenza vaccination is recommended for Hajj pilgrims. Accurate and rapid testing for MERS-CoV and other respiratory viruses, including influenza, is essential for identifying persons who may be contagious and for timely and effective antiviral drug treatment and, thus, should be considered for pilgrims with severe respiratory illness.

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Enterovirus D68 Infection, Chile, Spring 2014

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To the Editor: Enterovirus D68 (EV-D68) is an emergent viral pathogen associated with severe respiratory illness, especially in children with asthma (*1*). The ongoing epidemic in the United States has expanded to 47 states; as of November 25, 2014, a total of 1,121 persons were affected (http://www.cdc.gov/non-polio-enterovirus/outbreaks/EV-D68-outbreaks.html).

In Chile, because of clinical suspicion of infection caused by the same EV-D68 reported in the United States, we conducted further testing. We selected 6 children who were at Clinica Las Condes (CLC), Santiago, during September–October 2014, for whom nasopharyngeal samples were enterovirus positive according to multiplex PCR (CLART PneumoVir; Genomica, Madrid, Spain) or rhinovirus/enterovirus positive according to the FilmArray Respiratory Panel (BioFire Diagnostics, Salt Lake City, UT, USA). The CLC ethics committee authorized the study. CLC is the second largest private hospital in eastern Santiago, which has a population of nearly 400,000. Since 2008, detection of respiratory viruses by CLART PneumoVir has been performed for all hospitalized CLC patients with respiratory disease. This test detects 17 respiratory viruses, including enterovirus (generic detection). Since 2014, CLC also incorporated testing with the FilmArray Respiratory Panel, which does not distinguish enterovirus from rhinovirus.

We sent 6 nasopharyngeal samples from the children to the US Centers for Disease Control and Prevention for detection of EV-D68 by real-time reverse transcription PCR(http://www.cdc.gov/non-polio-enterovirus/dowloads/ev-d68-rt-pcr-protocol.pdf) and sequencing (*2*). Of the 6 patients, EV-D68 was confirmed for 2 patients.

Patient 1 was a boy, 7.5 years of age, who was hospitalized on September 21, 2014, with a history of asthma since 3 years of age. During the previous 3 months, his father had frequently traveled to the United States. The patient's current episode began with upper respiratory symptoms and low-grade fever (38°C) and was followed by intense vomiting. On admission, the child exhibited respiratory distress and an oxygen saturation of 88%, which led to his admission to the pediatric intensive care unit and management with noninvasive mechanical ventilation (bilevel positive airway pressure) for 48 hours. He was discharged home after 5 days of hospitalization, having required supplemental oxygen for 4 of those days.

Patient 2 was a 9-year-old boy who was hospitalized on October 1, 2014, with a history of severe asthma since 7 years of age. He had visited the emergency department multiple times for asthma crises. Both parents and his 2 brothers also had asthma. The episode reported here began 4 days before hospitalization, with a dry cough and progressive breathing difficulty, requiring 3 emergency department visits. During the third visit, low oxygen saturation (91%) led to hospitalization. The child had no fever during this entire episode. He received up to 50% fraction of inspired oxygen, intravenous methylprednisolone, intravenous magnesium sulfate, and inhaled salbutamol; he was discharged in good medical condition after 8 days of hospitalization, having received supplemental oxygen for 7 of those days.

These 2 EV-D68–positive patients had marked pulmonary hyperinsufflation and required prolonged oxygen

Table. Clinical features of enterovirus–positive patients, Chile, September–October 2013 and 2014*

Feature	2013, n = 6	2014, n = 27
Median age, y (IQR)	1.6 (0.25–5.0)	6.0 (2.5–7.0)
Male sex, %	50	52
Admitted to hospital, no.	5	24
Admission diagnosis, no. (%)		
Asthmatic crisis	1 (20)	16 (67)†
Obstructive bronchitis	2 (40)	5 (21)
Pneumonia	0	2 (8)
Other‡	2 (40)	1 (4)
Asthma as preexisting condition, no. (%)	1 (20)	13 (54)
Median oxygen saturation at admission (IQR)	96 (95.0–98.5)	91 (88.5–93.5)
Admitted to PICU, median no. (%)	0	9 (37)
Ventilation support required, no. (%)	0	5 (21)
Median days requiring supplemental oxygen (IQR)	0 (1–4)	2.5 (1–4.5)
Median days in hospital (IQR)§	3 (3–7)	4 (4–6.5)

*IQR, interquartile range; PICU, pediatric intensive care unit.
†p = 0.07 when considering only hospitalized and p = 0.05 when considering all 6 and 27 enterovirus-positive children, by 1-tailed Fisher exact test.
‡Febrile with seizure (n = 1), fever of unknown origin (n = 1), convulsion in child with epilepsy (n = 1).
§In 2014, one child was transferred to another hospital, and duration of hospital stay is unknown.

therapy. Sequence analysis of the viral protein 1 gene revealed that both of these viruses clustered with the major outbreak strain from the United States. Partial gene sequences of viral protein 1 were deposited in the GenBank database under accession nos. KP247599 and KP247600.

We next used CLART PneumoVir to retest samples that had been positive for enterovirus/rhinovirus by FilmArray during September–October 2013 and 2014. The number of overall samples tested for respiratory viruses did not increase from 2013 to 2014 (227 and 218, respectively), but the percentage of enteroviruses detected increased strikingly (from 2.6% to 14.6%). We then compared clinical characteristics and their frequency of occurrence among enterovirus-positive patients hospitalized during September–October 2013 and September–October 2014. The clinical features of 24 enterovirus-positive patients hospitalized during 2014 differed from those of 5 enterovirus-positive children hospitalized during 2013. Hospitalization in 2014 was mostly for asthmatic crisis in children 2.5 to 7 years of age; this pattern is less clear for the few patients hospitalized in 2013 (Table). A substantial proportion of patients hospitalized in 2014 required oxygen support and admission to the pediatric intensive care unit.

In conclusion, we report 2 confirmed cases of EV-D68 in a Southern Hemisphere country during the 2014 outbreak reported in the United States. That these cases are virologically and clinically related to those reported in the United States documents that the virus had been introduced to the Southern Hemisphere during the spring of 2014. A substantial increase in enterovirus cases displaying a notably similar clinical pattern (asthmatic crisis in children) strongly suggests that EV-D68 infections are increasingly rapidly. This virus has been previously identified in the region (*3*) but only sporadically. The virus could spread to other areas in Santiago and to other cities, and similar situations could occur in other Latin American countries, especially those with many residents who travel to the United States. Public health officials need to be notified of this potential, and appropriate surveillance and treatment strategies need to be implemented.

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Bat Coronavirus in Brazil Related to Appalachian Ridge and Porcine Epidemic Diarrhea Viruses

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To the Editor: *Tadarida brasiliensis* (I. Geoffroy, 1824) is a species of free-tailed bat that has resident and migratory populations in Brazil (*1*). This species has adapted to urban areas, occupying roofs, ceilings, and other human constructions, and often coexists with other bat species and humans (*2*), enabling epidemiologic risks (*3*). In recent studies, an alphacoronavirus has been detected in urban bat species *Molossus molossus*, *M. rufus*, and *Tadarida brasiliensi* in Brazil (*4*,*5*). Evidence suggests that alphacoronaviruses may use bats as hosts to spread human coronavirus (HCoV) NL63, which originated by evolution of Appalachian Ridge CoV strain 2 (ARCoV.2) (*6*).

[1]These authors contributed equally to this article.

In this study, a total of 20 anal and tracheal swab samples from 10 bats (*T. brasiliensis*) were collected at the Jequitibás Wood, in Campinas, São Paulo State, Brazil (22°54′31.34″S 47°02′58.01″W). We extracted viral genetic material using the RNA Extraction Mini Kit (QIAGEN, Hilden, Germany) and synthesized cDNA using random primers from the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol.

Samples were analyzed by conventional reverse transcription PCR assays using panCoV primers targeting a 215-bp replicase fragment as previously described (*7*) with slight modifications to include more cycles and less extension time in order to obtain more PCR products. Sequencing reactions on a Pancoronavirus-positive anal swab sample (*7*) were performed at Central Laboratory of High Performance Technologies in Life Sciences (LaCTAD) at UNICAMP (http://www.lactad.unicamp.br) using an automated sequencer (3730xl DNA Analyzer; Applied Biosystems).

The chromatograms were edited using the program UGENE version 1.14 (UGENE, http://ugene.unipro.ru/forum/YaBB.pl?num=1407749393) and evaluated using Phred scores for base calling. Alignment was made with ClustalW v.2.1 software (http://www.clustal.org> implemented on Linux command line interface, and a similarity matrix was generated with sequences retrieved from the GenBank database. A 144-nt fragment of the replicase gene was obtained after editing, and phylogenetic analysis was performed after determining the best evolution model by using the jModelTest2 software (https://code.google.com/p/jmodeltest2/). Different CoV sequences were included to represent the genera *Alpha-*, *Beta-*, *Gamma-*, and *Deltacoronavirus*. Clustering with the ARCoV.2 and porcine epidemic diarrhea virus (PEDV) was obtained using the maximum-likelihood (ML) method after 1,000 Shimodaira-Hasegawa–like support values with the general time-reversible model and category approximation in 20 rates category in a gamma distribution (online Technical Appendix Figure, panel A, http://wwwnc.cdc.gov/EID/article/21/4/14-1783-Techapp1.pdf) and neighbor-joining methods under Kimura-2-parameter and 1,000 replicates of bootstrap (online Technical Appendix Figure, panel B).

Subsequently, metagenomic analysis was made by creating a pool of the 10 bat samples. Samples were resuspended in Dulbecco Modified Eagle Medium (Life Technologies-GIBCO, Grand Island, NY, USA) and filtered through 0.22 μm. The recovered sample was then treated with DNase (Invitrogen, Carlsbad, CA, USA) to remove contaminating DNA and with Proteinase K (Invitrogen) to eliminate inhibitors and to disrupt viral capsids. Samples were then subjected to RNA extraction (QIAGEN) and sent to the sequencing core facility. Sequencing was performed on Illumina HiSeq2500 instrument by using the 2×100 bp kit according to manufacturer's instructions.

Through these analyses, we obtained 34,409,110 reads, of which 76.47% had quality index ≥30. The contigs were assembled by de novo genome assembly (blastx E-value $\leq 1^{-5}$) (*8*) generating 10.742 scaffolds: 35 matches for coronaviruses (using the Coronavirus Database, http://covdb.microbiology.hku.hk), 3 matches for PEDV, and 2 matches for HCoV-NL63 (both using the UniProt database, http://www.uniprot.org) (online Technical Appendix). The sequences obtained had 87.5% (126/144) nucleotide identity with ARCoV.2, an unclassified alphacoronavirus (GenBank accession no. JX537912) for which a zoonotic role has been suggested (*6*). Preliminary analysis indicated good coverage of the polymerase region of the ARCoV.2 reference sequence by the reads (quality index >40) by using reference assembly against CoV complete genomes. This finding reinforces the hypothesis of this viral agent in the specimens analyzed. Moreover, molecular assays are under way in our laboratory to elucidate the alternative hypothesis of PEDV presence in bats in Brazil.

In summary, we found that a CoV detected in *T. brasiliensis* bats in Brazil has close phylogenetic relationships to ARCoV.2 and PEDV. Considering the zoonotic impact of these viral agents on the emergence of new diseases in animal and human populations, we believe that both results may strongly contribute to a better understanding of the molecular eco-epidemiology of these alphacoronaviruses. The reconstruction of their evolutionary history to trace their occurrence in humans and in bat populations as well as in other animals is being conducted to clarify their evolutionary pathway.

Acknowledgments

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Tandem Repeat Insertion in African Swine Fever Virus, Russia, 2012

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To the Editor: The recent introduction of African swine fever virus (ASFV) into the European Union (http://www.oie.int/animal-health-in-the-world/the-world-animal-health-information-system/data-after-2004-wahid/) has caused serious concern in pig industries in Europe and their trade partners. African swine fever is one of the most feared infections that can affect pig industries because no vaccine is available and the socioeconomic effect of an outbreak would be serious (*1*). Therefore, early detection and coordinated countermeasures are urgently needed. For these countermeasures, information on disease dynamics and evolution is mandatory. In this respect, molecular epidemiology can be used to trace virus spread and transmission pattern.

Because it is a DNA virus, ASFV evolves rather slowly, and the use of routine genome fragments (variable region of the B646L gene and parts of the E183L gene) for partial sequencing has so far shown 100% identity among strains found in Russia (*2*) and the neighboring countries (*3*). Thus, the resolution power of these approaches is too low for in-detail analyses, which depend on information regarding larger genome fragments or whole genomes.

In 2014, an insertion of a tandem repeat sequence (TRS) in the intergenic region between the I73R and the I329L protein genes was found in ASFV strains from Poland and Lithuania (*3*). This TRS insertion was also found in ASFV strains from Ukraine in July 2012 and from Belarus in June 2013, but not in strains from Russia, Georgia, or Azerbaijan. Gallardo et al. (*3*) concluded that ASFV strains in Lithuania and Poland most likely originated from Belarus. However, these authors indicated that for a full understanding of evolution and spread, additional sequence analyses would be needed, especially from regions of Russia bordering Belarus and Ukraine. We report information for 3 additional sequences from ASFV strains from Russia that were analyzed for the previously-mentioned TRS insertion on the basis of full-genome sequences.

These ASFV strains originated from domestic pigs from the Tulskaya oblast (Tula06/2012), the Tverskaya oblast Kashinskiy district (Kashinskiy 09/2012), and the Tverskaya oblast (Tver06/2012) in 2012. Genome sequences were obtained by using a primer-walking method that was adapted from Portugal et al. (*4*). Resulting PCR products were subjected to next-generation sequencing by using the MiSeq platform (Illumina, San Diego, CA, USA). Raw sequence data were analyzed and assembled by using Genome Sequencer software version 2.6 (Roche, Mannheim, Germany). Additional sequences of the intergenic region of 17 virus isolates from domestic pigs and wild boar from Russia were obtained by using conventional PCR, and amplicons were directly sequenced by using a 3130x1 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations.

Chromatograms were manually edited and assembled by using CAP3 (http://pbil.univ-lyon1.fr/cap3.php). All nucleotide sequences of ASFV isolates obtained in this study were deposited in GenBank under accession nos. KP137625–KP137644. In the alignment, other published sequences available in GenBank from Poland, Lithuania, Belarus, Ukraine, Armenia, Azerbaijan, Russia, and Georgia were included (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/4/14-1792-Techapp1.pdf). Sequence alignment was performed by using the ClustalW algorithm (http://www.clustal.org) as implemented in Geneious version 7.1.7 (Biomatters Ltd., Auckland, New Zealand).

[1]These authors contributed equally to this article.

Figure. Locations where isolates of African swine fever virus were obtained in Russia during or after 2011. Black circles indicate isolates with tandem repeat insertions, and white circles indicate isolates without tandem repeat insertions. obl., oblast; Resp., respublika.

Spatial and temporal patterns were evaluated by using a map generated with the ArcMap package implemented in ArcGIS software 10.1 (ESRI CIS Ltd., Moscow, Russia). This map shows geographic locations of virus isolates from 2011 onwards (Figure). The alignment, as well as the geographic distribution of the available isolates, clearly shows that the TRS insertion was present in 2012, especially in the Russian Tulskaya oblast (Figure). The TRS insertion predominates in subsequent isolates, although isolates without the TRS insertion are still present. Furthermore, this TRS insertion was also present in Ukraine in 2012 but could not be found in any isolates obtained in the Tverskaya Oblast in 2011 and 2012 (Figure).

In conclusion, these findings confirm the suitability of the described TRS for a higher resolution of ASFV molecular epidemiology. However, this TRS insertion was already present in ASFV strains from Russia and is not restricted only to strains from central Europe. Thus, it can be hypothesized that viruses introduced into the European Union originated in Russia, emerged in 2012 or even earlier, and were transmitted through Belarus and Ukraine.

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Norovirus GII.21 in Children with Diarrhea, Bhutan

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To the Editor: Noroviruses are nonenveloped viruses of the family *Caliciviridae* with a single-stranded RNA genome. In developing countries, noroviruses cause >200,000 deaths annually among children <5 years of age (*1*). Noroviruses are divided into 6 genogroups, GI–GVI, which are further divided into genotypes. Twenty-nine of the genotypes cause human infections (*2*). Worldwide, most GII infections are caused by GII.4, followed by GII.3 or GII.6 and then other genotypes, such as GII.2, GII.12, GII.13, GII.17, and GII.7, in varying proportions. We report that GII.21 is the major genotype causing diarrhea in children in Bhutan.

During February 2010–December 2012, fecal samples were collected from children <5 years of age with watery diarrhea who were seen at the outpatient and inpatient clinics of the Department of Pediatrics, Jigme Dorji Wangchuk National Referral Hospital, Thimphu, Bhutan. We extracted RNA from rotavirus-negative fecal samples by using the QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany); norovirus was detected by reverse transcription PCR by amplifying capsid gene at C region (*3*). PCR results were confirmed by nucleotide sequencing of the amplicons (*4*). We determined genogroups and genotypes by submitting nucleotide sequences to the Norovirus Genotyping Tool (http://www.rivm.nl/mpf/norovirus/typingtool).

We collected 15 water samples (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/21/4/14-1856-Techapp1.pdf), including some from streams and water tanks that are sources of the Thimphu city water supply. Some samples were arbitrarily collected from taps from different locations in Thimphu. All samples were assayed for total coliforms, thermotolerant coliforms, and norovirus (*5*).

We performed a multiple sequence alignment using MUSCLE and conducted the phylogenetic analyses with the neighbor-joining method using MEGAS software (http://www.megasoftware.net). The branching patterns were evaluated statistically on the basis of bootstrap analyses of 1,000 replicates.

We tested 270 samples for norovirus. The mean age of children tested was 13.9 months. Sixty-four (23.7%) children were positive for norovirus. Results were positive for genotype GI in 4/210 samples (0/56 in 2010, 3/123 in 2011, and 1/31 in 2012); all positive samples were from boys in the outpatient clinic. Genotype distribution of norovirus GI was 1 GI.3 in 2012 and 1 GI.1 and 2 GI.9 in 2011.

Sixty of the 270 samples were positive for genotype GII norovirus (17/73 in 2010, 24/147 in 2011, and 19/50 in 2012). Of these, 32 (53.3%) were from boys. Forty-six children were outpatients. Most (85%) of the GII norovirus cases occurred in children <23 months of age; 63.3% of infection occurred in children 6–23 months of age (online Technical Appendix Figure 1). By patient age group, the pattern for norovirus GII.21 was similar; 76% of infections occurred in children <23 months, and 62% infections occurred in children 6–23 months.

We determined seasonal distribution of norovirus GII for 52 cases; 21 cases occurred during winter, and only 2 cases occurred during autumn. Nine cases each occurred during spring and summer, and 11 cases occurred during the rainy season (late June through late September). In 2010, GII.3 predominated, followed by GII.4 and GII.7; in 2011, GII.21 was dominant, followed by GII.6, GII.2, and GII.8, then others; and in 2012, GII.21 remained the dominant strain, followed by GII.2, GII.4, and GII.6 (Figure).

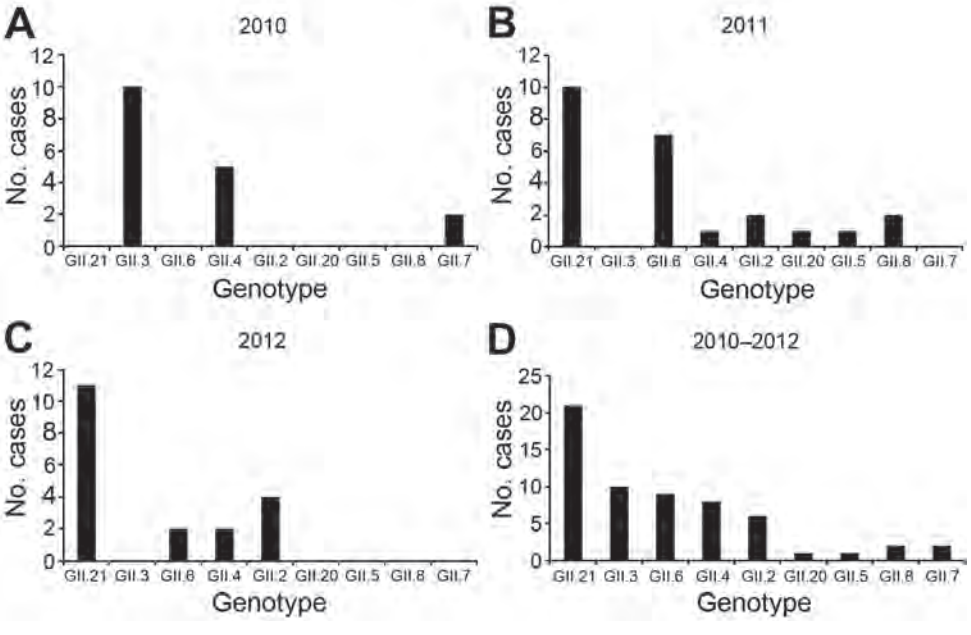

Figure. Yearly distributions of different genotypes of norovirus genogroup GII (A–C) and total numbers (D), Bhutan.

A Sydney variant of norovirus GII.4 was identified in 2012. Only 1 tap water sample (sample W8) from a house was positive for norovirus and coliform bacteria; and nucleotide sequencing of the norovirus amplicon confirmed it as GII.21. Phylogenetic analysis showed that non-GII.21 genotypes from Bhutan were closely associated with strains from Thailand and Korea (online Technical Appendix Figure 2).

In Bhutan, similar to other countries, genogroup GII is mainly responsible for norovirus infections, and most infections occur in younger children (*6*,*7*). In 2010, norovirus GII.3 was the dominant genotype in Thimphu, but in 2011, GII.21 became dominant and continued throughout 2012. GII.21 has been identified mostly in wastewater or rivers, and infections in human have been infrequently attributed to it (*8*). One GII.21 outbreak in a long-term care facility for elderly persons has been reported from the United States (*9*).

Why children in Thimphu were infected by GII.21 is not clear. The detection of norovirus GII.21 in 1 water sample suggests that the source of the outbreak might be tap water; however, GII.21 was not detected in the water related to the tap water supply system. Further examination using repeated samples from different sources is needed. Continuous dominance by GII.21 over 2 years indicates that norovirus of this genotype might have been established in the children of Thimphu, and human-to-human transmission might be ongoing. Determining the environmental source of norovirus GII.21 in Bhutan and developing prevention strategies to control the spread are urgently needed.

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Australia's War against Rabbits: The Story of Rabbit Haemorrhagic Disease

Brian D. Cooke

CISRO Publishing, Collingwood, Victoria, Australia, 2014

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Pages: 232; Price: US $79.95 (paperback)

In Australia's War against Rabbits, Brian Cooke details the emergence of rabbit hemorrhagic disease (RHD) in Asia and Europe and subsequent efforts to introduce RHD virus into Australia and New Zealand for rabbit control in the 1990s. The book documents the ecologic adaption between a virus and its animal host and provides cogent examples of our inability to contain the spread of (what was thought to be) a well-understood virus after it escaped into the environment.

The first failed containment effort ensued after the virus jumped from a quarantine compound on Wardang Island to the coast of South Australia 3 km away. Despite a program carefully designed to counter unplanned spread of RHD virus on the Australian mainland (Operation Garter), the virus prevailed. Uncontained spread also followed an intentional and illegal release of RHD virus in New Zealand in 1997 when a farmer with intractable rabbit problems allegedly smuggled the virus in from Australia. After the outbreak started, instead of helping the New Zealand government control RHD, farmers reportedly used blenders to make "rabbit smoothies" and actively spread RHD virus by applying these slurries to carrot baits.

The book is generally well written and heavily referenced and contains many anecdotes penned by a scientist obviously passionate about his work. The flow of the narrative can sometimes be erratic, however, veering from amusing personal stories to a didactic recounting of the taxonomy of rabbit parasites or legislation relevant to pest control in Australia. Australia's War against Rabbits is best suited for professionals with a keen interest in rabbits, fleas, and their pathogens.

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"Welcome to the World of the Plastic Beach"

Byron Breedlove

"Welcome to the World of the Plastic Beach" is the title and refrain of the second song from the 2010 concept album *Plastic Beach* by the British virtual band Gorillaz. Damon Albarn, who writes the songs, was moved to explore themes of disposable commodities when he noticed all the plastic mixed with the sand near his beach house.

That was four years after Pam Longobardi, an Atlanta-based artist and professor of art at Georgia State University, had encountered "mountainous piles of plastic the ocean regurgitated on remote Hawaiian beaches." Longobardi describes herself as a conceptual artist focused on exploring "the cultural archaeology of our time." This month's cover image, *Ghosts of Consumption/Archaeology of Culture (for Piet M.)*, is one of the most recognized works in her award-winning, ongoing Drifter's Project. It documents the impact of plastic marine debris as it journeys around the world's oceans, coalescing inside giant oceanic currents known as gyres and concentrating the plastic into areas such as the Great Pacific Garbage Patch that floats north of Hawaii.

Hundreds of plastic objects—taken from the tens of thousands of pounds of plastic debris Longobardi has removed

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from beaches—are on display in *Ghosts of Consumption*. Perched on steel pins, this assemblage of flotsam suggests an archeologic or forensic exhibition and invites the viewer to step forward and scrutinize each item. Displayed among this ocean-worn debris are household items, kitchen utensils, toys, and sporting goods; interspersed among them are the numerous black tubes that provide unity and rhythm to the installation. Many of these items are not recognizable; their original shapes have been distorted, smoothed, and rounded by the oceans' crucible of tides, currents, heat, friction, and chemistry.

Ghosts of Consumption, dedicated to the Dutch artist Piet Mondrian, recalls Mondrian's 1915 painting *Pier and Ocean*. Celebrated for its precise visual arrangement of intersecting horizontal and vertical lines, *Pier and Ocean* captures the pulse and rhythm of the ocean. The horizontal lines symbolize the ocean's surface, rendering its transient troughs and ridges uniformly, and the vertical lines denote the man-made pier that juts into the ocean, its form defined by the intersecting planes.

Longobardi's montage, representing the collision of nature and consumerism, also comprises an oval grid of horizontal and vertical lines, shapes, and forms. The intersection and contrast of the black tubes and distorted artifacts of collected rubbish disrupt the work's balance and harmony. According to the artist, the horizontal elements symbolize the natural world, and the vertical grid represents the human element, in particular, the consumer-based cultures of the world. "I created this work as an homage to Mondrian, an artist whom I admire. Mondrian's work was his response to the relationship between humans and the ocean in 1914, and mine is a response to our relationship to the ocean 100 years later."[1]

In *Ghosts of Consumption*, Longobardi uses art to engage our minds and our hearts in an unavoidable conversation about the consequences of disposability to the ocean environment. Longobardi describes her installations as "being preferentially in a transitive state, such that they may be reabsorbed into culture, commerce, or industry, as the technology develops to return plastic into oil."

Exactly how much plastic is in the ocean cannot be precisely measured. Plastics are estimated to comprise 60%–80% of all marine litter, perhaps 90%–95% in some areas. More than an eyesore, this debris harms marine biota and allows invasive species to hitchhike around the globe. The United Nations is among those who have flagged this problem, noting that "Communities of microbes have been discovered thriving on microplastics at multiple locations in the North Atlantic. This 'plastisphere' can facilitate the transport of harmful microbes, pathogens and algal species."

[1]Pamela Longobardi, personal communication, February, 10, 2015

Ghosts of Consumption focuses on the global consequences of disposability. The late Lewis Thomas wrote about the interdependence of life on earth and suggested the earth itself of being "most like a cell." This perspective offers another way to view Longobardi's installation, which resembles the contour of a cell, its myriad black tubes analogous to the microtubules within a cell's cytoskeleton. Once pristine and healthy (more like that Mondrian painting), the cell is now infested with various rods, filaments, and spheres of plastic debris and flotsam, items that emerged from the ocean reshaped and mutated from their original forms.

The ideas that fueled Longobardi's creation of *Ghosts of Consumption* appear in many of the articles in this issue and should also resonate with readers and researchers. Environmental degradation and encroachment, global travel and commerce, and climate are factors that provide opportunities for viruses to emerge in novel and expanded niches and to infect new host populations. Such human-derived factors allow these emerging pathogens greater dispersal and more opportunities to spill over to humans or other hosts.

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- Molecular Epidemiology of Malaria Outbreak, Tumbes, Peru, 2010–2012
- Delayed Severe Hemolytic Anemia in Patients with Severe Malaria Treated with Artesunate, France, 2011–2013
- Itaya virus, a Novel Orthobunyavirus Associated with Human Febrile Illness, Peru
- Canine Distemper in Endangered Ethiopian Wolves
- Canine Infections with *Onchocerca lupi* Nematodes, United States, 2011–2014
- Low-level Circulation of Enterovirus D68–associated Acute Respiratory Infections, Germany, 2014
- Postmortem Stability of Ebola Virus
- Transmission Potential of Influenza A(H7N9) Virus
- Novel Eurasian Highly Pathogenic Influenza A H5 Viruses in Wild Birds, Washington, USA, 2014
- Antimicrobial Drug Resistance of *Vibrio cholerae*, Democratic Republic of the Congo
- Pin-Site Myiasis Caused by Screwworm Fly, Colombia
- Ebola and Psychological Stress of Health Care Professionals
- Enterovirus D68–Associated Severe Pneumonia, China, 2014
- Probable Toxic Cause for Suspected Lychee-Linked Viral Encephalitis
- Enterovirus D68–Associated Acute Respiratory Distress Syndrome in Adult, United States, 2014
- Melioidosis in Trinidad and Tobago
- Acute Zika Virus Infection in a Traveler, Malaysian Borneo, September 2014

Complete list of articles in the May issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

April 12–15, 2015
9th International Symposium on Avian Influenza
Athens, GA, USA
http://www.georgiacenter.uga.edu/uga-hotel/conferences-events/register/9th-international-symposium-on-avian-influenza

April 20–23, 2015
EIS
Epidemic Intelligence Service Conference
http://www.cdc.gov/eis/conference.html
Atlanta, GA USA

May 14–17, 2015
SHEA
The Society for Healthcare Epidemiology of America
Orlando, FL, USA
http://shea2015.org/attendees/registration/

May 30–June 2, 2015
American Society for Microbiology General Meeting
New Orleans, LA, USA
http://gm.asm.org/

August 10–21, 2015
14th International Dengue Course
Havana, Cuba
http://instituciones.sld.cu/ipk/14thdenguecourse/

August 24–26, 2015
ICEID
International Conference on Emerging Infectious Diseases
Atlanta, GA, USA

August 29–September 2, 2015
IDBR
20th Annual Infectious Disease Board Review Course
McLean, VA, USA
http://smhs.gwu.edu/cehp/activities/courses/idbr

Announcements
To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to http://www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Animal-Associated Exposure to Rabies Virus among Travelers, 1997–2012

CME Questions

1. Your patient is a 52-year-old man planning a vacation to China. According to the study by Gautret and colleagues, which of the following statements about the demographic characteristics of travelers exposed to potentially rabid animals that might differentiate them from other ill travelers seeking medical care is correct?

A. Most persons who reported to GeoSentinel requiring rabies postexposure prophylaxis (PEP) were middle-aged adults
B. Most persons who reported to GeoSentinel requiring rabies PEP were business travelers visiting high-income regions
C. This study showed that only children were at risk for animal bites requiring PEP
D. Travelers exposed to potentially rabid animals did not have specific demographic characteristics differentiating them from other ill travelers seeking medical care

2. According to the study by Gautret and colleagues, which of the following travel-related characteristics is most likely to be associated with potential exposure to rabid animals?

A. Long duration of stay
B. Travel to South America
C. Travel to Asia
D. Travel during the winter

3. Which of the following statements about the clinical implications of findings from the study by Gautret and colleagues would most likely be correct?

A. Regardless of demographics or length of stay, international travelers to Asia and other rabies-endemic regions should be informed about potential rabies exposure and pretravel vaccination
B. Study findings support current recommendations from the US Centers for Disease Control and Prevention stating that preexposure rabies vaccine recommendations should be based, at least in part, on longer durations of stay
C. Bats are the leading animal responsible for exposure among travelers
D. Travelers exposed to nonhuman primates (NHPs) do not require rabies PEP because rabies cannot be transmitted from NHPs to humans

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to http://www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Deaths Associated with Respiratory Syncytial and Influenza Viruses among Persons ≥5 Years of Age in HIV-Prevalent Area, South Africa, 1998–2009

CME Questions

1. Which of the following general trends was identified in respiratory deaths in South Africa in the current study?

A. Gradual decline in the rate of respiratory deaths between 1998 and 2009
B. Gradual increase in the rate of respiratory deaths between 1998 and 2009
C. Sharp increase in the rate of respiratory deaths between 1998 and 2004, followed by a diminution
D. Sharp increase in the rate of respiratory deaths maintained after 2004

2. Which of the following statements regarding death related to influenza infection in the current study is most accurate?

A. The highest rates of death were found among young adults, who had the highest rates of concomitant HIV infection
B. The presence of HIV was associated with a nearly 8-fold increase in the risk for influenza-related death
C. Influenza A H1N1 particularly increased influenza-related mortality rates among older adults
D. There was limited difference in the rate of influenza-related mortality based on age

3. Which of the following statements regarding mortality related to respiratory syncytial virus (RSV) in the current study is most accurate?

A. Mortality rate related to RSV was highest among children and adolescents
B. There was a spike in RSV-related mortality rate among individuals 75 years and older
C. Concomitant HIV infection had an insignificant effect on the risk for RSV-related mortality
D. Nearly 90% of individuals who died of RSV had concomitant HIV infection

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 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.

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Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

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

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.