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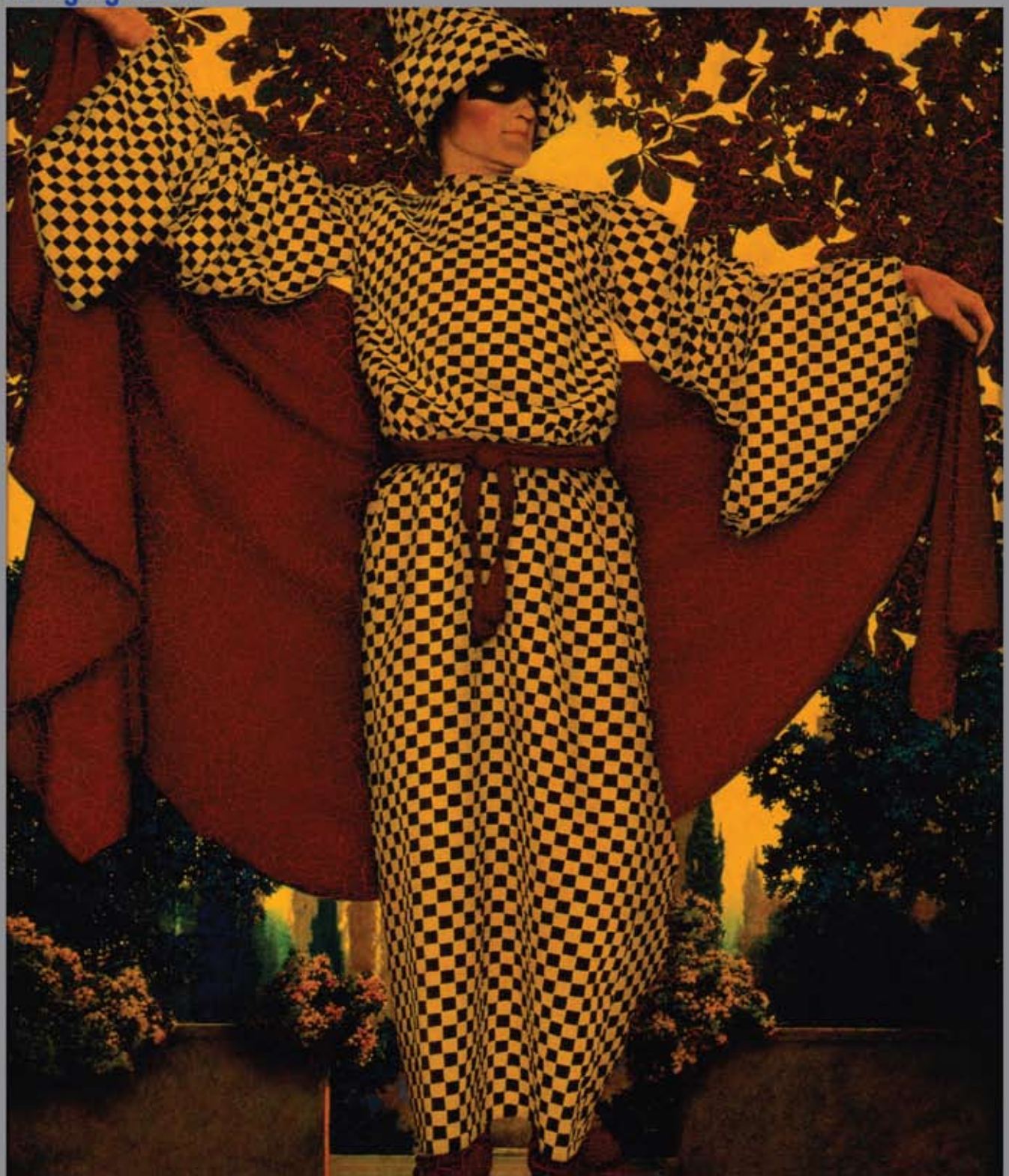
August 2011

Emerging Viruses



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On the Cover

Maxfield Parrish (1870–1966)
Masquerade (1922)
Oil on board (43.2 cm × 35.6 cm)
High Museum of Art, Atlanta, Georgia, USA
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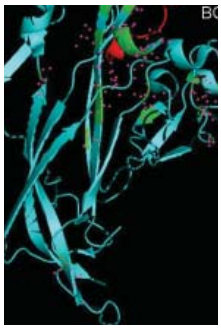

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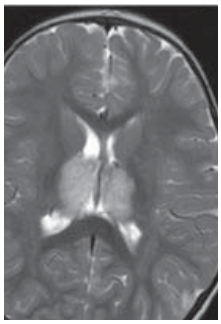

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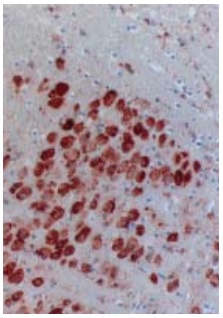

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Control and Prevention of Viral Gastroenteritis

Stephan S. Monroe

Diarrheal illness remains 1 of the top 5 causes of death in low-income and middle-income countries, especially for children <5 years of age. Introduction of universal childhood vaccination against rotaviruses has greatly reduced the incidence and severity of illness in upper-income and lower-income settings. For adults, norovirus is the leading cause of sporadic cases and outbreaks of diarrheal illness and is responsible for nearly 21 million episodes annually in the United States, of which 5.5 million are foodborne. Public health efforts to control and prevent norovirus illness have focused on rapid outbreak detection and source identification and control of transmission in institutional settings.

Diarrheal illness remains 1 of the top 5 causes of death in lower-income and middle-income countries (*1*), especially for children <5 years of age. In the ≈40 years since the initial detection of Norwalk virus (*2*) and rotavirus (*3*) by electron microscopy in stool samples of patients with gastroenteritis, there has been increased recognition of the role of enteric viruses as a major cause of diarrhea-associated illness and death in young children and adults. Unfortunately, standard improvements in water and sanitation that reduce the incidence of enterically transmitted bacteria do not appear to be equally effective for reducing the incidence of enterically transmitted viruses. Thus, other public health approaches have been pursued for the control and prevention of viral gastroenteritis.

For children <5 years of age, rotavirus is the leading cause of diarrhea-associated illness and death. Fortunately, safe and effective vaccines against rotavirus illness are now available in many countries. Introduction of universal childhood vaccination against rotaviruses greatly reduces the incidence and severity of illness in upper-and lower-income settings (*4*). As a result, the World Health Organization recommended in 2009 that rotavirus vaccines be included in all national immunization programs (*5*).

In adults, norovirus is now recognized as the leading cause of sporadic cases (*6*) and outbreaks of diarrheal illness and is responsible for ≈21 million episodes annually in the United States, of which 5.5 million are foodborne (*7*). Efforts to develop effective vaccines for norovirus have been hindered by lack of a cell culture system to propagate the virus, large genetic diversity of norovirus strains, and apparent lack of long-term immunity generated by natural infection. Recent work on characterizing the interaction between noroviruses and their putative cellular receptors, histo–blood group antigens, may provide insights for development of specific antiviral compounds (*8*).

Public health efforts to control and prevent norovirus illness have focused primarily on outbreak detection and control. The implementation of CaliciNet, as described by Vega et al. (*9*), provides a useful new public health tool for rapid identification of norovirus outbreaks. Similar to the successful PulseNet network for molecular typing of foodborne bacteria (*10*) and NoroNet in Europe (*11*), CaliciNet will enable linking of cases with identical sequence fingerprints into clusters of illness that may have a common exposure. This linking will be particularly useful in cases of illness related to food products with low levels of contamination in which identification of exposure to a common food source may be difficult by epidemiologic methods alone.

Because a large proportion of norovirus illness results from foodborne exposures, considerable effort has gone into development of methods for detecting and eliminating virus contamination from food items, particularly shellfish (*12*) and fresh produce (*13*). Additionally, because

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outbreaks of norovirus illness often occur in institutional settings, efforts are under way to standardize effective methods for disinfection of contaminated surfaces (*14*).

Finally, several other viruses, including astrovirus, sapovirus, and as described by Drexler et al. (*15*), Aichi virus, are also responsible for diarrheal illness in children and adults. Although the incidence and severity of illness caused by these pathogens may not warrant immediate development of vaccines, work continues to document their relative contributions to diarrhea-associated illness and death. Thus, although there is optimism for universal vaccination to prevent illness and death from severe rotavirus diarrhea and for reduction of norovirus illness by rapid outbreak detection and source identification, there are still many challenges remaining for the control and prevention of viral gastroenteritis.

Dr Monroe is director of the Division of High-Consequence Pathogens and Pathology at the Centers for Disease Control and Prevention. His primary research interests are the biology and epidemiology of high-consequence pathogens and public health policy regarding these pathogens.

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Dengue Virus Infection in Africa

Ananda Amarasinghe, Joel N. Kuritsky, G. William Letson, and Harold S. Margolis

Reported incidence of dengue has increased worldwide in recent decades, but little is known about its incidence in Africa. During 1960–2010, a total of 22 countries in Africa reported sporadic cases or outbreaks of dengue; 12 other countries in Africa reported dengue only in travelers. The presence of disease and high prevalence of antibody to dengue virus in limited serologic surveys suggest endemic dengue virus infection in all or many parts of Africa. Dengue is likely underrecognized and underreported in Africa because of low awareness by health care providers, other prevalent febrile illnesses, and lack of diagnostic testing and systematic surveillance. Other hypotheses to explain low reported numbers of cases include cross-protection from other endemic flavivirus infections, genetic host factors protecting against infection or disease, and low vector competence and transmission efficiency. Population-based studies of febrile illness are needed to determine the epidemiology and true incidence of dengue in Africa.

Dengue has emerged in recent decades as a worldwide public health problem, particularly in the Asia–Pacific and Americas–Caribbean regions (*1*–*3*). In Africa, the epidemiology and public health effect of dengue is not clear. *Aedes* spp. mosquitoes are widely distributed in Africa and can serve as vectors of dengue virus (DENV). When their distribution is combined with rapid population growth, unplanned urbanization, and increased international travel, extensive transmission of DENV is likely in Africa (Figure) (*2*,*4*). Over the past 5 decades, cases of epidemic or sporadic dengue have been reported in many countries in sub-Saharan Africa (*5*). Although sporadic cases of dengue hemorrhagic fever (DHF) have been reported in a few countries in Africa, no outbreaks have been reported (*1*). However, when compared with the Asia–Pacific and Americas–Caribbean regions, the epidemiology and effect of dengue in Africa has not been defined. A dengue outbreak in Cape Verde was recently reported (>3,000 cases), and the reappearance of dengue in Senegal after 20 years was also reported (*6*). To estimate the extent of DENV infection and dengue in Africa, we reviewed published literature and other sources for reports of this disease in persons 1iving in or traveling to this region.

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Search Methods

Published, peer-reviewed literature, published and unpublished country reports, and the World Health Organization (WHO) library database, including Dengue Net, were reviewed for 1960–2010 for the key terms "dengue" and "Africa." In addition, we examined peer-reviewed published literature and other sources to determine the extent of disease among travelers returning from Africa. We searched for publications in English by using MEDLINE and EMBASE electronic databases, Euro Surveillance, and ProMED-mail posts. A review for dengue reports in languages other than English did not find any reports that would change the conclusions of this article.

Additionally, references in each paper identified during searches were checked. Those references not already identified by the search were reviewed. Abstracts presented at international forums were included if they contained epidemiologic, entomologic, or virologic data pertaining to dengue in Africa.

Reported Dengue in Africa

Dengue was reported in Africa in the late 19th and early 20th centuries. Epidemics were reported in Zanzibar (1823, 1870), Burkina Faso (1925), Egypt (1887, 1927), South Africa (1926–1927), and Senegal (1927–1928) (*1*,*5*,*7*). The epidemic in South Africa was confirmed by retrospective neutralizing antibody testing in the mid-1950s, but the other reported epidemics were not laboratory

confirmed and therefore may not have been dengue. During the 50 years from 1960 through 2010, twenty laboratory-confirmed dengue outbreaks were reported in 15 countries in Africa; most were from eastern Africa. Nearly 300,000 cases were reported in 5 large epidemics in the Seychelles (1977–1979), Réunion Island (1977–1978), Djibouti (1992–1993), Comoros (1992–1993), and Cape Verde (2009) (*6*–*9*). DENV was first isolated in Africa in Nigeria in the 1960s (*10*). Subsequently, all 4 DENV serotypes have been isolated in Africa (*1*). DENV-2 has been reported to cause most epidemics, followed by DENV-1 (*8*,*9*).

Available data strongly suggest that DENV transmission is endemic to 34 countries in all regions of Africa (Figure; Table). Of these countries, 22 have reported local disease transmission, 20 have reported laboratory-confirmed cases, and 2 (Egypt and Zanzibar) have reported only clinical cases that were not laboratory confirmed. In the remaining 12 countries, dengue was diagnosed only for travelers returning to countries to which dengue was not endemic but never reported as occurring locally in these 12 countries (Table).

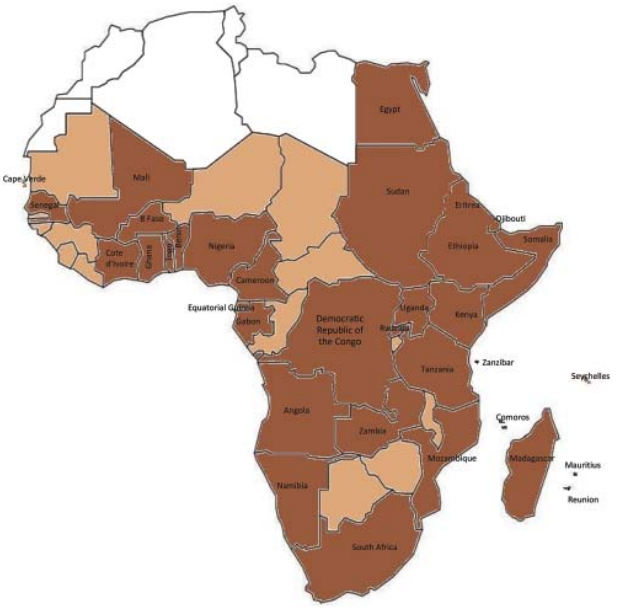

Figure. Dengue and *Aedes aegypti* mosquitoes in Africa. Brown indicates 34 countries in which dengue has been reported, including dengue reported only in travelers, and *Ae*. *aegypti* mosquitoes. Light brown indicates 13 countries (Mauritania, The Gambia, Guinea-Bissau, Guinea, Sierra Leone, Liberia, Niger, Chad, Central African Republic, Republic of the Congo, Malawi, Zimbabwe, and Botswana) in which dengue has not been reported but that have *Ae. aegypti* mosquitoes. White indicates 5 countries (Western Sahara, Morocco, Algeria, Tunisia, and Libya) for which data for dengue and *Ae. aegypti* mosquitoes are not available.

Dengue among Travelers/Expatriates Returning from Africa

The European Network on Imported Infectious Disease Surveillance and other published data have reported 27 countries in Africa as locations where travelers/expatriates from regions to which dengue was not endemic acquired dengue (Table) (*11*–*15*). Among travel-acquired dengue cases reported among persons from Europe, only 2%–8% had visited Africa (*11*–*14*). Although 54%–61% and 25%–31% of returning travelers with dengue returned from Asia and Latin America, respectively, Africa seems somewhat underrepresented with respect to dengue. However, this finding is not the result of a paucity of visits among travelers from countries to which dengue is not endemic.

Wilson et al. reported for the GeoSentinel Surveillance Network of the International Society of Travel Medicine and the Centers for Disease Control and Prevention that travelers reporting illness have disproportionately visited Africa (*15*). Febrile illness was more frequently reported for travelers to sub-Saharan Africa (371 febrile illnesses/1,000 patients) than to any other region, followed by Southeast Asia (248/1,000) and South America (143/1,000) (*11*). Similar to reported global dengue endemicity patterns by region, travelers with dengue came more frequently from Southeast Asia and South America than Africa (*11*).

Prevalence of DENV Infection in Africa

Although outbreaks of dengue have been reported, data on incidence or prevalence are not available for Africa. A study in Nigeria determined the prevalence of flavivirus infections among 1,816 children and adults from urban and rural areas in samples obtained mainly during the early 1970s. Virus-specific hemagglutination inhibition testing showed that the prevalence of immunity was 38% for DENV-1 infection, 45% for DENV-2 infection, 43% for yellow fever virus infection, and 49% for West Nile virus infection (*16*). Serum specimens were also tested by suckling mouse neutralization of DENV-2. The authors concluded that because a high proportion of specimens with antibody to DENV-2 were confirmed by neutralization and because many had only monotypic antibody, the prevalence results were not likely confounded by cross-reactive antibody to other flaviviruses. In addition, this study showed an increase in prevalence of antibodies against DENV with age, which suggests endemic infection (*16*). Collenberg et al. reported that the prevalence of antibodies against DENV determined by immunoglobulin G indirect ELISA among a sample of pregnant women and blood donors (n = 683) was 26.3% in rural settings and 36.5% in urban settings in Burkina Faso (*17*). However, in Cameroon, the prevalence of antibodies against DENV determined by neutralization testing among 256 adults was only 12.5% (*18*).

The prevalence of DENV infection found by these studies was considerably lower than that found in dengue-

Table. Countries in Africa with evidence of dengue virus transmission*

Type and country	Year	Serotype
Locally acquired, n = 7		
Cape Verde	2009†	3
Egypt	1779, 1887, 1927	Unknown
Eritrea	2005	Unknown
Mauritius	2009	Unknown
Réunion	1977–1978†	2
Seychelles	1977–1979†	2
Sudan	1984–1986	1 and 2
Locally and travel acquired, n = 15		
Angola	1986, 1999–2002‡	Unknown
Burkina Faso	1925	Unknown
	1983–1986	2
	2003–2004,§ 2007‡	Unknown
Cameroon	1987–1993,§ 1999–2002,‡ 2000–2003,§ 2006‡	Unknown
Comoros	1943–1948	Unknown
	1984, 1992–1993†	1 and 2
Djibouti	1991–1992†	2
Côte d'Ivoire	1982	2
	1998	1
	1999–2002‡	Unknown
	2008	3
Ghana	1932, 1987–1993§	Unknown
	2002–2005	2
Kenya	1982	2
	1984–1986	1 and 2
Madagascar	1943–1948	Unknown
	2006	1
Mozambique	1984–1985†	3
Nigeria	1964–1968	1
Senegal	1928	Unknown
	1979	1
	1980–1985	2 and 4
	1990, 1999	2
	2007‡	Unknown
	2009	3
Somalia	1982, 1985–1987	2
	1992–1993	2 and 3
South Africa	1927†	1
Zanzibar	1823, 1870, 2010‡	Unknown
Travel/expatriate acquired, n = 12		
Benin	1987–1993§	Unknown
DRC	1999–2001,‡ 2007§	Unknown
Ethiopia	1999–2002,‡ 2007§	Unknown
Equatorial Guinea	1999–2002‡	Unknown
Gabon	1999–2002‡	Unknown
Mali	2008	2
Namibia	1999–2002,‡ 2006‡	Unknown
Rwanda	1987–1993§	Unknown
Tanzania	1987–1993,§ 1999–2002,‡ 2006,‡ 2010‡	Unknown
Togo	1987–1993,§ 1999–2002‡	Unknown
Uganda	1999–2002‡	Unknown
Zambia	1987–1993§	Unknown

*DRC, Democratic Republic of the Congo.
†Large local outbreaks.
‡TropNet Europ Network (www.tropnet.net) and ProMED mail (www.promedmail.org).
§Seroprevalence study.

endemic areas of Asia and the Americas (*19*). However, it is difficult to generalize from the small number of studies in Africa because they had small sample sizes and noncomparative age groups. Although the testing method used for the studies in Nigeria and Cameroon studies would minimize overestimation of DENV infection prevalence because of cross-reactive antibodies to other flavivirus infections or yellow fever vaccination, use of an immunoglobulin G ELISA in the Burkina Faso study did not differentiate these infections.

Underrecognition of Dengue in Africa

In regions to which malaria is endemic, >70% of febrile illnesses are treated as presumptive malaria, often without proper medical examination and a laboratory diagnosis (*20*,*21*). In a setting where diagnostic testing is conducted, such as the GeoSentinel Surveillance Network, malaria was found to be the predominant cause of systemic febrile illness among travelers returning from sub-Saharan Africa (622/1,000 patients) compared with dengue (7/1,000) (*11*). This finding is not unexpected because malaria is more endemic to Africa than other febrile illnesses. However, overdiagnosis of malaria in areas of low transmission is well documented, and overestimation by clinical diagnosis is ≈61% (*20*,*21*). Many patients in Africa with fever are designated as having fever of unknown origin or malaria and remain without a diagnosis even if they fail to respond to antimalarial drugs. Under these prevailing practices, there is a real potential of misdiagnosing dengue as malaria.

In disease-endemic countries in the Asia–Pacific and Americas–Caribbean regions, dengue accounts for 3%–11% of febrile illnesses (*19*). Although dengue is well recognized as a public health problem in these regions, underreporting is common. Capture–recapture studies in Puerto Rico showed that the degree of underreporting and reporting in a passive surveillance system was ≈60% (*22*). Wichmann et al. showed that in Thailand and Cambodia underreporting of dengue was 1.4–9.6-fold (*23*). A virologic study conducted in the Sudan among 100 consecutive hospitalized patients with fever reported that 21 cases were caused by DENV infection (*24*). Coupled with the bias toward classifying most febrile illness as malaria, we expect that there is substantial underrecognition and underreporting of dengue in Africa.

During the 18th and 19th centuries, dengue was recognized almost exclusively among colonial settlers and military forces in Asia and the Americas and not among the local population, probably as a consequence of inadequate clinical investigation and surveillance (*25*). Similarly, except for some reported local outbreaks, many cases of dengue in Africa are more frequently reported among travelers than among the local population, which suggests lack of awareness, diagnostic facilities, and surveillance. In

addition, travelers with febrile illness are frequently given a misdiagnosis of malaria; a rate of misdiagnosis as high as 77% has been reported (*20*). Of 27 countries in Africa where travelers/expatriates acquired dengue, only 15 have reported local disease transmission (Table). Therefore, travel-acquired dengue appears to serve as a proxy for identifying the underrecognition of dengue in Africa.

Factors Potentially Affecting Sustained Transmission of DENV in Africa

Vector Ef ciency

Aedes aegypti mosquitoes, the principal DENV vector, originated in Africa and spread to other countries in Africa and other tropical countries in the 17th and 18th centuries (*1*,*3*). Several other *Aedes* species mosquitoes, including *Ae. albopictus*, *Ae. africanus*, and *Ae. luteocephalus*, are found in Africa and are potential DENV vectors.

Urbanization is a major factor in facilitating the increase of *Aedes* spp. mosquito populations (*1*). Accumulation of nonbiodegradable, human-made containers in and around living areas has provided the aquatic environment required by these mosquitoes (*25*). Since the 1950s, a 3-fold increase in urban human population density has occurred in Africa; larger increases have occurred in Asia and the Americas (*4*). With these demographic changes and subsequent increases in *Aedes* spp. populations, increased DENV transmission is likely to occur in Africa. For example, in Ghana, *Aedes* spp. mosquito densities and biting rates seem sufficient to result in outbreaks of yellow fever and dengue (*26*).

Susceptibility of different strains of *Aedes* spp. mosquitoes to DENV has been shown to vary geographically, and this variability may have implications for DENV transmission and the epidemiology of the disease in Africa. Mosquito strains in Africa have shown uniformly low susceptibility to all 4 DENV serotypes in laboratory settings (*27*–*29*). In addition, it has been well documented that there are different susceptibilities of the vector to different DENV genotypes; *Ae. aegypti* mosquitoes tend to be more susceptible to infection with DENV-2 of the Southeast Asian genotype than to the American genotype (*30*). Similar findings have been described for yellow fever virus, and the reduced vector competence of strains of *Ae. aegypti* mosquitoes from Asia has been suggested as an explanation for the absence of this disease in Asia (*2*,*31*). Reduced vector competence for DENV infection in Africa may be an explanation for some of the apparent low prevalence of DENV infection in Africa, although this explanation must be confirmed in appropriate studies.

Ae. albopictus mosquitoes are also potential DENV vectors in Africa where they are considered more anthropophilic than *Ae. aegypti* mosquitoes, more susceptible to DENV infection, and are responsible for some dengue outbreaks in Africa (*29*,*32*–*33*). However, similar to studies with *Ae. aegypti* mosquitoes, experimental studies with *Ae. albopictus* mosquitoes have demonstrated that geographic variations in susceptibility to DENV infection occur among different species (*28*,*29*). Furthermore, *Ae. albopictus* mosquitoes are believed to be less efficient as an epidemic vector largely because of their differences in host preferences and reduced vector competence, which decreases the probability of sustained disease transmission (*34*). Thus, appropriate ecologic studies are needed in Africa to determine the relative roles of each species in transmission of DENV.

Virus Infectivity

Dengue is caused by 4 genetically related but antigenically different viruses, and although it is uncertain where DENV evolved, maintenance of all 4 serotypes in enzootic cycles in Africa suggests that a progenitor virus most likely originated in Africa (*1*). Despite the apparent origin of DENV in Africa hundreds of years ago, the more recent reported outbreaks appear to be the result of virus introductions from Southeast Asia or the western Pacific region and not the result of spillover from forest transmission cycles. (*25*).

Vasilakis et al. reported that the rate of evolutionary change and pattern of natural selection are similar among endemic and sylvatic DENVs and suggested possible future reemergence of DENV from the sylvatic cycle (*35*). Recent experimental evidence suggests that emergence of endemic DENV-2 from sylvatic progenitors may not have required adaptation to replicate efficiently in humans, implying that sylvatic DENV-2 may reemerge (*35*). Existence of a silent zoonotic transmission cycle affords a potential mechanism for emergence of dengue in human populations and for selection of virus variants with altered host range and vector relationships (*25*).

Host Susceptibility

Host genetic factors influencing pathogenesis have been suggested to account for some variability in susceptibility of DENV infection and disease expression among different races. Halstead et al. provided evidence of a dengue resistance gene in the black population (*36*). During the 1981 and 1997 dengue epidemics in Cuba, blacks were hospitalized with DHF/dengue shock syndrome at lower rates than whites (*37*). This potential decreased susceptibility to severe disease among the black population and similar observations in Haiti have been used to support the hypothesis that specific genomic difference among different racial groups is a risk factor for DHF (*36*,*38*). This hypothesis may provide an explanation for the observation that, to our knowledge, outbreaks of DHF/dengue shock syndrome have not been reported in Africa.

Other prevailing diseases in Africa could provide another hypothesis to explain the apparently low incidence of dengue. Malaria, tuberculosis, and HIV infections are endemic to many parts of Africa. Prevailing socioeconomic and environmental factors may make populations in Africa more vulnerable to these diseases than to dengue. Monath (*31*) and Gubler (*2*) hypothesized that immunologic cross-protection from heterotypic antibodies to other flavivirus infections (DENV and Japanese encephalitis virus) could explain the absence of yellow fever virus in Asia. A similar argument could be made to explain the low rate of DENV infection caused by cross-protection from other endemic flaviviruses in Africa, but the extent to which it may exist is unknown.

Conclusions

Dengue is a highly underrecognized and underreported disease even in areas of the world where there is a high level of public health and clinical awareness and diagnostic capacity. In Africa, most febrile illness is not assessed by laboratory diagnostics and is assumed to be malaria. Sustained, systematic surveillance for dengue-like illness combined with laboratory diagnostics and education of health care providers has been the source of the information about the public health role of dengue in Asia and the Americas. This surveillance is needed in Africa to determine the epidemiology and public health role of dengue.

The 2004 WHO Global Epidemiology of Infectious Diseases Study estimated that 2.4% of global DHF cases occurred in Africa and that 20% of the population in Africa was at risk for dengue (*39*). However, because these estimates were only for DHF and not dengue fever (DF), a conservative approach to estimate DF in Africa would be to apply the expected DHF to DF ratio of 1% to 5% to this WHO study estimate. Thus, 0.2–1.0 million cases of DF could be expected to occur in Africa on the basis of WHO estimates of 10,000 cases of DHF in Africa, although no DHF outbreaks have been reported.

Although there is some uncertainty about estimates of cases provided by various sources, these estimates provide a strong argument that DENV transmission is present in Africa but likely underreported. Reported outbreaks and dengue acquired by travelers to Africa from regions to which dengue is not endemic indicate that local transmission of DENV occurs in Africa. Furthermore, the apparent increase of dengue in the region is the result of an increase in the disease, consequence of improved disease reporting, or both. Nevertheless, the epidemiology of DENV transmission and the incidence of dengue in Africa are poorly defined.

Dengue is usually not among the differential diagnoses of acute febrile illness in Africa. Reasons for this lack of inclusion are as follows: 1) malaria is the most prominent endemic febrile illness in Africa and does not require complex clinical and laboratory diagnostic facilities; 2) a low awareness of dengue may contribute to health care workers not considering the disease; 3) dengue is not a reportable disease in most countries in Africa; 4) dengue surveillance and diagnostics are not widely and consistently available throughout Africa; and 5) funding for surveillance and other research activities pertaining to dengue in Africa are limited (*8*,*9*). For these reasons, improved surveillance and laboratory diagnosis of fevers in Africa is a priority and first step in assessing the incidence of dengue in Africa.

Whether populations in Africa are susceptible to DENV infection and disease at rates comparable with those in populations in Asia or the Americas and the true incidence of dengue in these countries cannot be determined from data obtained from occasional reports of disease outbreaks. Given that safe and effective dengue vaccines should become available within the next decade (*40*), questions regarding dengue incidence and epidemiology in Africa must be answered by using appropriately designed surveillance studies. Studies to determine the extent of DENV infection among persons of all ages with febrile illness could be included in other studies (e.g., malaria) being conducted in the region and would provide answers to speculation about dengue in Africa that has existed for many years.

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Seroprevalence of Trichodysplasia Spinulosa–associated Polyomavirus

Els van der Meijden, Siamaque Kazem, Manda M. Burgers, Rene Janssens, Jan Nico Bouwes Bavinck, Hester de Melker, and Mariet C.W. Feltkamp

We identified a new polyomavirus in skin lesions from a patient with trichodysplasia spinulosa (TS). Apart from TS being an extremely rare disease, little is known of its epidemiology. On the basis of knowledge regarding other polyomaviruses, we anticipated that infections with trichodysplasia spinulosa–associated polyomavirus (TSV) occur frequently and become symptomatic only in immunocompromised patients. To investigate this hypothesis, we developed and used a Luminex-based TSV viral protein 1 immunoassay, excluded cross-reactivity with phylogenetically related Merkel cell polyomavirus, and measured TSV seroreactivity. Highest reactivity was found in a TS patient. In 528 healthy persons in the Netherlands, a wide range of seroreactivities was measured and resulted in an overall TSV seroprevalence of 70% (range 10% in small children to 80% in adults). In 80 renal transplant patients, seroprevalence was 89%. Infection with the new TSV polyomavirus is common and occurs primarily at a young age.

Trichodysplasia spinulosa (TS) is a rare disease of the skin seen in solid organ transplant patients receiving immunosuppressive therapy (*1*–*5*) and in lymphocytic leukemia patients (*4*,*6*–*8*). A total of 15 TS cases have been described, of which 3 were identified in 2010 (*9*–*11*). The disease is characterized by development of follicular papules and keratin spines (spicules) predominantly in the face, often accompanied by alopecia of the eyebrows and eyelashes. Histologically, TS is characterized by abnormal maturation and marked distention of hair follicles. The inner root sheath cells are highly proliferative and contain excessive amount of trichohyalin (*1*). Transmission electron microscopy showed virus particles 40–45 nm in diameter within these cells (*1*,*4*,*6*).

In plucked spicules of a TS patient, we recently identified a new human polyomavirus virus known as TS-associated polyomavirus (TSV) (*9*). This finding has been recently confirmed by Matthews et al. (*12*). Recent analyses by our group have shown high copy numbers of TSV in lesions from other TS patients (S. Kazem and M.C.W. Feltkamp, unpub. data), underscoring the concept that TSV is the causative infectious agent. Phylogenetic analysis showed that TSV forms a tight cluster with a Bornean orangutan polyomavirus and among human polyomaviruses is most closely related to Merkel cell polyomavirus (MCV; also known as MCPyV) (*9*).

Eight human polyomaviruses have been identified: BKV (*13*), JCV (*14*), KIV (*15*), WUV (*16*), MCPyV (*17*), human polyomavirus type 6 (HPyV6) and type 7 (HPyV7) (*18*), and TSV (*9*). Infections with BKV and JCV are common and occur primarily in childhood without symptoms, after which the person remains persistently infected. Reactivation occurs only in immunocompromised patients and can cause serious disease, such as BKV-associated nephropathy and progressive multifocal leukoencephalopathy, and probably TS.

In immunocompetent populations, high seroprevalence values of 82%–98% for BKV (*19*–*22*) and 39%–77% for JCV (*19*–*22*) have been reported. For KIV and WUV identified in airway specimens, calculated seroprevalences are high in the general population (55%–90% and 69%–98%, respectively) (*20*,*21*,*23*,*24*). For MCPyV, which is present in ≈80% of rare but aggressive cutaneous Merkel

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cell carcinomas (MCCs) (*17*,*25*–*27*), seroprevalence among healthy persons was shown to be 42%–77% (*20*,*21*,*28*,*29*). A recent study reported higher serologic responses in MCC patients than in healthy controls (*30*).

Seroepidemiologic data for BKV, JCV, and MCPyV indicate that human polyomavirus infections are ubiquitous and generally occur without apparent disease. TSV seems to fit this profile, but no seroepidemiologic data to confirm this hypothesis are available. We report development and performance of a multiplex immunoassay to measure seroreactivity against TSV in immunocompetent persons, immunosuppressed persons, and a TS patient. Seroprevalences of TSV infection were calculated for persons of different ages and immune status. We show that TSV is a common infection in the general population and in immunocompromised patients, and discuss the relevance of our findings with respect to TSV-induced disease.

Materials and Methods

Generation of pGEX-TSV VP1 Expression Plasmid and GST-VP1 Fusion Protein Expression

To express TSV viral protein 1 (VP1) as a glutathione-S-transferase (GST) fusion protein, we created a pGEX4t3-TSV VP1.tag plasmid. For cloning of TSV VP1, sense (5′-GGATCCGGATCCGCCCCCAAAAGAAAAGG-3′) and antisense (5′-GTCGACGTCGACATAAAGCCGGGC GGGGAAG-3′) primers (*Bam*HI and *Sal*I restriction sites are underlined) were generated (Eurogentec, Cologne, Germany). Using these primers, we performed a PCR on the pUC19-TSV plasmid (*9*). A 2-step AmpliTaq gold PCR program was performed as described (*9*). TOPO TA cloning (Invitrogen, Carlsbad, CA, USA) of the amplified PCR product resulted in a construct used for cloning TSV VP1 into the pGEX4t3-BKV VP1.tag plasmid (*18*) after removal of the BKV VP1 sequence. The pGEX4t3-TSV VP1.tag construct was verified by sequencing using the BigDye Terminator Kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

We sequenced VP1 from MCV isolate 344 and verified amino acid residues aspartic acid (D) and arginine (R) at positions 288 and 316 as found in MCV isolates 339 and 162. These residues are likely involved in proper folding of the VP1 for conformation-dependent epitope recognition (*21*). A pGEX4t3-tag plasmid was included to express tagged GST alone, which is necessary for serologic background determinations. In every construct, the tag sequence included codes for the 11-aa KPPTPPPEPET epitope of simian virus 40 (SV40) large T-antigen (*31*,*32*). GST and GST-fusion proteins of TSV, BKV, and MCPyV VP1 were expressed in the Bl21 Rosetta *Escherichia coli* strain as described (*21*,*31*–*33*).

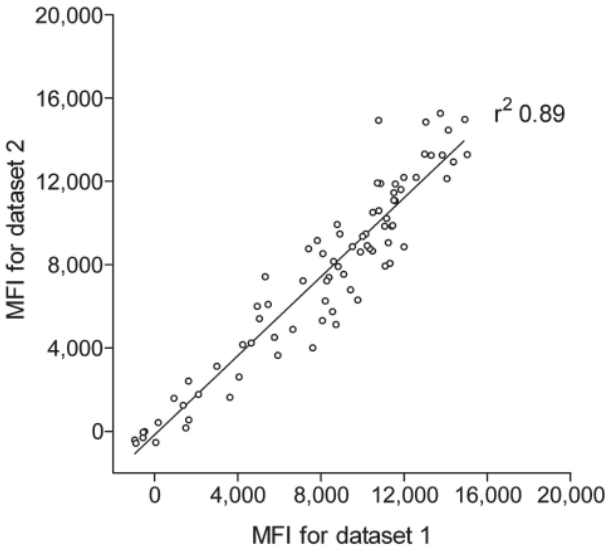

Figure 1. Reproducibility of trichodysplasia spinulosa–polyomavirus (TSV) viral protein 1 (VP1) immunoassay. Seroreactivity against TSV VP1 in 80 renal transplant patients, the Netherlands, was analyzed twice by using the Bio-Plex 100 analyzer (Bio-Rad Laboratories, Hercules, CA, USA). Datasets 1 and 2 were obtained during a 3-month interval by using freshly coupled identical glutathione–casein bead sets coupled independently with the same crude TSV VP1 bacterial extract. Each circle represents 1 serum sample, and the line represents results of linear regression analyses. Correlation coefficient (r^2) was determined by using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). MFI, median fluorescent intensity.

Expression of the GST-fusion proteins was analyzed by using Western blotting.

Samples

A total of 528 serum samples from a population-based serum bank of healthy persons from the Province of Utrecht in the Netherlands were analyzed. This serum bank was set up in 1994 as a pilot study, the prePienter study, for a nationwide serum bank that would be used to evaluate long-term seroepidemiologic changes of diseases included in the Dutch National Immunization Program (*34*) (M.A. Conyn van Spaendonk et al., pilot study for Pienter project, logistical evaluation (part 1), RIVM-report no. 213675001/1995). Approval of the prePienter study was obtained from the Medical Ethical Committee of the Dutch Organization for Applied Scientific Research (TNO) (Leiden, the Netherlands), and every participant provided written informed consent. The age distribution within the population was <1–9 years, n = 79; 10–19 years, n = 66; 20–29 years, n = 51; 30–39 years, n = 64; 40–49 years, n = 76; 50–59 years, n = 54; 60–69 years, n = 79; and 70–79 years, n = 56.

We also tested 80 serum samples obtained in 1995 from immunocompromised renal transplant patients who

came to a specialized dermatologic outpatient clinic at Leiden University Medical Center. These samples were obtained after informed oral consent was obtained from the patients, which was documented in patient files. The Medical Ethics Committee of Leiden University Medical Center reviewed and approved this study. The average age of the patients was 45 years (range 26–64 years).

A serum sample was also obtained from a 16-year-old immunocompromised heart transplant patient with TS. A detailed description of this patient was reported by van der Meijden et al. (*9*). The TS patient and his mother provided oral informed consent for the patient to provide serum for detection of antibodies against TSV, which was recorded in the patient's medical file. The Medical Ethics Committee of the Leiden University Medical Center declared in writing that no formal ethical approval was needed to analyze this sample for viral diagnosis.

Multiplex Serologic Analysis

Samples were analyzed for polyomavirus seroreactivity by using the multiplex antibody-binding assay developed and described by Waterboer et al. (*33*). Briefly, glutathione–casein (GC) coupled Bio-Plex polystyrene beads (Bio-Rad Laboratories, Hercules, CA, USA) containing a combination of fluorescent dyes were coupled to either GST-TSV VP1.tag, GST-BKV VP1.tag, GST-MCV VP1.tag, or GST.tag. For each antigen, 3,000 GC-coupled beads per sample were loaded with crude bacterial lysates containing relevant GST fusion protein. Samples were preincubated with GST.tag containing bacterial crude lysates (2 mg/mL) in blocking buffer to reduce nonspecific GST binding. For cross-reactivity studies, samples were preincubated with GST-TSV VP1.tag, GST-MCV VP1.tag, or GST-BKV VP1.tag. After preincubation, antigen-coated bead mixtures were incubated with samples diluted 1:100. For detection of bound serum antibodies, beads were incubated with goat anti-human total immunoglobulin G–biotin (1:1,000 dilution; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), streptavidin R–phycoerythrin (1:1,000 dilution; Invitrogen), and washed. Beads were analyzed in a Bio-Plex 100 analyzer (Bio-Rad Laboratories). Results are presented as median fluorescent intensity (MFI) units. For each sample, antigen-specific binding was obtained by subtracting the MFI for beads coated with GST alone from those of beads coated with GST VP1.

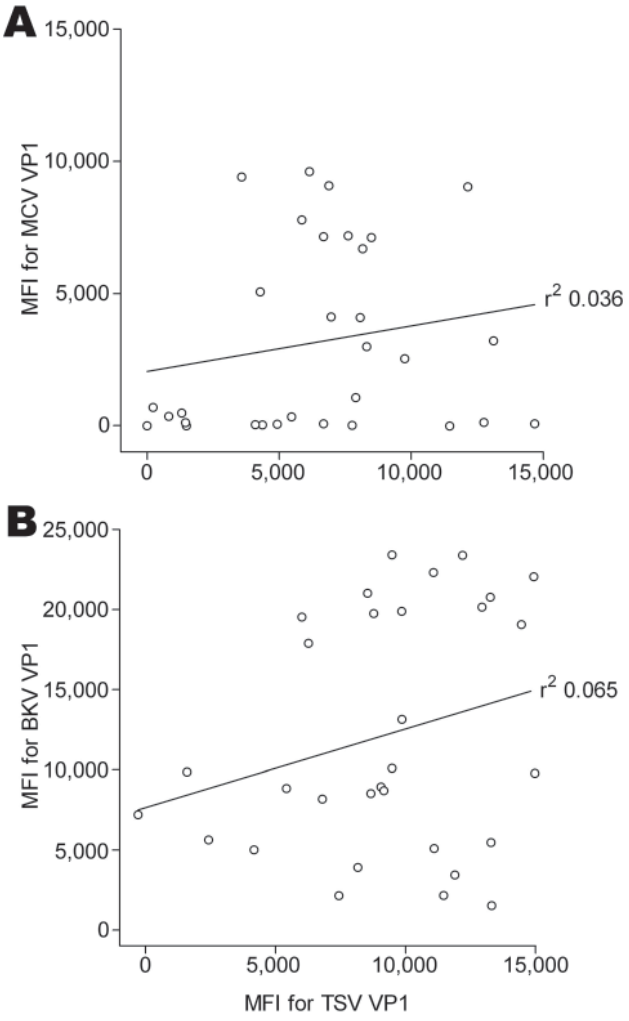

Figure 2. Cross-reactivity between trichodysplasia spinulosa–polyomavirus (TSV), Merkel cell polyomavirus (MCV), and BKV polyomavirus viral protein 1 (VP1). Correlation between seroreactivity against TSV VP1 and MCV VP1 (A) and BKV VP1 (B) was analyzed by using Bio-Plex 100 analyzer (Bio-Rad Laboratories, Hercules, CA, USA) with 30 serum samples from renal transplant patients, the Netherlands. Each circle represents 1 serum sample, and the line represents results of linear regression analyses. Correlation coefficients (r^2) were determined by using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). MFI, median fluorescent intensity.

Results

Development of the TSV VP1 Immunoassay

To measure seroreactivity against TSV, an immunoassay was developed with TSV VP1 antigen expressed as a GST-fusion protein in *E. coli*. The TSV VP1 immunoassay was developed according to the Luminex-based assay described by Waterboer et al. for simultaneous measurement of seroresponses against different human papillomavirus types (*33*). We first analyzed the reproducibility of the new assay with 80 serum samples from renal transplant patients. These samples were tested 3 months apart by using GC-coated beads coupled independently to the same crude TSV VP1 bacterial extract. This comparison showed reproducible results with a correlation coefficient of r^2 0.89 (Figure 1).

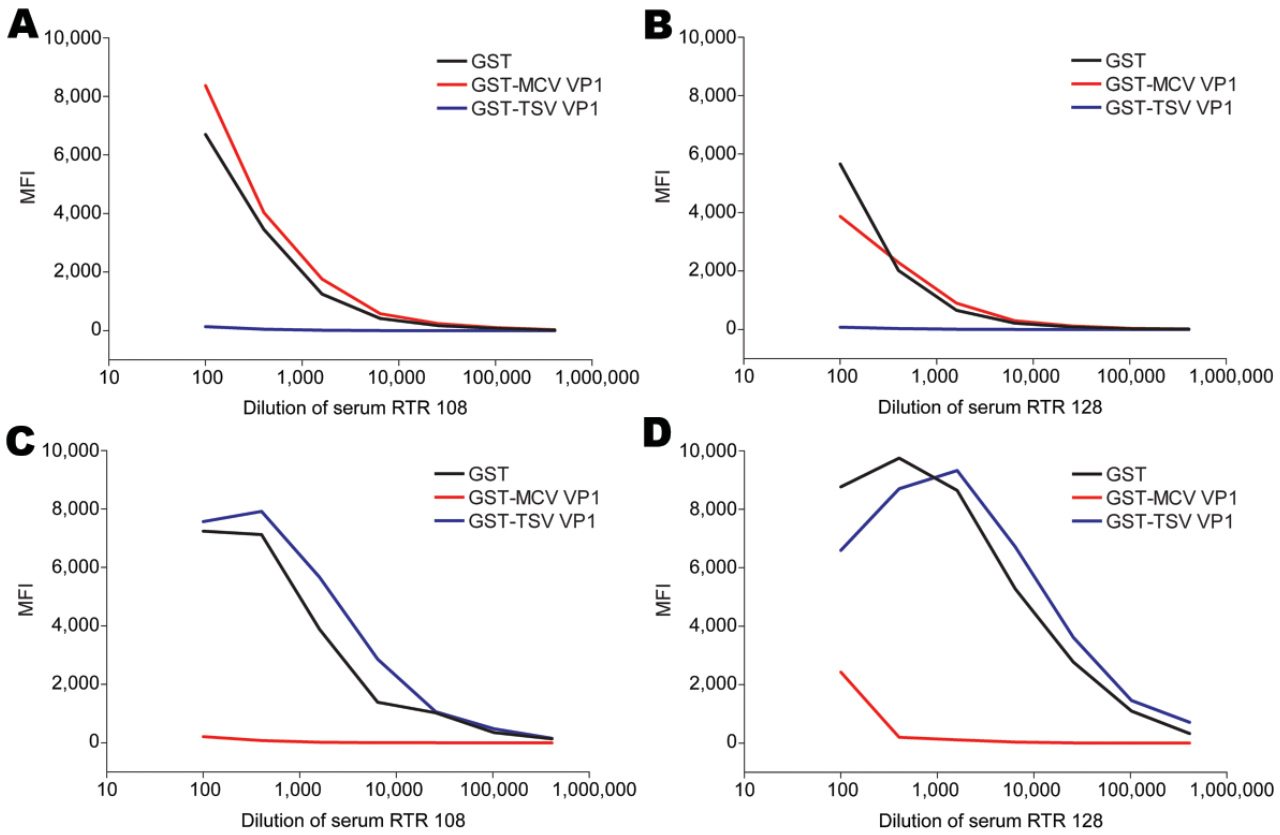

Figure 3. Cross-competition between trichodysplasia spinulosa–associated polyomavirus (TSV) and Merkel cell polyomavirus (MCV) viral protein 1 (VP1) in serial dilutions of serum samples RTR 108 and RTR 128 from renal transplant recipient patients reactive against TSV VP1 and MCV VP1, the Netherlands. Reactivity was determined by using the VP1 multiplex antibody-binding assay. Samples were preincubated with soluble recombinant glutathione-S-transferase (GST) (black line), GST-MCV VP1 (red line), or GST-TSV VP1 (blue line). Values are median fluorescent intensity (MFI) for seroreactivity against TSV VP1 (A and B) or MCV VP1 (C and D).

Although not expected on the basis of amino acid sequence comparison (RefSeq TSV NC_014361 and MCV: NC_010277) for a randomly selected subset of 30 renal transplant serum samples, we investigated a possible association between seroreactivity against VP1 of TSV and that of MCPyV because MCPyV is phylogenetically the closest related human polyomavirus to TSV (*9*). No association between TSV and MCPyV VP1 seroresponses was observed (r^2 0.036; Figure 2, panel A). Similar findings were obtained when TSV VP1 seroresponses were compared with those against the more distantly related BKV VP1 (r^2 0.065; Figure 2, panel B).

We also evaluated potential cross-reactivity between TSV VP1 and MCPyV VP1 in detail for 2 TSV- and MCPyV-reactive serum samples. These samples were titrated and preincubated with soluble GST, GST-TSV VP1, or GST-MCV VP1. Subsequently, TSV VP1 and MCPyV VP1 seroresponses were measured. In both samples tested, TSV VP1 reactivity was inhibited by preincubation with TSV VP1 only and not with MCPyV VP1, whereas MCPyV VP1 reactivity could be inhibited by preincubation with MCPyV VP1 only (Figure 3). Similar results were obtained in a TSV VP1 and BKV VP1 competition experiment with TSV-reactive and BKV-reactive samples (Figure 4).

TSV VP1 Seroresponse in a TS Patient

TSV seroreactivity was determined in a TS patient previously reported (*9*). A serum sample was obtained 6 months after detection of TSV and daily facial treatment with cidofovir-containing cream had started. At the time the sample was obtained, treated lesions had resolved but untreated skin (e.g., of the legs) still had typical spicules indicative of active TSV infection.

Serial dilutions of the TS serum sample were tested by using the TSV VP1 assay. High reactivities were observed (Figure 5, panel A). This response could be exceeded by using soluble GST-TSV VP1 but not with GST-BKV VP1. Conversely, the BKV seroresponse observed in this patient was exceeded only by using soluble recombinant GST-BKV VP1 and not by using GST-TSV VP1 (Figure 5,

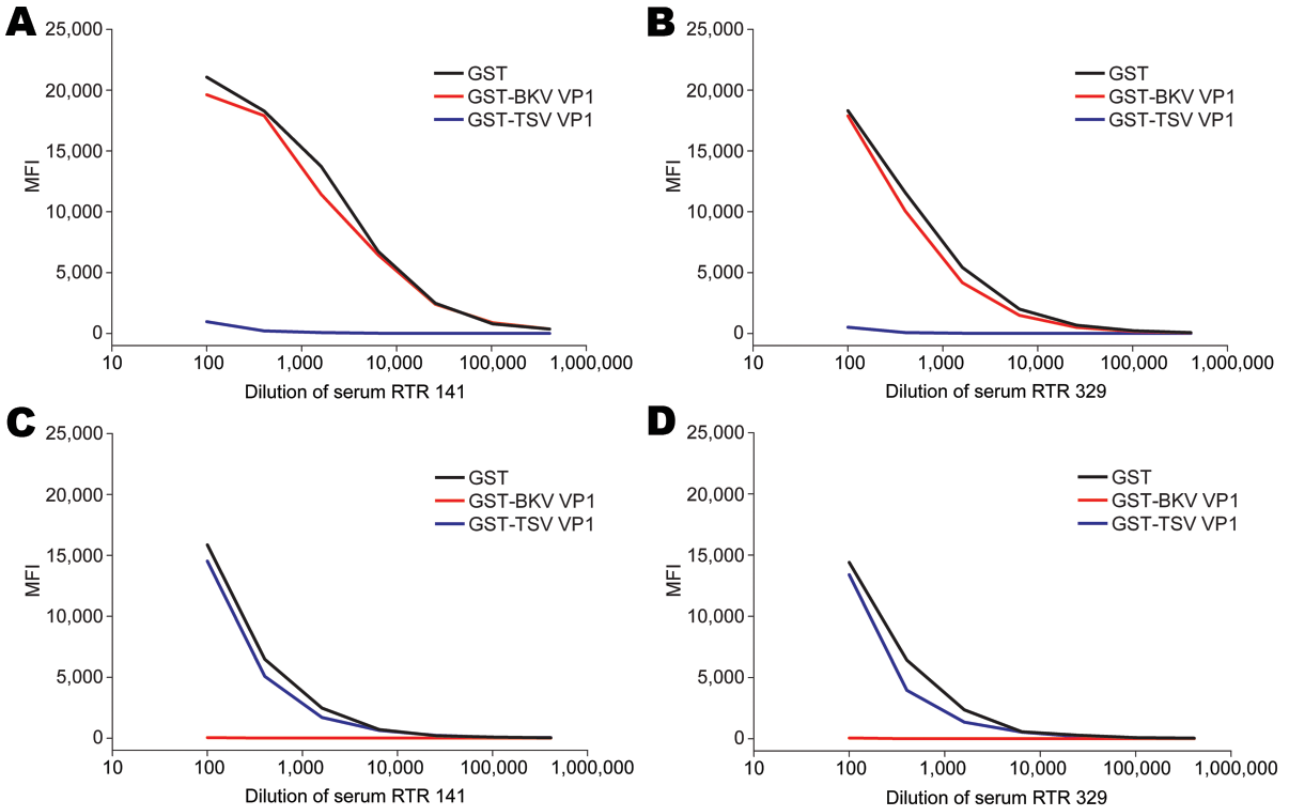

Figure 4. Cross-competition between trichodysplasia spinulosa–associated polyomavirus (TSV) and BKV polyomavirus viral protein 1 (VP1) in serial dilutions of serum samples RTR 141 and RTR 329 from renal transplant recipient patients reactive against TSV VP1 and BKV VP1, the Netherlands. Reactivity was determined by using the VP1 multiplex antibody-binding assay. Samples were preincubated with soluble recombinant glutathione-S-transferase (GST) (black line), GST-BKV VP1 (red line), or GST-TSV VP1 (blue line). Values are median fluorescent intensity (MFI) for seroreactivity against TSV VP1 (A and B) or BKV VP1 (C and D).

panel B). No seroresponse against MCV VP1 was detected for this patient.

TSV Seroresponses in Healthy and Immunocompromised Populations

TSV VP1 seroreactivity was determined for 528 healthy persons and 80 immunosuppressed renal transplant patients. BKV VP1 was included in the analyses as a positive control because of the known high BKV seroprevalence in the general population (*19–21*). In every experiment, a panel of 3 reference serum pools was also included, which showed little variance over time. Results for TSV VP1 and BKV VP1 are shown in Figure 6.

To investigate age-specific TSV seroreactivity and to calculate a cutoff value to determine TSV seropositivity, we subdivided the healthy population into different age groups (Figure 7, panel A). For persons <1–9 years of age, a clear distinction could be made between patients who were seronegative for TSV (MFI ≈0) and children with TSV seroreactivities of 4,000–12,000 MFI units. To calculate TSV seropositivity, a cutoff value of 877 MFI units was calculated on the basis of mean seroreactivity of the TSV serononresponders from the lowest age group + 3 SD. Although the distinction between seronegative persons and seropositive persons in the first age group was less clear for BKV (Figure 7, panel B), a similar strategy was used for BKV and resulted in a cutoff value of 1,051 MFI units.

TSV Seroprevalence in Healthy and Immunocompromised Populations

Using the calculated cutoff values, we determined the age-specific seroprevalence for TSV in each age group of the immunocompetent population. In the children <10 years of age, the seroprevalence for TSV was 41% (Figure 7, panel C). This percentage increased to ≈75% at 30 years of age, and remained stable for higher age groups. BKV seroprevalences were calculated and showed values between 75% and 97% (Figure 7, panel C). Analyses of the youngest age group showed an increasing trend for TSV and BKV seropositivity starting at 10% for TSV in children 1–2 years of age (Figure 7, panel D).

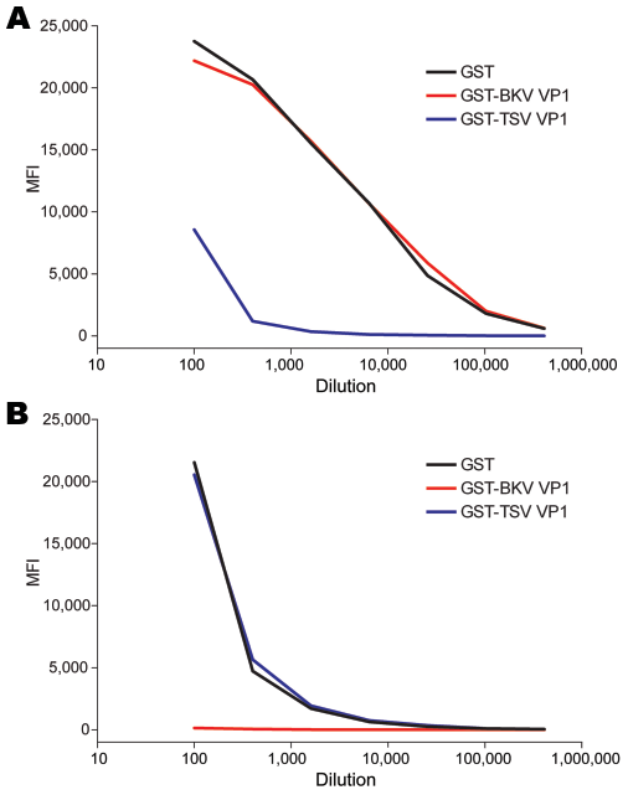

Figure 5. Seroresponses against trichodysplasia spinulosa–associated polyomavirus (TSV) (A) and BKV polyomavirus (B) for a patient with trichodysplasia spinulosa, the Netherlands. Serial dilutions of serum from a TS patient were tested for reactivity against TSV viral protein 1 (VP1) or BKV VP1 by using the VP1 multiplex antibody-binding assay. Samples were preincubated with soluble recombinant glutathione-S-transferase (GST) (black line), GST-BKV VP1 (red line), or GST-TSV VP1 (blue line). MFI, median fluorescent intensity.

On the basis of these calculations, overall seroprevalence for TSV VP1 was 70% for the healthy population and 89% for the immunocompromised population (Figure 6, panel A). For BKV VP1, the overall seroprevalence for both groups was somewhat higher (85% and 99%, respectively) (Figure 6, panel B).

Discussion

To investigate the seroepidemiologic aspects of TSV infection, we developed a multiplex immunoassay. This approach was based on Luminex technology and shown to be a reliable method for seroepidemiologic studies of papillomavirus and polyomavirus infections (*19*,*21*,*33*,*35*,*36*). The choice for VP1 as antigen of interest was governed by results of studies on BKV, JCV, and SV40 polyomavirus, which showed that the major capsid protein is immunodominant (*19*). However, the less immunogenic large T-antigen may also be useful in discriminating active

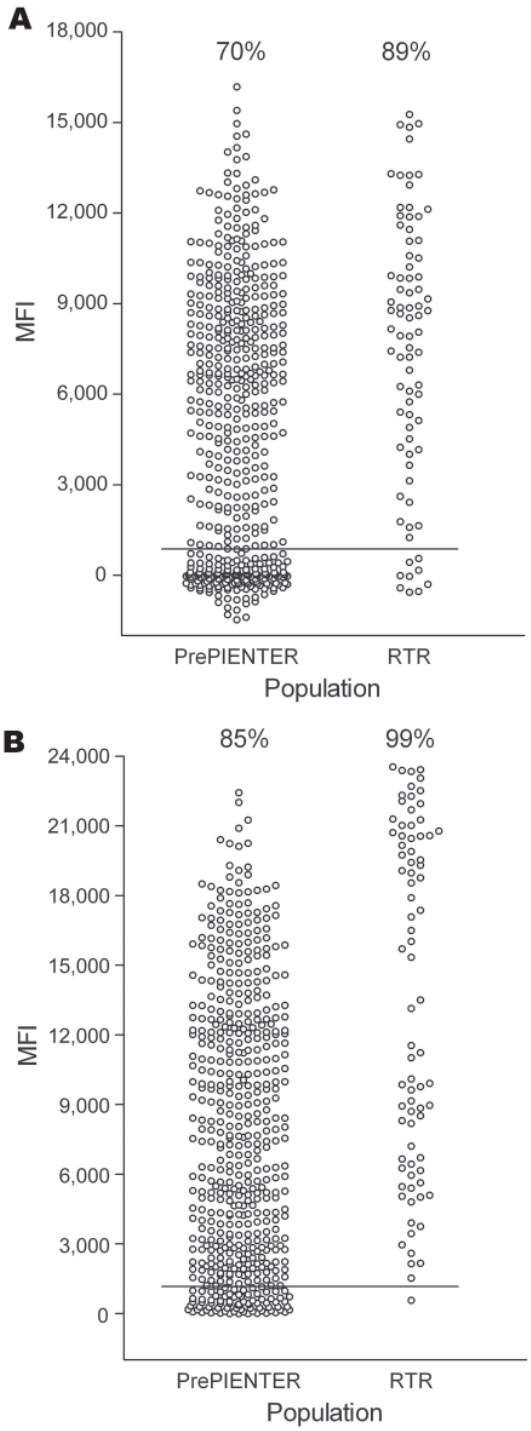

Figure 6. Seroresponses to trichodysplasia spinulosa–associated polyomavirus (TSV) and BKV polyomavirus in healthy and immunocompromised populations, the Netherlands. Serum samples were obtained from 528 healthy persons (PrePIENTER) and 80 renal transplant recipients (RTR) and screened for reactivity against TSV viral protein 1 (VP1) (A) and BKV VP1 (B) by using the VP1 multiplex antibody-binding assay. Each circle represents 1 sample, and horizontal lines represent cutoff values. Percentage values indicate seropositivity. MFI, median fluorescent intensity.

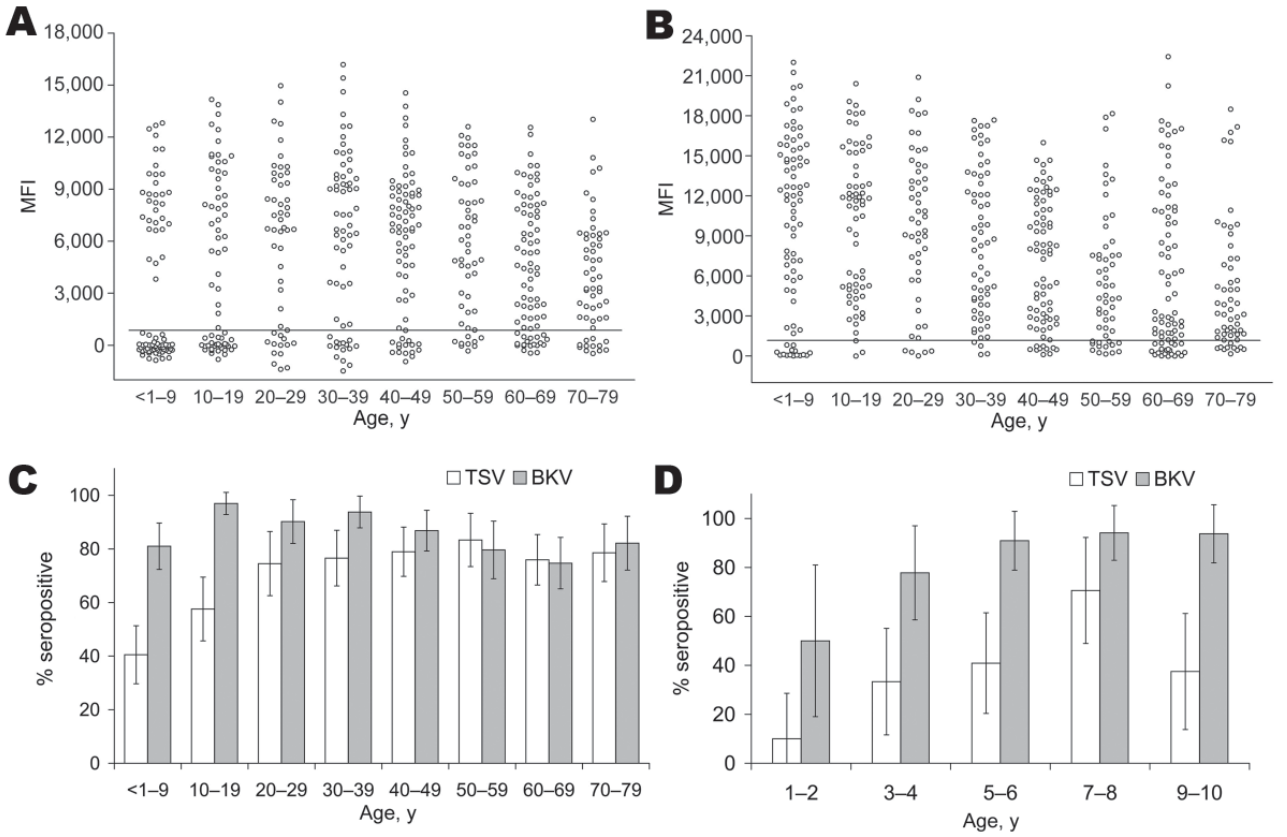

Figure 7. Age-related seroprevalence of trichodysplasia spinulosa–associated polyomavirus (TSV) viral protein 1 (VP1) (A) and BKV polyomavirus VP1 (B) in a healthy population, the Netherlands. The population was divided into 8 age groups: <1–9 years of age (n = 79), 10–19 (n = 66), 20–29 (n = 51), 30–39 (n = 64), 40–49 (n = 76), 50–59 (n = 54), 60–69 (n = 79), and 70–79 (n = 56). Each circle represents 1 serum sample, and the horizontal lines represent cutoff values. MFI, median fluorescent intensity. C) Seroprevalence of TSV VP1 (white bars) and BKV VP1 (gray bars), by age. D) Seroprevalence of TSV VP1 and BKV VP1 in youngest age group. Population was divided into 5 smaller age groups: 1–2 years of age (n = 10); 3–4 (n = 18); 5–6 (n = 22); 7–8 (n = 17); 9–10 (n = 16). Error bars indicate 95% confidence intervals.

TSV infections from latent infections because it has been reported that antibodies against MCPyV T antigens reflect the tumor incidence for MCC patients (*37*).

The TSV VP1 immunoassay was reproducible and showed minimal signs of TSV cross-reactivity with MCPyV. Cross-reactivity studies have shown correlations between serorecognition of SV40 and BKV only, and to a lesser extend between SV40 and JCV (*20*,*38*,*39*), all of which are more closely related than TSV and MCPyV (*9*). Detailed comparison of antigenic VP1 loop regions of TSV, MCPyV, and BKV, as performed for KIV, WUV, MCPyV, and lymphotropic polyomavirus by Kean et al. (*20*), also showed little similarity. On the basis of the new polyomavirus phylogenetic tree that was recently published (*9*), only cross-reactivity between TSV and the closely related Bornean orangutan polyomavirus 1 might have been expected. However, this animal virus was not included in this human study.

Seroreactivity of the symptomatic TS patient was the highest of all participants in the study. Even at a dilution of 1:100,000, some reactivity above background was detected, which indicates a high concentration of TSV-specific antibodies in this patient. This interpretation was also suggested by the observation that at the highest serum concentration, competition with soluble GST-TSV VP1 did not result in complete inhibition of TSV seroreactivity. This finding might be unexpected because immunosuppressed patients are often considered less immunoreactive. However, the immunosuppressive regimens are aimed to decrease cellular immunity to prevent donor organ rejection. It is anticipated that polyomavirus-specific cellular immunity will be decreased by such a regimen, which would increase the pool of infected cells and produce larger amounts of virus, even viremia. As a result, memory B cells may become activated and production of TSV-specific antibodies will increase accordingly.

The seroprevalence we calculated for TSV among the healthy population was high and comparable with that found for other human polyomaviruses (*20–24*,*28*,*29*). Therefore, TSV seems to be a ubiquitous virus that frequently causes infection in the general human population. A total of 41% of the children <1–9 years of age were seroreactive to TSV and therefore likely infected. Whether TSV infections persist is not known, but this persistence is likely on the basis of results for other polyomavirus infections.

The calculated overall TSV seroprevalence was higher for the immunocompromised group than for healthy persons. When age was taken into account, we observed that the difference in TSV prevalence between both populations was of borderline significance ($p = 0.03$). As discussed for the TS patient, this seemingly paradoxical phenomenon might be explained by increased humoral immunity against TSV as a result of increased viral activity under (cellular) immunosuppression. Whether this hypothesis involves TSV reactivations or reinfections is not known. However, it is also not known whether overt TS reflects a fulminant primary TSV infection or a symptomatic reactivation.

In conclusion, by using a newly developed immunoassay, we were able to measure TSV seroreactivity with high reproducibility and low cross-reactivity. We calculated the seroprevalence of TSV in healthy persons and provided evidence that TSV is a common circulating virus in the general population in the Netherlands that preferentially infects persons at an early age. Additional studies will need to determine whether TSV infections remain persistent in the host, as shown for other polyomaviruses, and what triggers TSV reactivation. The fact that symptomatic TS is such a rare condition suggests that there are more factors involved in this condition than immunosuppression alone.

Acknowledgment

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Ms van der Meijden is a PhD student at the Leiden University Medical Center. Her research interests are the epidemiology of human polyomavirus TSV and virologic characterization of this virus.

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Human Polyomavirus Related to African Green Monkey Lymphotropic Polyomavirus

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While studying the virome of the skin surface of a patient with a Merkel cell carcinoma (MCC) by using unbiased, high-throughput sequencing, we identified a human polyomavirus nearly identical to human polyomavirus 9, a virus recently reported in blood and urine of renal transplantion patients and closely related to the African green monkey lymphotropic polyomavirus. Specific PCR analysis further identified this virus in 2/8 patients with MCC but in only 1/111 controls without MCC. This virus was shed for ≥20 months by the MCC index patient and was on the skin of the spouse of the index patient. These results provide information on the viral ecology of human skin and raise new questions regarding the pathology of virus-associated skin disorders.

Polyomaviridae is a family of nonenveloped viruses with a circular double-stranded DNA genome. Natural hosts for *Polyomaviridae* are primates, including humans and monkeys, cattle, rabbits, rodents, and birds (*1*). Currently, viruses in this family that infect humans are the opportunistic JC polyomavirus (JCPyV) associated with progressive multifocal leukoencephalopathy in immunocompromised person; BK polyomavirus (BKPyV) associated with interstitial nephritis and hemorrhagic cystitis; KI polyomavirus (KIPyV) identified in respiratory secretions of patients with respiratory symptoms at the Karolinska Institute (Stockholm, Sweden); WU polyomavirus (WUPyV), isolated from patients with similar symptoms at Washington University (St. Louis, MO, USA); Merkel cell polyomavirus (MCPyV), associated with the rare but aggressive Merkel cell carcinoma (MCC); and trichodysplasia spinulosa–associated polyomavirus (TSPyV), associated with a rare skin condition in immunocompromised persons (*2*).

Two recently identified polyomaviruses, human polyomaviruses 6 and 7 (HPyV6 and HPyV7) have been detected in cutaneous swab specimens of healthy persons (*3*). HPyV9 has been identified by consensus PCR in blood and urine of asymptomatic renal transplant recipients (*4*). In this study of the skin virome of a patient with MCC, using high-throughput sequencing (HTS) and comparing sequences from a patient with MCC with sequences from healthy controls, we identified a human polyomavirus strain nearly identical to HPyV9, a virus species closely related to the lymphotropic polyomavirus (LPV).

Methods

Patients and Sample Collection

For analysis by HTS, 6 DNA samples extracted from cutaneous swabs obtained from the skin surface of facial areas (forehead and eyebrows) of patients previously studied by PCR for MCPyV sequences were selected (*5*). These samples included 1 from an index patient with an MCC on his elbow and 5 from the skin of 5 healthy persons.

For investigation of prevalence by specific nested PCR, 120 skin specimens were similarly obtained from 120 volunteers. The median age of these persons was 48 years (range 19–96 years); 30 persons were 57–96 years of age (median age 71 years). This group of 120 volunteers was composed of 40 patients hospitalized or attending outpatient

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clinics at the dermatology unit at Montpellier University Hospital for various skin disorders (including 8 patients with MCC; median age 75 years, range 57–86 years), 20 immunocompromised patients without skin lesions (10 patients infected with HIV-1 without skin symptoms and 10 renal transplant recipients receiving immunosuppressive regimens [steroids, mycophenolate mofetil, and calcineurin inhibitors]), and 60 healthy controls.

Respiratory samples tested were composed of 46 bronchoalveolar lavage samples obtained from hospitalized patients in intensive care units with acute respiratory failure of unknown origin and 46 nasopharyngeal aspirates from children in the pediatric emergency unit at Montpellier University Hospital with various respiratory tract disorders. An additional 92 fecal samples were obtained from children hospitalized in the pediatric unit for gastroenteritis.

Extraction and Amplification of DNA

DNA from all samples was extracted as described (*5*). For HTS, DNA was amplified by using a bacteriophage ϕ29 polymerase-based rolling circle amplification assay and random primers. The protocol of the QIAGEN REPLI-g Midi Kit (QIAGEN, Courtaboeuf, France) was followed as recommended by the manufacturer.

High-Throughput Sequencing

HTS was performed by using the Illumina HiSeq 2000 apparatus (Illumina Inc., San Diego, CA, USA) at GATC Biotech AG (Konstanz, Germany). Five micrograms of high molecular weight amplified DNA was divided into 200–350-nt fragments to which adaptors were ligated. These adaptors included a nucleotide tag that enabled multiplexing several samples per lane or channel. Sequencing was conducted at a mean depth per sample of 8.9×10^6 paired-end reads of 100 nt (range $7.6–10.3 \times 10^6$ reads).

Sequence Analysis

Sequences were first sorted by using a subtractive database comparison procedure. Several assembly programs dedicated to short or medium reads were used to generate contigs: Velvet (www.ebi.ac.uk/~zerbino/velvet), SOAPdenovo (http://soap.genomics.org.cn), and CLC Genomics Workbench (www.clcbio.com) (J. Cheval et al., unpub. data). Comparison of single reads and contigs with known genomic and taxonomic data was performed by using dedicated specialized viral, bacterial, and generalist databases created and maintained at the Institut Pasteur (GenBank viral and bacterial databases). Aforementioned databases were screened by using BLASTN and BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi). We used BLAST software (Paracel, Pasadena, CA, USA) capable of executing searches on multiple nonshared memory processors simultaneously.

The entire sequence of the Institut Pasteur polyomavirus (IPPyV) strain genome was analyzed and annotated by using CLC Genomics Workbench (CLC Bio, Aarhus, Denmark). GenBank reference sequences of other members of the family *Polyomaviridae* used were JCPyV (NC_001699), BKPyV (NC_001538), KIPyV (NC_009238), WuPyV (NC_009539), MCPyV (NC_010277), SV40 (NC_001669), TSPyV (NC_014361), and LPV (M30540). Protein structures were visualized by using Pymol (Delano Scientific LLC, San Francisco, CA, USA).

Phylogenetic Analysis

Phylogenetic reconstructions were based on separate analyses of nucleotide sequences from viral protein 1 (VP1) and large T antigen (LT). A 974-nt region of monkey B-lymphotropic papovavirus (reference sequence M30540.1 from the VP1 coding sequence) was aligned with corresponding regions from the polyomaviruses available in GenBank. For the LT matrix, a 1,453-nt region (same reference sequence as for VP1) was used for analysis. Sequences were aligned by using SeaView version 4 (*6*) and the Muscle algorithm (*7*). Only partial but contiguous parts of each alignment were included in final matrixes because alignment in some regions was not possible. The 742–1715 and 2904–4356 nt regions were included in matrixes. The matrixes were based on monkey B-lymphotropic papovavirus M30540 and African green monkey polyomavirus NC_004763 sequence annotations for VP1 and LT genes, respectively.

Phylogenetic analyses were performed by using a probabilistic (Bayesian) approach implemented in BEAST (*8*). Matrixes were tested against 88 substitution models by using jModelTest software (*9*). On the basis of results obtained with jModelTest software, the generalized time reversible substitution model (with invariant sites and a gamma site heterogeneity distribution) was used for analysis. The 3-codon partition model of evolution and the Yule speciation process were also specified as priors. A Markov chain Monte Carlo method was used to set 30,000,000 states to obtain an adequate posterior effective sample size >300. Pertinence of nodes was evaluated by using posterior probabilities. Sequences used in phylogenetic reconstructions were obtained from the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov/nucleotide). Confidence intervals for proportions were calculated according to the efficient-score method (corrected for continuity) (*10*) (http://dogsbody.psych.mun.ca/VassarStats).

PCR

For sequencing the IPPyV by the Sanger method, 9 primer pairs were designed to amplify the entire genome by reference to the contigs assembled from HTS data

acquired in the first phase (see Results). Primer sequences and protocols are available upon request. After the genome was sequenced, we developed a specific nested PCR for detection of IPPyV in samples by using primers based on the IPPyV genome sequence and designed by using PrimerPro 3.4 software (www.changbioscience.com): VP1_354F (5′-ACCATATCAGTAGGATAGGTA-3′) and VP1_354R (5′-TGAATTGTATGGCTACAGTGC-3′) for the outer PCR, and VP1_198F (5′-CACTGGGA TAGTTCCTGAGG-3′) and VP1_198R (5′-CCTA ATGCTACTACCCTCCCT-3′) for the inner PCR. These primers were designed to avoid amplification of other known human polyomaviruses.

Ethical Approval

The study was reviewed and approved by the Institut Pasteur Comité de Recherche Clinique and the French Commission Nationale Informatique et Libertés (09.465). Consent was provided by participants for obtaining human samples according to French regulations.

Results

Identification of the IPPyV Strain of HPyV9

Using 8,052,770 Illumina reads obtained from DNA extracted from the skin surface of the MCC index patient, we assembled the complete genome of MCPyV. We found numerous papillomavirus contigs and contigs covering more than half of the genomes of HPyV6 and HPyV7. Additionally, 14 other contigs were assembled that showed a better homology with LPV (NCBI accession no. M30540, version M30540.1, GI:333282) than with any other virus present at that time in the NCBI database, including other human or animal members of the family *Polyomaviridae*. On the basis of sequence of 8 of the 14 obtained contigs, which were distributed along the LPV genome, we defined a set of 9 primer pairs encompassing the entire target genome. These primers enabled amplification of the entire genome by PCR and analysis of its sequence of 5,028 nt by using the Sanger method, which confirmed the circular nature of the genome.

Whole genome organization of the IPPyV strain, which exhibits general molecular characteristics of polyomaviruses, is shown in Figure 1. It encodes analogs of small T antigen, LT, and structural proteins VP1, VP2, and VP3 and does not appear to encode an agnoprotein. Pairwise amino acid identity was 100% between IPPyV and HPyV9 proteins, 72%–80% between IPPyV and LPV proteins, and much lower for other known family *Polyomaviridae* members (Table). Because the nucleotide sequence of IPPyV is nearly identical to that of HPyV9, with a difference of only 2 nt in a noncoding region at nt 4449, it appears that IPPyV should be considered a strain of HPyV9. Its sequence have been was deposited in GenBank (accession no. FR823284).

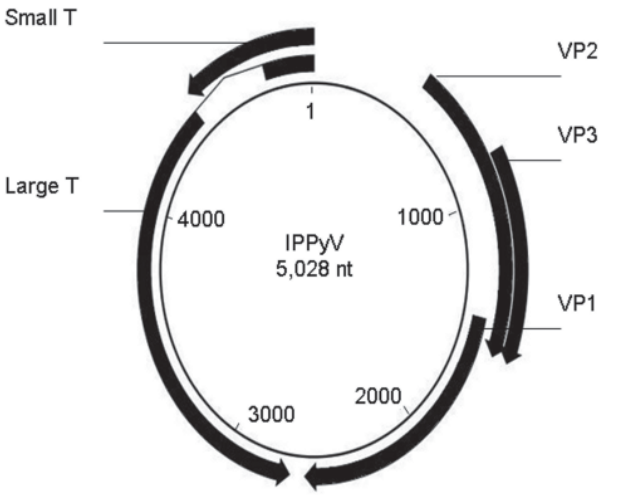

Figure 1. Genomic map of the circular genome of the Institut Pasteur polyomavirus (IPPyV) strain of human polyomavirus 9. Arrows indicate open reading frames. Small T, small T antigen; VP, viral protein; Large T, large T antigen.

Phylogenetic Analysis

Reconstructions of VP1 and LT phylogenies on the basis of nucleotide sequences clustered the mammalian polyomaviruses and placed the species *Avipolyomavirus* in basal position when rooting with the oldest known *Avipolyomavirus* (Budgerigar fledging virus) (Figure 2). Despite the divergences described below, VP1 and LT of HPyV9 were closely related to those of the monkey B-lymphotropic polyomavirus. Moreover, VP1 and LT phylogenies consistently identify several monophyletic groups among mammalian polyomaviruses (Figure 2). Nevertheless, the incongruence of VP1 and LT signals induce notable differences in the topology of these 2 phylogenies. For instance, LT of bovine polyomavirus is closely related to one of the common ancestors of all other mammalian polyomaviruses, whereas its VP1 is closely related to VP1 of the sea lion polyomavirus. Incongruence between VP1 and T phylogenies has been observed for HPyV6 and HPyV7. Nomenclature described in proposals of the International Committee on Taxonomy of Viruses is shown in Figure 2, even though the species *Orthopolyomavirus* is not monophyletic and therefore should be considered cautiously.

Comparison of VP1 from LPV and HPyV9

We compared the secondary structure of VP1 from LPV and HPyV9 because the external capsid protein of *Polyomaviridae* is known to interact with the cell

Table. Amino acid identity between putative proteins encoded by IPPyV and proteins of *Polyomaviridae* deduced by using pairwise sequence alignment*

Protein	Putative open reading frame	Frame	No. amino acids	Amino acid identity, %								
				JCV	BKV	KIV	WuV	MCyV	TSV	SV40	LPV	HPyV9
VP1	1443–2558	+3	371	53.9	53.2	28.3	28.3	54.8	60.6	52.,9	87.1	100.0
VP2	503–1561	+2	352	32.3	32.6	23.8	20.8	26.1	43.5	33.1	74.9	100.0
VP3	860–1561	+2	233	34.1	35.9	24.5	20.3	15.1	41.3	33.7	72.5	100.0
ST antigen	5028–4459	–1	189	35.1	34.0	39.5	34.6	40.1	42.5	31.8	81.0	100.0
LT antigen	5028–4792, 4437–2632	–1	680	40.4	41.2	44.2	42.0	39.9	49.3	40.0	80.5	100.0

*IPPyV, Institut Pasteur polyomavirus; JCV, JC polyomavirus; BKV, BK polyomavirus; KIV, KI polyomavirus; WUV, WU polyomavirus; MCyV, Merkel cell polyomavirus; TSV, trichodysplasia spinulosa–associated polyomavirus; SV40, Simian virus 40; LPV, lymphotrophic polyomavirus; HPyV9, human polyomavirus 9; VP, viral protein; ST antigen; small T antigen; LT antigen, large T antigen. Pairwise sequence alignment was performed by using EMBOSS Needle Software (http://emboss.sourceforge.net/apps/release/5.0/emboss/apps/needle.html).

receptor and because antibodies cross-reacting with LPV VP1 have been detected in a large proportion of humans. Overall amino acid identity was 87.1%. The VP1 monomer consists of antiparallel β-strands folded into a jelly roll β-barrel structure. Three outer loops (BC, DE, and HI) are exposed outside the pentamer core and are most likely recognized by antibodies. Using the crystal structure of SV40 VP1 (3BWQ), we mapped the amino acids that differed between the 2 proteins. Polymorphic residues are present in the 3 VP1 loops, and the BC and HI loops appear more conserved than DE loop, which thus shows the major differences (Figure 3).

Detection of HPyV9 in Human Samples

We first confirmed by specific nested PCR the presence of HPyV9 in the skin swab specimen of the index patient in which the virus had been identified by HTS. We also identified this virus by nested PCR in a second cutaneous sample from the same index patient case obtained 20 months after the first sample. Because HPyV9 was identified in a patient who had MCC, we analyzed the skin surface of 7 other MCC patients. HPyV9 was detected in 1 patient 80 years of age. The overall prevalence in the MCC group was thus 2/8 (25%, 95% confidence interval 4.4%–64%; p = 0.05).

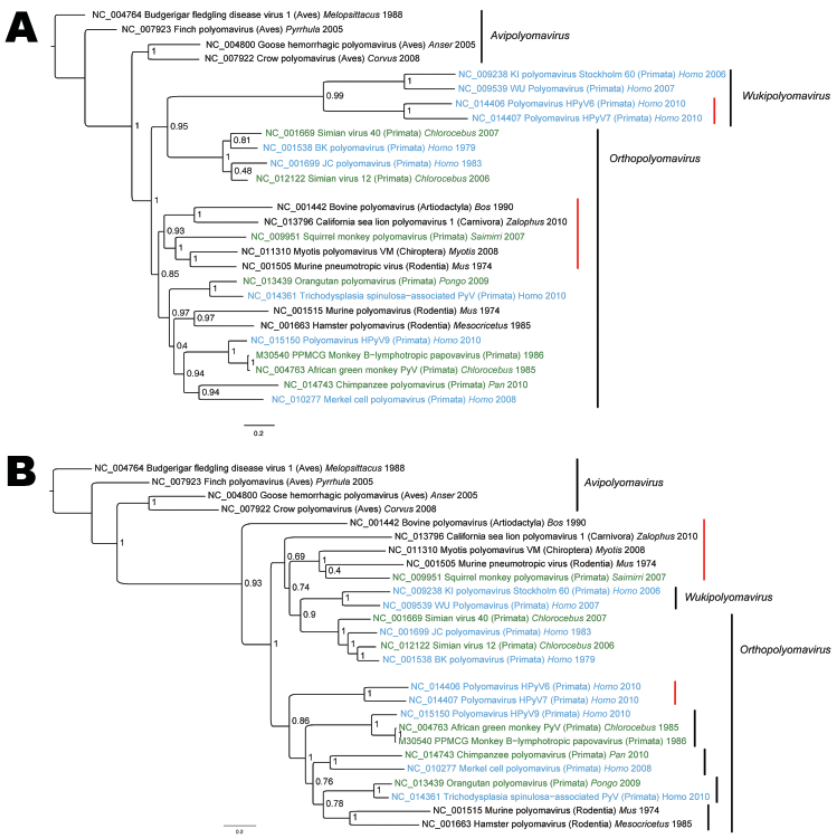

Figure 2. A) Viral protein 1 (VP1) and B) large T antigen (LT) nucleotide-based phylogenetic reconstructions of polyomaviruises inferred by using a Bayesian method. Taxa annotations include reference number, name of the virus, host taxonomic order (in parentheses), host genus whenever available, and reported collection date. Human viruses are indicated in blue, and monkey viruses are indicated in green. Red vertical bars highlight groups for which VP1 and LT signals are incongruent. Posterior probabilities are indicated at each node. GenBank identification numbers are indicated directly on trees for each sequence. Scale bars indicate nucleotide substitutions per site.

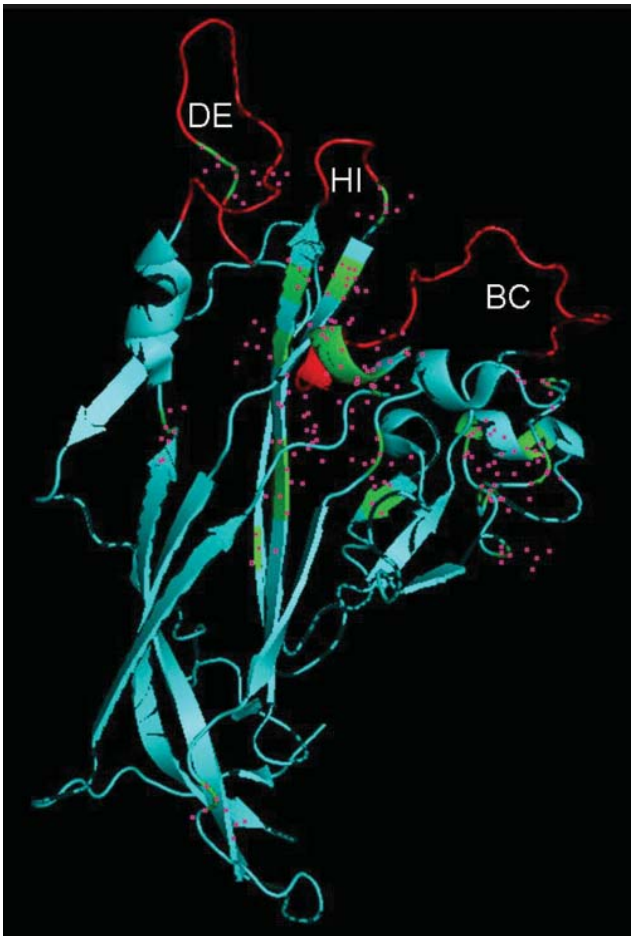

Figure 3. Identification of viral protein 1 (VP1) residues differing between human polyomavirus 9 (HPyV9) and lymphotropic polyomavirus (LPV). The DE, HI, and BC loops that extend outward from VP1 are indicated. The crystal structure of simian virus VP1, derived from strain 3BWQ, was used as a template. The red region in the center indicates part of a β strand, which is mostly hidden. Residues differing between HPyV9 and LPV are indicated by pink squares.

Because we were interested in possible interhuman transmission of HPyV9, a skin swab specimen from the wife of the first index patient was analyzed and found to be positive for this virus. We sampled 111 skin swab specimens from healthy persons or non-MCC patients who had no known contact with MCC patients and screened them with the same nested PCR. Only 1 healthy 30-year-old person had HPyV9, which demonstrated low prevalence in this control group of 1/111 (0.9%, 95% confidence interval 0.05%–5.6%; p = 0.05). Age range of the MCC population was 57–86 years (median 75 years), and because we were unable to detect HPyV9 among the 30 older controls (age range 57–96 years, median 71 years), this finding suggests that the rate of detection in MCC samples was not biased by older ages of these patients. None of the 92 respiratory and 92 stool specimens was positive for HPyV9.

Discussion

There is much evidence indicating that healthy human skin harbors numerous viruses. This finding has been extensively reported for cutaneous human papillomavirus (HPV), which is commonly present on the superficial layers of the skin of most persons (*11*). The recent description of new human viruses belonging to the family *Polyomaviridae* suggests that some of these viruses share the cutaneous tropism of β-HPV and γ-HPV. MCPyV associated with MCC has also detected on the surface of healthy skin of most persons (*5*,*12*), and HPyV6 and HPyV7 have been identified on the surface of the skin of healthy persons (*13*). Detection of an additional human polyomavirus in cutaneous samples reinforces the perception of the skin as a complex microecosystem colonized by many viruses, and polyomaviruses represent part of this viral microbiota.

The existence in humans of a polyomavirus closely related to LPV, whose natural host is the African green monkey, has been anticipated (*14*). An African green monkey polyomavirus, also known as monkey B-lymphotropic papovavirus, or LPV, was isolated >30 years ago (*15*) from a lymphoblastoid cell line derived from the African green monkey. The existence of a virus closely related to LPV has been suspected for >30 years because of cross-reacting antibodies in humans who had no known contact with monkeys (*14*,*16*). PCR amplification of short sequences matching those of LPV has been reported, but the length and overlap of these sequences were insufficient for characterizing this virus (*13*,*17*). The phylogenetic position of HPyV9 we described makes it the sister group of LPV. Moreover, we have shown that the sequence of this virus makes it a good candidate to be the target of antibodies found in humans.

The BC and HI loops of VP1 appear conserved between HPyV9 and LPV; the DE loop shows major changes. Thus, human antibodies cross-reacting with LPV may preferentially recognize the BC and HI loops, and antibodies specific for the DE loop probably cross-react only weakly with the LPV VP1. With the availability of HPyV9-specific reagents, analysis of the specificity of antibodies found in humans will provide useful information.

HPyVs are ubiquitous viruses that infect most of a given population, remain latent, and show frequent reactivations that lead to asymptomatic shedding (*1*). The VP1 and T proteins of HPyV6, HPyV7, and several polyomaviruses from wild animals show different pathways of evolution (Figure 2). The close relationship between the HPyV6–HPyV7 group and the WUPyV–KIPyV group could result from convergence caused by common host pressure on

VP1 domains (involved in virus–host interactions) or from horizontal gene transfer (recombination).

In immunocompromised persons, reactivation of HPyV often results in specific diseases such as progressive multifocal leukoencephalopathy caused by JCPyV and tubular nephropathy and hemorrhagic cystitis caused by BKPyV. Most newly identified HPyVs are also detected frequently in healthy persons. In addition, most HPyVs (KIPyV, WUPyV, HPyV6, and HPyV7) have not been clearly associated with any human diseases; however, MCPyV has been associated with MCC (*18*) and TSPyV has been associated with a rare cutaneous disorder (*2*); both occurred in immunocompromised patients.

HPyV9 was detected in skin of healthy persons who did not have any obvious immunodeficiency and thus might have asymptomatically shed HPyVs. However, among the 3 persons in whom we detected HPyV9, 2/8 had MCC in cutaneous samples, yielding an apparent higher rate of detection (2/8) in this subset of patients, compared with 1/111 in a control and heterogeneous population. This difference in prevalence rate was not biased by age within each group (0/30 had HPyV in the age-matched part of the control group). Although this unexpected result should be interpreted with caution, given the small number of MCC samples and the retrospective nature of our analysis, MCC patients might be prone to reactivation of HPyV9 shedding from their skin or HPyV9 might be involved in the pathophysiology of MCC. We are currently exploring expression of HPyV9 LT antigen in MCC and other cancer tissues. Long-term carriage of HPyV9 for ≥20 months observed in the index patient with MCC is consistent with this hypothesis.

Chronic shedding of HPyV from skin is similar to a well-known feature of cutaneous HPVs that replicate in keratinocytes and are likely to be transmitted environmentally or through person-to-person contact. In our study, detection of HPyV9 in skin of the wife of the index patient suggests a similar route of transmission.

It has been proposed that MCPyV and HPyV6 or HPyV7 may infect superficial cells of the epidermis and that production of virions may be, as for HPVs, linked to differentiation of the epidermis (*3*). We cannot rule out a similar scenario for HPyV9. However, because its closest relative (LPV) has been described as a lymphotropic virus, on the basis of its in vitro growth ability, the ability of HPyV9 to infect lymphoid precursors and its putative role in various lymphoproliferative disorders in humans are worth coinvestigating.

HPyV9 was detected in cutaneous samples but not in respiratory and fecal samples, and the rate of detection appeared lower than that reported for MCPyV (*19*) or HPyV6 and HPyV7 (*3*). The sampling site was chosen because of methods useful for detection of HPyVs on the skin (*3*,*12*). Furthermore, we have observed a specific pattern for MCPyV shedding because face swab specimens yielded a higher rate of viral detection than limb specimens (*5*). However, because shedding of HPyV was not as extensively studied as that of HPVs, we cannot rule out a similar pattern of excretion, which results in underestimating detection of HPyV9 on face swab specimens. The exact prevalence of HPyV9 should be investigated by using serologic and PCR assays, notably to investigate the relevance of published data suggesting that ≈30% of humans have antibodies specific for an LPV-like virus.

Because clinical manifestations associated with HPyV infections dramatically increase in immuocompromised patients, clinical manifestations caused by HPyV9, if they exist, are also more likely to occur in these patients. Furthermore, HPyV9 infection might not remain restricted to the cutaneous area in immunocompromised patients, and reactivation might lead to systemic dissemination and in some cases clinical signs. This hypothesis is supported by identification of HPyV9 in blood and urine of asymptomatic renal transplant recipients (*4*). Further investigations will be required to decipher the potential role of HPyV9 in homeostatic and pathologic processes.

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Asymptomatic Primary Merkel Cell Polyomavirus Infection among Adults

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Merkel cell polyomavirus (MCV) is a recently discovered virus that causes 80% of Merkel cell carcinomas. We examined data for 564 gay/bisexual male participants >18 years of age in the Multicenter AIDS Cohort Study in Pittsburgh, Pennsylvania, USA, and found that 447 (79.3%) were MCV-antibody positive at initial enrollment. Of the 117 MCV-seronegative men, 31 subsequently seroconverted over a 4-year follow-up period, corresponding to a 6.6% annual conversion rate. MCV immunoglobulin G levels remained detectable up to 25 years after exposure. No signs, symptoms, or routine diagnostic test results were associated with MCV infection, and no correlation between HIV infection or AIDS progression and MCV infection was noted. An initial correlation between chronic hepatitis B virus infection and MCV prevalence could not be confirmed among MCV seroconverters or in studies of a second hepatitis B virus–hyperendemic cohort from Qidong, China. In adults, MCV is typically an asymptomatic, common, and commensal viral infection that initiates rare cancers after virus (rather than host cell) mutations.

Merkel cell carcinoma (MCC) is a rare but aggressive skin cancer most commonly occurring among the elderly and among immunosuppressed persons, including AIDS patients (*1*–*3*). By using digital transcriptome subtraction, Feng et al. recently discovered Merkel cell polyomavirus (MCV) clonally integrated in the tumor cell genome of ≈80% of MCC (*4*). This association between MCV and MCC has subsequently been confirmed by other investigators (*5*–*8*). MCV in MCC tumors possesses specific mutations that disable virus replication (*9*), which indicates that MCV is not a passenger virus and provides an explanation for how a common infection can lead to a rare tumor. MCV T antigen is specifically expressed in MCV-positive MCC tumor cells (*10*). T-antigen knockdown studies show that MCV T antigen is needed for the tumor phenotype in MCV-positive tumor cells (*11*), and the extent of tumors in the patient is correlated with levels of antibodies to MCV T antigen (*12*), leaving little doubt that MCV is the infectious cause for most but not all MCC tumors.

Serologic studies have been the primary tool to investigate the prevalence of various polyomaviruses in human populations (*13*–*16*). BK virus (BKV) and John Cunningham virus (JCV), for example, are ubiquitous human polyomavirus infections. Seroconversion for both occurs largely in childhood, with BKV seroprevalence reaching 75% among children >9 years of age and JCV seroprevalence estimated at >23% among those >10 years of age (*17*). The seroprevalence of Washington University and Karolinska Institute polyomaviruses plateau at 56% and 54%, respectively, for children 5–9 years of age (*17*). Longitudinal studies measuring immunoglobulin (Ig) G to BKV show stable levels throughout life with a slight tendency to decline after age 40–50 years, while JCV seropositivity tends to increase slowly from childhood into late adulthood (*13*,*14*,*17*,*18*). A serologic study of adult commercial blood donors that used human polyomavirus 6 and 7 virus-like particles showed that these viruses are also widely established in the human population with 69% and 35% seroprevalence, respectively (*19*).

Despite numerous reports describing seroprevalence for human polyomaviruses, less is known about

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seroconversion or signs and symptoms of primary polyomavirus infection (*13*,*14*,*20*). Two studies have reported JCV and BKV seroconversion among adults (*13*,*20*), but no data were presented describing signs or symptoms associated with infection. Bohl et al. demonstrated that antibody titers among kidney transplant donors reflect the activity and transmissibility of BKV infection (*21*,*22*). Randhawa et al. reported an inverse correlation between serum anti-BKV IgG optical density (OD) and peak urine viral load in kidney transplant recipients, suggesting a possible protective role of serum antibodies, which may impact the clinical outcome of postransplant BKV infection (*20*). These reports demonstrate that measurements of antiviral adaptive immune responses may provide prognostic value and reflect the clinical course of polyomavirus infection.

Serologic surveys have examined MCV prevalence in various groups, including children (*16*,*17*,*23*), blood donors (*16*,*24*), MCC patients (*15*,*16*,*24*), and the general population (*15*), demonstrating that MCV infection is widespread among healthy adults. Further supporting widespread infection, Chen et al. recently reported evidence for high rates of MCV seroconversion among children 3–13 years of age (*23*). Significantly elevated anti-MCV capsid IgG levels are present in blood from MCC patients compared with healthy controls, suggesting the possibility of resurgent MCV replication among patients before MCC development (*15*,*16*). Antibodies against MCV large T and small T antigens are less sensitive for detecting exposure to this virus but may be useful in monitoring tumor progression among some virus-positive MCC patients (*12*).

To examine signs, symptoms, and diagnostic test results associated with primary MCV infection, we examined participants of the Pittsburgh Men's Study, a component of the Multicenter AIDS Cohort Study (MACS). MACS recruited gay and bisexual men in 1984 and followed up at ≈6-month intervals with extensive symptom histories and physical examinations.

Methods and Materials

Study Population and Recruitment

For study population 1, stored samples from the Pittsburgh Men's Study were examined. MACS was begun in 1984 to characterize the natural history of HIV infection in the United States. All study participants were homosexual and bisexual men >18 years of age who were followed up every 6 months with physical examinations and collection of serum, plasma, and peripheral blood mononuclear cells. To be eligible for our analysis, participants had to have remained in the Pittsburgh Men's Study for at least 4 years. A subset of 564 study participants was selected for this study.

For study population 2, to search for a correlation between MCV and hepatitis B virus (HBV) infection, a set of 200 samples from participants of a community study of HBV infection in Qidong, People's Republic of China, was examined. Persons in this study (73 male and 127 female) ranged in age from 12–35 years (median age 30 years). Roughly equal numbers of serum samples were selected for hepatitis B surface antigen (HBsAg) positivity and negativity. Samples were matched for participant age and gender. Written informed consent was obtained, and all procedures and protocols were approved by the Institutional Review Board of the University of Pittsburgh.

Seroconversion Analysis

Blood samples at study entry were examined for MCV IgG positivity. For those participants initially seronegative, a sample from the end of the study (≈4 years later) was tested, and, if MCV seropositive, intervening samples were tested to determine the time of seroconversion. MCV seroconversion was defined as the midpoint between last negative and first positive serum sample. For many of these seroconverters, additional follow-up samples were available in the years after the formal end of our 4-year cohort study. A corresponding study visit was randomly chosen from 82 MCV negative controls for comparison. Specifically, the distribution of visits for MCV seroconversion was used: 10 at visit 2 (V2), 1 each at V3, V4, V8, and V10; 4 at V5; 3 at V6; 8 at V7; and 2 at V9 (total 31). Using a 4:1 match at V2 and a 2:1 match for all other seroconversion visits, we selected 82 controls, and their corresponding visit data were compared with the data at the first MCV-seropositive visit for the MCV seroconverters. Thus, MCV seroconverters and controls were temporally matched so that long-term events (e.g., AIDS progression) over the course of the cohort study would not bias the results.

Virus-like Particle Production

Virus-like particles were produced in human embryonic kidney 293TT cells (*25*) (a kind gift of Chris Buck) as previously described (*26*). Viral protein (VP) 1 and VP2 genes were designed according to a silent codon modification scheme (GenBank accession nos. FJ548568–FJ54871) (*27*) and synthesized by Blue Heron Biotechnology (Bothell, WA, USA) based on MCV339 (accession no. EU375804) (*4*). BKV VLP produced in a baculovirus system (*28*) was a kind gift of John T. Schiller.

ELISA

Serum or plasma samples were tested at 1:100 dilution in a blinded and randomized fashion for MCV VLP reactivity by using Immulon HB2 plates (Thermo Fisher Scientific, Waltham, MA, USA) coated overnight

at 4°C with MCV virus-like particles at 100 ng of the protein per well in phosphate-buffered saline (PBS) and blocked with 0.5% nonfat dry milk for 2 hours at room temperature. Paired analysis of serum samples and plasma showed no significant differences on MCV ELISA of 100 μL of serum, diluted with PBS/0.5% milk, added to wells, and incubated at room temperature for 2 hours. Anti-MCV antibody was detected by using horseradish peroxidase–conjugated rabbit anti-human IgG or anti-human IgM (Dako, Glostrup, Denmark) diluted 1:6,000 with PBS/0.5% milk (100 μL incubated for 1 hour). TMB (3.3′,5.5′-tetramethylbenzidine) substrate (Sigma, St. Louis, MO, USA) was used to detect absorbance signal at 405 nm with reference wavelengths of 620 nm after 45 minutes of incubation. Assay optimization by using MCV virus-like particles for protein saturation curves were performed as previously described (*16*). Each determination was performed in duplicate, and OD values were adjusted by background subtraction by using wells without antigen as previously described (*29*). A detailed protocol for this assay is available from www.tumorvirology.pitt.edu/mcvtools.html.

MCV ELISA Cutoff Determination

Cutoff values were based on results reported previously, which provides a detailed description (*16*). In brief, in this study, all samples with MCV IgG ELISA reactivity >0.2 OD units were found to have MCV-specific IgG specific for MCV virus-like particles but not heterologous polyomavirus virus-like particles by competition. Samples in the current study with MCV IgG OD values >0.2 units were considered positive without further testing. Previously, we also found human serum samples to have nonspecific reactivity up to 0.05 OD units that showed no specific competition. Thus, patient samples with MCV IgG ELISA <0.05 OD units in our current study were considered negative. Patient samples with MCV IgG ELISA results between 0.05 and 0.2 OD units were subjected to competition with MCV virus-like particles to determine if these titers were specific for MCV infection. If MCV reactivity for the sample was reduced by ≥50% by MCV virus-like particles competition, the sample was considered positive. Otherwise, the sample was considered negative for MCV antibodies.

Virus-Like Particle Competition Assays

Competition experiments were performed by mixing soluble MCV or BK virus–like particles with diluted serum or plasma (100 ng per 100 μL) in 1.5-mL microcentrifuge tubes followed by incubation for 1 hour at room temperature. After incubation, serum samples were directly added to MCV VLP–coated plates, and ELISA was performed as described above.

HBsAg and Core Antigen Antibody ELISA

Testing for HBsAg and hepatitis B core antigen antibody (HBc) were performed by using commercial ELISA kits (Abazyme, Needham, MA, USA, and Abnova, Taipei, Taiwan). Serum samples were diluted 1:100 with PBS and tested in duplicate. Results of the tests were

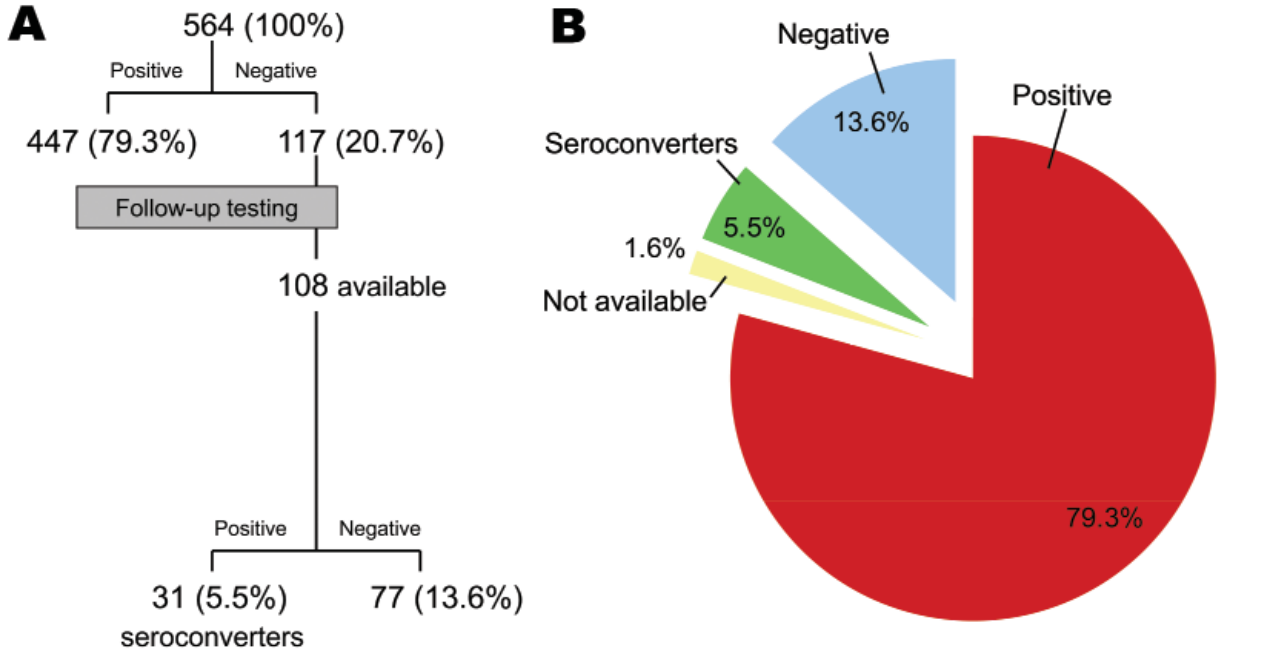

Figure 1. Merkel cell polyomavirus immunoglobulin G reactivity among Multicenter AIDS Cohort Study participants, Pittsburgh, Pennsylvania, USA. A) Flowchart of test results for 564 participants and for 108 participants with initial negative tests who were available for follow-up testing. B) Combined results for initial and follow-up testing.

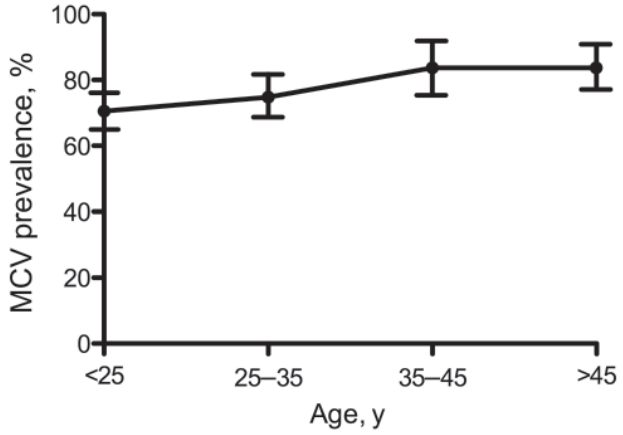

Figure 2. Age-dependent prevalence of Merkel cell polyomavirus antibodies among the Multicenter AIDS Cohort Study participants, Pittsburgh, Pennsylvania, USA. A small but significant linear trend for Merkel cell polyomavirus positivity with age among adult gay and bisexual men plateaued in the 35–45-year-old age group. Whiskers represent 95% confidence intervals.

interpreted by using cutoffs determined with negative and positive controls provided by manufacturers.

Statistical Methods

All analyses were conducted by using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) and the Epi Info statistical calculator (wwwn.cdc.gov/epiinfo). Continuous data were analyzed by using a nonparametric Mann-Whitney test, and categorical data were analyzed by using a 2-sided Fisher exact test and by χ^2 tests for trend.

Results

MCV Seroprevalence in MACS Participants

Study participants were 18–69 years old and were mostly white (96.2%). The mean age at baseline for participants was 32.8 years. Blood samples collected at entry were subjected to a battery of laboratory tests as described elsewhere (*30*,*31*). Patients were asked about symptoms during the preceding 6-month interval and evaluated for physical signs.

Of 564 MACS participants, at enrollment 447 (79.3%) were positive for MCV IgG (Figure 1). The median MCV ELISA value for positive samples was 0.313 (range 0.013–2.451 OD units). The remaining 117 (20.7%) participants provided specimens that were MCV-antibody negative. A weak but significant increasing trend for MCV seropositivity with age (p = 0.036, χ^2 test for trend) was found for young adults (25–35 years, n = 278) with a study entry prevalence of 74.8% that rose to 83.7% among men older than 45 years (n = 147) (Figure 2).

Absence of Signs and Symptoms Associated with Prevalent MCV Infection

Comparing MCV-seropositive and seronegative men, we found no significant differences in HIV, hepatitis

Table 1. Routine blood test characteristics of MCV-infected and MCV-uninfected men at initial visit for Multicenter AIDS Cohort Study, Pittsburgh, Pennsylvania, USA*

	MCV infected		MCV uninfected		
Characteristic	No. tested	Value	No. tested	Value	p value
HIV positive, no. (%)	447	138 (31)	117	33 (28)	0.58
AIDS, subsequent, no. (%)	447	58 (13)	117	12 (10)	0.43
Hepatitis B surface antigen positive, no. (%)	446	31 (7)	117	1 (1)	0.01
Hepatitis B core antibody, no. (%)	446	231 (52)	117	55 (47)	0.36
RPR reactive, no. (%)	446	19 (4)	117	4 (3)	0.68
Leukocyte count, $\times 10^3/mm^3$	443	6793.5	116	7111.2	0.22
Erythrocyte count, $\times 10^3/mm^3$	443	5.0	116	5.0	0.53
Hemoglobin, g/dL	443	15.5	116	15.5	0.86
Hematocrit, %	443	45.8	116	46.0	0.45
Platelet count, $\times 10^3/mm^3$	443	262.6	116	270.3	0.12
Neutrophils, %	443	59.1	116	59.5	0.38
Lymphocytes, %	443	35.6	116	35.5	0.41
Monocytes, %	300	4.0	69	3.7	0.33
Eosinophils, %	318	2.5	79	2.8	0.24
Cytomegalovirus Ab titer	447	239.9	117	232.1	0.86
Rubella Ab titer	447	92.0	117	87.6	0.16
IgA, mg/dL	447	360.6	117	361.4	0.44
IgG, mg/dL	447	2016.0	117	1945.9	0.91
IgM, mg/dL	447	220.3	117	227.6	0.62
CD4+, T-cells/μL	441	917.8	116	1004.2	0.09
CD8+, T-cells/μL	441	680.6	116	668.7	0.65

*MCV, Merkel cell polyomavirus; RPR, rapid plasma reagin; Ab, antibody; Ig, immunoglobulin.

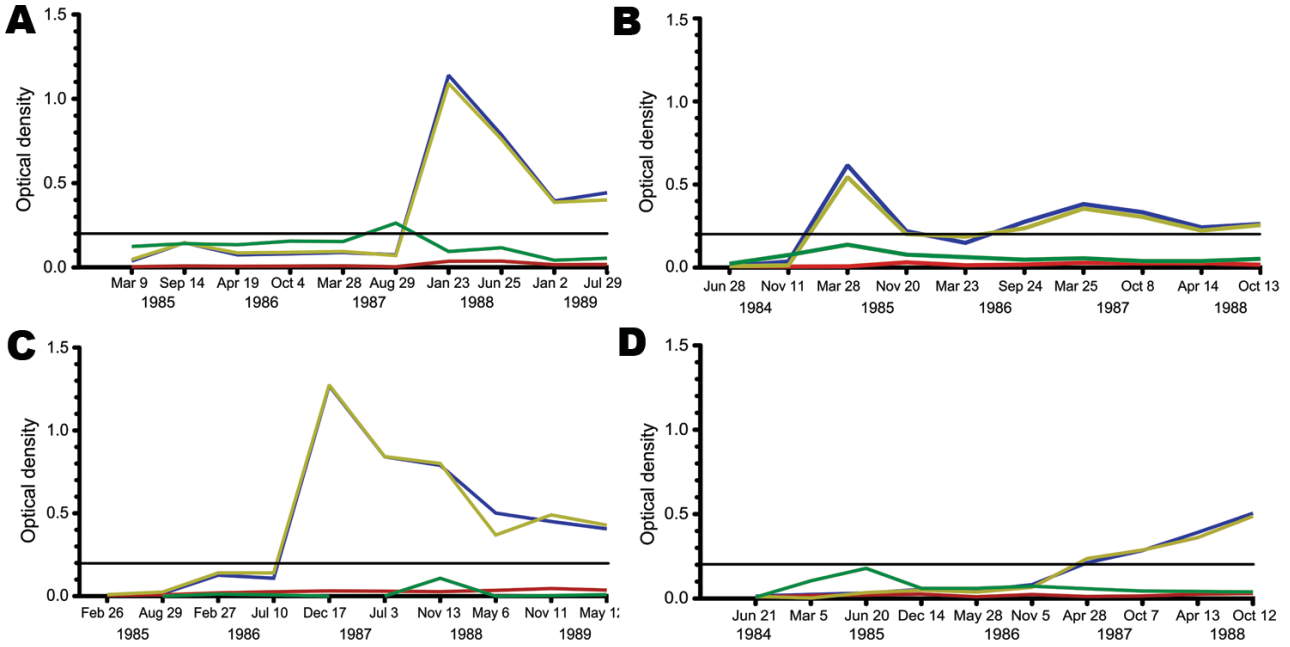

Figure 3. Representative patterns of Merkel cell polyomavirus (MCV) seroconversion among participants in the Multicenter AIDS Cohort Study, Pittsburgh, Pennsylvania, USA. Most participants showed MCV immunoglobulin (Ig) M (green line) and IgG (blue line) patterns similar to patient 1 (A) (MCV IgM peak immediately preceding IgG seroconversion) or patient 2 (B) (MCV IgM and IgG are concordant). For patient 3 (C), no IgM peak was detected during MCV IgG seroconversion. Delayed MCV IgG seroconversion, as seen with patient 4 (D), could also occur 1–2 years after an initial IgM spike. The black line represents the 0.2 optical density threshold value for MCV IgG positivity. The specificity of this test is shown by MCV virus-like particle (VLP) competition (red line) and BK virus (BKV) competition (gold line), in which MCV IgG titers are measured after plasma are preincubated with VLP antigen from the respective viruses. MCV IgG reactivity is markedly reduced by MCV competition but not BKV competition.

C virus, or syphilis status; blood count values; or other immunologic markers (Table 1). No correlation between MCV positivity and reported sexual activity was identified (data not shown). A significant association between MCV seropositivity and chronic hepatitis B virus infection was found at study entry: 31 (7.2%) of 447 MCV-seropositive participants were also positive for HBsAg as compared with 1 (0.8%) of 117 MCV-seronegative participants (odds ratio [OR] 8.1, 95% confidence interval [CI] 1.1–58.8). No significant association was found, however, between MCV and HBV exposure: 231 (51.7%) of 447 MCV-positive men, compared with 55 (47.0%) of 117 of MCV-negative men, were positive for HBc at study entry (OR 1.1, 95% CI 0.9–1.4). No other specific symptoms, signs, or blood test results were significantly associated with MCV positivity at entry, including rashes, diarrhea, fever, respiratory symptoms, or changes in total leukocyte count or cellular subpopulations (not shown).

MCV Seroconversion Asymptomatic among Adult Men

To determine seroconversion, we tested 108 (92.3%) available plasma samples taken 4–5 years after study enrollment from the 117 initially MCV-seronegative persons. Of the 108 initially MCV-negative persons, 31 (5.5%) seroconverted (Figure 1), resulting in an incidence of 6.62/100 person-years.

MCV seroconversion at longitudinal follow-up visits was determined and infection was defined, for the purposes of this study, to occur at the midpoint between the last MCV-negative and the first MCV IgM- or IgG-positive blood sample. In general, MCV IgM peak levels preceded IgG seroconversion by 1 study visit or were concurrent with IgG seroconversion. Peak IgM levels (OD range 0.12–0.47), when present, were consistently lower than peak IgG levels (OD range 0.18–1.16). Once IgG seroconversion had occurred, none of the subsequent samples from these patients reverted to MCV IgG seronegativity.

Typical patterns for seroconversion are shown in Figure 3. Patient 1 shows the most common pattern, in which IgM peaks immediately before a rise in IgG levels, while the next most common pattern, exemplified by patient 2, shows a concurrent IgM peak with IgG seroconversion. For 6 seroconverters, rises in IgG were not accompanied by a preceding increase in IgM reactivity (patient 3), possibly due to transient IgM peaks that resolved between the 2 blood collections. Finally, for 3 seroconverters, an MCV IgM peak was detected 1–2 years before the rise in MCV IgG titers, which suggests

Table 2. Signs and symptoms at seroconversion study visit or during previous 6 months for MCV seroconverters and controls matched by study visit among participants in the Multicenter AIDS Cohort Study, Pittsburgh, Pennsylvania, USA*

	Seroconverters		Controls		
Signs or symptom	No. evaluated	No. (%) with sign or symptom	No. evaluated	No. (%) with sign or symptom	p value
Shortness of breath ≥2 weeks	31	1 (3.2)	75	8 (10.7)	0.28
Shortness of breath at time of visit	28	1 (3.6)	73	6 (8.2)	0.67
Cough ≥2 weeks	31	1 (3.2)	75	4 (5.3)	1
Cough now	31	0	75	3 (4.0)	0.55
Sore throat ≥2 weeks	31	1 (3.2)	75	5 (6.7)	0.67
Sore throat now	31	1 (3.2)	75	2 (2.7)	0.50
Rash ≥2 weeks	31	2 (6.5)	75	4 (5.3)	1
Rash now	31	3 (9.7)	75	4 (5.3)	0.41
Bruising ≥2 weeks	31	0	75	4 (5.3)	0.32
Bruising now	31	0	75	3 (4.0)	0.55
Fatigue ≥2 weeks	31	0	75	5 (6.7)	0.32
Fatigue now	31	1 (3.2)	75	4 (5.3)	1
Weight loss ≥10 pounds	31	0	75	1 (1. 3)	1
Weight loss now	31	0	75	0	NA
Diarrhea ≥2 weeks	31	2 (6.5)	75	0	0.08
Diarrhea now	31	1 (3.2)	75	0	0.29
Fever ≥2 weeks	31	0	75	0	N/A
Fever now	31	0	75	0	N/A
Lymphadenopathy ≥2 weeks	31	3 (9.7)	75	5 (6.7)	0.69
Lymphadenopathy now	31	3 (9.7)	75	5 (6.7)	0.69
Night sweats ≥2 weeks	31	0	75	4 (5.3)	0.32
Night sweats now	31	0	75	2 (2.7)	1.0
Headache ≥2 weeks	31	1 (3.2)	75	3 (4.0)	1.0
Headache now	31	1 (3.2)	75	2 (2.7)	1.0

*MCV, Merkel cell polyomavirus; NA, not applicable.

a prolonged period of infection before Ig class switching (patient 4).

To determine signs or symptoms associated with primary MCV infection, the date of seroconversion was identified for each of the seroconverters. Signs, symptoms, and laboratory values reported at the first MCV-seropositive visit were then determined. Corresponding study visits (selected as described in Methods) from MCV-seronegative controls were then examined. Symptoms, such as fever, rash, weight loss, fever, diarrhea, and cough present at the study visit were similar for MCV seroconverters and the control group (Table 2). Self-reported symptoms lasting ≥2 weeks during the 6 months before the MCV seroconversion visit were also not significantly different between patients and controls. Moreover, no statistically significant differences in erythrocyte, leukocyte, CD4+, CD8+ cell counts, or other clinical test values were identified between the 2 groups at the first MCV-seropositive visit for patients or at comparable visits for controls (Table 3). Although not significant, a greater proportion of the MCV seroconverters were hepatitis B core antibody positive (63.3% vs. 44.4%) and had a marginally lower hemoglobin level (15.5 vs. 16.0 g/dL) as compared with controls. No DNA of the virus was detected by real-time PCR in the plasma for 4 participants at the time of seroconversion (data not shown).

Longevity of MCV IgG Responses

Plasma from 17 of the 31 MCV seroconverters with known dates of infection was available for long-term follow-up (ranging from 7–25 years). Two general patterns of anti-MCV antibody reactivity were seen: 11 (64.7%) patients demonstrated robust MCV seroconversion, with slowly increasing levels of MCV IgG over time (Figure 4). The second pattern (6 patients) revealed a transient increase in MCV IgG >0.2 OD units at the time of seroconversion, which declined over 1–2 years and generally remained <0.2 OD units. Despite this decline, all 6 retained readily detected MCV IgG as measured by MCV VLP competition assays.

Lack of Correlation between Hepatitis B Virus Infection and MCV Status

Prevalent MCV seropositivity at study entry was only significantly associated with a positive test result for chronic HBsAg carriage. To examine the possible role of MCV infection in influencing chronic HBV infection, we tested 93 HBsAg-positive and 107 negative serum samples collected in a convenience sample from Qidong residents. Among these samples the prevalence of MCV antibody positivity was 66% with mean OD 0.452 (range 0.01–2.19). Rates of MCV positivity for HBsAg positive

Table 3. Routine blood test characteristics for MCV seroconverters and controls matched by study visit among participants in the Multicenter AIDS Cohort Study, Pittsburgh, Pennsylvania, USA*

Characteristic	Seroconverters		Controls		p value
	No. tested	Value	No. tested	Value	
HIV positive, no. (%)	31	12 (38.7)	82	19 (23.2)	0.10
AIDS, subsequent, no. (%)	31	5 (16.1)	82	7 (8.5)	0.31
Hepatitis B surface antigen positive, no. (%)	30	0 (0)	81	1 (1.2)	1.00
Hepatitis B Core antigen antibody positive, no. (%)	30	19 (63.3)	81	36 (44.4)	0.08
RPR reactive, no. (%)	31	1 (3.2)	73	1 (1.4)	0.50
Leukocytes count, $\times 10^3/mm^3$	31	7174.2	75	6978.7	0.26
Erythrocytes count, $\times 10^3/mm^3$	31	5.1	75	5.2	0.13
Hemoglobin, g/dL	31	15.5	75	16.0	0.07
Hematocrit, %	31	46.7	75	47.7	0.15
Platelet count, $\times 10^3/mm^3$	31	247.3	75	259.6	0.14
Polymorphonuclear lymphocytes, %	31	58.7	75	58.1	0.61
Lymphocytes, %	31	32.6	75	32.9	0.83
Monocytes, %	29	5.9	72	5.5	0.22
Eosinophils, %	30	2.6	74	3.1	0.39
Atypical lymphocytes, %	29	1.2	71	1.1	0.56
Cytomegalovirus Ab titer	12	168.5	44	306.4	0.34
Rubella Ab titer	12	73.6	45	75.4	0.72
IgA, mg/dL	12	308.2	45	295.1	0.70
IgG, mg/dL	12	1,117.2	45	1151.5	0.93
IgM, mg/dL	12	178.8	45	172.5	0.75
CD4+, T-cells/μL	26	925.1	65	900.1	0.85
CD8+, T-cells/μL	26	637.7	65	564.5	0.22

*MCV, Merkel cell polyomavirus; RPR, rapid plasma reagin; Ab, antibody; Ig, immunoglobulin.

and negative samples were 61% and 70%, which were not statistically different (Table 4). Among the 107 HBsAg negative participants, MCV prevalence was not statistically significantly different for those exposed (HBc negative, n = 9) and not exposed (HBc negative, n = 84) to HBV infection (75% vs. 71%, respectively).

Discussion

Our study makes use of the MACS cohort study to evaluate MCV seroprevalence and seroconversion among adult men at risk for HIV infection. While MCC occurrence is elevated among HIV-positive persons (*32*), it is still an uncommon cancer and no cases of MCC were reported among the participants selected from MACS for this study.

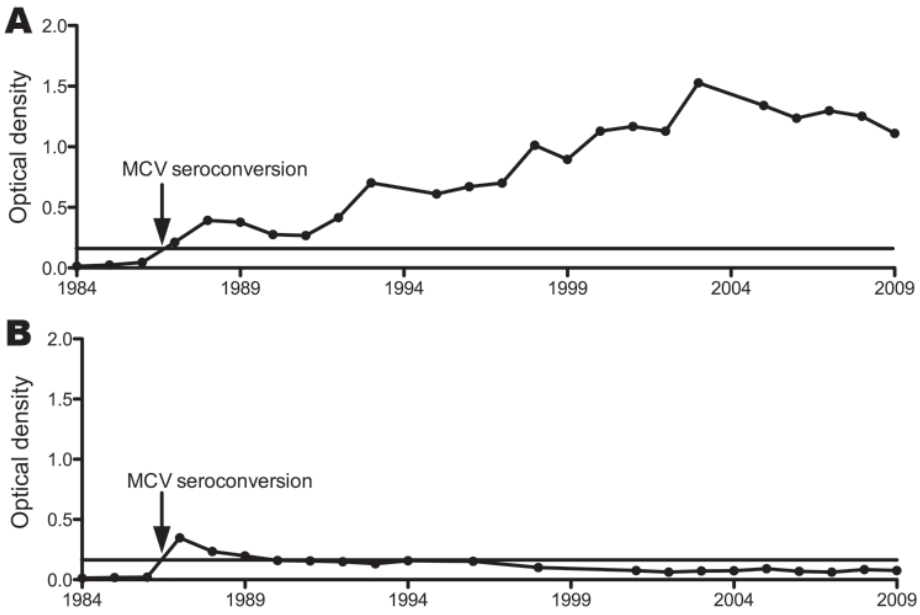

Figure 4. Two general patterns for Merkel cell polyomavirus (McV) immunoglobulin G levels after seroconversion among participants in the Multicenter AIDS Cohort Study, Pittsburgh, Pennsylvania, USA: a gradual increase over the 25-year period (A, patient 5) or a multiyear decline during 25-year follow up (B, patient 6). Horizontal line represents the 0.2 optical density threshold value for positivity.

Table 4. MCV prevalence among 200 Qidong, China, residents with and without hepatitis B virus surface antigen and hepatitis B virus core antibodies*

Characteristic†	No. residents	MCV positive, no. (%)	MCV negative, no. (%)
HBsAg positive/B core antibody positive	84	51 (61)	33 (39)
HBsAg positive/B core antibody negative	9	5 (56)	4 (44)
HBsAg negative/B core antibody positive	8	6 (75)	2 (25)
HBsAg negative/B core antibody negative	99	70 (71)	29 (29)

*MCV, Merkel cell polyomavirus; HBsAg, hepatitis B virus surface antigen.
†NS for comparison of either HBsAg or hepatitis B core antibody with MCV positivity.

We found no correlation between MCV infection and HIV status or AIDS in our study. MCV seroconversion was not associated with signs or symptoms of illness in adult gay and bisexual men. MCV prevalence plateaued for men 35–45 years of age in our study, which is consistent with primary MCV infection occurring mainly among children and young adults (*23*). We found MCV prevalence among participants was 79.3% with a 6.6% annual seroconversion rate, which suggested widespread circulation of the virus. Our study suggests that MCV infection is a highly prevalent infection among adults that is often asymptomatic. We cannot exclude rare illnesses occurring from primary MCV infection, however, or illness mild enough not to be reported by our cohort participants. These results, and those of others, indicate that active MCV transmission is common even though MCV-related cancer is rare (*33*).

Signs and symptoms for primary MCV infection were not found in our study. An important caveat is that MACS participants self-reported symptoms at ≈6-month intervals, and minor symptoms may have been forgotten between study visits. MACS is a closely monitored cohort study designed to study risk factors and natural history of HIV in homosexual and bisexual men in the United States. Participants in this study were all sexually active adult men, most of whom were already positive for MCV, and so caution is needed in generalizing our results to other populations (e.g., women, children, non-US populations). We cannot exclude, for example, the possibility of symptoms or disease after primary pediatric MCV infection. Weak correlations that did not reach a level of significance in our study, such as lower hemoglobin and hematocrit values after MCV seroconversion, might be reconciled by testing in other cohorts.

We did find an unexpected correlation between prevalent MCV infection and chronic HBV carriage for MACS participants. HBc positivity, however, was not elevated. When MCV seroconverters were examined, no correlation was found between MCV infection and HBsAg positivity, and only a weak but nonsignificant association was present for HBc values. It is likely that most of the MACS men were exposed to HBV as adults through unprotected sex or parenteral exposure. None of our other comparisons suggest that either of these routes of infection is significant for MCV, although we can infer that MCV infection (a childhood infection that primarily occurs before onset of sexual activity) (*23*) likely preceded HBV infection in most participants.

To further investigate the relationship between MCV and HBV infection, we examined HBV-hyperendemic samples from eastern China that likely represent mainly vertical or early childhood horizontal HBV infections. No correlation with MCV infection was found. Because of selection to ensure sufficient numbers of HBV-exposed participants, the Qidong study group cannot be assumed to represent a community serosurvey. Nonetheless, our results indicate widespread MCV infection among Asian adults similar to that seen for North Americans. It is unlikely that MCV and HBV are biologically linked in any significant manner, but caution is needed in interpreting these results since modes of HBV infection for MACS and Qidong participants are different.

MCV appears to be a life-long, chronic infection that may cause continuous antigen stimulation. Recent studies have shown that detection of MCV antibodies is improved by use of conformational epitopes present in VLP ELISA (*16*,*23*,*34*). Detection of MCV IgG by virus-like particles ELISA is persistent for up to 25 years after seroconversion, making it unlikely that seronegative participants were exposed to MCV and subsequently lost detectable antibodies. While only a portion of skin samples from healthy persons have viral DNA detectable by PCR (*35*,*36*), more sensitive techniques show persistent viral DNA in skin samples over a time scale of months and possibly years (*19*).

Our study indicates that MCV is one of a burgeoning number of newly recognized viruses that are part of the normal human flora (*19*,*37*–*39*). MCV infection among adults is generally silent and not associated with common signs, symptoms, or laboratory measures for infection. This virus, nonetheless, directly contributes to one of the most deadly human skin cancers, which illustrates that common commensal viral infections can contribute to the etiology of chronic diseases under unusual circumstances, such as virus mutation together with loss of immune surveillance.

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Incidence of Acute Gastroenteritis and Role of Norovirus, Georgia, USA, 2004–2005

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Approximately 179 million cases of acute gastroenteritis (AGE) occur annually in the United States. However, lack of routine clinical testing for viruses limits understanding of their role among persons seeking medical care. Fecal specimens submitted for routine bacterial culture through a health maintenance organization in Georgia, USA, were tested with molecular diagnostic assays for norovirus, rotavirus, astrovirus, sapovirus, and adenovirus. Incidence was estimated by using national health care utilization rates. Routine clinical diagnostics identified a pathogen in 42 (7.3%) of 572 specimens; inclusion of molecular viral testing increased pathogen detection to 15.7%. Community AGE incidence was 41,000 cases/100,000 person-years and outpatient incidence was 5,400/100,000 person-years. Norovirus was the most common pathogen, accounting for 6,500 (16%) and 640 (12%) per 100,000 person-years of community and outpatient AGE episodes, respectively. This study demonstrates that noroviruses are leading causes of AGE among persons seeking medical care.

Acute gastroenteritis (AGE), defined as diarrheal disease of rapid onset potentially accompanied by nausea, vomiting, fever, or abdominal pain, is a major cause of illness in the United States. Approximately 179 million episodes of AGE occur each year and result in ≈600,000 hospitalizations and 5,000 deaths (*1*,*2*). A specific etiology is attributed to only ≈20% of AGE cases, although viruses are recognized as the most common of the known agents (*1*,*3*). Noroviruses, in particular, have been estimated to cause ≈21 million cases of AGE annually, including >56,000 hospitalizations and 570 deaths (*3*). However, these estimates are based on US estimates of AGE and extrapolation of etiologic fractions from studies in other industrialized countries because few laboratory-based data are available on the role of noroviruses in sporadic AGE in the United States.

Development of more precise disease incidence estimates for noroviruses and other viral causes of AGE have been hampered, in part, by the lack of diagnostic assays available in clinical settings. With the exception of an enzyme immunoassay (EIA) for rotavirus, diagnosis of viral AGE in the United States is made largely on the basis of clinical signs and symptoms. Molecular techniques used for definitive diagnosis, specifically PCR, are available mostly in public health laboratories and research settings. Commercial EIA kits for norovirus have been developed but are not widely available in the United States and are not cleared by the US Food and Drug Administration for diagnosis of sporadic AGE cases. Evaluation of viral AGE incidence is further limited by the fact that most AGE patients do not seek medical care, and, of those who do, <20% submit fecal specimens for diagnostics (*2*). Lastly, general perception is that norovirus gastroenteritis is a self-limiting mild illness that rarely requires medical attention, despite several reports of serious illness and death in various settings (*3*–*7*).

Better understanding of the relative role of specific viral causes of AGE among persons seeking medical care is needed to help guide clinical management and ultimately to develop more appropriate AGE prevention strategies. We sought to determine the prevalence of viral pathogens among AGE patients who sought medical care and identify their trends in seasonality and molecular epidemiology. Using a similar strategy as that used for common bacterial

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and parasitic causes of AGE (*3*), we further sought to estimate incidence of viral agents of AGE in community and outpatient settings.

Methods

Study Population and Sample Selection

Kaiser Foundation Health Plan of Georgia, Inc. (KP) is a health maintenance organization with ≈280,000 members in Georgia, USA, who almost exclusively seek care with KP. One microbiology laboratory serves the entire population of Georgia KP members and receives ≈140 fecal specimens each month from outpatients seeking medical care. Upon order of fecal diagnostics by an outpatient clinician, patients were provided fecal collection kits and instructions for their in-home collection. Patients were instructed to keep specimens refrigerated after collection and to return them as soon as possible to the clinic, typically within 48 hours. Specimens were then transported by same-day courier to the KP microbiology laboratory for processing.

Each week during March 15, 2004–March 13, 2005, a total of 11 specimens from different patients were randomly selected for inclusion in the study. A target sample size of 572 was selected to identify 10% norovirus prevalence with a 95% confidence interval of ± 5%, assuming patients with AGE of short duration were 4× less likely to submit samples than those with AGE of longer duration. Only patients for whom a specimen was submitted after a clinician order for routine culture were eligible for inclusion, although additional diagnostics (e.g., ova and parasites, *Clostridium difficile*, rotavirus EIA) may have also been ordered for some patients. The following data were obtained for each specimen: days from outpatient visit to receipt at the KP laboratory, week of receipt by the KP laboratory, patient age group in 5-year intervals, patient sex, fecal consistency, and results of any diagnostic tests performed by the KP laboratory. Data and specimens sent to the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA, were anonymous and had no identifiable information that could be linked to the patient. Thus, our study did not require review by an institutional review board.

Laboratory Testing

All specimens submitted for routine culture were tested for *Campylobacter*, *Shigella*, and *Salmonella* spp. at the KP laboratory. When requested, specimens were also tested for *Giardia* and *Cryptosporidium* spp. by using ProSpec T Microplate Assays (Remel, Lenexa, KS, USA), for rotavirus by using the Immunocard STAT Rotavirus Test (Meridian Bioscience Inc., Cincinnati, OH, USA), and for *C. difficile* toxigenic strains by using the *C. difficile* Tox A/B 11 EIA (Wampole Laboratories LLC, Princeton, NJ, USA). Specimens submitted in Cary Blair medium (i.e., routine bacterial culture) then underwent molecular testing for norovirus, rotavirus, sapovirus, astrovirus and enteric adenovirus at the CDC laboratory.

Viral nucleic acid was extracted by using RNA spin columns (Omega Bio-Tek, Doraville, GA, USA). TaqMan real-time reverse transcription PCR and PCR were used for initial sample screening for genogroup I (GI) and GII noroviruses, sapoviruses, and adenoviruses, as described (*8–10*). Conventional PCR was conducted to screen samples for astrovirus and rotavirus group A and to determine the genotype of norovirus-, sapovirus-, and adenovirus-positive samples (*11–15*). PCR products were purified by using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA) and sequenced by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. For genotyping, detected virus sequences were compared with sequences in the CDC reference sequence databases.

Disease Incidence Calculations

The prevalence of each pathogen in specimens was used to calculate pathogen-specific incidence rates of acute gastroenteritis among the study population. We used pooled data on self-reported health care utilization practices of persons with acute diarrheal disease obtained from Foodborne Diseases Active Surveillance Network (FoodNet) population surveys in 2000–2001, 2002–2003 (*2*), and 2006–2007 (CDC, unpub. data). These 3 population-based telephone surveys were conducted in selected sites located throughout the United States, including Georgia, by using a probability sample design.

The weighted proportion of survey respondents with AGE (defined as diarrhea [≥3 loose stools in a 24-hour period] beginning within the past month) who sought medical care in person and the proportion of those respondents who submitted a specimen were calculated and stratified by age group (Table 1). Because viral diarrhea is generally of shorter duration than diarrhea of other etiologies (*4*), data from respondents with diarrhea lasting <3 days at the time of interview were used for estimates of viral etiologies, and data from respondents with diarrhea of any duration were used for estimates of bacterial, parasitic, and other etiologies. These data were used to generate age-group–specific rates of fecal specimen submission among all those in the community with diarrhea and among only those seeking medical care. Respondents with a chronic disease for which diarrhea is a major sign (e.g., celiac disease, Crohn disease, diverticulitis, irritable bowel syndrome, ulcerative colitis) were excluded from analysis.

To adjust for variations in health care utilization practices by age, we weighted age-group–specific FoodNet Population Survey data proportional to the age distribution

Table 1. Health care utilization practices among persons with acute gastroenteritis, by age group, FoodNet Population Surveys, USA, 2000–2007*

	% Persons with acute gastroenteritis who sought medical care		% Persons who submitted a fecal specimen of those with acute gastroenteritis who sought medical care	
Age group, y	<3 d illness duration	Any illness duration	<3 d illness duration	Any illness duration
<5	16.8	28.5	5.8	15.8
5–15	16.8	20.1	6.9	10.9
16–25	6.2	12.4	1.6	0.6
26–45	6.4	10.7	1.5	13.7
46–65	5.4	9.5	7.5	21.5
>65	10.3	15.7	17.4	13.0
Total	9.3	14.6	6.1	13.3

*Acute gastroenteritis is defined as diarrhea (≥3 loose stools in a 24-h period) beginning within the past month and in the absence of a chronic disease for which diarrhea is a major sign. Data were obtained from the Foodborne Diseases Active Surveillance Network (FoodNet) Population Survey, cycles 3–5 (*2*; Centers for Disease Control and Prevention, unpub. data).

of persons with specimens positive for each pathogen by using the following age groups: <5, 5–15, 16–25, 26–45, 46–65, and >65 years. These age groups were broadly selected for clinical relevance and consistency in health care utilization rates. Community and outpatient incidence of each pathogen was calculated on the basis of prevalence of that pathogen in sampled specimens (P_i), pathogen-specific fecal specimen submission rates among all respondents with AGE ($ComSS_i$) and among those seeking medical care ($OutSS_i$), the total number of specimens submitted to the KP laboratory during the study period (S = 1,825), and the total Kaiser membership in Georgia (i.e., study catchment population, N = 280,000) (Figure 1).

Thus, incidence was calculated on the basis of extrapolation of pathogen prevalence by using fecal specimen submission and medical care–seeking rates for community estimates and only fecal specimen submission rates for outpatient estimates. A simulation approach was used to generate 90% credible intervals (CIs). For each pathogen or group of pathogens, P_i, $ComSS_i$, and $OutSS_i$ were randomly drawn assuming a β distribution for each, and the 2 incidence equations were recalculated. We report 5th and 95th centiles of 100,000 simulations. Incidence estimates weighted for monthly fluctuations in the number of specimens received by the KP laboratory were also evaluated, but because they did not differ appreciably with unadjusted estimates, the simpler unadjusted approach was used. Chi-square tests were used to evaluate trends among categorical variables. Analyses were performed by using SAS version 9.2 (SAS Institute, Cary, NC, USA), Stata version 11.0 (StataCorp LP, College Station, TX, USA), and Epi Info version 3.4.3 (CDC).

Results

A total of 572 specimens were included in the study. Routine bacterial culture and viral PCR were performed on all specimens, and 375 (65.6%) were also tested for ova and parasites, 161 (28.1%) for *C. difficile*, and 28 (4.9%) for rotavirus by using EIA. Specimens not tested for ova and parasites or *C. difficile* were considered not positive for those pathogens. Adult patients 26–65 years of age provided 325 (56.8%) of the specimens tested and 316 (55.2%) were from female patients. Clinical diagnostic testing at the KP laboratory identified a pathogen in 42 (7.3%) specimens. Subsequent PCR testing at CDC identified ≥1 virus in 53 (9.3%) specimens, for a total of 88 (15.4%) specimens with a detected pathogen (includes 5 specimens previously positive for rotavirus by EIA and 2 specimens positive for bacteria and virus). Among these 88 specimens, viruses were detected in 53 (60.2%), bacteria in 30 (34.1%), and parasites in 7 (8.0%). Multiple pathogens were identified in only 4 specimens (Table 2). Norovirus was the most frequently identified pathogen overall, detected in 25 (4.4%) of all specimens and 28.4% of the 88 specimens with any pathogen identified. Detection of any pathogen in feces was most likely in children <5 years of age (32.1%)

$$\frac{P_i \times S \times 100{,}000}{ComSS_i \times N} = \text{community incidence/100,000 person-years}$$

$$\frac{P_i \times S \times 100{,}000}{OutSS_i \times N} = \text{outpatient incidence/100,000 person-years}$$

$$ComSS_i = \frac{\sum_a (S_{ia} \times ComSS_a)}{\sum_a S_{ia}} \qquad OutSS_i = \frac{\sum_a (S_{ia} \times OutSS_a)}{\sum_a S_{ia}}$$

Figure 1. Equations used for calculation of community and outpatient incidence of each pathogen in patients with acute gastroenteritis (AGE), Kaiser Foundation Health Plan of Georgia, Inc., USA, March 15, 2004–March 13, 2005. P_i, prevalence of pathogen *i* in stool samples; S, total no. stool samples submitted; $ComSS_i$, pathogen-specific fecal specimen submission rates among all respondents with AGE; N, total Kaiser membership; $OutSS_i$, pathogen-specific fecal specimen submission rates among those seeking medical care; S_{ia}, no. of stools positive for pathogen *i* in age group *a*; $ComSS_a$, proportion of those in age group *a* with AGE who seek care and submit stool specimen; $OutSS_a$, proportion of those in age group *a* seeking care for AGE who submit stool specimen.

Table 2. Pathogens detected among fecal specimens submitted by outpatients, by age group, to Kaiser Foundation Health Plan of Georgia, Inc., USA, March 15, 2004–March 13, 2005

Pathogen	Age group, y, no. (%) positive						Total, n = 572
	<5, n = 81	5–15, n = 63	16–25, n = 47	26–45, n = 190	46–65, n = 135	>65, n = 56	
Virus	19 (23.5)	5 (7.9)	6 (12.8)	14 (7.4)	7 (5.2)	2 (3.6)	53 (9.3)
Norovirus	6 (7.4)	1 (1.6)	3 (6.4)	8 (4.2)	7 (5.2)	0	25 (4.4)
Astrovirus	5 (6.2)	1 (1.6)	1 (2.1)	3 (1.6)	0	0	10 (1.7)
Rotavirus	4 (4.9)	2 (3.2)	1 (2.1)	0	0	0	7 (1.2)
Sapovirus	4 (4.9)	0	1 (2.1)	0	1 (0.7)	1 (1.8)	7 (1.2)
Adenovirus	1 (1.2)	1 (1.6)	0	3 (1.6)	0	1 (1.8)	6 (1.0)
Bacteria	8 (9.9)	3 (4.8)	3 (6.4)	8 (4.2)	8 (5.9)	0	30 (5.2)
*Clostridium difficile**	0	0	1 (2.1)	6 (3.2)	7 (5.2)	0	14 (2.4)
Salmonella spp.	6 (7.4)	1 (1.6)	1 (2.1)	0	0	0	8 (1.4)
Shigella spp.	2 (2.5)	1 (1.6)	0	2 (1.1)	0	0	5 (0.9)
Campylobacter spp.	0	1 (1.6)	1 (2.1)	0	1 (0.7)	0	3 (0.5)
Parasite†	1 (1.2)	1 (1.6)	1 (2.1)	4 (2.1)	0	0	7 (1.2)
Giardia spp.	0	1 (1.6)	0	4 (2.1)	0	0	5 (0.9)
Cryptosporidium spp.	1 (1.2)	1 (1.6)	0	0	0	0	2 (0.3)
Multiple‡	3 (3.7)	0	0	0	1 (0.7)	0	4 (0.7)
Any pathogen	26 (32.1)	9 (14.3)	10 (21.3)	26 (13.7)	15 (11.1)	2 (3.6)	88 (15.4)
Unidentified	55 (67.9)	54 (85.7)	37 (78.7)	164 (86.3)	120 (88.9)	54 (96.4)	484 (84.6)

**C. difficile* testing performed on 161 (28.1%) of 572 total specimens; those not tested were classified as not positive.
†Parasite testing performed on 375 (65.6%) of 572 total specimens; those not tested were classified as not positive.
‡Combinations observed were *Salmonella* spp./norovirus, *Salmonella* spp./sapovirus, norovirus/sapovirus, and rotavirus/sapovirus.

and decreased significantly with age to 3.6% for persons >65 years of age (p<0.001).

Most (62.4%) specimens were received for laboratory testing within 3 days of the patient's medical visit. However, 90 (15.7%) were received ≥1 week later. Time lag did not differ by age group. Pathogens were detected most frequently among specimens received by the laboratory within 3 days of outpatient visit (18.2%) and were significantly less likely to be detected with increasing delay, decreasing to 6.7% among specimens received after >1 week (p<0.001). This time lag effect was more pronounced for viruses than bacteria and was likely caused by the decrease in viral shedding and test sensitivity after the acute phase of illness. For example, norovirus was identified in 5.0% of specimens received ≤7 days of outpatient visit, but in only 1.1% of those received >1 week after outpatient visit; overall detection of bacteria decreased from 5.6% to 3.3% during the same time frame. Most (81.4%) specimens were unformed (i.e., took on the shape of the collection cup), indicating that the patients were still symptomatic at the time of specimen collection. No differences in rates of pathogen detection were found between formed and unformed specimens.

Overall rate of pathogen detection and the relative distribution of pathogens showed apparent seasonal variation, although insufficient sample size precluded identification of significant temporal trends. Overall, viruses predominated during winter and spring, and bacteria predominated during summer and fall (Figure 2). Relatively low prevalence of parasites was observed year round with no discernible seasonal pattern. Pronounced winter peaks in prevalence were observed for norovirus (10.5%) and astrovirus (4.2%). All rotavirus-positive specimens were received during winter and spring, during which prevalence was 1.4% and 3.5%, respectively. Similarly, peak prevalence of sapovirus (2.8%) and adenovirus (2.1%) was during spring.

Overall estimated community incidence of AGE of any etiology was 41,000 cases/100,000 person-years (90% CI 38,000–44,000 cases), of which 13,000 (32%, 90% CI 10,000–20,000 cases) were caused by identified agents (Table 3). Estimated incidence of AGE in outpatients was 5,400 cases/100,000 person-years (90% CI 4,400–6,700 cases), of which 1,600 (30%, 90% CI 1,300–2,400 cases) could be attributed to a specific pathogen. Norovirus was the leading identifiable cause of illness in community and outpatient settings and was associated with 16% of all community AGE (6,500 cases/100,000 person-years, 90% CI 3,700–12,000 cases) and 12% of all outpatient AGE (640/100,000 person-years, 90% CI 360–1,200 cases).

In comparison with estimated community incidence of AGE caused by bacteria (1,700 cases/100,000 person-years, 90% CI 1,100–2,300) and parasites (420 cases/100,000 person-years, 90% CI 200–790), the community incidence of norovirus was ≈4-fold and >15-fold greater, respectively. Likewise, the incidence of norovirus infection prompting medical care was ≈3-fold greater than that of bacterial pathogens (240 cases/100,000 person-years, 90% CI 160–320 cases) and >10-fold greater than that of parasites (60 cases/100,000 person-years, 90% CI 29–110 cases). Astrovirus was the second leading identifiable pathogen associated with

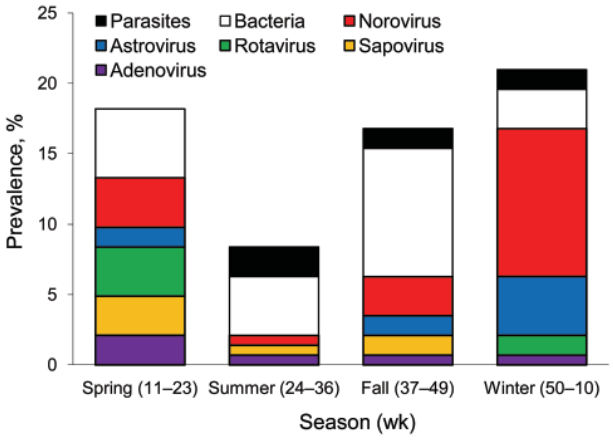

Figure 2. Pathogen prevalence in acute gastroenteritis outpatients by season, Kaiser Foundation Health Plan of Georgia, Inc., USA, March 15, 2004–March 13, 2005. Viral pathogens predominated during winter and spring, and bacteria predominated during summer and fall.

AGE in community and outpatient settings; estimated incidences were 1,800 cases (90% CI 880–3,400 cases) and 270 (90% CI 130–590 cases) per 100,000 person-years, respectively.

Sequence analysis of the 25 norovirus-positive samples identified 24 (96%) as GII strains and 1 (4%) as a GI strain (Figure 3). Of all strains, approximately one third could be typed as GII.4, specifically the Farmington Hills and Hunter subclusters. GII.3 viruses were equally prevalent in this population. Other norovirus genotypes identified included GI3b, GII.2, GII.14, and GII.17.

Discussion

This study provides US laboratory–based incidence rates for norovirus disease determined by using direct outpatient surveillance of routinely collected fecal specimens. Despite examination of only fecal specimens submitted for bacterial culture, our data demonstrate that viruses, and noroviruses specifically, were the leading cause of AGE among persons of all ages seeking medical care. We estimate that 24% of persons seeking medical care for AGE do so because of viral infection, including 12% because of norovirus infection. Overall, viruses accounted for 27% of AGE episodes in the community and noroviruses accounted for 16%.

In contrast to bacterial and parasitic pathogens, viral AGE pathogens were primarily detected during winter and spring months, which is consistent with previous descriptions of norovirus and rotavirus seasonality in temperate climates (*16*). Norovirus strains identified in this study were similar to those identified in studies of sporadic AGE (*17*) and those implicated in norovirus outbreaks confirmed by CDC during the same period (*18*). The predominance of GII.4 strains in epidemic and sporadic norovirus disease demonstrates the need for including this

Table 3. Estimated incidence of pathogens causing acute gastroenteritis in community and outpatient settings, Kaiser Foundation Health Plan of Georgia, Inc., USA, March 15, 2004–March 13, 2005*

	Outpatient†		Community‡	
Pathogen	Incidence (90% CI)§	% Total	Incidence (90% CI)§	% Total
Virus	1,300 (750–2,200)	24.1	11,000 (6,800–19,000)	26.8
Norovirus	640 (360–1,200)	11.9	6,500 (3,700–12,000)	15.9
Astrovirus	270 (130–590)	5.0	1,800 (880–3,400)	4.4
Rotavirus	150 (65–330)	2.8	880 (400–1,700)	2.1
Sapovirus	110 (49–220)	2.0	900 (420–1,800)	2.2
Adenovirus	120 (49–260)	2.2	970 (410–2,100)	2.4
Bacteria	240 (160–320)	4.4	1,700 (1,100–2,300)	4.1
Clostridium difficile	96 (61–150)	1.8	960 (590–1,500)	2.3
Salmonella spp.	69 (35–120)	1.3	250 (130–440)	0.6
Shigella spp.	41 (17–85)	0.8	200 (86–410)	0.5
Campylobacter spp.	31 (9–76)	0.6	240 (71–590)	0.6
Parasite	60 (29–110)	1.1	420 (200–790)	1.0
Giardia spp.	43 (18–86)	0.8	350 (150–720)	0.9
Cryptosporidium spp.	17 (4–48)	0.3	68 (14–190)	0.2
Any pathogen	1,600 (1,300–2,400)	29.6	13,000 (10,000–20,000)	31.7
Unidentified	3,800 (3,200–4,600)	70.4	28,000 (23,000–34,000)	68.3
Total	5,400 (4,400–6,700)	100	41,000 (38,000–44,000)	100

*Incidence is per 100,000 person-years. Acute gastroenteritis is defined as diarrhea (≥3 loose stools in a 24-h period) beginning within the past month and in the absence of a chronic disease for which diarrhea is a major sign. CI, credible interval.
†Outpatient incidence calculated from prevalence in fecal specimens sampled, age-adjusted fecal specimen submission rates among health care seekers, number of fecal specimens submitted to the Kaiser laboratory annually (1,825), and number of Kaiser memberships in Georgia (280,000).
‡Community incidence calculated from prevalence in fecal specimens sampled, age-adjusted medical care seeking and fecal specimen submission rates, number of fecal specimens submitted to the Kaiser laboratory annually (1,825) and total Kaiser memberships in Georgia (280,000).
§90% CI calculated from the 5th and 95th percentiles of 100,000 simulations, assuming a β distribution of variables.

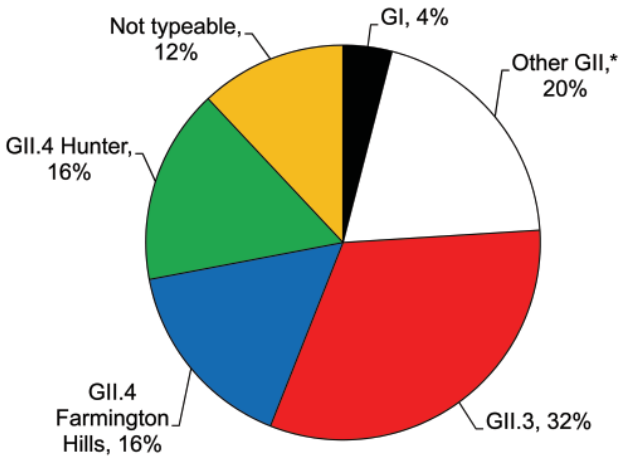

Figure 3. Distribution of norovirus genotypes among 25 outpatients with acute gastroenteritis, Kaiser Foundation Health Plan of Georgia, Inc., USA, March 15, 2004–March 13, 2005. Genogroup II (GII) was more prevalent than GI. *Includes GII.2 (2 specimens), GII.14 (2 specimens), and GII.17 (1 specimen).

genotype in vaccine development efforts.

Our findings are consistent with those of studies conducted in other industrialized countries, which demonstrated the relative role of viral pathogens in causing AGE (*19*–*23*). A recent systematic literature review that included 13 etiologic studies of community and outpatient cases of sporadic diarrhea, most of which focused on children, determined that norovirus was responsible for 12% (range 5%–36%) of AGE cases (*17*). In a community-based study in Germany, the incidence of AGE requiring medical consultation attributable to norovirus was 626 cases/100,000 person-years (*19*). In England, the incidence of general practice consultations for norovirus-associated infectious intestinal disease was 540 cases/100,000 person-years (*24*). The estimated incidence of norovirus among outpatients in our study (640 cases/100,000 person-years) supports the findings of the studies in England and Germany. A similar study in the Netherlands reported a considerably lower incidence of gastroenteritis in general practices (797 cases/100,000 person-years), in which 5% (40 cases/100,000 person-years) were caused by norovirus (*20*). However, standardized gastroenteritis incidence reported in the Dutch community of 28,300 cases/100,000 person-years is more similar to that estimated in our study (41,000 cases/100,000 person-years) (*25*). Dutch guidelines for general practitioners, which recommend that cases of uncomplicated AGE be handled by telephone consultation (http://nhg.artsennet.nl), may explain the apparent difference in health care utilization practices.

Prior US estimates of the incidence of norovirus have been based on a bottom-up approach of using overall incidence of AGE from population surveys and estimating the proportion attributable to norovirus on the basis of data from other countries, given the lack of laboratory-based data available at the time (*3*). This approach yielded an estimate of ≈21 million cases of norovirus illness annually, corresponding to a community incidence similar to that estimated in our study (6,974 cases/100,000 person-years vs. 6,500 cases/100,000 person-years). Similarly generated bottom-up estimates for astrovirus, rotavirus, and sapovirus (each 1,024 cases/100,000 person-years) were likewise within 90% CIs of our community incidence estimates (*3*).

Although markedly different approaches were used, each subject to considerable uncertainty, concordance of the respective incidence estimates generated is reassuring. However, given the inclusion of only specimens submitted for routine bacterial diagnostics, our study likely underestimated the true incidence of norovirus disease in the community. Furthermore, use of health care utilization multipliers based primarily on diarrhea may underestimate the incidence of pathogens that can cause other AGE signs (e.g., vomiting) in the absence of diarrhea, as has been reported for norovirus (*26*). Previous studies have demonstrated that seeking medical care is influenced by disease severity and social factors (*27*,*28*), which leads to a smaller proportion of viral AGE patients in the community who seek medical care, compared with bacterial and parasitic AGE (*22*). Identifying laboratory-confirmed cases of norovirus infection and extrapolating community incidence by using pathogen-specific health care utilization multipliers in a top-down approach may be preferable when such data are available and would likely yield more reliable estimates.

The primary limitation of this study results from use of routinely collected specimens, as opposed to systematic, active patient recruitment. The study specimens may have therefore been obtained from patients with noninfectious or chronic diseases, which may have contributed to the low detection rate for pathogens in this study. Delays in collection of specimens may have also contributed to the low rates of pathogen detection. Routinely collected samples also overrepresent some age groups, as demonstrated by differential fecal submission rates (Table 1), which may not reflect age groups at greatest risk for infection by specific pathogens or risk for the overall population.

We have adjusted for this sampling artifact by development of pathogen-specific, age-adjusted health care utilization multipliers. These multipliers resulted in the observed increase from pathogen prevalence in specimens (e.g., 4.4% for norovirus) to pathogen prevalence among estimated community AGE cases (e.g., 16% for norovirus). In addition to age, pathogen-specific health care utilization multipliers also accounted for duration of illness and yielded different pathogen distributions in community and outpatient settings. These results are consistent with those

of previous studies (*20*,*22*,*25*). In contrast to a previous study that estimated US incidence of AGE (*3*), our study did not adjust for differential health care utilization rates based on illness severity (i.e., bloody vs. nonbloody diarrhea) to maintain adequate power to enable the adjustment for age group. Nonetheless, there was insufficient sample size within each age group to generate age group–specific disease incidence estimates.

Attributing causality of AGE to norovirus infection is complicated by the fact that asymptomatic infections may occur; healthy persons used in previous studies as controls have shown background rates of infection of 1%–16% (*17*). However, low prevalence (1.1%) of norovirus in specimens received >1 week after outpatient visit and low number of mixed infections identified in our study suggest that norovirus was likely the etiologic agent when detected. Incidence estimates were not adjusted for test sensitivity because the molecular methods used for viral diagnostics generally show extremely high sensitivities if appropriate and timely specimens are tested. Prevalence and incidence of parasites and *C. difficile* may be underestimated given that not all specimens were tested for these pathogens and indications for clinicians requesting specific tests are unknown and likely varied. Finally, given that the study population included only Georgia residents and health maintenance organization members, which tends toward younger age and higher socioeconomic status, the results may not be generalizable to the overall US population. Restriction of FoodNet data to only respondents in Georgia who had health insurance was evaluated for comparison but did not differ. Therefore, all respondents were ultimately included to enable adequate power for age stratification.

Development of sensitive clinical assays for identification of viral agents of AGE, such as norovirus, and more widespread use of such assays may help close the diagnostic gap on sporadic AGE cases and guide more appropriate case management. The demonstrated predominance of viruses among medically attended AGE cases should help prevent the unnecessary use of antimicrobial drugs and spur development of novel interventions specific for the unique transmission pathways of viruses. Compared with bacterial AGE etiologies, many of which result from foodborne transmission from infected animal sources, viral AGE pathogens originate in human reservoirs and usually involve direct or indirect person-to-person spread. Although occasionally implicated in outbreaks (*29*,*30*), the role of foods contaminated during processing in the overall norovirus disease incidence remains largely unknown. Further assessment of the incidence of enteric viruses, including hospital-based studies of risk factors for severe disease and attribution to specific transmission pathways, are needed to improve control measures and assess future potential of vaccines.

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Novel Surveillance Network for Norovirus Gastroenteritis Outbreaks, United States[1]

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CaliciNet, the outbreak surveillance network for noroviruses in the United States, was launched in March 2009. As of January 2011, twenty state and local health laboratories had been certified to submit norovirus sequences and epidemiologic outbreak data to CaliciNet. During the network's first year, 552 outbreaks were submitted to CaliciNet, of which 78 (14%) were associated with foodborne transmission. A total of 395 (72%) outbreaks were typed as GII.4, of which 298 (75%) belonged to a new variant, GII.4 New Orleans, which first emerged in October 2009. Analysis of the complete capsid and P2 region sequences confirmed that GII.4 New Orleans is distinct from previous GII.4 variants, including GII.4 Minerva (2006b).

Noroviruses are the primary cause of epidemic viral gastroenteritis and the leading cause of foodborne outbreaks in the United States (*1*–*3*). Although the course of disease is in most cases self-limiting, young, elderly, and immunocompromised persons are at risk for complications caused by severe vomiting and diarrhea (*4*–*8*). In addition to the clinical impact of norovirus disease, the economic effects in lost wages, time, and intervention procedures (e.g., clean-up costs and recalls) can be significant (*9*–*11*). Although norovirus outbreaks occur year-round, they are more common during the winter months (*12*–*14*).

Noroviruses are genetically classified into 5 genogroups, GI–GV, with GI and GII strains responsible for most human disease (*2*,*15*). GII viruses can be further divided into at least 19 genotypes, of which GII.4 is responsible for >85% of outbreaks (*14*,*16*), although other genotypes and viruses continue to circulate and cause sporadic disease in children (*17*–*19*). Over the past 15 years, new GII.4 variants have been identified; several have been associated with a global increase in the number of outbreaks (*15*). The last pandemic GII.4 variant, GII.4 2006b or GII.4 Minerva, was identified in late 2005/early 2006 and has been the predominant outbreak strain in the United States since then. The successive displacement of GII.4 variants suggests that population immunity is driving the evolution of GII.4 viruses (*20*,*21*), and the emergence of a new variant will cause an increase in the number of outbreaks in an immunologically naive population.

It is not fully understood why some GII.4 variants become pandemic whereas others do not. The combination of novel antigenic sites in protruding regions of the capsid (centered around amino acids 295 and 396) and the change or expansion of a susceptible population may be responsible for the emergence of pandemic variants (*20*,*22*). The latter theory has been supported by the discovery that different norovirus strains may have different histo–blood group antigen (HBGA) binding patterns and that nonsecretors are not susceptible to infection with certain genotypes or variants (*23*). Most mutations between genotypes and variants occur in the P2 region of the major capsid viral protein (VP), VP1, which contains the HBGA binding sites.

Since 2008, all 50 states have had the laboratory capacity for norovirus testing; the Centers for Disease Control and Prevention (CDC) National Calicivirus Laboratory (NCL) provides laboratory support to states that do not have in-

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[1]Additional members of the CaliciNet participating laboratories who contributed data are listed at the end of this article.

house capacity for norovirus strain typing. Recent studies on the molecular epidemiology of norovirus in the US have been based on specimens from a subset of outbreaks that were submitted to CDC (*13*,*24*,*25*). To enhance and harmonize norovirus outbreak surveillance, CDC and its state partners have developed a national norovirus outbreak surveillance network, CaliciNet. CaliciNet was developed to improve standardized typing of norovirus outbreaks, assist in linking geographically different clusters of norovirus illness, allow rapid classification and identification of new norovirus strains, and establish a comprehensive strain surveillance network in the United States. In this article, we describe the CaliciNet network and report first-year results, including the identification of a new GII.4 norovirus variant.

Materials and Methods

CaliciNet

CaliciNet is a novel electronic laboratory surveillance network of local and state public health laboratories in the United States, coordinated by CDC. CaliciNet participants perform molecular typing of norovirus strains by using standardized laboratory protocols for reverse transcription PCR (RT-PCR) followed by DNA sequence analysis of the amplicons. A customized CaliciNet database developed in Bionumerics version 5.1 (Applied Maths, Austin, TX, USA) includes norovirus sequence and basic epidemiologic information (Table 1), which are submitted electronically via a secure connection to the CaliciNet server at CDC. Both epidemiologic and sequence data can then be used to help link multistate outbreaks to a common source (e.g., contaminated food). To ensure high-quality data entry, submissions to the CaliciNet server are performed by certified laboratory personnel of the participating state or local health laboratories, and final quality assurance/quality control is performed at CDC.

CaliciNet certification for participants is a 2-step process that involves evaluation of data entry and analysis of sequences and a laboratory panel test. Each laboratory must pass an annual proficiency test. The laboratory certification and proficiency test consists of analyzing a panel of fecal samples by real-time RT-PCR and conventional RT-PCR analysis followed by bidirectional sequencing as described below. Certified participants are then authorized to upload norovirus outbreak data consisting of ≥2 samples per outbreak to the national CaliciNet database (Table 1). GII.4 sequences with >2% and 3% difference in region C or D, respectively, and >10% difference with all other noroviruses are further analyzed at CDC by amplification of the VP1 or P2 region.

Table 1. Epidemiologic data fields required for upload to CaliciNet*

Required CaliciNet fields	Description
LabOBNumber	Year, outbreak, and number
Outbreak date	Begin date of outbreak
Outbreak city	City where outbreak occurred
Outbreak state	State where outbreak occurred
Outbreak setting	Select outbreak setting†
Outbreak country	Country where outbreak occurred
Transmission	Foodborne, person-to-person, waterborne
Conventional RT-PCR	Results of RT-PCR‡
Sequence experiment	Sequence of region D§

*RT-PCR, reverse transcription PCR.
†Child care center, cruise ships, hospital, long-term care facility, party or event, restaurant, school and community, correctional center.
‡Region C or D.
§Region D is the preferred sequence, but region C is also accepted.

Outbreaks

All outbreaks submitted to CaliciNet and the NCL from October 2009 through March 2010 were genotyped by region D analysis (*26*). To verify GII.4 New Orleans variants, a subset of outbreaks from CaliciNet participating laboratories and 2 specimens from each outbreak received at the NCL from October 2009 through May 2010 were analyzed by using the P2 region as described below.

Viral RNA Extraction

Viral RNA was extracted from clarified 10% fecal suspensions in phosphate-buffered saline with the MagMax-96 Viral RNA Isolation Kit (Ambion, Foster City, CA, USA) on an automated KingFisher magnetic particle processor (Thermo Fisher Scientific, Pittsburgh, PA, USA) according to the manufacturer's instructions and eluted into 100 μL of elution buffer (10 mmol/L Tris pH 8.0 and 1 mmol/L EDTA). Extracted RNA was stored at –80°C until further use.

Real-time RT-PCR

Viral RNA was tested for GI and GII noroviruses in a duplex format by using the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) on a 7500 Realtime PCR platform (Applied Biosystems). The final reaction mix of 25 μL consisted of 400 nmol/L of each oligonucleotide primer, Cog1F, Cog1R, Cog2F, and Cog2R, and 200 nmol/L of each TaqMan Probe Ring 2 (*27*) and Ring 1C (*28*) (Table 2). Cycling conditions included reverse transcription for 10 min at 45°C and denaturation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

Region D RT-PCR

The QIAGEN One-Step RT-PCR Kit (QIAGEN, Valencia, CA, USA) was used for region D amplification in a 25-μL reaction volume. RNAse Inhibitor (Applied

Table 2. Oligonucleotide primers and probes used for detection and genotype identification of norovirus strains submitted to CaliciNet*

Primer or probe name	RT-PCR target	Sequence, 5′ → 3′	Reference
TVN-L1	ORF2–ORF3	GGG TGT GTT GTG GTG TTG T_{26}VN	(*29*)
L1	ORF2–ORF3	GGG TGT GTT GTG GTG TTG	This study
EVP2F	P2 (GII.4 specific)	GTR CCR CCH ACA GTT GAR TCA	This study
EVP2R	P2 (GII.4 specific)	CCG GGC ATA GTR GAY CTR AAG AA	This study
Cap D1	Region D GII	TGT CTR STC CCC CAG GAA TG	(*26*)
Cap C	Region D GII	CCT TYC CAK WTC CCA YGG	(*26*)
Cap D3	Region D GII	TGY CTY ITI CCH CAR GAA TGG	(*26*)
Cog 2F	ORF1–ORF2 junction (GII)	CAR GAR BCN ATG TTY AGR TGG ATG AG	(*27*)
Cog 2R	ORF1–ORF2 junction (GII)	TCG ACG CCA TCT TCA TTC ACA	(*27*)
Ring 2	ORF1–ORF2 junction (GII)	Cy5-TGG GAG GGC GAT CGC AAT CT-BHQ	(*27*)
Ring 1C	ORF1–ORF2 junction (GI)	FAM-AGA TYG CGI TCI CCT GTC CA-BHQ	(*28*)
Cog 1F	ORF1–ORF2 junction (GI)	CGY TGG ATG CGI TTY CAT GA	(*27*)
Cog 1R	ORF1–ORF2 junction (GI)	CTT AGA CGC CAT CAT CAT TYA C	(*27*)

*RT-PCR, reverse transcription PCR; ORF, open reading frame.

Biosystems) was added to a final concentration of 15–20 units/reaction. Oligonucleotide primers CapD1, CapD3, and CapC were added to a final concentration of 1 μmol/L each (Table 2). RT-PCR conditions included reverse transcription at 42°C for 30 min and denaturation at 95°C for 15 min, followed by 40 cycles of 30 s at 94°C, 30 s at 40°C, and 30 s at 72°C. A final elongation step was run for 10 min at 72°C.

P2 Region Amplification

The P2 region was amplified by using the SuperScript III One-Step RT-PCR with Platinum Taq High Fidelity Kit (Invitrogen, Carlsbad, CA, USA). The final reaction volume of 25 μL consisted of 4 μmol/L of EVP2F and EVP2R (Table 2). RT-PCR conditions included reverse transcription at 55°C for 30 min and denaturation at 94°C for 2 min, followed by 40 cycles of PCR at 94°C for 15 s, 55°C for 30 s, 68°C for 1 min, and a final extension step of 68°C for 5 min.

Amplification and Cloning of GII.4 New Orleans

Novel GII.4 New Orleans sequences were identified by region D sequence analysis and further analyzed by amplification of complete open reading frame 2. Extracted RNA from fecal samples underwent cDNA synthesis with a TVN-L1 primer (*29*) (Table 2) for 60 min at 50°C by using the Superscript III cDNA synthesis kit (Invitrogen). The reaction mixture was purified by using the DNA Clean and Concentrator-5 (Zymo Research, Orange, CA, USA). The cDNA was amplified by using oligonucleotides (0.5 μmol/L each) L1 and Cog2F (Table 2), using the Phusion PCR Kit with the addition of 3% dimethyl sulfoxide (Finnzymes, Woburn, MA, USA). PCR conditions included denaturation at 98°C for 30 s followed by 40 cycles of 98°C for 10 s, 48°C for 30 s, and 72°C for 1.5 min. A final elongation step was run at 72°C for 10 min.

PCR products of ≈2.5 kb were gel purified and cloned by using a TOPO-TA Cloning Kit (Invitrogen). Five clones of each strain were fully sequenced bidirectionally and their respective consensus sequences were submitted to GenBank. The accession no. for GII.4 New Orleans is GU445325.

DNA Sequencing

All amplicons were purified with the QIAquick Gel Extraction or PCR Purification Kits (QIAGEN) and sequenced by using the BigDye Terminator Kit version 1.1 (Applied Biosystems). Sequence reactions were cleaned up by using the BigDye Xterminator Kit (Applied Biosystems) and analyzed on a 3130XL Automated Sequencer (Applied Biosystems).

Phylogenetic Analysis

VP1 or P2 sequences were aligned by using MEGA4 software (*30*). Maximum-likelihood phylogenetic analysis of VP1 amino acids were run in PhyML version 3.0 (www.atgc-montpellier.fr/phyml/binaries.php) by using the LG amino acids replacement matrix (*31*). The initial tree was the best of 5 random trees, and branches were supported by 100 bootstrap replicates. Branches with bootstrap support <60 were collapsed. The P2 sequence of representative GII.4 variants (GII.4 NOLA, GII.4 Osaka, GII.4 Yerseke [2006a], GII.4 Minerva [2006b], and GII.4 New Orleans) were also included in the analysis. Maximum-likelihood scores were generated for all models available in jModelTest by using the Akaike information criterion or the Bayesian information criterion (*32*) to select the best nucleotide replacement matrix for phylogenetic analysis. A transitional model with rate variation between sites, TIM3+G, had the best maximum-likelihood scores. The custom model was then run in PhyML version 3.0 with the approximate-likelihood ratio test calculated for branch support (*33*).

Results

As of February 2011, public health laboratories in 20 states have been CaliciNet certified (Figure 1); these

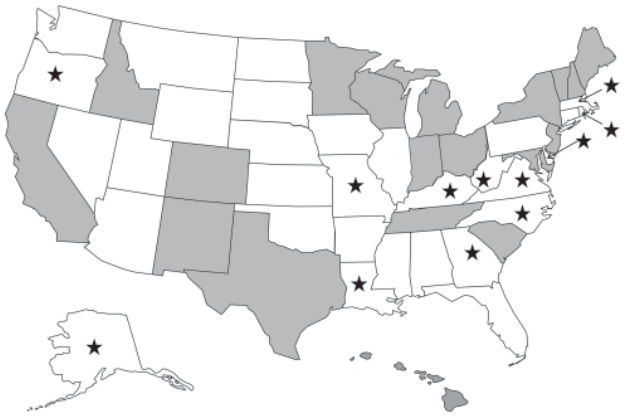

Figure 1. CaliciNet participating states (gray), nonparticipating states (white), and 12 states that submitted norovirus-positive specimens to Centers for Disease Control and Prevention for P2 analysis (stars).

states represent 53% of the US population (*34*). From the inception of CaliciNet in March 2009 through May 2010, 552 outbreaks were uploaded to the national CaliciNet database. Foodborne and person-to-person transmission were reported for 78 (14%) and 340 (62%) of the outbreaks, respectively, whereas the transmission route for the 134 remaining outbreaks was not reported.

GII.4 viruses caused 395 (73%) of the 552 outbreaks. The number of outbreaks increased from 4 in October 2009 to a peak of 110 in January 2010 and then decreased to 31 in May 2010 (Figure 2). A novel GII.4 variant (GII.4 New Orleans) was first identified in October 2009 and caused 56% of the outbreaks in November, compared with 11% caused by GII.4 Minerva. This novel variant remained the dominant strain in December 2009 and January 2010, causing 48% and 65% of the outbreaks, respectively. In February 2010, the number of outbreaks decreased to 84, but the proportion of GII.4 New Orleans outbreaks remained high (60%). In March 2010, GII.4 New Orleans accounted for 75% of the outbreaks. In April, total outbreaks decreased to 43, with 67% caused by GII.4 New Orleans and 7% by GII.4 Minerva; in May, total outbreaks were 31, with 52% caused by GII.4 New Orleans and 13% caused by GII.4 Minerva.

Because region C and region D analyses are not able to differentiate genetically closely related GII.4 variants, we further analyzed the P2 region of 20 GII.4 New Orleans region D–positive strains from 4 CaliciNet states and sequenced the complete VP1 gene from a representative outbreak (Figure 3). Compared with recent GII.4 variants, GII.4 New Orleans had several amino acid substitutions, which were located near protruding regions (aa 294 and 396) and HBGA interaction sites (aa 339–341) (Figure 4).

In addition to the 20 strains from the CaliciNet-certified laboratories, the P2 region from 75 GII.4 outbreaks submitted by 12 non-CaliciNet states was sequenced (Figure 1). Of these, 72 (96%) outbreaks had P2 sequences with ≤2% nt difference compared with the prototype GII.4 New Orleans strain. Sequences from 3 outbreaks were closely related to a GII.4 variant first detected in Australia in 2008 (Figure 3).

Discussion

The launch of CaliciNet in March 2009 was a milestone in the surveillance of norovirus gastroenteritis in the United States. CaliciNet enables standardized genotyping of norovirus strains, comparison of sequences from outbreaks that have a common source, and identification of new strains in real-time. The usefulness of CaliciNet was demonstrated during the winter of 2009–2010 when the emergence of a new GII.4 variant (GII.4 New Orleans) was identified. This new variant caused 60% of the outbreaks and replaced GII.4 Minerva as the predominant GII.4 strain. In addition, a new GII.12 strain caused 17% of the outbreaks during the winter of 2009–2010 (*35*).

GII.4 New Orleans was first detected in October 2009, and the proportion of all norovirus outbreaks it caused increased gradually to >50% during the winter months. Compared with known GII.4 viruses, GII.4 New Orleans had several changes in key amino acids in the P2 region of VP1 and around the sites that have been shown to be important in HBGA binding (*20*). Because most GII.4 variants that have been identified since 2004 are conserved at these sites, it has been speculated that mutations that change the HBGA binding pattern would decrease the fitness of the virus (*36*). During the last transitional period

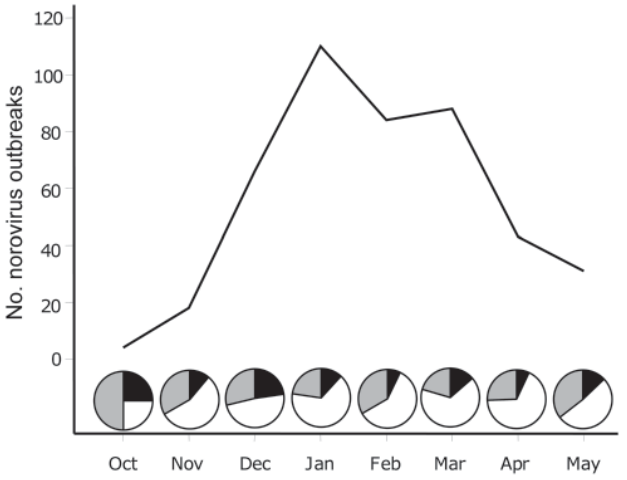

Figure 2. Gastroenteritis outbreak data submitted to CaliciNet from October 2009 through May 2010. Pie graphs represent the proportion of outbreaks reported as norovirus GII.4 New Orleans (white), norovirus GII.4 Minerva (black), and all other norovirus genotypes (gray).

when GII.4 Minerva (GII.4 2006b) was identified, another GII.4 variant was co-circulating (*21*,*37*).

CaliciNet uses the same software as the US bacterial enteric pathogen surveillance network (PulseNet) (*38*), but it is customized with plug-ins to add CaliciNet-specific functionality. CaliciNet uses sequence data, whereas PulseNet is based on pulsed-field gel electrophoresis restriction digestion patterns of bacterial enteric pathogens. Current typing regions of CaliciNet target small regions of the norovirus genome, which makes it difficult to discern closely related norovirus strains, although the implications to human health may be significant. Our data and data from other studies (*39*) demonstrated that P2 region analysis enables more sensitive identification of new GII.4 variant strains compared with currently used CaliciNet regions. Use of these analyses would increase the sensitivity of outbreak surveillance to track strains that are part of a single outbreak and likely to have a common source. Hence, P2 is under consideration to be included in CaliciNet.

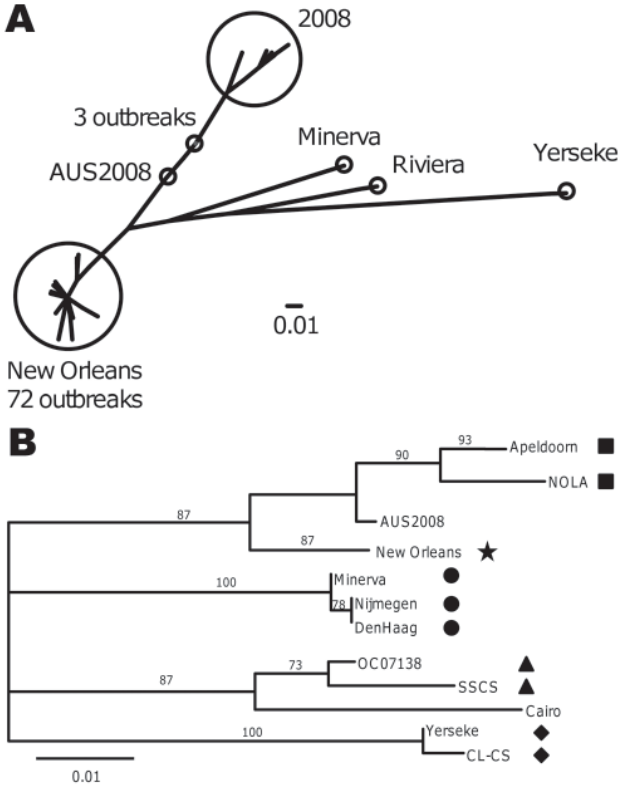

Figure 3. Unrooted phylogenetic tree of the P2 region from all norovirus GII.4 New Orleans strains submitted to CaliciNet and identified by region D analysis from October 2009 through May 2010 (A) and of the complete major capsid protein viral protein 1 of selected norovirus GII.4 variants (B). Numbers on branches represent bootstrap support out of 100. Symbols represent GII.4 variant types (nomenclature proposed by NoroNet in parentheses): black squares, GII.4 NOLA variant; star, GII.4 New Orleans (2010) variant; circles, GII.4 Minerva (2006b) variant; triangles, GII.4 Riviera (2007) variant; and diamonds, Yerseke (2006a) variant. AUS2008 (GenBank accession no. GQ845367) and Cairo (accession no. EU876888) are unidentified variant types. GenBank accession numbers of GII.4 sequences included in the analysis: Apeldoorn (AB445395), NOLA (GU270580), New Orleans (GU445325), Minerva (EU078417), Nijmegen (EF126966), DenHaag (EF126965), OC07138 (AB434770), SSCS (FJ411171), Yerseke (EF126963), and CL-CS (EU078419). Scale bars represent number of nucleotide (A) or amino acid (B) substitutions per site. P2 sequences can be provided upon request.

Like CaliciNet, the Foodborne Viruses in Europe network (FBVE) uses a central database to which users can submit norovirus sequences (*40*). Compared with the FBVE network, CaliciNet focuses primarily on noroviruses, is not web-based, and is based on a secured network connection to CaliciNet servers at CDC where the states log on as clients, enabling them to upload, view, and query outbreak data submitted by other states. CaliciNet also organizes training workshops and sends standardized protocols and annual proficiency panels to its members. The benefit of the FBVE network is that it can be more easily expanded to include laboratories outside its network, whereas to date CaliciNet allows only participants from state and local health laboratories in the US to participate.

The success of CaliciNet in linking multistate outbreaks to a common source (e.g., contaminated food) will depend on joint efforts of state and local epidemiologists to rapidly identify the likely common source and on CaliciNet laboratories for the timely upload of outbreak sequences to the national CaliciNet database. Although CaliciNet has selected region D as its preferred sequence region, a region C and soon a P2 region sequence database will be maintained to enable exchange of information with other norovirus surveillance networks. Because the region D assay targets a genetically highly heterogeneous region of VP1, the performance of this assay will be closely monitored over time, and necessary changes will be implemented to improve assay sensitivity and specificity. Future CaliciNet expansion will include other gastroenteritis viruses, such as sapovirus and astrovirus, as well as add capability for CaliciNet members to submit fecal samples from patients involved in norovirus-negative outbreaks to CDC for further testing, including novel pathogen discovery sequencing technologies (*18*).

CaliciNet launched in March 2009 and helped in the rapid identification of a new GII.4 variant. P2 analysis confirmed that this variant was divergent from previous GII.4 viruses. The widespread presence of GII.4 New Orleans across the US coupled with the decreasing prevalence of the GII.4 Minerva variant, which has been the major cause of outbreaks during 2006–2009, suggests gradual strain displacement. Data from the 2009–2010 winter season showed the importance of CaliciNet and its future potential for norovirus surveillance in the US. To enhance norovirus surveillance globally, CaliciNet will collaborate with other norovirus surveillance networks,

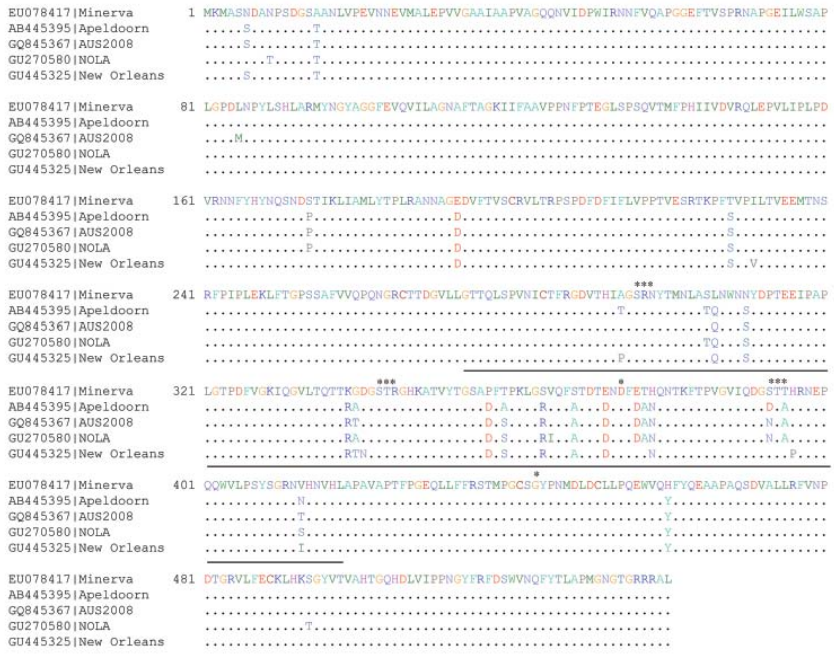

Figure 4. Amino acid substitutions in the major capsid viral protein 1 of norovirus New Orleans GII.4 strains compared with recent GII.4 variants. The P2 hypervariable region is underlined. *Protruding regions and histo–blood group antigen interacting sites. Dots indicate sequence identity.

such as ViroNet in Canada and the global norovirus network, NoroNet (*15*), to better predict or determine norovirus epidemiologic or outbreak trends. International surveillance of viral foodborne outbreaks is essential because of the increasing globalization of the food industry.

Additional members of the Calicivirus network who contributed data (state represented): Chao-Yang Pan, Tasha Padilla (CA); Justin Nucci, Mary-Kate Cichon (CO); Gregory Hovan (DE); Precilia Calimlim, Cheryl-Lynn Daquip (HI); Edward Simpson (IN); Amanda Bruesch, Kari Getz (ID); Jonathan Johnston, Julie Haendiges (MD); Heather Grieser, John Martha (ME); Laura Mosher (MI); Elizabeth Cebelinski (MN); Alisha M. Nadeau, Fengxiang Gao (NH); Ondrea Shone (NJ); Frederick Gentry (NM); Gino Battaglioli (NY), Eric Brandt, Rebekah Carmen, Steven York (OH); Andrea Maloney (SC); Amy M. Woron, Christina Moore (TN); Chun Wang (TX); Valarie Devlin (VT); Tim Davis, Tonya Danz, and Jose Navidad (WI).

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Spread of Measles Virus D4-Hamburg, Europe, 2008–2011

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A new strain of measles virus, D4-Hamburg, was imported from London to Hamburg in December 2008 and subsequently spread to Bulgaria, where an outbreak of >24,300 cases was observed. We analyzed spread of the virus to demonstrate the importance of addressing hard-to-reach communities within the World Health Organization European Region regarding access to medical care and vaccination campaigns. The D4-Hamburg strain appeared during 2009–2011 in Poland, Ireland, Northern Ireland, Austria, Greece, Romania, Turkey, Macedonia, Serbia, Switzerland, and Belgium and was repeatedly reimported to Germany. The strain was present in Europe for >27 months and led to >25,000 cases in 12 countries. Spread of the virus was prevalently but not exclusively associated with travel by persons in the Roma ethnic group; because this travel extends beyond the borders of any European country, measures to prevent the spread of measles should be implemented by the region as a whole.

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The 53 member states of the World Health Organization (WHO) European Region (EUR) have set a goal to eliminate measles and rubella virus transmission by 2015 in Europe (*1*). Elimination targets include 95% vaccination coverage with 2 doses of measles virus–containing vaccine (MVCV), an incidence of <1 measles case per million population, 80% of outbreaks associated with <10 cases, and transmission of indigenous or imported measles virus for no longer than 12 months in a defined region (*2*). Thus, monitoring transmission chains of measles virus is an indispensable tool to assess elimination progress, although the specific boundaries of the region have not yet been defined for the WHO EUR.

To comply with the goal of eliminating measles virus, Germany implemented a national intervention program against measles, mumps, and rubella (MMR) in 1999 (*3*). Since then, measles incidence in Germany has declined. Molecular surveillance showed that endemic genotypes C2 (MVi/Kempten.DEU/23.00) and D6 (MVi/Berlin.DEU/47.00) (*4*) were replaced rapidly by genotype D7 (MVi/Mainz.DEU/06.00), which circulated until the beginning of 2003 (*5*). Imported measles virus of genotypes B3, D4, D5, D6, D8, D9, and H1 appeared in Germany from 2005 onward. In 2009 and 2010, most cases were linked to measles virus of genotype D4, of which several distinct subvariants were detected.

Elimination targets have not yet been met in Germany. Vaccination coverage in Germany, routinely assessed in children 5–6 years of age during an examination before school entry, is still below the required 95% for the second dose of MVCV. Recent outbreaks showed an immunization gap in adolescents and young adults (*6*). Consequently, outbreaks still occur in Germany every year, although recently they have been more limited in number of cases, length of time, and extent of national transmission (*7*,*8*). A total of 915 measles cases were reported in 2008, 571 in 2009, and 780 in 2010; incidence was 7–10 cases/1 million population (www3.rki.de/SurvStat/QueryFormaspx).

The reasons for Germany's malperformance are complex. Measles virus vaccination is not mandatory, and some groups within the German population do not comply with official vaccination recommendations (*9*) because of philosophical or religious beliefs or fear of adverse effects (*10*). As in other countries in Europe, strategies to address hard-to-reach populations and improve access to medical care, preventive measures, and vaccination campaigns have not yet been developed. In this article, we describe exportation of a measles D4 variant from Germany and its subsequent circulation in Europe.

Material and Methods

Serum, urine, and oral fluid or throat swabs were sent to the WHO Regional Reference Laboratory in Berlin according to the procedures outlined in the WHO LabNet manual (*11*). Immunoglobulin (Ig) M and IgG serologic testing was performed as described previously (*12*). Sequencing was performed according to WHO recommendations (*13*). Sequences were aligned by ClustalW (*14*) and further analyzed by SeqScape 2.5 and MEGA4 DNA analysis software (*15*). Phylogenetic trees were constructed by using the neighbor-joining method. Genotype assignment was performed by phylogenetic comparison with the measles virus reference strains as designated by WHO (*16*). The obtained sequence data, the genotype, the official WHO measles virus sequence name, and relevant epidemiologic data were submitted to the WHO measles sequence database, MeaNS (www.hpa-bioinformatics.org.uk/Measles/Public/Web_Front/main.php) or to GenBank.

Results

Outbreak Hamburg/Lower Saxony

At the end of December 2008, a 27-year-old man residing in the southern part of Hamburg was hospitalized with measles. Five other adults contracted the virus while waiting for treatment in the emergency room. The resultant outbreak first affected a southern quarter (Hamburg-Harburg) of the city of Hamburg. It spread subsequently into a Roma group residing in central Hamburg and to the neighboring federal state of Lower Saxony. The probable index case-patient was identified as a 19-year-old Roma man living in Hamburg. He had stayed in London from May through November 2008 and became ill at the beginning of December with measles-like symptoms. The outbreak in Hamburg lasted from the end of December 2008 through June 2009 (*17*); a 4-week peak occurred in February and comprised 216 cases in the city of Hamburg.

For 69% of the reported cases, the diagnosis was laboratory confirmed. Complications leading to hospitalization were seen in 40% of the patients (pneumonia or otitis media). The affected age group ranged from a 1-day-old newborn to a 54-year-old adult (median age 13.5 years). Most frequently affected were young children, then young adults. Data with respect to vaccination status were available for 196/216 case-patients. No vaccination was documented for 167 persons (85%); 28 had not yet reached the age of vaccination (>11 months). Twenty-six (13%) previously unvaccinated persons had received MVCV after being exposed to measles virus. Three patients had received 2 doses of MVCV. Several of the 216 cases occurred in the Roma ethnic community.

Seventy-two cases of measles were reported during the same time in Lower Saxony. Fifty-three cases were clearly related to the outbreak in Hamburg. The first cases in Lower Saxony were reported during week 2 (January) and the last case occurred during week 17 (April) of 2009; the peak of the outbreak occurred during week 14 (April). The connection to the Hamburg outbreak was suggested either by the presence of patients in the emergency department of a Hamburg hospital at the time in question, an epidemiologic link, or the result of the sequencing. Many cases occurred in the Roma ethnic group.

Case-patients ranged from 7 months to 42 years of age (median age 15 years); adolescents and younger adults were the main affected age group. Forty-two (79%) case-patients had received no measles vaccination, 10 (19%) had received 1 dose of MVCV, and 1 (2%) had been vaccinated 2 times. In the latter case, primary infection with measles virus was confirmed by PCR and IgM, but IgG was not detected. Five patients received vaccination after exposure, which did not prevent clinical symptoms. Overall, 47 (89%) of measles cases were confirmed by laboratory testing. Eleven (21%) case-patients were admitted to a hospital with complications (e.g., pneumonia, otitis media).

Specimens from 12 cases in Hamburg and 18 cases in Lower Saxony were genotyped. All case-patients were infected with the same D4 measles virus variant (MVs/Hamburg.DEU/03.09/[D4], MVs/Harburg.DEU/06.09/[D4], and MVs/Wildeshausen.DEU/21.09/[D4]), none of which were published in the GenBank and MeaNS databases.

D4-Hamburg showed 1 mismatch to other D4 measles virus sequences published in GenBank, MVs/Raichur.IND/38.06/[D4]), MVi/Kolar.IND/03.07/1[D4], and MVs/Enfield.GBR/14.07/[D4]; the latter is a strain endemic to the United Kingdom and responsible for the large outbreak there during 2007–2009 (Figure 1). Sequences identical to the Hamburg strain were subsequently identified in London (MVs/London.GBR/5.09/[D4]).

Transmission to Bulgaria

In April 2009, after an absence of 7 years, measles cases began occurring in Bulgaria (*18*). Sequencing of 3 specimens from the National Measles Laboratory in Sofia identified MVs/Shumen.BGR/15.09/1-3(D4), identical to D4-Hamburg. The index case-patient in the Bulgaria outbreak was a Roma who worked as a builder in Hamburg and who had visited Razgrad district in northeastern Bulgaria. The outbreak in Bulgaria proceeded from the northeast to the southwest of the country; in 2009, a total of 2,249 cases were reported. A marked increase in case numbers was reported at the end of 2009 and in the beginning of 2010 (*19*). From the start of the measles epidemic in April 2009 through the end of week 10 (mid-March) of 2011, a total of 24,379 cases were reported; 24 were fatal (*20*).

The WHO Regional Reference Laboratory in Berlin received 20 specimens at regular intervals from hospitalized persons. Genotype information was obtained for 19/20 case-patients (online Appendix Table, www.cdc.gov/EID/content/17/8/101994-appT.htm). All viruses detected showed the same sequence (MVs/Shumen.BGR/15.09/1-3[D4], MVs/Silistra.BGR/21.09/1-4[D4], MVs/Blagoevgrad.BGR/02.10[D4], MVs/Plovdiv.BGR/03.10/1-3[D4], MVs/Plovdiv.BGR/23.10/1-5[D4]), and MVs/VelikoTarnovo.BGR/10.11/1-2[D4]), with the exception of MVs/Plovdiv.BGR/23.10/6[D4], characterized by 1 mismatch (Figure 1).

Laboratory Investigation of Measles Virus Samples from Bulgaria

Measles virus infection was reconfirmed for all 20 case-patients by positive test results for IgM, PCR, or both (online Appendix Table). Results were correlated with the clinical data for each case-patient that had been compiled during hospitalization. For 12 case-patients, vaccination status was unknown; a 7-month-old baby was unvaccinated. Seven case-patients (1, 7, 12, 14, 15, 17, and 18) presented vaccination cards that stated the date of 1 or 2 vaccinations with MVCV (online Appendix Table). All had positive IgM and PCR results; 2 had measles virus–specific IgG (case-patients 7 and 14). IgG avidity testing showed low avidity and thus a vaccination failure for case-patient 7. The equivocal IgM and the mediocre avidity of IgG in patient 14 did not indicate a primary infection. In summary, lack of immunologic response despite documented vaccination was apparent in 6 of 7 case-patients.

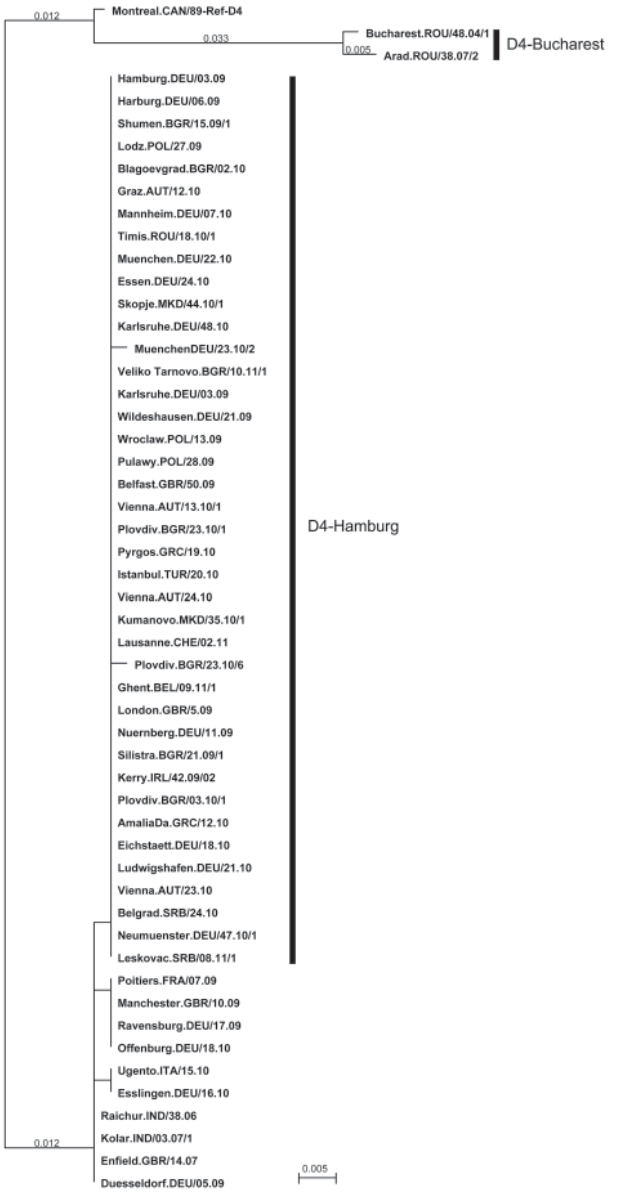

Figure 1. Phylogenetic relationships between measles viruses of genotype D4 recently detected in Europe. The measles virus variant D4-Hamburg initiated a long-lasting transmission chain spreading to several European countries during 2008–2011. D4-Hamburg belongs to the D4-Enfield lineage, which is genetically distinct from the previously widespread lineage D4-Bucharest. Phylogenetic analysis is based on a 456-nt sequence encoding the C-terminus of the measles virus nucleocapsid protein. The tree was constructed by the neighbor-joining method by using MacVector version 11.1.2 software (www.macvector.com). Scale bar indicates number of 5-nt deviations per 1,000-nt sequence. GenBank accession numbers are available online (www.cdc.gov/EID/content/17/8/101994-F1.htm).

Transmission of D4-Hamburg Strain in Europe

WHO Regional Reference Laboratories in Berlin, Luxembourg, and London receive either specimens or sequence information from the national measles laboratories of 41 European countries. Sequencing of the 450-nt fragment of the N gene showed that the D4-Hamburg strain had further spread in Europe (Figure 2). Samples taken in Poland during the summer of 2009 showed infection with a virus identical to D4-Hamburg (Figure 1); a total of 54 cases were recorded during 2009, the first in June and the last in October. All were linked to 3 outbreaks among Roma residents in the towns of Lodz, Pulawy, and Olpole Lubelskie (MVs/Lodz.POL/27.09[D4], MVs/Pulawy.POL/28.09[D4]) (*21*). The virus was also exported to Ireland (MVs/Kerry.IRL/40.09[D4]) from the Roma population and from there into Northern Ireland (MVs/Belfast.GBR/50.09[D4]), with small clusters of associated cases in both countries.

In Austria, 4 cases classified as D4-Hamburg–associated were detected in March and June 2010. A first sporadic case occurred in Graz in March. A person of Bulgarian nationality who was a member of the Roma ethnic group was infected; he was staying in Austria at the time (MVs/Graz.AUT/12.10[D4]). Three additional cases belonged to a cluster observed among persons in Vienna who spoke Bulgarian (MVs/Vienna.AUT/13.10[D4], MVs/Vienna.AUT/23.10[D4], MVs/Vienna.AUT/24.10[D4]). D4-Hamburg was also seen in Greece, where the first cases and clusters at the beginning of 2010 were identified among families of Roma communities of Bulgarian nationality (MVs/Amaliada.GRC.12.10[D4], MVs/Pyrgos.GRC/19.10[D4]). The virus was then spread to persons of Greek nationality, mainly from Roma communities, reaching 91 laboratory-confirmed measles cases in 2010. Moreover, 2 sporadic cases of D4-Hamburg were observed in 2010 in Romania (MVs/Timis.ROU/18.10/1[D4]).

In Turkey, D4-Hamburg was detected in a tourist who stayed in Romania and Bulgaria before visiting Turkey (MVs/Istanbul.TUR/20.10/[D4]). In Serbia, D4-Hamburg was detected in a person with a sporadic case (MVs/Belgrad.SRB/24.10/[D4]) and in the Roma population during an outbreak in Leskovac (MVs/Leskovac.SRB/08.11/1[D4]); 13 persons were infected, of which 3 were hospitalized. The index case-patient was a person who returned at the end of November from Germany (Duisburg). Nearly 400 cases were detected in Macedonia (MVs/Kumanovo.MKD/35.10/1[D4], MVs/Skopje.MKD/44.10/1[D4]). Although we cannot be sure that D4-Hamburg is the only virus contributing to the current outbreaks in Serbia and Macedonia, ongoing transmission of D4-Hamburg is indicated by the recent detection of a sporadic case of D4-Hamburg in Switzerland (MVs/Lausanne.CHE/02.11[D4]; this person probably became infected in Serbia) and by an outbreak of >40 cases in Belgium (MVs/Ghent.BEL/09.11/1[D4]).

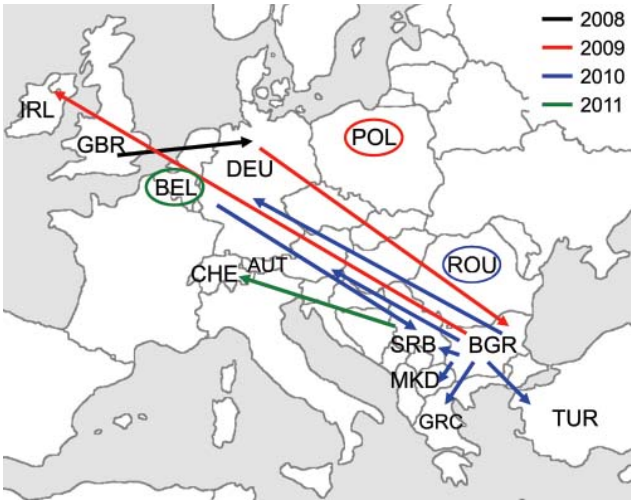

Figure 2. Transmission of the D4-Hamburg measles virus strain in Europe, 2008–2011. Arrows mark transmission with known epidemiologic link; ellipsoids mark detection without verified epidemiologic data. IRL, Ireland; GBR, Great Britain; BEL, Belgium; DEU, Germany; POL, Poland; CHE, Switzerland; AUT, Austria; ROU, Romania; SRB, Serbia; BGR, Bulgaria; MKD, Macedonia; GRC, Greece; TUR, Turkey.

Reimportation of the D4-Hamburg Strain to Germany

In 2010, D4-Hamburg measles virus was reimported to Germany. It appeared first in February in Mannheim, where specimens from 3 case-patients showed a sequence identical to D4-Hamburg (MVs/Mannheim.DEU/07.10[D4]). The virus was introduced by 8 persons from Bulgaria who belonged to a Turkish-speaking minority population, had acquired the infection in Dobrich (Bulgaria), and transmitted the virus to 3 relatives who were living in Mannheim. During June–August 2010, 48 measles cases were reported in Munich; 28 cases occurred among Bulgarian Roma residents in a migrant camp in eastern Munich. Several of these residents worked as cleaning staff at hotels in Munich. From these persons and other hospitalized members of the affected Roma group, the virus spread into the general population.

The age of case-patients in Munich ranged from 9 months to 36 years; 7 case-patients were <7 years of age, and 23 were >18 years of age. One case-patient was hospitalized because of encephalitis. Interviews with the help of an interpreter showed that none of the case-patients had MMR vaccination documents. Therefore, vaccination was offered to all inhabitants of the camp. Twenty-eight cases were investigated at the WHO Regional Reference Laboratory in Berlin. Twenty-three cases were associated with MVs/Muenchen.DEU/22.10[D4] identical with

D4-Hamburg, and specimens from 5 members of the same group were closely related to MVs/Muenchen. DEU/23.10/2[D4]. Moreover, clusters and sporadic cases of D4-Hamburg were detected in several German cities, e.g., Eichstaett (2 cases, MVs/Eichstaett.DEU/18.10[D4]) and Ludwigshafen (1 case, MVs/Ludwigshafen. DEU/21.10[D4]) in May 2010. These cases were linked to importation of D4-Hamburg from Bulgaria and did not initiate virus spread within Germany. The same virus variant was also detected in a cluster of cases observed in the city of Essen during June–July 2010. This variant was imported from Bulgaria by a citizen of Bulgaria (MVs/Essen.DEU/24.10[D4]) and spread to another citizen of Bulgaria (MVs/Essen.DEU/25.10/1[D4]) and 6 persons of the general population (MVs/Essen.DEU/25.10/2[D4], MVs/Essen.DEU/28.10[D4]).

From week 47 (the end of November) on, 8 cases of infection with D4-Hamburg occurred in Neumuenster in northern Germany. This outbreak occurred in a home for migrants mainly from Afghanistan and Serbia (MVs/Neumuenster.DEU/47.10/1[D4]). From week 48 on, 6 cases were seen in another home for migrants in Karlsruhe (MVs/Karlsruhe.DEU/48.10[D4]).

Discussion

A combination of epidemiologic data and genotyping results enabled us to trace the spread of measles virus D4-Hamburg in Europe. It was imported from London at the end of 2008 to northern Germany (288 cases), then transmitted from Hamburg to Bulgaria, where, after a 7-year absence of measles, an outbreak of 24,379 cases occurred. This was the largest outbreak seen in Europe since an outbreak in the Ukraine in 2006 (*22*).

Twenty cases from the outbreak in Bulgaria were sampled at different times (April and June 2009, January and June 2010, and March 2011) from persons in distinct districts. The samples were collected initially in northeastern and later in southwestern Bulgaria, thereby following the course of the outbreak. The cases were associated with measles virus sequences such as MVs/Shumen.BGR/15.09[D4], corresponding to D4-Hamburg. The only exception was MVs/Plovdiv.BGR/23.10/6[D4], which showed 1 mismatch but in all probability developed from MVs/Plovdiv.BGR/23.10/1–5[D4]. Because the samples had been obtained at different times and regions, our analysis provides substantial evidence that D4-Hamburg is responsible for the outbreak in Bulgaria, despite the small number of samples. Samples from 6 of 7 persons showed diagnostic markers of a primary measles infection, although these persons had a certificate of prior measles vaccination. Our results therefore demonstrate an urgent need to investigate the vaccination procedures for ethnic minorities.

D4-Hamburg was detected subsequently in Poland (54 cases) (*21*), Ireland, Northern Ireland, Austria (4 cases), Greece (149 cases) (*23*), Serbia (14 cases), Belgium (>40 cases), and Macedonia (>400 cases). Sporadic cases were detected in Romania, Turkey, and Switzerland. More than 70 D4-Hamburg–associated cases were detected in Germany after 8 separate reimportations. Taken together, D4-Hamburg was present in Europe from December 2008 to March 2011—that is, at least 27 full months—and caused >25,300 cases. Because sequencing results are not available quickly in most countries, this transmission chain is probably still ongoing. Circulation of imported measles virus for no longer than 12 months (and therefore endemic transmission according to the WHO definition) is a marker for successful elimination. We suggest, therefore, that the length of a given transmission chain should not be assessed on a national level but at the level of the all 53 countries within the WHO EUR.

Epidemiologic data showed that the spread of D4-Hamburg across Europe involved predominantly persons from the Roma ethnic group in Bulgaria. Another transmission chain affecting the Roma population in particular was recorded in 2004 in Romania. An outbreak of >8,000 cases associated with MVs/Bucharest. ROU/48.04[D4] commenced in the Roma population. Subsequent spread of D4-Bucharest by traveling Roma persons was observed until 2007 (*4*). The pronounced sequence deviation of D4-Bucharest and D4-Hamburg indicates the presence of at least 2 distinct and successive transmission chains in the Roma population. Both chains were long lasting and associated with a high number of cases, as well as several fatalities. This and other recent outbreaks in Roma communities (*7*,*24*,*25*) underline the need for the development of strategies to address this ethnic minority at the regional level and to improve their integration into the respective national health services.

The lack of strategies to address reaching the hard-to-reach communities in Europe will clearly have an adverse effect on the measles elimination process. In this context, we want to make clear that elimination of measles virus should not be seen exclusively as a Roma-associated problem. Measles virus is a highly infectious agent and will infect any population with low immunity rates. If itinerant groups are underserved by the national health sector, spread of measles virus is highly probable. Because measles outbreaks in western European countries occur mainly in undervaccinated groups (*26*), reaching the hard-to-reach is not the only important challenge. Thus, closing vaccination gaps in a setting of optional vaccination and vaccine skepticism is another important prerequisite that must be met on Europe's path toward elimination of measles virus.

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Deaths Associated with Human Adenovirus-14p1 Infections, Europe, 2009–2010

Michael J. Carr, Adriana E. Kajon, Xiaoyan Lu, Linda Dunford, Paul O'Reilly, Paul Holder, Cillian F. De Gascun, Suzie Coughlan, Jeff Connell, Dean D. Erdman, and William W. Hall

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Release date: July 22, 2011; Expiration date: July 22, 2012

Learning Objectives

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

- Describe demographic, genetic, and transmission-related factors associated with human adenovirus-14p1 (HAdV-14p1) infections recently detected in Ireland
- Describe clinical characteristics and complications associated with HAdV-14p1 infections recently detected in Ireland
- Describe the clinical and public health implications of these findings.

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Human adenovirus (HAdV) serotype 14 is rarely identified. However, an emerging variant, termed HAdV-14p1, recently has been described in the United States in association with outbreaks of acute respiratory disease with high rates of illness and death. We retrospectively analyzed specimens confirmed positive for HAdV by immunofluorescence, virus culture, or real-time PCR during July 1, 2009–July 31, 2010, and describe 9 cases of HAdV-14p1 infection with characteristic mutations in the fiber and E1A genes that are phylogenetically indistinguishable from the viruses previously detected in the United States. Three patients died; 2 were immunocompromised, and 1 was an immunocompetent adult. We propose that surveillance should be increased for HAdV-14p1 and recommend that this virus be considered in the differential diagnosis of sudden-onset acute respiratory disease, particularly fatal infections, for which an etiology is not clear.

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Human adenoviruses (HAdVs) were identified independently by 2 groups during the early 1950s (*1*,*2*). HAdVs are nonenveloped, linear double-stranded DNA viruses encapsidated within a protein shell and have been categorized into 6 species (A–F) that contain 51 immunologically distinct serotypes (*3*). HAdVs most commonly cause acute respiratory disease; however, depending on the infecting HAdV serotype and tropism resulting from differential host receptor use, the wide variety of symptoms can include pneumonia, febrile upper respiratory illness, conjunctivitis, cystitis, and gastroenteritis (*4*). The severity of disease appears dependent on the immunocompetence and cardiopulmonary health of the host, and the spectrum of disease can range from subclinical to severe respiratory distress and death (*4*). Immunocompromised patients (especially bone marrow transplant [BMT] recipients) are particularly susceptible to HAdV infection, resulting in severe illness and deaths, whereas illness in immunocompetent patients generally resolves without major complication.

HAdV species B comprises 2 subspecies: B1 (including HAdV-3, -7, -16, -21, and -50) and B2 (HAdV-11, -14, -34, and -35). The subspecies B2 HAdV-14 (agent de Wit) was originally identified as an etiologic agent of acute respiratory disease in military recruits in the Netherlands during 1955 (*5*). Despite reports of subsequent outbreaks in Europe and Asia during the 1950s and early 1960s (*6*), global surveillance in the subsequent decades had not identified circulation of this serotype until spring 2006, when HAdV-14 emerged as a cause of a major proportion of acute febrile respiratory illness in military bases across the United States (*7*–*9*). In 2007, community-associated outbreaks were identified in California and New York (*10*), and during March–June 2008, ≈140 cases of HAdV-14 were identified in Oregon, Washington, and Texas. Overall, 38% of patients were hospitalized, 17% were admitted to intensive care units, and 5% died. During September 2008, an outbreak was reported with 32 cases of pneumonia on Prince of Wales Island off the coast of Alaska (*11*). In the United States, the earliest documented case of HAdV-14 infection, by retrospective testing, occurred in California during December 2003, and the most recent evidence of HAdV-14 had been in Pennsylvania during June 2009 (*7*). The virus has circulated uninterrupted in some military recruit camps (A.E. Kajon, unpub. data).

Sequence analysis of the fiber gene of HAdV-14 associated with the recent outbreaks has shown a 6-bp deletion resulting in a 2-aa deletion (lys-glu) at positions 251 and 252 in the knob region compared with the de Wit HAdV-14p prototype strain (*10*). The 252glu site is conserved in all other species B HAdVs and is located in the F–G loop of the fiber protein knob near a putative host receptor binding site. Further sequencing of the hexon gene hypervariable regions 1–7 and the E1A genes demonstrated that this emerging HAdV-14 was clearly separable from the reference strain de Wit (genome restriction type HAdV-14p) (*9*,*11*). This new genomic variant has been designated HAdV-14p1 on the basis of novel restriction profiles. Viral receptor binding and internalization studies that used recombinant HAdV-14p1 fiber regions containing the 2-aa deletion did not demonstrate substantial phenotypic differences between the new agent and the prototype virus (*12*). This finding suggests that HAdV-14p1 could be an immune escape mutant that has lost a potential neutralizing epitope, has modified postinternalization steps, or has enhanced binding to host cells through a yet-unidentified viral receptor.

However, no evidence suggests that HAdV-14p1 has reemerged because of altered virulence. Despite the mutations in the fiber and EIA genes, no other genetic differences with the prototype HAdV-14 clearly explain differing disease severity in US outbreaks (*13*). This observation suggests that reemergence of this agent might be more likely to have resulted from spread of an infectious agent in immunologically naive populations and changes in the rate of specific immunity in host populations over time. The recent whole-genome analysis of HAdV-14p1 in mild and severe infections supports this idea (*13*). In addition, reports of recent HAdV-14 and HAdV-14–11 outbreaks in Asia and the United States have detailed similar dynamics (*14*,*15*). The observed characteristics (including periods of little activity punctuated by distinct outbreaks with occasional severe disease and death) may simply be the natural pattern of adenovirus species B2 respiratory pathogens.

Whether HAdV-14p1 had circulated elsewhere before, or at the same time as, the outbreaks in the United States and whether it is currently circulating in Europe is unclear. In this report, we show that recent infection and deaths associated with HAdV-14p1 infection have occurred in Ireland and that this virus is genetically indistinguishable from HAdV-14p1 described in the United States.

Materials and Methods

Study Period

The study period was July 1, 2009–July 31, 2010. The study comprised 29 cases confirmed adenovirus positive by indirect immunofluorescence assay (IFA), viral culture, or real-time quantitative PCR (qPCR) at the National Virus Reference Laboratory (NVRL, Dublin, Ireland).

Virus Culture and Restriction Enzyme Analysis

HAdVs were cultured at NVRL and Lovelace Respiratory Research Institute (Albuquerque, NM, USA). Isolates were passaged once in human embryonic lung

carcinoma cells in 25-cm^2 flasks for detection of cytopathic effects and then subsequently expanded in 75-cm^2 flasks for extraction of viral DNA for restriction enzyme analysis as described (*16*). Viral DNA was digested with the same panel of endonucleases used to characterize the North American strain of HAdV-14 (*7*).

Indirect Immunofluorescence

We performed IFA for influenza A virus, influenza B virus, parainfluenza viruses 1–3, respiratory syncytial virus, and HAdV. We used the respiratory virus panel kit (Biotrin, Dublin, Ireland) and followed the manufacturer's instructions.

Molecular Analysis

We performed qPCR for HAdV as described (*17*). Adenoviruses were initially typed by using partial hexon gene sequencing as described (*17*,*18*). We extracted 200 μL of serum, plasma, or nasopharyngeal aspirate by using the QIAamp DNA Mini Kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions or by using the MagNA Pure automated extraction platform (Roche, Lewes, UK) with an external lysis step. The final elution volumes for both methods was 50 μL. Real-time PCR specific for HAdV-11 and HAdV-14 (*19*) provided by the Centers for Disease Control and Prevention (Atlanta, GA, USA) was performed by using the ABI7500 SDS platform (Applied Biosystems, Foster City, CA, USA) with 5 μL of template and the Platinum qPCR SuperMix-UDG kit (Invitrogen, Paisley, UK) with 0.5-μmol/L forward and reverse primers and 0.1-μmol/L probe labeled at the 5′ end with 6-carboxyfluorescein and a 3′ quencher dye in 25-μL single-plex assays performed with the following cycling conditions: 50°C for 2 min, then 95°C for 2 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min, with data acquisition in the anneal/extension phase. Complete HAdV-14 fiber, E1A, and hexon genes were amplified as described (*7*) and sequenced bidirectionally. Nucleotide sequence data for HAdV-14p1 fiber, E1A, and hexon genes were submitted to GenBank (accession nos. HQ163915, HQ163916, and HQ265808).

Phylogenetic Analysis

The fiber, E1A, and hexon genes of the Ireland HAdV-14p1 strains were compared with recently described US HAdV-14p1 strains (*7*) and reference sequences from HAdV B2 subgenera obtained from GenBank. The accession numbers for all sequences are included on the tree (Figure 1). Lasergene version 8 (DNASTAR, Madison, WI, USA) was used for contiguous assembly (*20*), and the sequences were aligned by using ClustalW (*21*) implemented in Bioedit version 7.05 (*22*). Phylogenetic trees were constructed by using the maximum-likelihood

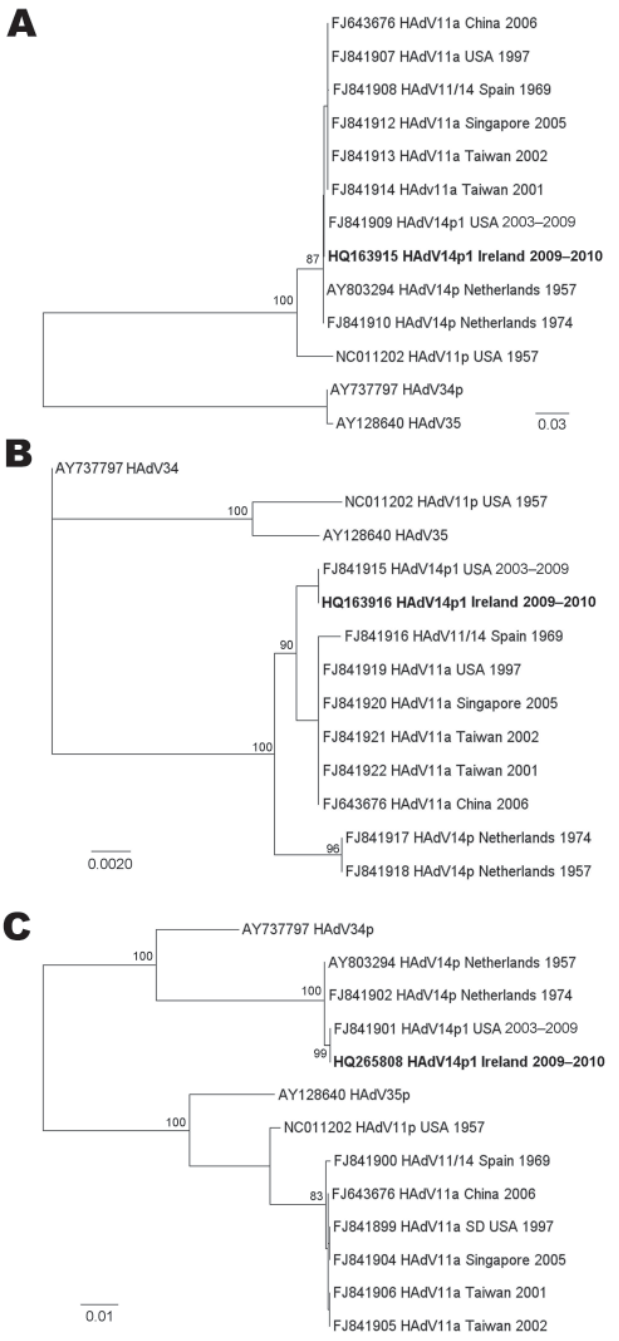

Figure 1. Maximum-likelihood trees of the full-length fiber (A), E1A (B), and hexon (C) open reading frames of adenovirus B2 subgenera. Phylogenetic analysis was performed by using reference sequences from GenBank for the adenovirus B2 subgenera, including prototype reference strains. The query sequences from this study are identical and are represented in **boldface**. The tree was built in PAUP* (*23*) on the basis of the HKY85 model of evolution and for the fiber tree also with a β distribution and used midpoint rooting. Bootstrap resampling (n = 1,000) was performed by using the neighbor-joining algorithm. Scale bars indicate nucleotide substitutions per site.

method under the HKY85 substitution model that used PAUP* version 4.0 β 10 (*23*), and bootstrapping with 1,000 replicates was used to analyze the stability of the tree topology.

Results

We retrospectively tested clinical specimens received by NVRL from 29 patients with positive results for HAdV by IF, virus culture, or qPCR during the study period by using specific HAdV-11 and -14 real-time assays. All 29 samples were negative for HAdV-11; however, 9 (31%) samples tested positive for HAdV-14 (Table). All patients sought care or were previously hospitalized during November 2009–July 2010. Of the 9 patients, 7 (78%) were male, 6 (67%) patients were ≤4 years of age, and the remaining 3 patients were ≥34 years of age. No epidemiologic links were known between any of the patients. No geographic clustering was observed; the cases were distributed throughout Ireland and involved several hospitals. All respiratory samples were IFA negative for the following respiratory viruses: influenza A, influenza B, parainfluenza viruses 1–3, and respiratory syncytial virus. Our earliest identified HAdV-14–positive specimen (November 10, 2009; case-patient 1 in Table) was positive for influenza A virus by real-time reverse transcription PCR and confirmed as pandemic influenza A (H1N1) 2009 on October 29. No influenza A was detected in a specimen from this patient on November 8 and a nasopharyngeal aspirate collected 2 days later; however, this later specimen was positive for HAdV-14.

Fiber, E1A, and hexon gene sequences derived from the 9 HAdV-14 case-patients from Ireland during the study period were 100% identical for each gene analyzed. These sequences were then compared with subspecies B2 HAdV reference sequences from GenBank, including the prototype de Wit strain, HAdV-14p (*5*). Phylogenetic analysis demonstrated that the Ireland and US HAdV-14p1 fiber, E1A, and hexon sequences formed a monophyletic group (Figure 1, panels A–C). The sequences from Ireland were 100% identical in the fiber, E1A, and hexon open reading frames (ORFs) to the recently described HAdV-14p1 strains isolated in the United States during 2003–2009 (*7*–*9*).The fiber ORF sequences analyzed from the isolates from Ireland were 972 nt, and all contained the 6-nt deletion identified in the recent US HAdV-14p1. This deletion, unique among HAdV B2 strains, corresponds to amino acid residues lysine and glutamic acid (AAA/GAA) at codon positions 251/252 located in the F–G loop of the fiber protein knob. Other than this deletion, the fiber sequences were highly conserved and had 99.3% nt identity to the HAdV-14p de Wit type strain, 99.1% identity to the 11a strains, 99% identity to the Spain 11a/14 strain in 1969, and only 93%–94% similarity to the HAdV-11p strains. The de Wit prototype strain had 99%–99.1% identity to the Spain 11/14 strain 273 from 1969 and other 11a strains, which have been identified as intertypic recombinants with a HAdV-11 hexon gene and a HAdV-14-like fiber gene (*7*).

The 870 bp of the E1A ORF from the Ireland sequences had higher sequence identity to HAdV-11a strains (99.8%) and the prototype HAdV-14p de Wit strain (99.1%) than to HAdV-11p strains (97%) or any other HAdV B2 reference strains. Furthermore, the Ireland E1A sequences contain a 3-nt insertion, GTG, which is also present in all of the 11a and 14p1 strains but not in the 14p strains. The HAdV-14p1 E1A sequences were most closely associated with the 11a sequences from Taiwan, Singapore, Spain, and the United States, and the fiber sequences from Ireland clustered close to the 11a and 14p sequences. The hexon ORFs analyzed from the samples from Ireland were 2,838 nt and were almost identical (99.86%) to the prototype HAdV-14p de Wit strain. Compared with the E1A and fiber gene sequences,

Table. Clinical characteristics of patients with confirmed HAdV-14p1, Ireland, July 1, 2009–July 31, 2010*

Case no.	Age/sex	Location	Sample date	Sample type	Previous condition	Clinical characteristics	Outcome
1	4 y/M	Dublin	2009 Nov	NPA	Pierre Robin syndrome (craniofacial abnormality)	Fever, tachypnea, cough	Survived
2	7 mo/M	Dublin	2010 Mar	NPA	None known	Bronchiolitis	Survived
3	1 mo/M	Dublin	2010 May	NPA	None known	Bronchiolitis, diarrhea, vomiting	Survived
4	8 d/F	Dublin	2010 May	Serum (×2), urine	Preterm birth at 35 weeks' gestation	Hypotonic, abnormal LFT results	Died
5	46 y/F	Kilkenny	2010 May	Other/lung biopsy	Smoking, high BMI	Community-acquired pneumonia, sepsis	Died
6	34 y/M	Dublin	2010 May	BAL	Post-BMT, neutropenic	Unilateral pulmonary infiltrate, fever	Died
7	4 mo/M	Dublin	2010 Jul	NPA	None known	Bronchiolitis, diarrhea, vomiting	Survived
8	14 d/M	Cork	2010 Jul	Plasma (×2)	None known	ARDS	Survived
9	48 y/M	Dublin	2010 Jul	BAL	HIV+ (RNA <50) HCV+ (RNA not detected); CD4 436 (15%)	Pneumonia	Survived

*HAdV-14p1, human adenovirus serotype 14p1; NPA, nasopharyngeal aspirate; LFT, liver function test; BMI, body mass index; BAL, bronchoalveolar lavage; BMT, bone marrow transplant; ARDS, acute respiratory distress syndrome; +, positive; HCV, hepatitis C virus; CD4, CD4 cells/mL.

in the hexon region the Ireland sequences exhibited much lower sequence identity to the HAdV-11a and -11p strains analyzed in the study; the HAdV-11a and -11p hexon genes were much more similar to each other and branched separately on the tree (Figure 1, panel C). Viral DNA extracted from each of the 8 isolates of HAdV-14 yielded profiles identical to each other and identical to those reported for the North American strain of HAdV-14. The Ireland isolates were therefore identified as corresponding to genome type 14p1 on the basis of the distinct *Bcl*I, *Bst*EII, and *Pst*I restriction patterns (Figure 2).

Of the 9 HAdV-14p1–infected patients, 8 (90%) had respiratory symptoms: pneumonia was diagnosed for 4, bronchiolitis for 3, and acute respiratory distress syndrome (ARDS) for 1. The other patient (case-patient 4; a premature infant born at 35 weeks) was hypotonic at birth with abnormal liver function (Table). Major underlying medical conditions were reported in 4 patients: a BMT recipient; a premature neonate; a patient co-infected with HIV and hepatitis C virus; and a patient with the craniofacial abnormality Pierre Robin syndrome, who had a prior tracheostomy and percutaneous enteral gastrostomy. ARDS developed in the BMT recipient (case-patient 6) 56 days posttransplant; lymphocyte count had not recovered and was at undetectable levels (<0.3/mm^3). The patient co-infected with HIV and hepatitis C virus was receiving antiretroviral therapy and had undetectable HIV RNA (<50 copies/mL), a CD4 count of 436 cells/mL (15%), and a resolved hepatitis C virus infection. No underlying conditions were reported for the 5 remaining patients. Three patients died in May 2010: the premature neonate, the BMT recipient, and a 46-year-old woman. The woman had no known notable medical history but had a high body mass index and smoked cigarettes (Table). Two patients (case-patients 4 and 8) received extracorporeal membrane oxygenation. Case-patient 4 had a natural killer (NK) cell deficiency and died. The nature of the NK cell defect in the premature infant was not defined, and bone marrow was analyzed with no pathologic findings noted. Leukocyte screen, however, showed a lack of NK cells; additional screen for immunodeficiencies (such as severe combined immunodeficiency) proved inconclusive, and a human leukocyte antigen–B57–negative haplotype was recorded. Case-patient 8, however, for whom ARDS had been diagnosed, received prolonged (>6 weeks) extracorporeal membrane oxygenation and recovered. Four patients had an unremarkable full recovery.

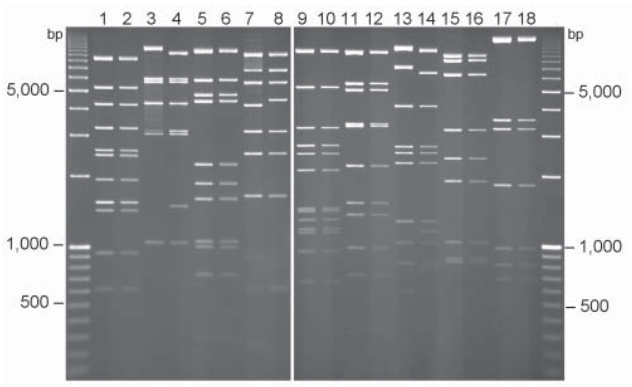

Figure 2. Comparative restriction enzyme analysis of viral DNA extracted from the prototype human adenovirus (HAdV) 14 de Wit strain and the first detected HAdV-14 case isolated in Dublin, Ireland, November 2009. All odd-numbered lanes (e.g., 1, 3) contain the de Wit strain and all even-numbered lanes (e.g., 2, 4) contain the Dublin 2009 strain, with restriction enzyme digests as follows: lanes 1 and 2 with *BamH*I; lanes 3 and 4 with *Bcl*I; lanes 5 and 6 with *Bgl*II; lanes 7 and 8 with *BstE*II; lanes 9 and 10 with *Dra*I; lanes 11 and 12 with *Hind*III; lanes 13 and 14 with *Pst*I; lanes 15 and 16 with *Sma*I; lanes 17 and 18 with *Xba*I. Outer lanes are molecular markers (1 Kb +100 bp; BioRad, Hercules, CA, USA).

Of the 9 patients positive for Ireland HAdV-14p1, serum or plasma samples of 2 were submitted for HAdV DNA quantification. The preterm infant (case-patient 4) had a HAdV load in serum of 8.18 $\log_{10}$ viral genomes/mL, and the infant with ARDS (case-patient 8) had a plasma adenoviral load of 8.83 $\log_{10}$ viral genomes/mL. The immunocompetent 46-year-old woman (case-patient 5) had a viral load of 4.80 $\log_{10}$ genomes/g from a postmortem lung biopsy specimen.

Discussion

We have demonstrated that the HAdV-14p1 strain first identified in the United States is now circulating in Ireland and is associated with substantial illness and with 3 deaths: a BMT recipient, a neonate, and (most notably) an immunocompetent, apparently otherwise healthy adult. The role of HAdV-14p1 in the deaths remains to be elucidated because 2 of the 3 deaths occurred in substantially immunocompromised patients. Conversely, other patients who were also immunocompromised recovered unremarkably, and fatal severe pneumonia and ARDS are known to occur in immunocompetent adults infected with other HAdV serotypes (*24*). Nevertheless, of concern in the cohort described here is the third death, which occurred in a previously well patient. The immunocompetent woman was a smoker, and smoking has been identified as an independent risk factor possibly facilitating transmission of HAdV-14p1 (*11*). However, the low rate of infections (5%) observed by Esposito et al. (*11*) in household contacts of persons infected with the emerging virus suggests that infection is unlikely to spread in the community. Efficient transmission of HAdV-14p1 appears to require close physical contact, as supported by the observation that in barracked communities used for military trainees in the United States, antibody titers demonstrated recent exposure after entry to the facility (*25*). Of interest in the HAdV-14p1 cases identified in the present study is the higher frequency of male patients (78%) infected;

this observation has been reported by other groups (*19*,*26*), although the numbers identified in our study are insufficient to make definitive conclusions. Previous reports have noted that prior vaccination with HAdV-7, a subspecies B1 virus, of military recruits may confer cross-protection, presumably from heterotypic immunity, and that this vaccine could potentially be used to reduce HAdV-14p1 disease severity (*27*,*28*). Trei et al. have reported on the spread of HAdV-14 in 2007 from a large military training facility in Texas to secondary sites across the United States and to South Korea, despite the institution of active surveillance for acute respiratory disease and prevention and control measures (*29*). Despite evidence of decreased spread from military personnel to family members outside the training population, many recent military graduates may have been asymptomatic and incubating the virus, which presents a possible scenario for the subsequent dissemination of this virus.

Our findings suggest that clinicians and other health care workers should consider HAdV-14p1 in the differential diagnosis of community-acquired pneumonia. No evidence indicates that HAdV-14p1 infection is substantially more severe than that by other HAdV serotypes; however, we propose increased surveillance for this emerging agent in Europe and elsewhere in immunologically naive populations. In addition, we suggest the retrospective investigation of HAdV-positive specimens for HAdV-14p1, particularly from untyped viruses from patients who had severe disease and who died without an established etiology to help determine the time and location of reemergence of this HAdV in Europe.

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Case–Control Study of Risk Factors for Hospitalization Caused by Pandemic (H1N1) 2009

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We conducted a case–control study to identify risk factors for hospitalization from pandemic (H1N1) 2009 virus infection among persons >16 years of age in Sydney, Australia. The study comprised 302 case-patients and 603 controls. In a logistic regression model, after adjusting for age and sex, risk factors for hospitalization were pregnancy (odds ratio [OR] 22.4, 95% confidence interval [CI] 9.2–54.5), immune suppression (OR 5.5, 95% CI 2.8–10.9), pre-existing lung disease (OR 6.6, 95% CI 3.8–11.6), asthma requiring regular preventive medication (OR 4.3, 95% CI 2.7–6.8), heart disease (OR 2.3, 95% CI 1.2–4.1), diabetes (OR 3.8, 95% CI 2.2–6.5), and current smoker (OR 2.0, 95% CI 1.3–3.2) or previously smoked (OR 2.0, 95% CI 1.3–3.0). Although obesity was not independently associated with hospitalization, it was associated with an increased risk of requiring mechanical ventilation. Public health messages should give greater emphasis to the risk for severe disease among pregnant women and smokers.

The emergence of pandemic (H1N1) 2009 virus (*1*,*2*) was associated with a large increase in the number of persons requiring hospitalization for severe influenza disease in many parts of the world (*3–5*). In response, in an effort to reduce the impact of the pandemic on their communities and health services, public health agencies developed recommendations for persons at increased risk for disease to seek early treatment. However, these recommendations were based on studies of seasonal influenza (*6–10*) and descriptive case reports (*11–20*).

The first cases of pandemic (H1N1) 2009 infection from New South Wales (NSW) were reported in May 2009. In NSW, laboratories were required to notify all pandemic (H1N1) 2009 diagnoses to the NSW Department of Health under Public Health Act 1991 (*21*). Australian public health management protocols recommended laboratory testing for all persons with influenza-like illness (fever and cough or sore throat) admitted to a hospital (*22*). Public health follow-up was required to ascertain hospitalization status for all notified cases at the time of diagnosis; this information was collated on a statewide database. Within Sydney, the capital city of NSW (population 4.4 million), there are 4 Area Health Services (AHSs) responsible for the provision of local public health and clinical services.

By the end of June 2009 (before a vaccine was available), community transmission was widespread in Australia, and public health efforts were focused on protecting those at greatest risk for severe disease. The groups considered most vulnerable were pregnant women; indigenous people; very obese persons; persons with pre-existing chronic medical conditions, including lung, heart, and kidney disease; and persons with blood, metabolic or neurologic disorders, immunosuppressive conditions, or asthma (*23*). Persons in these risk groups and those with severe disease were urged to seek medical attention early if influenza-like symptoms appeared. Doctors were provided with free antiviral medication for patients who were seen within 48 hours of symptom onset. A vaccine became publicly available for distribution in September 2009. To help reach populations who would most benefit from prevention and early intervention, we sought to identify independent risk factors for moderate to severe disease from pandemic (H1N1) 2009 infection among adults and to describe the characteristics of those who sought early medical treatment to determine the effectiveness of public health messages.

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Methods

The study population was defined as persons >16 years of age residing in metropolitan Sydney during July 1–August 31, 2009. All interviews were conducted during September and October 2009.

Cases

We defined a case as a person with influenza-like illness admitted (for a minimum of an overnight stay) to a Sydney metropolitan hospital from July 1 through August 31, 2009, who had laboratory confirmation of pandemic (H1N1) 2009 by PCR that was notified to the Department of Health. Patients <16 years of age or residing outside metropolitan Sydney were excluded from the study because we assumed the threshold for hospital admission may have been different for this age group and for patients in regional and rural areas. To ensure complete case ascertainment, all cases notified in the study period were cross-matched with the Department of Health database containing all hospital admissions in NSW for the study period; additional identified cases were included in the study. If case-patients were not able to complete a telephone interview because of ill health or disability, another household member completed the interview on their behalf. Up to 30 attempts were made to contact case-patients by telephone.

Case–Control Study

In a case–control study, we compared demographic and health information reported by case-patients with those of controls. We defined a control as a person >16 years of age residing in metropolitan Sydney who had not been hospitalized for influenza in 2009. Telephone numbers (stratified by AHS) were used to randomly select potential control households. Within each AHS in Sydney, we selected 2 households per case. A single control was randomly selected from within each selected household for interview. Up to 12 attempts were made to contact selected households. Fewer attempts were made to contact controls than case-patients as a result of random-digit generation methods described in detail elsewhere (*24*). The age and sex distribution of participating controls was compared with that of the metropolitan Sydney population.

Study Questionnaire

We used a standard questionnaire to ask case-patients and controls about influenza symptoms, pregnancy or delivery within the previous 28 days, weight and height, smoking history, current and previous medications, and past hospitalizations. In addition, information was collected regarding diagnosis of general health conditions, including asthma, lung disease (defined as emphysema, chronic lung problems and/or chronic bronchitis), heart disease (defined as heart problems from birth, rheumatic heart disease, angina, heart attack, and/or heart failure excluding hypertension), diabetes (type I or II), other metabolic disorders, kidney disease (defined as kidney transplant, renal failure, and/or dialysis), liver disease, blood disorders (defined as sickle cell disease, thalassemia, or hemoglobin problems), mental health diagnoses, neurologic conditions (defined as conditions that involve muscles, nerves, or the brain), immune suppression (defined as cancer, HIV infection, or immunosuppressive medication), and obstructive sleep apnea. Ethics approval was not required because information was collected under NSW Public Health Act 1991 (*21*)

Statistical Analyses

In univariate analysis, the proportions of characteristics among case-patients and controls were compared by using χ^2 tests. In multivariate analysis, independent risk factors for hospitalization were assessed through logistic regression by using backward elimination. All variables with a univariate level of significance $p<0.25$ were selected for inclusion in the base model, and variables were excluded if the p value was >0.05 and did not meaningfully alter the point estimates of the remaining variables. Because a similar proportion of case-patients and controls reported a diagnosis of asthma not requiring regular medication, only a history of asthma requiring regular medication was included in the final logistic regression model.

Additional logistic regression models were constructed to compare case-patients with controls for women of childbearing age (16–45 years), case-patients who received mechanical ventilation compared with all controls, case-patients who sought medical attention within 48 hours of onset of symptoms compared with case-patients who sought medical attention after 48 hours, and controls who reported symptoms of influenza-like illness during the study period (defined as self-reported fever and either cough or sore throat) with controls who reported no illness during the study period. Mechanical ventilation (rather than intensive care unit admission) was used as a measure of severity of illness because cases were reported from several hospital facilities with varying criteria for patient admission to intensive care. The model fit the data well by the Hosmer-Lemeshow goodness-of-fit test (χ^2 = 15.85, 9 df, p = 0.07). Seventy-nine percent of pairs were concordant, and c = 0.804.

Results

In total, 402 hospitalized patients were identified as eligible for inclusion in the study. Of these, 302 (75%) participated in the study, 27 (7%) refused interview, 66 (16%) were unable to be contacted, and 7 (2%) were excluded because of language difficulties. In univariate analysis, there was no significant difference between participating patients and nonparticipating patients with

respect to sex (p = 0.226), geographic location of residence (p = 0.341), indigenous status (p = 0.123), length of stay in hospital (p = 0.477), or ventilation status (p = 0.890). However, interviewed patients were significantly younger (median 45 years, range 16–88 years) than patients who were not interviewed (median 51 years, range 17–88 years) (p = 0.007).

Of 1,252 potential controls, 603 (48%) participated in the study. Of those remaining, 357 (28%) refused to participate, 153 (12%) were excluded because of language difficulties, and 139 (11%) were unavailable for interview. Of the controls, 25% were ages 16–35 years, 36% were 36–55 years, and 37% were >55 years. This compares with the adult metropolitan Sydney population of 38% ages 16–35 years, 36% 36–55 years, and 28% >55 years (Australian Bureau of Statistics, unpub. data).

Descriptive Epidemiology

The median age of case-patients was 45 years (range 16–88 years). Of the 302 case-patients, 125 (41%) were male, 68 (23%) were admitted to a high dependency or intensive care unit, and 37 (12%) required mechanical ventilation. The median length of hospital stay was 4 nights (range 1–91 nights). Reported risk factors among case-patients included a history of asthma (38%, including 29% who required regular medication), lung disease (19%), diabetes (19%), mental health diagnosis (18%), heart disease (14%), pregnancy (13%), obstructive sleep apnea (12%), immune suppression (10%), neurologic condition (8%), liver disease (8%), kidney disease (3%), blood disorders (5%), and metabolic conditions (1%) (online Appendix Table, www.cdc.gov/EID/content/17/8/100842-appT.htm).

None of the case-patients reported giving birth in 28 days before symptom onset. Of the 40 patients who were pregnant at the time of symptom onset, 28 (70%) were in their third trimester. Sixty-six (22%) case-patients were current smokers, and 91 (30%) were ex-smokers. Among the 91 ex-smokers, the median time smoked was 20 years (range 1–60 years, median 15 cigarettes/day); 39 reported cessation >5 years prior to illness, 15 patients between 12 months and 5 years, and 37 patients within the past 12 months. Among the 66 current smokers, the median time smoked was 20 years (range 1–60 years; median 10 cigarettes/day) (online Appendix Table).

Case–Control Study

There were no significant differences in characteristics of case-patients and controls by place of residence, receipt of 2009 seasonal influenza vaccination, or history of neurologic disorders (online Appendix Table). In univariate analysis, compared with controls, case-patients were more likely to be male, aged 16–35 years and 46–55 years, have higher body mass index (BMI), and report a history of asthma, heart disease, mental health diagnosis, immune suppression, obstructive sleep apnea, lung disease, diabetes, liver disease, blood disorder, and pregnancy, and smoking (online Appendix Table).

In the logistic regression model, age, sex, asthma (requiring regular medication), smoking (current or ex-smoker), heart disease, immune suppression, lung disease, diabetes, and pregnancy were independently associated with hospitalization for pandemic (H1N1) 2009 (Table 1). Similar results were found when analysis was restricted to men only or women only. The factor most strongly associated with hospitalization was pregnancy, followed by lung disease, immune suppression, and ages 16–25 and 46–55 years (Table 1). Overall, 262 (86%) case-patients reported ≥1 independent risk factor (asthma, heart disease, immune suppression, lung disease, diabetes, pregnancy, or smoking) compared with 315 (52%) of controls (p<0.0001). The risk for hospitalization increased with increasing number of reported significant risk factors.

In the logistic regression model for women of childbearing age, asthma (requiring regular medication), lung disease, diabetes, and pregnancy were independently associated with hospitalization for pandemic (H1N1) 2009 (Table 2). In total, 88% of women of childbearing age

Table 1. Independent risk factors for hospitalization from pandemic (H1N1) 2009 influenza, all case-patients and controls, Sydney, Australia, 2009*

Patient characteristic	Adjusted OR (95% CI)	p value
Sex		
F	Referent	
M	1.8 (1.2–2.5)	0.0017
Age, y		
16–25	5.4 (2.5–11.4)	
26–35	4.1 (2.0–8.3)	
36–45	3.9 (2.0–7.6)	<0.0001
46–55	5.1 (2.7–9.6)	
56–65	1.9 (1.0–2.5)	
>65	Referent	
Underlying condition		
Asthma, regular medication	4.3 (2.7–6.8)	<0.0001
Heart disease	2.3 (1.2–4.1)	0.0083
Immunosuppression	5.5 (2.8–10.9)	<0.0001
Lung disease	6.6 (3.8–11.6)	<0.0001
Diabetes	3.8 (2.2–6.5)	<0.0001
Pregnancy	22.4 (9.2–54.5)	<0.0001
Smoking status		
Nonsmoker	Referent	
Current smoker	2.0 (1.3–3.2)	0.002
Former smoker	2.0 (1.3–3.0)	
No. significant risk factors		
0	Referent	
1	3.9 (2.6–5.8)	
2	9.3 (5.9–14.6)	<0.0001
3	20.4 (10.4–40.2)	
≥4	80.3 (10.1–638.7)	

*OR, odds ratio; CI, confidence interval.

hospitalized for pandemic (H1N1) 2009 reported 1 of the risk factors compared with 39% of controls (p <0.0001). Pregnancy was the only risk factor reported for 25% of hospitalized women of childbearing age.

In univariate analysis, compared with controls, case-patients who required mechanical ventilation were more likely to report a history of lung disease, asthma (requiring regular medication), have a higher BMI, be pregnant, have an influenza vaccination in the previous 12 months, and be 26–45 years of age (Table 3). In the logistic regression model for ventilated case-patients, a history of lung disease, diabetes, pregnancy, high BMI, or status as a current or ex-smoker were independently associated with mechanical ventilation (Table 3).

Of the 603 controls, 113 (19%) reported an influenza-like-illness during the study period. There was no significant difference in underlying risk factors between controls who reported influenza-like-illness and controls who did not report any respiratory symptoms. However, influenza-like-illness was significantly more common among controls aged 16–25 (odds ratio [OR] 3.3, 95% confidence interval [CI] 1.4–7.5, p = 0.005) and 36–45 years (OR 3.0, CI 1.4–6.2, p = 0.004) compared with controls aged >65 years.

Information on the time from onset of illness to medical attention was available for 295 (98%) case-patients. Of these, 238 (81%) reported having ≥1 risk factor listed in the public message campaigns. Overall, the proportion of case-patients seeking medical attention was similar for

Table 2. Risk factors for hospitalization caused by pandemic (H1N1) 2009 influenza for female case-patients and controls of childbearing age, Sydney, Australia, 2009*

Patient characteristic	No. (%) case-patients, n = 99	No. (%) controls, n = 181	OR (95% CI)	p value	Adjusted OR (95% CI)	p value
Age, y						
16–25	28 (28)	43 (24)	1.4 (0.8–2.6)			
26–35	37 (37)	64 (35)	1.3 (0.7–2.2)	0.5233		
36–45	34 (34)	74 (41)	Referent			
Aboriginal status						
Nonindigenous	95 (98)	177 (96)	Referent	0.3864		
Indigenous	4 (4)	4 (2)	1.9 (0.5–7.6)			
Body mass index†						
Underweight (<18.5)	8 (9)	9 (5)	2.8 (1.0–8.0)			
Normal (18.5–24.9)	31 (33)	99 (57)	Referent			
Overweight (25.0–29.9)	15 (16)	35 (20)	1.4 (0.7–2.8)	0.0003		
Obese 1 (30.0–34.9)	17 (18)	18 (10)	3.0 (1.4–6.6)			
Obese 2 (35.0–39.9)	10 (11)	4 (2)	8.0 (2.3–27.3)			
Obese 3 (>40)	12 (13)	8 (5)	4.8 (1.8–12.8)			
Health condition‡						
Asthma	46 (46)	35 (19)	Referent			
No regular medication	7 (7)	21 (12)	0.9 (0.4–2.3)	<0.001	10 (4.0–21.0)	<0.0001
Regular medication	39 (39)	14 (8)	7.7 (3.9–15.3)			
Heart disease§	0	1 (0.5)	<0.001	0.9871		
Kidney disease	0	1 (0.5)	<0.001	0.987		
Mental health problem	17 (17)	19 (11)	1.8 (0.9–3.6)	0.1139		
Neurologic problem	4 (4)	8 (4)	0.9 (0.3–3.1)	0.8809		
Immunosupression	1 (1)	2 (1)	0.9 (0.1–10.2)	0.942		
Obstructive sleep apnea	10 (10)	4 (2)	5.0 (1.5–16.3)	0.0081		
Lung disease	10 (10)	4 (2)	5.0 (1.5–16.3)	0.0081	7 (2.0–28.0)	0.0043
Diabetes	14 (14)	2 (1)	14.7 (3.3–66.3)	0.0005	20 (4.0–103.0)	0.0003
Metabolic disorder	1 (1)	2 (1)	0.9 (0.1–10.2)	0.942		
Liver disease	5 (5)	0	>999.9	0.9798		
Blood disorder	4 (4)	8 (4)	0.9 (0.3–3.1)	0.8809		
Pregnancy	40 (40)	7 (4)	16.9 (7.2–39.6)	<0.001	28 (11.0–70.0)	<0.0001
Smoking status						
Nonsmoker	53 (54)	130 (72)	Referent			
Current smoker	24 (24)	32 (18)	1.8 (1.0–3.4)	0.0057		
Former smoker	22 (22)	19 (11)	2.8 (1.4–5.7)			
Influenza vaccine in 2009¶	20 (20)	30 (17)	1.3 (0.7–2.4)	0.4262		

*OR, odds ratio; CI, confidence interval.
†8 controls and 6 case-patients missing data.
‡Groups are not mutually exclusive.
§1 control and 3 case-patients missing data.
¶1 case-patient missing data.

Table 3. Risk factors for mechanical ventilation because of pandemic (H1N1) 2009 infection, Sydney, Australia, 2009*

Patient characteristic	No. (%) case-patients, n = 37	No. (%) controls, n = 603	OR (95% CI)	p value	Adjusted OR (95% CI)	p value
Sex						
M	11 (30)	207 (34)				
F	26 (70)	396 (66)	1.2 (0.6–2.6)	0.5674		
Age, y						
16–25	3 (8)	61 (10)	3.7 (0.7–19)		5.5 (0.7–42.0)	
26–35	11 (30)	92 (15)	9.1 (2.5–33.3)		12.2 (2.5–58.2)	
36–45	12 (32)	111 (18)	8.2 (2.3–29.7)		10.4 (2.3–48.3)	
46–55	8 (22)	111 (18)	5.5 (1.4–21.0)		10.2 (2.3–45.9)	
>55	3 (8)	228 (38)		0.0114		0.0176
Aboriginal status						
Nonindigenous	36 (97)	596 (99)				
Indigenous	1 (3)	7 (1)	2.4 (0.3–19.7)	0.426		
Body mass index†						
Underweight (<18.5)	3 (9)	15 (3)	8.6 (2.0–37.9)		10.6 (1.9–58.9)	
Normal (18.5–24.9)	6 (17)	259 (45)		0.0004		0.0022
Overweight (25.0–29.9)	10 (29)	175 (30)	2.5 (0.9–6.9)		3.2 (1.0–10.3)	
Obese 1 (30.0–34.9)	5 (14)	79 (14)	2.7 (0.8–9.2)		1.8 (0.5–7.4)	
Obese 2 (35.0–39.9)	5 (14)	29 (5)	7.4 (2.1–25.9)		9.8 (2.4–40.1)	
Obese 3 (>40)	6 (17)	21 (4)	12.3 (3.7–41.6)		11.6 (2.7–49.3)	
Health condition‡						
Asthma	13 (65)	93 (15)				
No regular medication	5 (14)	48 (8)	2.2 (0.8–6.1)	0.0061		
Regular medication	8 (22)	45 (7)	3.8 (1.6–8.9)			
Heart disease§	3 (9)	41 (7)	1.3 (0.4–4.3)	0.7136		
Kidney disease¶	1 (3)	8 (1)	2.1 (0.3–16.9)	0.5013		
Mental health problem	6 (16)	49 (8)	2.2 (0.9–5.5)	0.0958		
Neurologic problem	3 (8)	42 (7)	1.2 (0.3–4.0)	0.792		
Immunosuppression	1 (3)	22 (4)	0.7 (0.1–5.6)	0.7655		
Obstructive sleep apnea	2 (5)	23 (4)	1.4 (0.3–6.4)	0.6296		
Lung disease	6 (16)	31 (5)	3.6 (1.4–9.2)	0.0084	8.6 (2.6–28.5)	0.0005
Diabetes	5 (14)	38 (6)	2.3 (0.9–6.3)	0.0977	4.4 (1.2–15.6)	0.0383
Liver disease	2 (5)	20 (3)	1.7 (0.4–7.4)	0.503		
Pregnancy	8 (22)	7 (1)	23.4 (8.0–69.2)	<0.001	40.5 (9.7–168.1)	<0.0001
Smoking status						
Nonsmoker	17 (46)	372 (62)			2.4 (1.0–5.5)	0.0325
Current or former smoker	20 (54)	231 (38)	1.9 (1.0–3.7)	0.0605		
Influenza vaccine in 2009	20 (54)	202 (34)	0.3 (0.1–0.8)	0.0163		

*OR, odds ratio; CI, confidence interval.
†Data missing for 2 case-patients and 25 controls.
‡Groups are not mutually exclusive.
§Date missing for 2 case-patients and 12 controls.
¶Data missing for 3 controls.

both those with reported risk factors and those with no risk factors (80% and 79%, respectively). There was no significant difference in individual underlying risk factors between case-patients who sought medical attention within 48 hours of symptoms and those who did not (Table 4).

Discussion

We found that pregnancy, lung disease, immune suppression, asthma, diabetes, heart disease, and a history of smoking were associated with hospitalization from pandemic (H1N1) 2009 infection. Among women of childbearing age, pregnancy was the single greatest risk factor for hospitalization, followed by diabetes, history of asthma requiring regular medication, and lung disease. Obesity was not an independent risk factor for hospitalization although it was a risk factor for mechanical ventilation. The majority of case-patients sought medical attention within 48 hours. This study did not identify any particular risk groups that were less likely to seek early medical attention.

Our study was designed to identify risk factors for moderate to severe illness resulting from influenza (as measured by requirement for hospital admission), not risk factors for acquiring influenza. Controls for the study were therefore selected from the community rather than nonhospitalized case-patients. When controls who reported

influenza-like-illness were compared with controls without these symptoms, there was no significant difference in risk factors other than age. This finding suggests that apart from age (which is likely to reflect past infection with influenza strains that protected against pandemic [H1N1] 2009) (*25*), other participant characteristics were not important for determining susceptibility for infection.

Our data are subject to several limitations. First, compared with the adult population in Sydney, controls were older and a higher proportion were women, introducing the possibility of bias if the groups were not otherwise similar. However, our analysis adjusted for age and sex, and the findings were consistent with those of similar studies examining risk factors for seasonal influenza (*3*–*5*,*6*). When the model was restricted to gender, the results were similar to the final model. These findings suggest that the potential bias from control selection on the final model was minimal. Second, risk factor status was determined by self-report for case-patients and controls. However, public messaging during the pandemic relied on

Table 4. Comparison of characteristics of patients with cases of pandemic (H1N1) 2009 influenza by time from symptom onset to medical attention, Sydney, Australia, 2009*

	Medical attention within 48 h			
Patient characteristic	Yes, no. (%) patients, n = 235	No, no. (%) patients, n = 60	OR (95% CI)	p value
Sex				
M	97 (41)	24 (40)		
F	138 (59)	36 (60)	1.1 (0.6–1.9)	0.8576
Age, y				
16–25	33 (14)	5 (8)	1.1 (0.3–4.3)	
26–35	44 (19)	9 (15)	0.8 (0.3–2.8)	
36–45	47 (20)	11 (18)	0.7 (0.2–2.3)	
46–55	50 (21)	20 (33)	0.4 (0.1–1.3)	
56–65	32 (14)	10 (17)	0.6 (0.2–1.8)	
>65	29 (12)	5 (8)		0.3677
Aboriginal status				
Nonindigenous	229 (97)	58 (97)		0.7409
Indigenous	6 (3)	2 (3)	0.8 (0.1–3.9)	
Body mass index†				
Underweight (<18.5)	13 (6)	2 (3)	1.6 (0.3–7.8)	
Normal (18.5–24.9)	65 (28)	16 (27)		0.4457
Overweight (25.0–29.9)	67 (29)	10 (17)	1.6 (0.7–3.9)	
Obese 1 (30–34.9)	38 (16)	12 (20)	0.8 (0.3–1.8)	
Obese 2 (35–39.9)	23 (10)	6 (10)	0.9 (0.3–2.7)	
Obese 3 (>40)	19 (8)	8 (13)	0.6 (0.2–1.6)	
Health condition‡				
Asthma	89 (38)	24 (40)		0.3607
No regular medication	16 (7)	9 (15)		
Regular medication	73 (31)	15 (25)	1.4 (0.7–2.6)	
Heart disease§	31 (13)	7 (12)	1.2 (0.5–2.8)	0.703
Kidney disease	8 (3)	2 (3)	1.0 (0.2–4.9)	0.9785
Mental health problem	34 (14)	17 (28)	0.4 (0.2–0.8)	0.0129
Neurologic problem	14 (6)	8 (13)	0.4 (0.2–1.0)	0.0586
Immunosuppression	22 (9)	6 (10)	0.9 (0.4–2.4)	0.8803
Obstructive sleep apnea	26 (11)	10 (17)	0.6 (0.3–1.4)	0.2399
Lung disease	46 (20)	11 (18)	1.1 (0.5–2.2)	0.828
Diabetes	48 (20)	10 (17)	1.3 (0.6–2.7)	0.5141
Liver disease	16 (7)	5 (8)	0.8 (0.3–2.3)	0.6823
Pregnancy	33 (14)	7 (12)	1.3 (0.5–3.2)	0.6008
Smoking status¶				
Nonsmoker	113 (48)	26 (43)		
Current or former smoker	121 (51)	34 (57)	0.8 (0.5–1.5)	0.493
Influenza vaccine in 2009#	86 (37)	19 (32)	1.3 (0.7–2.3)	0.4507

*OR, odds ratio; CI, confidence interval.
†Data missing for 10 case-patients who received medical attention within 48 h and 6 who did not.
‡Groups are not mutually exclusive.
§Data were missing for 6 case-patients who received medical attention within 48 h.
¶Data were missing for 1 case-patient who received medical attention within 48 h.
#Vaccination information was missing for 2 case-patients and 1 control.

the persons recognizing that they were in a high-risk group. For this reason, self-reported risk factors were thought to be a good indication of identified risk. However, undiagnosed or unacknowledged medical conditions would not have been captured in our study and could underestimate the effect of some risk factors. Third, the underlying reason for admission to hospital for patients (whether specifically caused by the infection or because of preexisting illness or both) was not determined in this study. Case-patients were selected for inclusion in the study if they met the pandemic (H1N1) 2009 case definition, were hospitalized, and had onset of symptoms >2 days before admission. Fourth, although pregnancy was identified as an independent risk factor for hospitalization, the magnitude of this risk may be biased upward if clinicians had a lower threshold for admitting pregnant women as a precaution, particularly in later stages of pregnancy. Fifth, patients who died following pandemic (H1N1) 2009 infection were excluded from the study, thereby excluding those with the most severe disease. Although patients who died were obviously unable to be interviewed, information regarding the presence of underlying medical conditions was collected from the treating clinicians. During the study period, there were 23 deaths that met the study's case definition. Similar to interviewed case-patients, 20 (87%) of the patients who died were reported to have ≥1 significant risk factor. However, a higher proportion of patients who died were reported as having lung disease (60% vs. 19%), being a current smoker (35% vs. 22%), and having immune suppression (25% vs. 10%) when compared with study participants. Deceased patients were significantly older (median 59 years, range 23–85 years) compared with surviving patients (median 45 years, range 16–88 years) included in the study ($p<0.001$).

Descriptive studies of pandemic (H1N1) 2009 infection have reported obesity, heart disease, diabetes, pregnancy (*26–28*), kidney disease, neurologic disease, immune suppression, lung disease, asthma, smoking, and relatively young age (*29*,*30*) as the most common concurrent conditions for hospitalized patients (*11–20*). Given that many of these underlying medical conditions do not occur in isolation, our analytical study was able to ascertain which of these were independently associated with hospitalization from pandemic (H1N1) 2009.

Although high rates of pandemic (H1N1) 2009 infection have been reported from indigenous people in other reports (*31*), our study lacked sufficient power to explore the independent impact of being Aboriginal on the risk factor of moderate to severe disease. However, among those Aboriginal case-patients included, all reported a history of other independent risk factors. These data may suggest that it is the high prevalence of risk factors for severe disease that place Aboriginal people at an increased risk rather than genetic susceptibility.

BMI was not independently associated with increased risk for hospitalization with pandemic (H1N1) 2009. Obesity appears to be a confounder of other risk factors for overweight patients. Of the 58 case-patients with BMI >35 (very obese), 55 (95%) reported ≥1 significant risk factor, including smoking (35/55, 64%), asthma (32/55, 58%), and diabetes (18/55, 33%). Further analysis suggested that obesity was independently associated with increased risk of ventilation in our study. Of the 35 ventilated case-patients, 11 reported a BMI >35, and all but 2 patients reported other significant risk factors.

Our study highlights the increased risk of moderate to severe illness from pandemic (H1N1) 2009 for pregnant women and introduces smoking as an independent risk factor for hospitalization from pandemic (H1N1) 2009. In addition, our study provides evidence to support the continuation of influenza prevention efforts (including vaccination) targeted to persons with lung disease, immune suppression, asthma, diabetes, and heart disease. Although Aboriginal status and obesity may not be independent risk factors for severe disease, they indicate the likely presence of other risk factors, and so prevention messages should continue to be directed to these groups.

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Novel Arenavirus Infection in Humans, United States

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Immunoglobulin G against Whitewater Arroyo virus or lymphocytic choriomeningitis virus was found in 41 (3.5%) of 1,185 persons in the United States who had acute central nervous system disease or undifferentiated febrile illnesses. The results of analyses of antibody titers in paired serum samples suggest that a North American Tacaribe serocomplex virus was the causative agent of the illnesses in 2 persons and that lymphocytic choriomeningitis virus was the causative agent of the illnesses in 3 other antibody-positive persons in this study. The results of this study suggest that Tacaribe serocomplex viruses native to North America, as well as lymphocytic choriomeningitis virus, are causative agents of human disease in the United States.

The arenaviruses (family *Arenaviridae*, genus *Arenavirus*) known to occur in North America include Whitewater Arroyo virus (WWAV), 7 other members of the Tacaribe serocomplex (Table 1), and lymphocytic choriomeningitis virus (LCMV, the prototypic member of the lymphocytic choriomeningitis–Lassa serocomplex). Specific members of the order Rodentia are the principal hosts of the arenaviruses, for which natural host relationships have been well characterized. For example, the hispid cotton rat (*Sigmodon hispidus*) in Florida is the principal host of Tamiami virus (*6*,*7*), and the ubiquitous house mouse (*Mus musculus*) is the principal host of LCMV (*9*).

Five South American members of the Tacaribe serocomplex, LCMV, and Lassa virus are etiologic agents of severe febrile illnesses in humans (*10*,*11*). The human health significance of the North American Tacaribe serocomplex viruses has not been rigorously investigated (*12*).

Studies since the mid-1990s have shown that Tacaribe serocomplex viruses are widely distributed in the United States and Mexico and that woodrats (*Neotoma* spp.) and other members of the family Cricetidae are natural hosts of these viruses (*1–5*,*8*,*13*,*14*). The purpose of this study was to investigate whether humans have been infected with North American Tacaribe serocomplex viruses.

Materials and Methods

Samples of serum (n = 1,305), plasma (n = 2), and cerebrospinal fluid (n = 70) from 1,185 persons in the United States with acute central nervous system disease or undifferentiated febrile illnesses were tested for immunoglobulin (Ig) G against the WWAV prototype strain AV 9310135 and LCMV strain Armstrong by using an ELISA as described (*15*). The samples were diagnostic specimens submitted to the Arbovirus Diseases Branch, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention (CDC) (Fort Collins, CO, USA) during 1989–2000 by public health laboratories in the United States. The samples had been tested selectively by CDC laboratorians for evidence of infection with St. Louis encephalitis virus, western equine encephalomyelitis virus, and other arthropod-borne agents of human disease. These tests had not yielded a specific diagnosis for any of the cases in this study.

Information about each case was limited to patient age, sex, date of illness onset, and state from which the samples were submitted. Most (634 [53.5%]) of the 1,185 case-patients were male. Ages at illness onset ranged from 0.2 months to 93 years (median 35 years), and 982 (82.0%) of the case-patients were ≥10 years of age at illness onset.

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Table 1. Natural hosts and geographic distribution of the North American Tacaribe serocomplex viruses

Virus	Natural host(s)	Location	Reference
Bear Canyon	Large-eared woodrat (*Neotoma macrotis*), California mouse (*Peromyscus californicus*)	California, USA	(*1*)
Big Brushy Tank	White-throated woodrat (*N. albigula*)	Arizona, USA	(*2*)
Catarina	Southern plains woodrat (*N. micropus*)	Texas, USA	(*3*)
Rio Catorce	White-toothed woodrat (*N. leucodon*)	San Luis Potosí, Mexico	(*4*)
Skinner Tank	Mexican woodrat (*N. mexicana*)	Arizona, USA	(*5*)
Tamiami	Hispid cotton rat (*Sigmodon hispidus*)	Florida, USA	(*6,7*)
Tonto Creek	White-throated woodrat (*N. albigula*)	Arizona, USA	(*2*)
Whitewater Arroyo	White-throated woodrat (*N. albigula*)	New Mexico, USA	(*8*)

The period between illness onset and sample collection ranged from 0 days to 10.1 years (median 31 days). At least 1 sample from each of 580 case-patients was collected before the end of week 4 of illness; for 108 case-patients multiple samples, representing different time points, were available. Cases were geographically distributed as follows: New England, 72 cases; Mid-Atlantic, 50; South Atlantic, 141; East North Central, 96; West North Central, 73; East South Central, 78; West South Central, 42; Mountain, 177; Pacific, 96; and unknown, 360.

A 1:80 dilution and 1:320 dilution of each sample was tested against the WWAV antigen, LCMV antigen, and corresponding comparison (negative-control) antigens. The adjusted optical density (AOD) of a sample-antigen reaction was the optical density of the well coated with the test antigen minus the optical density of the well coated with the corresponding control antigen. A sample was considered positive if the AOD at 1:80 was ≥0.250, the AOD at 1:320 was ≥0.250, and the sum of the AOD at 1:80 and AOD at 1:320 was ≥0.750. Endpoint titers against each antigen were measured in the positive samples by using serial 2-fold dilutions from 1:320 through 1:40,960. The antibody titer of a positive sample was the reciprocal of the highest dilution for which the AOD was ≥0.250. Titers <320 were 160 in comparisons of titers to WWAV and LCMV in individual samples. The apparent homologous virus in an antibody-positive sample was the virus associated with the highest titer if the absolute value of the difference between the titers to WWAV and LCMV was ≥4-fold.

Results

We detected antibody against an arenavirus in 41 (3.5%) of the 1,185 case-patients. Of the antibody-positive case-patients, most (27 [65.9%]) were male. Ages ranged from 4 years to 85 years (median 39 years). Antibody-positive samples were submitted from Florida, Massachusetts, and Wyoming (3 samples each) and Arizona, Idaho, Kansas, Maryland, Michigan New Mexico, New York, North Carolina, Ohio, Rhode Island, Tennessee, Washington, and Wisconsin (1 sample each). For 19 samples, state of submission was unknown.

Twelve persons had positive test results for WWAV but not LCMV; 28 for LCMV but not WWAV; and 1 for WWAV and LCMV (Table 2). In the positive samples, endpoint titers against WWAV and LCMV ranged from <320 to 10,240 and from <320 to 20,480, respectively. The apparent homologous virus was WWAV in 10, LCMV in 24, and indeterminate in 7 of antibody-positive persons (Table 2).

Ages of the 10 persons in whom WWAV was the apparent homologous virus ranged from 5 to 70 years (median 43 years). Samples from these persons were submitted from Arizona, New Mexico, and North Carolina (1 sample each) and Florida and Wyoming (2 samples each); for 3 samples, state of submission was unknown.

The ELISA included paired samples from 8 antibody-positive persons. Time from onset of illness to the first samples from these persons ranged from 0 to 47 days. In side-by-side tests, the endpoint titer to WWAV in the second sample was ≥4-fold higher than that to WWAV in the first sample in paired samples from 2 persons, and the endpoint titer to LCMV in the second sample was ≥4-fold higher than that to LCMV in the first sample in paired samples from 3 of the 6 other antibody-positive persons (Table 3).

Table 2. Antibody (immunoglobulin G) titers against WWAV and LCMV in 1,185 cases of acute central nervous system disease or undifferentiated febrile illnesses, United States*

No. cases	Antibody titer		Apparent homologous virus
	WWAV	LCMV	
5	640	<320	WWAV
1	1,280	<320	WWAV
3	2,560	<320	WWAV
1	10,240	<320	WWAV
7	<320	640	LCMV
3	<320	1,280	LCMV
5	<320	2,560	LCMV
4	<320	5,120	LCMV
2	<320	10,240	LCMV
3	<320	20,480	LCMV
2	320	<320	Indeterminate
1	640	1,280	Indeterminate
4	<320	320	Indeterminate
1,144	<320	<320	None

*WWAV, Whitewater Arroyo virus; LCMV, lymphocytic choriomeningitis virus.

Table 3. Antibody (immunoglobulin G) against WWAV and LCMV in paired serum samples from humans with acute central nervous system disease or undifferentiated febrile illnesses, United States*

Case-patient no.	Age, y, at illness onset	Days after illness onset		Antibody titer, WWAV		Antibody titer, LCMV		Apparent homologous virus
		S1	S2	S1	S2	S1	S2	
1	32	14	44	<320	640	<320	<320	WWAV
2	65	15	61	<320	2,560	<320	<320	WWAV
3	38	14	33	<320	<320	5,120	5,120	LCMV
4	51	2	68	<320	<320	320	20,480	LCMV
5	59	24	38	<320	<320	320	5,120	LCMV
6	72	0	15	<320	<320	<320	640	LCMV
7	12	25	33	<320	<320	320	320	Indeterminate
8	25	47	123	<320	<320	320	320	Indeterminate

*WWAV, Whitewater Arroyo virus; LCMV, lymphocytic choriomeningitis virus; S1, first sample; S2, second (last) sample in paired samples.

Discussion

Previously, antibody to Tamiami virus was found in 5 (3.8%) of 131 Seminole Indians sampled in southern Florida (*16*), and antibody to a Tacaribe serocomplex virus was found in 2 (0.24%) of 829 persons who had worked with cricetid rodents in North America (*15*,*17*). The results of our current study strengthen the notion that Tacaribe serocomplex viruses enzootic in North America are infectious in humans. The increase in antibody titer against WWAV in cases 1 and 2 in this study (Table 3) suggests that a North American Tacaribe serocomplex virus caused the illnesses in these persons.

The WWAV strain AV 9310135 was originally isolated from a white-throated woodrat (*N. albigula*) captured in northwestern New Mexico (*8*). A recent study demonstrated a high level of diversity among the amino acid sequences of the structural proteins of the North American Tacaribe serocomplex viruses (*5*). Hypothetically, human IgG against some North American Tacaribe serocomplex viruses is not strongly reactive against WWAV in ELISA. If so, the prevalence of antibody to Tacaribe serocomplex viruses in this study actually might be >3.5%.

The severity of human disease caused by LCMV ranges from mild febrile illness to severe encephalitis and disseminated disease (*18*). The results of this study suggest that the illnesses in case-patients 4–6 (Table 3) were caused by LCMV. Whether samples from these 3 persons were tested for anti-LCMV antibody (IgM or IgG) by clinical laboratories could not be determined from records maintained at CDC.

Specimens from 33 of the antibody-positive persons in this study were limited to single specimens. Perhaps these illnesses were caused by a North American Tacaribe serocomplex or by LCMV. The antibody titer to WWAV in the antibody-positive person from New Mexico was 10,240 in a serum sample collected on day 22 day after illness onset.

Future studies on the relevance to human health of the North American Tacaribe serocomplex viruses should include defining the clinical spectrum and epidemiology of human disease caused by these viruses. Some of these viruses may cause aseptic meningitis, encephalitis, or meningoencephalitis. Thus, human disease caused by North American Tacaribe serocomplex viruses may be confused with severe encephalitis caused by LCMV, especially in persons who report recent exposure to rodents.

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Cost-effectiveness of Sick Leave Policies for Health Care Workers with Influenza-like Illness, Brazil, 2009

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We describe the effect of influenza-like illness (ILI) during the outbreak of pandemic (H1N1) 2009 on health care worker (HCW) absenteeism and compare the effectiveness and cost of 2 sick leave policies for HCWs with suspected influenza. We assessed initial 2-day sick leaves plus reassessment until the HCW was asymptomatic (2-day + reassessment policy), and initial 7-day sick leaves (7-day policy). Sick leaves peaked in August 2009: 3% of the workforce received leave for ILI. Costs during May–October reached R$798,051.87 (≈US $443,362). The 7-day policy led to a higher monthly rate of sick leave days per 100 HCWs than did the 2-day + reassessment policy (8.72 vs. 3.47 days/100 HCWs; p<0.0001) and resulted in higher costs (US $609 vs. US $1,128 per HCW on leave). ILI affected HCW absenteeism. The 7-day policy was more costly and not more effective in preventing transmission to patients than the 2-day + reassessment policy.

During mid-April 2009, Mexico reported 1,918 cases of influenza-like illness (ILI) and 84 deaths. In July 2009, the World Health Organization (WHO) declared an influenza pandemic on the basis of widespread pandemic influenza A (H1N1) 2009 observed globally (*1*). On July 16, the Brazilian Ministry of Health notified transmission within the country and declared the epidemic to be widespread (*2*). During the 2009–10 season, pandemic (H1N1) 2009 was the main contributor to influenza infections.

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In 2009, WHO reported 12,799 deaths from pandemic (H1N1) 2009. South America was affected during the winter season (June–September). Brazil reported 48,978 confirmed cases of pandemic (H1N1) 2009, with 2,051 deaths (*3*). In São Paulo, the most populated state in the country (≈40 million inhabitants), the reported incidence was higher than anywhere else in the country (15.17 cases/100,000 inhabitants); 479 persons died (*4*).

Hospital das Clínicas (HC), the largest hospital in Brazil, was assigned by the State Health Department as 1 of the reference hospitals for persons with severe pandemic (H1N1) 2009 in the city of São Paulo. During the pandemic, specific sick leave policies were instituted at HC for health care workers (HCWs) who had influenza.

Considerable concern exists among HCWs about the risks of working during an influenza epidemic. Although they feel responsible to care for patients, they also are concerned about their own and their families' health (*5*,*6*). Transmission of influenza from HCWs to patients under their care is also a concern (*7*). Isolation precautions needed to prevent transmission of pandemic (H1N1) 2009 virus were heavily debated, and recommendations from the US Centers for Disease Control and Prevention (Atlanta, GA, USA) and WHO conflicted (*8*). Guidance on appropriate sick leave policies to avoid transmission from HCWs varies and is not well established (*9*).

Interim guidelines for protecting HCWs from pandemic (H1N1) 2009 (*9*) suggest that HCWs in whom fever and respiratory symptoms develop should be excluded from work for at least 24 hours after defervescence. HCWs caring for severely immunocompromised patients should be reassigned or excluded from work for 7 days after symptom onset or until resolution of symptoms, whichever is longer.

Workforces at large tertiary care hospitals functioning as reference hospitals for persons with influenza may be substantially affected during pandemics, particularly in regard to absenteeism and associated costs. Effectiveness and costs of sick leave policies should be evaluated to guide hospital managers and public health officials, particularly during epidemics and pandemics.

The objectives of this study were to describe the effects of ILI during the pandemic (H1N1) 2009 outbreak on HCW absenteeism and the associated costs. Furthermore, we aimed to compare effectiveness and cost of 2 policies for HCW sick leave during the first wave of pandemic (H1N1) 2009 in a large urban tertiary care hospital.

Methods

Location and Setting

The study was conducted at HC, in the city of São Paulo, São Paulo state, Brazil. São Paulo is among the largest cities in Latin America, with 11 million inhabitants. HC, a tertiary care teaching hospital complex affiliated with the University of São Paulo, is a government hospital predominantly for patients covered by the publicly funded Brazilian National Health Service. During the outbreak of pandemic (H1N1) 2009 in Brazil, HC was assigned by the State of São Paulo Health Department as a state reference hospital for persons with severe pandemic (H1N1) 2009.

HC has ≈2,000 beds distributed in 7 institutes. The Central Institute (main building) has 894 beds, including 100 intensive care unit (ICU) beds. The other centers are the Heart Institute (444 beds); Orthopedics Institute (152 beds); Children's Institute (174 beds); Psychiatry and Neurosurgery Institute (90 beds); Cancer Institute (213 beds); and Radiology Institute, without inpatients, which serves all other institutes. Although also part of the HC complex, the administration building, 2 long-term care facilities (234 beds total), and 1 outpatient rehabilitation center were not included in this study.

Influenza Triage, Diagnosis, and Treatment

As recommended by international guidelines, triage for suspected influenza cases was put in place at all of HC´s institutes. Triage occurred in the existing emergency departments in the Central, Heart, and Children's Institutes. Persons with confirmed influenza were then referred to designated units within HC for hospitalization. Included in the Central Institute were an ICU (7 beds), an influenza-specific ward (11 beds for semi-intensive care), and a specific ward for pregnant influenza patients (15 beds). In the Children's Institute, an ICU (2 beds) , a ward (14 beds), and 2 rooms at the emergency department were assigned. Later into the influenza season (August 2009), 2 additional influenza-specific wards were designated in the Heart Institute, with 16 rooms and 32 beds. Although persons with influenza should have been hospitalized preferentially in the above-designated units, a few patients in whom influenza was only suspected after admission and who could not be transferred were hospitalized in nondesignated units.

Laboratory testing for confirmatory diagnosis was conducted for all hospitalized persons suspected to have influenza. Rapid diagnostic tests for influenza were not performed during the pandemic. Before July 2, 2009, samples were collected and sent to the Adolfo Lutz Reference Laboratory, where real-time PCR (rt-PCR) testing was conducted. By July 2, 2009, HC Central Hospital Laboratory started the influenza A (H1N1) rt-PCR. Although available for patients admitted to the hospital, laboratory confirmation of pandemic (H1N1) 2009 infection was not available for HCWs. Thus, suspected cases among HCWs were triaged according to the attending physician's evaluation. The rt-PCR for suspected influenza in HCWs was made available only on August 24, 2009. The PCR protocol in place was developed by the US Centers for Disease Control and Prevention (*10*).

During the entire pandemic period, active surveillance for nosocomial pandemic (H1N1) 2009 was performed by the infection control teams. A suspected influenza case was defined, according to national guidelines (*11*), as fever and respiratory symptoms such as cough or sore throat in the absence of other diagnoses, and confirmed cases were defined as those with positive test results for pandemic (H1N1) 2009.

Infection Control Policies

Following global and national guidelines (*8*,*12*), infection control policies for prevention and control of nosocomial transmission of influenza were in place in the hospital. These included contact and droplet precautions taken by HCWs during the care of patients with suspected and confirmed influenza and use of N95 masks only during aerosol-generating procedures.

HC has a centralized occupational health service for workers of all institutes. We assessed ILI in and ILI-associated sick leave for HCWs who are overseen by the hospital's occupational health service, i.e., physicians; nurses and nurse assistants; and pharmacy, nutrition, laboratory, and administration workers. Cleaning and security services are furnished by third-party service providers; therefore, their staff are not overseen by HC's occupational health service.

Starting on July 16, 2009, in HC institutes except the Heart Institute, an HCW with suspected influenza received an initial 2-day leave and was reassessed every 2 days until asymptomatic, when he or she returned to work (2-day leave + reassessment policy). In the Heart Institute, a

different sick leave policy was adopted in which an HCW with suspected pandemic (H1N1) 2009 infection received a 7-day leave (7-day leave policy). During the prepandemic period, sick leave for respiratory infection was determined on a case-by-case basis after evaluation by a physician (Figure).

Study Design

We retrospectively evaluated the effect of ILI on HCW absenteeism during the pandemic (H1N1) 2009 outbreak. The main outcome of interest was HCW absenteeism.

We estimated the economic effects of HCW-associated illness from the hospital´s perspective. Costs were estimated retrospectively. Among the various techniques for measuring costs, 1 of the most commonly used is the accounting approach (the conventional costing method). This approach can be divided into 2 categories: the first uses detailed, bottom-up, step-down analyses of accounting to distribute shared costs across the activities considered (also called ingredients approach). The second uses a top-down approach, which makes less detailed estimates of high-level average costs on the basis of aggregate expenditure records. In this study, we used the bottom-up approach as we considered individual ingredients to estimate costs. We compared the effectiveness and cost of the 2 sick leave policies: initial 2-day + reassessment policy and 7-day.

Study Period

The study was conducted during the first wave of pandemic (H1N1) 2009 in the Southern Hemisphere winter season (May–October 2009). The prepandemic period was defined as August 2008–April 2009. HCW was defined as a professional on the hospital's payroll, even if the worker did not work directly with patients. This definition excluded students and residents/fellows, as well as workers of third-party service providers (cleaning and security).

Data Analysis

We calculated the monthly number, average duration, and rate of influenza sick leaves issued in HC by institute and HCW profession. We determined the proportion of sick leaves taken for ILI in relation to the total number of sick leaves during the pandemic (H1N1) 2009 outbreak (May–October). Rate of influenza-associated sick leaves issued during the pandemic (H1N1) 2009 outbreak (August–October 2009) were compared with the prepandemic period (August–October 2008).

To assess the effectiveness of the 2 sick leave policies, we evaluated the number of nosocomial pandemic (H1N1) 2009 cases. We calculated the total number of sick leaves issued, their average duration, and total days of ILI-associated sick leave for HCWs working under the 7-day and 2-day + reassessment policies during the pandemic period. In addition, monthly rates of ILI-associated sick leave days per 100 HCWs were compared for the 2 sick leave policies by using χ^2.

Cost Analysis

The cost analysis considered only ILI and absenteeism in physicians, nurses, and nurse assistants because of the availability of information. Direct medical costs of diagnosis, treatment, and follow-up for HCWs with suspected and confirmed pandemic (H1N1) 2009 were estimated. Direct medical costs also included the cost of replacement HCWs during sick leave. Direct nonmedical costs assessed were transportation of symptomatic HCWs to and from HC for assessment. Indirect costs comprised productivity losses of HCWs during sick leave. Costs for outpatient treatment of HCWs suspected to have influenza comprised medical assessment by the occupational health clinic, specimen collection and rt-PCR (only after August 24, 2009) for laboratory diagnosis, and treatment.

Figure. Schematic representation of the sequence of events that occurred each time symptoms consistent with influenza developed in a health care worker during the pandemic (H1N1) 2009 outbreak, Hospital das Clínicas, São Paulo, Brazil, May–October, 2009.

We assumed that 100% of HCWs with suspected influenza received antiviral medication (oseltamivir) according to the official hospital guideline. Twenty percent received antimicrobial drugs (amoxicillin) for secondary bacterial infection, and 70% received symptomatic medication.

HCW hospitalization costs associated with pandemic (H1N1) 2009 were estimated by considering the average

Table 1. Evaluation of the effect of ILI-associated absenteeism among HCWs during the pandemic (H1N1) 2009 outbreak, Hospital das Clínicas, São Paulo, Brazil, May–October 2009*

Input	Value	Data source	Assumptions		
			Baseline cost	Lowest cost scenario	Highest cost scenario
No. physicians, nurses, and nurse assistants receiving ILI-associated sick leave	415 for 2-d + reassessment; 169 for 7-d	Centralized occupational health database	NA	NA	NA
Costs					
Medical consultation	R$7.90/ consultation	National Health Care System	1.1 consultations/ HCW for 7-d; 2.5/HCW for 2-d + reassessment	1 consultation/HCW for 7-d; 1.5/HCW for 2-d + reassessment	2.5 consultations/HCW for 7-d; 4/HCW for 2-d + reassessment
Transportation for consultation	R$10.00/round trip	Public transport fare	10% returned for consultation for 7-d 1.5 returns/HCW for 2-d + reassessment	No return visits for 7-d; 0.5 returns/HCW for 2-d + reassessment	1.5 returns for consultations for 7-d; 3 return visits for 2-d + reassessment
Oseltamivir treatment	R$112.40/ treatment	Central pharmacy	Received by 100% of HCWs with suspected influenza	Received by 100% of HCWs with suspected influenza	Received by 100% of HCWs with suspected influenza
Amoxicillin treatment	R$2.40/ treatment	Central pharmacy	Received by 20% of HCWs with suspected influenza.	Received by 5% of HCWs with suspected influenza	Received by 60% of HCWs with suspected influenza
Medication for symptoms	R$2.95/ treatment	Central pharmacy	Received by 70% of HCWs with suspected influenza	Received by 20% of HCWs with suspected influenza	Received by 100% of HCWs with suspected influenza
Diagnostic rt-PCR†	R$100.00/test	Central laboratory	NA	NA	NA
Swab for collecting specimen for rt-PCR	R$0.46/test	Central laboratory	NA	NA	NA
No. HCWs undergoing diagnostic rt-PCR†	244	Central laboratory	NA	NA	NA
No. HCWs hospitalized†	None	Nucleus of Information on Health Care	NA	NA	NA
No. d hospitalization of HCWs†	30 d for 2-d + reassessment; 4 d for 7-d	Direct review of patient records	NA	NA	NA
Daily cost					
Hospitalization	R$1,196.39 for 2-d + reassessment; R$1,871.06 for 7-d	Administration			
Staff replacement	Nurse: R$257.07; nurse assistant: R$167.09; physician: R$858.51	Human resource department	70% of nurses, 70% of nurse assistants, 50% of physicians replaced	30% of nurses, 30% of nurse assistants, 10% of physicians replaced	100% of nurses, 100% of nurse assistants, 80% of physicians replaced
Productivity losses	Nurse: R$307.15; nurse assistant: R$157.18; physician: R$381.23	Human resource department	NA	NA	NA

*ILI, influenza-like illness; HCW, health care worker; 2-d + reassessment, 2-day sick leave plus reassessment every 2 days until asymptomatic policy; 7-d, 7-day sick leave policy; NA, not applicable; R$, Brazilian reals (R$1.80 = US $1.00); rt-PCR, real-time PCR.
†For pandemic (H1N1) 2009.

daily cost of hospitalization in HC. No information about complications or secondary bacterial infection in hospitalized HCWs with influenza was available for the cost analysis.

When evaluating cost of staff replacement, we assumed that 70% of nurses and nurse assistants and 50% of physicians on sick leave were replaced. We also assumed that HCWs under the 2-day + reassessment policy would return on average 1.5 times for reassessment, thus incurring medical consultations and transportation costs for 1.5 return visits to HC. Under the 7-day policy, we assumed that 10% of HCWs would return for medical consultations during leave; thus, only 10% of HCWs required transportation for reassessment.

We estimated productivity losses by considering the daily 12-hour average salaries for each HCW category based on an average of 5 years of work in HC. Wages and mandatory Social Security and health care contributions were considered. Monthly salary was Brazilian real (R$) 3,071.54 for nurses working 30 h/week; R$2,541.55 for physicians working 20 h/week; and R$1,571.80 for nurse assistants working 30 h/week. One US dollar was equivalent to ≈R$1.80.

We also recalculated the costs for each sick leave policy on the basis of a lowest cost scenario and a highest cost scenario. For the lowest cost scenario, we assumed that HCWs on leave would have returned on average 0.5 times for reevaluation if under the 2-day + reassessment policy and would not have returned for the 7-day policy. We also assumed that 5% of HCWs received antimicrobial drugs and 20% received treatment for symptoms. Staff replacement was assumed to have been 30% for nurses and nurse assistants and 10% for physicians. For the highest cost scenario, we assumed that each HCW had 3 return consultations for the 2-day + reassessment policy and 1.5 return visits for the 7-day policy, that 60% of HCWs received antimicrobial drugs, and that 100% received treatment for symptoms. Staff replacement in the highest cost scenario was assumed to be 100% for nurses and nurse assistants and 80% for physicians.

Data Sources

The inputs, values, and data sources are presented in Table 1. HC maintains a centralized occupational health database in which sick leave of HCWs since August 2008 is registered by International Statistical Classification of Diseases and Related Health Problems (*13*). The number of HCWs placed on sick leave during August 2008–October 2009 because of diseases of the respiratory system reported as codes J10 (Influenza due to other identified influenza virus) and J11 (Influenza, virus not identified), by using the International Statistical Classification of Diseases and Related Health Problems, 10th Revision, was evaluated (*13*). Cost data were obtained from the hospital administration.

Results

During June–September 2009, a total of 796 persons with suspected influenza were hospitalized at HC, for which 214 infections were laboratory confirmed as pandemic (H1N1) 2009 (*2*). In July 2009, HCW sick leaves began to increase and peaked in August, when 3% of the workforce received leave for ILI (Table 2). HCWs received 884 ILI-associated sick leaves during August–October 2009, compared with 96 during the same period in 2008 ($p<0.00001$).

Table 2. Total and ILI-associated monthly number, duration, and rates of sick leave by HCWs, Hospital das Clínicas, São Paulo, Brazil, August 2008–October 2009*

Date	Total no. HCWs	ILI-associated			No. sick leaves from all causes	% Sick leaves from ILI
		No. sick leaves	Average duration, d	Days/100 HCWs		
2008						
Aug	17,890	27	1.63	0.25	1,609	1.7
Sep	16,243	33	1.61	0.33	1,931	1.7
Oct	18,064	36	1.36	0.27	1,741	2.1
Nov	18,294	40	1.90	0.42	1,567	2.6
Dec	18,370	47	1.43	0.29	1,555	2.4
2009						
Jan	18,500	31	1.65	0.03	1,586	2.0
Feb	18,697	18	2.11	0.20	1,444	1.2
Mar	18,426	37	1.78	0.36	1,982	1.9
Apr	17,804	30	1.47	0.25	1,815	1.7
May	17,994	40	1.35	0.30	1,884	2.1
Jun	18,102	79	1.66	0.72	1,899	4.2
Jul	18,216	279	3.36	5.14	2,306	12.1
Aug	18,400	548	4.23	12.60	2,716	20.2
Sep	18,544	240	3.37	4.36	2,180	11.0
Oct	18,476	96	2.15	1.11	1,887	5.1

*ILI, influenza-like illness; HCW, health care worker. Includes entire hospital complex.

Table 3. ILI-associated sick leaves for HCWs during pandemic (H1N1) 2009 outbreak, by hospital institute, Hospital das Clínicas, São Paulo, Brazil, May–October, 2009*

Hospital institute†	No. sick leaves	Average duration of sick leave, d	Monthly average no. HCWs in institute	Monthly average no. d of sick leave day/100 HCWs‡	No. hospitalized patients with confirmed pandemic (H1N1) 2009 influenza
Heart	357	5.14	3,507	8.72	34
All other	776	2.89	10,760	3.47	186
Central	436	2.81	5,874	3.48	94
Children's	65	3.15	1,241	2.75	78
Orthopedics	50	2.18	959	1.89	1
Cancer	145	3.47	1,474	5.69	12
Psychiatry/neurosurgery	42	1.98	679	2.04	1
Radiology	38	3.08	533	3.66	§

*ILI, influenza-like illness; HCW, health care worker.
†Excludes the 2 long-term care facilities, the outpatient rehabilitation institute, and the administration building.
‡p<0.0001 comparison between Heart Institute and other Institutes.
§The Radiology Institute does not contain inpatient beds.

Of 244 HCWs tested for pandemic (H1N1) 2009, a total of 52 (21%) received positive results. The mean monthly rate of influenza sick leaves per 100 HCWs was significantly higher in the Heart Institute, which had a different sick-leave policy from the other institutes (p<0.0001) (Table 3). The distribution of ILI-associated sick leave of HCWs varied by professional category (Table 4).

Three HCWs were hospitalized in HC because of pandemic (H1N1) 2009, resulting in a total of 34 days of hospitalization. One HCW was hospitalized in a private hospital; related costs were covered by private medical insurance and thus not included in our analysis. Total cost in all HC institutes was R$798,051.87 (≈US $443,362). At the Heart Institute (7-day sick leave policy), 169 staff (physicians, nurses, and nurse assistants) received sick leave because of ILI, resulting in a total cost of R$343,082.94 (≈US $190,602). At the remaining 6 institutes (2-day + reassessment policy), a total of 415 staff (physicians, nurses and nurse assistants) received ILI-associated sick leaves, resulting in a total cost of R$454,968.92 (≈US $252,761) (Table 5). Thus, for each HCW on leave, cost was R$1,096.31 (≈US $609.06) for the 2-day + reassessment policy and R$2,030.08 (≈US $1,127.82) for the 7-day policy (Table 6).

In the lowest cost scenario, total cost was R$617,135.45 (≈US $342,853.02). In the highest cost scenario, total cost was R$942,588.49 (≈US $523,660.27).

During the study period, active surveillance was conducted for hospital-acquired pandemic (H1N1) 2009 infections. No cases of influenza were documented or suspected in patients.

Discussion

Pandemic (H1N1) 2009 substantially affected HCW absenteeism with significantly higher ILI-associated sick leave during August–October 2009 than during the same period in 2008. The 2-day + reassessment policy was less costly (approximately half the cost per HCW) and as effective for preventing transmission of pandemic (H1N1)

Table 4. ILI-associated sick leaves for HCWs during the pandemic (H1N1) 2009 outbreak, by professional category, Hospital das Clínicas, São Paulo, Brazil, May–October, 2009*

Professional category†	No. ILI-associated sick leaves	Average leave duration, d	Average no. HCWs working in hospital	Total no. sick leave days/total no. HCWs/100 HCWs
Physician	32	5.44	2,284	1.27
Nurse	147	3.88	1,113	8.55
Nurse assistant	416	3.28	3,235	7.03
Nurse technician	72	3.76	520	8.69
Physiotherapist	19	3.79	240	5.00
Nutrition assistant	61	2.52	437	5.87
Laboratory technician	28	4.14	398	4.86
Pharmacy or ECG technician	27	2.19	266	3.70
Janitor, doorman, telephone or elevator operator, etc.	144	2.24	1,339	4.01
Specialized maintenance, e.g., painter, driver, mechanic, plumber, electrician	21	2.95	293	3.53
Administrative officer	97	2.79	1,200	3.76
Administrative assistant	19	4.21	282	4.73

*ILI, influenza-like illness; HCW, health care worker; ECG, electrocardiogram.
†Does not include all professional categories.

Table 5. Costs associated with HCW absenteeism during the pandemic (H1N1) 2009 outbreak, by type of sick leave policy, Hospital das Clínicas, São Paulo, Brazil, May–October, 2009*

	2-d + reassessment, n = 415 HCWs			7-d, n = 169 HCWs		
Cost category	Unit cost, R$	No. units	Cost, R$	Unit cost, R$	No. units	Cost, R$
Direct costs						
Diagnosis						
Medical consultation	7.90	1,037.50	8,196.25	7.90	185.90	1,468.61
Real-time PCR	100.00	184.00	18,400.00	100.00	60.00	6,000.00
Respiratory swab	0.46	184.00	84.64	0.46	60.00	27.60
Outpatient care						
Oseltamivir	112.40	415	46,646.00	112.40	169	18,995.60
Antimicrobial drugs	2.40	83	199.20	2.40	34	81.60
Medication for symptoms	2.95	291	858.45	2.95	118	348.10
Hospitalization, d	1,196.39	30.00	35,891.70	1,871.06	4.00	7,484.24
Staff replacement, d						
Physician	858.51	50.50	43,354.76	858.51	17.50	15,023.93
Nurse	257.07	131.60	33,830.41	257.07	166.60	42,827.86
Nurse assistant	167.09	421.40	70,411.73	167.09	419.30	70,060.84
Nonmedical	10.00	1.50	6,225.00	10.00	0.10	169.00
Indirect costs						
Productivity losses, d						
Physician	381.23	101.00	38,504.23	381.23	35.00	13,343.05
Nurse	307.15	188.00	57,744.20	307.15	238.00	73,101.70
Nurse assistant	157.18	602.00	94,622.36	157.18	599.00	94,150.82
Total			454,968.92			343,082.94

*HCW, health care worker; 2-d + reassessment, 2-day sick leave plus reassessment every 2 days until asymptomatic policy; 7-d, 7-day sick leave policy; R$, Brazilian reals (R$1.80 = US $1.00).
†Round-trip bus fare for reassessment.

2009 as the 7-day policy because no cases of nosocomial acquisition occurred among patients.

The effect on society of influenza leading to absenteeism is well documented. A systematic review of studies in this field showed a loss of 1.5–4.9 workdays per episode of laboratory-confirmed influenza (*14*), but this review did not focus on HCWs. The impact of influenza epidemics on sickness-associated absence in hospitals is difficult to define. One study involving a hospital in the United Kingdom during 2 influenza seasons (1993–94 and 1996–97) showed that although the number of ILI-associated medical consultations in the population increased markedly during the outbreaks, they did not affect absences from work of hospital staff (*15*). Contrary to that study, our study demonstrated an effect of the pandemic: the number of HCW sick leaves increased greatly during the pandemic (H1N1) outbreak.

The lack of evidence-based policies for HCW sick leaves during influenza pandemics led us initially to consider that recommendations for patients (*9*) applied to HCWs, based on potential transmission for 7 days or even longer if symptoms persist. If we had followed this policy, our workforce would have been substantially reduced; thus our capacity to respond adequately to the pandemic would have been diminished. Because of that, we decided to apply the 2-day + reassessment policy. One of the HC Institutes did not comply with the official policy, which allowed us to compare the policies. Active surveillance for hospital-acquired influenza among patients was essential to validate and allow continued reassessment of the policy. Although costs and cost savings may seem moderate by some standards, it is necessary to remember that the minimum monthly salary in Brazil is R$510, equivalent to ≈US $283.

Table 6. Costs related to HCW absenteeism resulting from ILI during the pandemic (H1N1) 2009 outbreak, Hospital das Clínicas, São Paulo, Brazil, May–October 2009*

Cost	Baseline cost, R$ (US $)	Lowest cost scenario, R$ (US $)	Highest cost scenario, R$ (US $)
Total	798,051.86 (443,362.14)	617,135.45 (342,853.02)	942,588.49 (523,660.27)
2-d + reassessment	454,968.92 (252,760.51)	352,527.28 (195,848.49)	537,563.93 (298,646.63)
7-d	343,082.94 (190,601.64)	264,608.17 (147,004.54)	405,024.56 (225,013.64)
Per HCW			
2-d + reassessment	1,096.31 (609.06)	849.46 (471.92)	1,295.33 (719.63)
7-d	2,030.08 (1,127.82)	1,565.73 (869.85)	2,396.60 (1,331.44)

*HCW, health care worker; ILI, influenza-like illness; R R$, Brazilian reals (R$1.80 = US $1.00); 2-d + reassessment, 2-day sick leave plus reassessment every 2 days until asymptomatic policy; 7-d, 7-day sick leave policy.

Because diagnostic testing was not available for HCWs during most of the season, most of the decisions regarding sick leave were based on clinical evaluation, and we could not evaluate whether absenteeism resulted from influenza infections themselves or from other factors, such as the psychological impact of the pandemic and concerns about self and family health related to occupational exposure at work. A few studies have evaluated the effect a pandemic of respiratory disease might have on the attitudes of HCWs (*16*,*17*) and on the distribution of resources within hospitals (*18*). In Australia, only 50% of HCWs questioned said they would come to work during an avian influenza pandemic (*16*), and only 25% said they believed that their department was prepared to handle an influenza pandemic (*17*). In our hospital, diagnostic testing (rt-PCR) was available for HCWs at the end of the season, and only 21% of HCWs tested received positive results. Whether this percentage reflects the previous period, when the test was not available, is not known.

These issues suggest that to mitigate concerns of HCWs, clear infection control strategies and hospital policies to protect HCWs and immediate testing of symptomatic HCWs and patients would reassure the workforce. Thus advanced planning and preparedness for implementation of such policies during epidemics and pandemics is needed.

Our study had several limitations. Part of the costs had to be estimated because of lack of data. We tried to counteract this limitation by estimating highest and lowest cost scenarios. In addition, diagnostic testing was available for HCWs only at the end of the season.

Some positive aspects were observed during the pandemic (H1N1) 2009 outbreak. Awareness increased that HCWs should not work when sick with respiratory infections. Usually HCWs tend to underestimate their health problems and work when ill, placing patients at risk (*7*). Another positive effect was the widespread use of alcohol-gel solutions within and outside the health care environment to halt transmission of infection. The pandemic presented an unprecedented opportunity for education of HCWs, children, and the general public about hand hygiene.

In conclusion, our retrospective study evaluated the effect on HCWs and associated costs of different sick leave policies implemented during the pandemic (H1N1) 2009 outbreak. The 7-day policy was more costly but not any more effective than the 2-day + reassessment policy in preventing transmission to patients. Decisions about HCW sick leave policies during pandemics should account for multiple factors, including effectiveness, cost, and feasibility of implementation during emergency conditions.

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Enterovirus 68 among Children with Severe Acute Respiratory Infection, the Philippines

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Enterovirus 68 (EV68) is a rare enterovirus associated with respiratory illness that, unlike other enteroviruses, has been identified only from respiratory specimens. We identified EV68 from respiratory specimens of children hospitalized with a diagnosis of severe pneumonia in Leyte, Republic of the Philippines. Twenty-one samples showed high similarity with EV68 by sequencing of 5′ nontranslated region; 17 of these samples were confirmed as EV68 by sequencing of viral protein 1 capsid coding region. Most previously reported EV68 cases had been identified as sporadic cases. All 21 patients we identified had severe illness, and 2 died, possibly the first reported fatal cases associated with EV68 infection. Our study suggests that EV68 may be a possible causative agent of severe respiratory illnesses.

The genus *Enterovirus* (family *Picornaviridae*) contains 10 species: *Human enterovirus* (HEV) A, HEV-B, HEV-C, HEV-D, *Simian enterovirus A*, *Bovine enterovirus*, *Porcine enterovirus B*, *Human rhinovirus* (HRV) A, HRV-B, and HRV-C. To date, only 3 serotypes have been found for HEV-D: enterovirus 68 (EV68), EV70, and EV94. EV70 is associated with acute hemorrhagic conjunctivitis (*1*), and EV94, a newly found serotype in HEV-D, was identified among enteroviruses associated with acute flaccid paralysis (*2*,*3*). The first EV68 was isolated from hospitalized children with lower respiratory infection in California in 1962 (*4*). Since then, EV68 has been identified sporadically from respiratory specimens (*5*,*6*). EV68 is one of the most rarely isolated enteroviruses; only 26 strains were identified during 36 years of enterovirus surveillance in the United States (*7*). All documented EV68 to date have been reported from the United States and Europe (*3*–*6*); little epidemiologic information is available from Asia and nonindustrialized countries. The clinical significance of EV68 is also not well defined.

Enteroviruses are normally acid resistant and grow at an optimal temperature of ≈37°C, which enables enterovirus to amplify efficiently in the alimentary tract. However, EV68 shares the main characteristics of HRV, which is acid sensitive and grows at a lower optimal temperature (*5*,*8*). These characteristics may explain why EV68 had been isolated only from the respiratory tract (*5*). EV68 and HRV also share high similarity in the 5′ nontranslated region (5′ NTR) (*5*,*8*,*9*). We report a cluster of EV68 infections among hospitalized children with severe acute respiratory illness in the Eastern Viasayas Region of the Philippines during 2008–2009.

Materials and Methods

Patients

This retrospective study was conducted at Eastern Visayas Regional Medical Center (EVRMC) in Tacloban City as part of a pediatric pneumonia study. EVRMC is a tertiary government hospital for Eastern Visayas Region, which has a population of ≈3.9 million.

Nasopharyngeal swabs were collected from patients between 7 days and 14 years of age who visited the outpatient clinic at EVRMC and were hospitalized because they met the criteria for a diagnosis of severe pneumonia

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as defined by the World Health Organization, that is, "a child with cough or difficult breathing and with any of the following signs—any general danger signs (child unable to drink or breastfeed, child is lethargic or unconscious, child vomits everything, or convulsions), chest indrawing or stridor in a calm child—is classified as having severe pneumonia or very severe disease" (*10*). Clinical specimens were collected from 816 children from mid-May 2008 to mid-May 2009. The median age was 9 months; 53% were boys. The study protocol was approved by the institutional review boards of Tohoku University Graduate School of Medicine, Research Institute for Tropical Medicine, and EVRMC. Parents or guardians gave written informed consent for their children to participate in the study.

Molecular Analysis

RNA was extracted from clinical specimens by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized by using random primers (Invitrogen, Carlsbad, CA, USA) and M-MLV Reverse Transcriptase (Invitrogen).

Samples were screened by PCR targeting the 5′ NTR of rhinovirus by using primer pairs DK001 (*11*) and DK004 (*12*) (Table 1). PCR amplicons were purified by using a SUPREC-PCR Kit (TaKaRa Bio Inc., Shiga, Japan) and used as templates in cycle sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1; Applied Biosystems, Foster City, CA, USA) in automated sequencers (3130/3130xl Genetic Analyzer, 3730/3130xl DNA Analyzer; Applied Biosystems). For the samples that showed high identity with previously reported EV68 in 5′ NTR sequences, PCR and sequence analysis targeting viral protein (VP) 1 were conducted by using primer pairs 484 and 222 (*5*) and EV68-VP1F and EV68-VP1R (Table 1).

Sequence Analysis

Sequence analysis was done by using MEGA3.1 software (wwwmegasoftware.net). Phylogenetic trees were generated by using the neighbor-joining method, with maximum-composite likelihood as a substitution model. Similarity was calculated for each genome region by using MEGA3.1 software. Strains of sequences from previous studies that were used for this study are listed in Table 2.

Results

Sequences of EV68 Strains from the Philippines

Among 816 clinical specimens, a total of 274 were positive by PCR targeting for the 5′ NTR of rhinoviruses, and, of these, 245 were identified as rhinovirus by sequencing of 5′ NTR. Among the remaining 29 specimens, 21 samples had 95.2%–100% similarity to previously reported EV68 by 5′ NTR sequencing. However, sequences of these samples had similarity of <86% with those of EV70 and EV94 (data not shown). Among 8 remaining specimens, 1 specimen was classified as coxsackie virus A16, and other specimens were not identified as any viruses because of poor quality of sequence data. EV68 sequences among the study samples were 96.1%–100% identical to one another (data not shown). These 21 samples were subjected to PCR for the VP1 region. The VP1 region was amplified only for 17 of 21 positive samples. The similarity that was calculated for the VP1 region was compared with the sequences of EV68 from the Philippines, EV68 strains from other countries, EV70, and EV94 (Table 3). VP1 sequences from the Philippines had similarity of 86.2%–95.3% with those of the strains from other countries and had similarity of 90.6%–100% to the viruses in the Philippines, while they had similarity of ≤65% with EV70 and EV94.

The sequence data described in this paper have been deposited in the GenBank sequence database under accession nos. AB569257–AB56924. Because of high similarity of VP1 sequences among the analyzed samples, RNA extraction, PCR, and sequencing for VP1 were repeated for selected samples to exclude a possibility of contamination. All retested samples showed identical results.

Phylogenetic trees were based on the 5′ NTR and VP1 gene sequences, including the sequences of previously reported EV68. On the phylogenetic tree based on VP1 sequences, sequences from the Philippines fit into the EV68 cluster in which all EV68 stains from other countries are located. This cluster is clearly distinguishable from clusters of other enteroviruses, including EV70 and EV94

Table 1. Primers used for detection and analysis of EV68, the Phillipines*

Primer (reference)	Primer sequence, 5′ → 3′	Location (location no.)†
DK001 (*11*)	CAAGCACTTCTGTTTCCC	5′ NTR (164–168)
DK004 (*12*)	CACGGACACCCAAAGTAGT	5′ NTR (483–501)
484 (*5*)	GGRTCYCAYTACAGGATGT	VP1 (2197–2215)
222 (*5*)	CICCIGGIGGIAYRWACAT	VP1 (2933–2951)
EV68-VP1F	ACCATTTACATGCAGCAGAGG	VP1 (2393–2413)
EV68-VP1R	GACAAGAACTTTTTCAAATGGACAA	VP1 (2683–2707)

*EV, enterovirus; NTR, nontranslated region; VP, viral protein; F, forward; R, reverse.
†Location numbers correspond to the genome of EV68 Fermon strain (GenBank accession no. AY426531).

Table 2. Sequence data used for analysis of EV isolates, the Philippines*

Strain	GenBank accession no.	Location	Year
EV68.FR37-99	EF107098	France	
EV68.CA62-1	AY426531	United States	1962
EV68.TX99	AY426527	United States	1999
EV68.TX03	AY426526	United States	2003
EV68.NY93	AY426525	United States	1993
EV68.MN98	AY426524	United States	1998
EV68.MD99	AY426523	United States	1999
EV68.MN89	AY426522	United States	1989
EV68.TX02-1	AY426520	United States	2002
EV68.MD02-1	AY426519	United States	2002
EV68.WI00	AY426517	United States	2000
EV68.MO00	AY426516	United States	2000
EV70	DQ201177	Japan	ND
EV94	DQ916376	Egypt	ND
PV1	DQ792910	Greece	ND

*Sequences of 15 referential strains from previous studies, which were used for genetic analysis in this study, are listed above: 12 sequences of EV68, 1 sequence of EV70, EV94 and PV1. EV, enterovirus; ND, no data; PV, polio virus.

(Figure 1, panel B). Among 17 EV68 strains from the Philippines, no significant variation of VP1 sequences was observed, except for Ph561, which was not grouped together with strains from the Philippines but was grouped with EV68.TX03, which was identified in Texas (United States) in 2003, and EV68.MD99, which was identified in Maryland (United States) in 1999 (Figure 1, panel B). On the phylogenetic tree based on 5′ NTR sequences, 21 sequences from the Philippines fit into the EV68 cluster together with other EV68. There were variations among viruses from the Philippines in 5′ NTR, especially Ph451 and Ph569, which formed a separate branch from other EV68 viruses. Ph561 appears to be closely related to TX03. This sample was also grouped into the same distinct lineage with TX03 on the phylogenetic tree based on 5′ NTR (Figure 1, panel A). Moreover, Ph561 had 94.9% similarity to TX03, while it was ≤90.9% identical to EV68 strains from other countries.

Within the enterovirus species, serotype classification is based on nucleotide similarity in the VP1 region (*13*–*15*). It was proposed that they should be classified into the same serotype if they have >75% nucleotide similarity in the VP1 region (>85% amino acid similarity) (*13*,*15*). Sequence analysis of VP1 revealed that EV68 detected in the study had similarity of >86.2% with previously identified EV68, which matched the proposed criteria. Phylogenetic tree of VP1 sequences also confirmed that EV68 detected in the study were located among EV68 clusters with other EV68 strains reported in previous studies.

VP1 sequences were not obtained for 4 specimens among 21 that were positive for the 5′ NTR region, probably due to the low virus RNA content in the samples. Reports have shown that sequencing of the 5′ NTR is not reliable for serotype classification due to high frequency of recombination in this region (*16*–*18*). However, similarity and phylogenetic analysis of the 5′ NTR indicated that all 21 specimens had high sequence similarity with previously identified EV68. These facts indicate that all 21 patients had EV68 infection.

On the phylogenetic tree based on VP1 sequences, only Ph561 did not cluster with other strains from the Philippines, but instead clustered with strains from the United States (TX03 and MD99). Ph561 was ≤91.9% identical to other strains from the Philippines, while other strains from the Philippines were ≥97.6% identical to one another. This finding suggests that the particular virus had a different origin from others and that ≥2 genetically different EV68 with divergent VP1 sequences were circulating. However, there was no unique geographic or temporal characteristic of Ph561, because this virus was identified from the patient from Tacloban City in December 2008.

Descriptive Epidemiology of EV68

EV68 was detected in 21 of 816 samples by molecular methods. These samples represent 2.6% of 816 samples collected in this study.

Table 3. Percentage similarity among viral protein 1 sequences of EV68 from the Philippines and reference strains of EV68, EV70, and EV94 from other countries*

Strain (reference)	FR37-99 (*3*)	TX03 (*5*)	NY93 (*5*)	CA62-1 (*5*)	Ph343	Ph451	Ph513	Ph561	Ph575	Ph43	EV70 (*12*)	EV94 (*12*)
FR37-99 (*3*)		92.8	90.7	88.1	93.2	93.2	93.2	89.4	94.1	94.1	62.3	67.8
TX03 (*5*)			93.6	89.0	92.8	92.8	92.8	94.1	93.6	93.6	63.6	67.8
NY93 (*5*)				90.3	92.4	92.4	92.4	90.3	93.2	93.2	64.8	66.9
CA62-1 (*5*)					89.8	91.9	91.9	88.1	91.9	91.9	64.4	66.1
Ph343						97.5	97.5	91.1	98.3	98.3	62.3	66.1
Ph451							100	91.9	99.2	99.2	63.1	66.1
Ph513								91.9	99.2	99.2	63.1	66.1
Ph561									92.8	92.8	63.6	65.7
Ph575										100	63.1	66.5
Ph43											63.1	66.5

*In the table, homology of viral protein 1 sequences between the strains are listed horizontally, and those listed vertically are indicated in the box where the rows for each strain meet. EV, enterovirus.

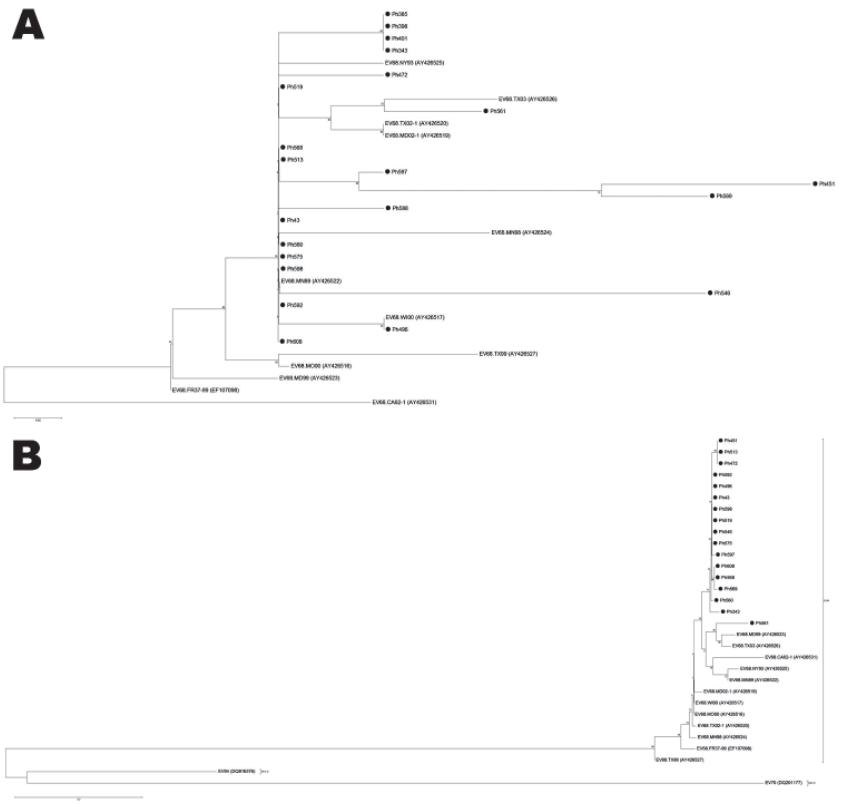

Figure 1. Phylogenetic trees of selected enterovirus (EV) 68 strains, based on the nucleotide sequence of 2 genomic regions: A) partial 5′ nontranslated region and B) partial viral protein 1. EV68 strains analyzed in this study are indicated by black circles. Phylogenetic analysis was performed by using nucleotide alignments and the neighbor-joining method, as implemented in MEGA software (www.megasoftware.net). Poliovirus 1, EV70, and EV94 sequences were used as outgroups. Scale bar indicates number of nucleotide substitutions per site.

Geographic distribution of the patients who had positive EV68 samples is shown in Figure 2. Among 21 patients found to have EV68 positive specimens, 12 were identified in Tacloban City, and the 7 remaining were from surrounding areas (Figure 2). Patients with EV68 infection sought treatment beginning in the third week of October 2008, the number of patients peaked in the 1st week of December, and EV68-positive cases were found after March 2009 (Figure 3).

The patients in whom EV68 was detected were from 1 month to 9 years in age; median age was 21 months. Eight of 21 patients were girls, and 13 were boys. Common signs and symptoms the patients had included cough (100%), difficulty in breathing (85.7%), wheeze (66.7%), and chest in-drawing (100%). Fifteen patients were discharged, but 2 patients died during hospitalization. The outcome of 4 patients was not obtained. The 2 deaths represented 9.5% of 21 patients in whom EV68 was detected, while the rate of deaths associated with HRV infection in the study period was 6.1%. Among 816 patients with severe pneumonia, 70 died in the study period. The 2 patients who died with EV68 infection represented 2.9% (2/70) of the total deaths of patients with pneumonia.

Discussion

We reported a cluster of EV68 infections among hospitalized children with a diagnosis of severe pneumonia in Leyte province, the Philippines. EV68 was identified in 21 cases by PCR and sequencing between October 2008 and February 2009. The number of reported cases of EV68 is limited, and most cases have been reported as sporadic cases (*4*–*6*). As far as we know, clusters of EV68 reported to date include only 2 reports: 4 cases among hospitalized children with lower respiratory tract infections in California in 1962 (*5*) and 7 cases with febrile respiratory illness among military recruits in San Diego during 2004 and 2005 (*6*). We report a large cluster of EV68 that includes fatalities.

Limitations of this study include the fact that we only tested samples from hospitalized patients with severe cases of pneumonia. There might have been many more cases among patients with milder illness in the community. Most of the cases were identified in patients from Tacloban City, the biggest city in the region, but additional cases were also identified from neighboring communities. It indicates that the virus was circulating in a relatively large geographic area during an extended period of 5 months. It is possible that a rare outbreak of EV68 happened to be detected during this study period. It is also possible that EV68 is endemic and causing annual or cyclic outbreaks in this area. Further studies are necessary to define epidemiology of EV68 in the Philippines.

EV68 shares several phenotypic characterizations with rhinovirus, including acid lability and a lower optimal growth temperature (*5*,*8*). The virus that was previously

Figure 2. Geographic distribution of residences of patients in whom enterovirus 68 was detected in the Philippines, May 2008–May 2009. A) Eastern Visayas Region in the Philippines; B) expanded Eastern Visayas Region. Address information was obtained from parents of the children. Locations for 6 patients were unknown.

classified as HRV87 was shown to be identical to EV68 (*8*,*9*). It was also shown that EV68, like other HRV, replicates well in the bronchial epithelial cells (*5*). Human enteroviruses are commonly isolated from stool specimens; however, all previously identified EV68 had been isolated from respiratory specimens (*4*,*6*,*13*). Clinical spectrum of EV68 infection is still not well defined. However, EV68 may have similar clinical illness with HRV. HRV was thought to only cause mild upper respiratory infection. Recently it has been shown that HRV is commonly associated with lower respiratory infection (*19*) and exacerbations of asthma (*20*–*23*). The first isolates of EV68 were detected in hospitalized children with lower respiratory infection (*4*). In the present study, all EV68-positive cases were in hospitalized children with a diagnosis of severe pneumonia, and 2 children died. Acute respiratory infection, particularly pneumonia, is still a major cause of child deaths in nonindustrialized countries (*24*,*25*). The clinical importance of EV68, including its etiologic role in severe respiratory infection, should be further defined.

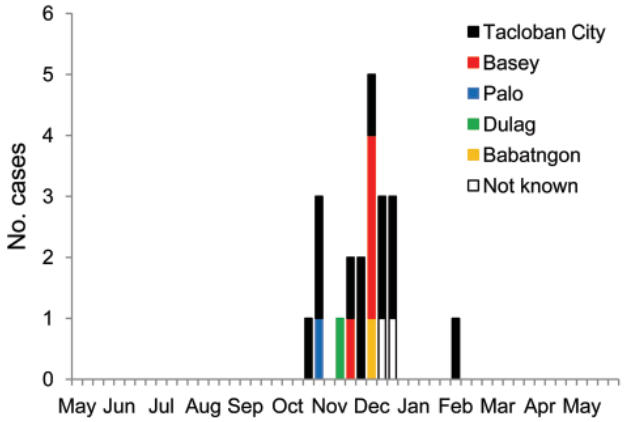

Figure 3. Temporal and geographic distribution of enterovirus (EV) 68 cases in Eastern Visayas Region in the Philippines, May 2008–May 2009. Address information was obtained from parents of the pediatric patients. The graph shows the number of reported EV68 cases of each week in Eastern Visayas Region, and a report from a different city in the region is indicated with a bar of different color. The weeks with no bars indicate no reported cases of EV68.

The sequences of 5′ NTR are similar between HRV and EV68 (*8*). Therefore, EV68 was detected by reverse transcription PCR (RT-PCR) targeting 5′ NTR of HRV (*6*). However, the sensitivity of EV68 detection by RT-PCR by using primers for 5′ NTR of HRV has not been validated. In our study, samples were screened by RT-PCR using primers for 5′ NTR of HRV, which may have missed some EV68 positive cases.

In conclusion, our study highlighted the potential importance of EV68 as a causative agent of severe respiratory infection, which is a leading cause of pediatric deaths in nonindustrialized countries. Clinical and public health impact of EV68 may be underestimated because isolation of EV68 is relatively difficult and requires the use of fibroblast cells. Sporadic cases of EV68 have been detected by virologic surveillance, which suggests that EV68 is circulating in the community. A careful laboratory testing approach may be able to detect more EV68 among patients with respiratory infections.

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Novel Human Reovirus Isolated from Children with Acute Necrotizing Encephalopathy

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For many encephalitis cases, the cause remains unidentified. After 2 children (from the same family) received a diagnosis of acute necrotizing encephalopathy at Centre Hospitalier Universitaire (Tours, France), we attempted to identify the etiologic agent. Because clinical samples from the 2 patients were negative for all pathogens tested, urine and throat swab specimens were added to epithelial cells, and virus isolates detected were characterized by molecular analysis and electron microscopy. We identified a novel reovirus strain (serotype 2), MRV2Tou05, which seems to be closely related to porcine and human strains. A specific antibody response directed against this new reovirus strain was observed in convalescent-phase serum specimens from the patients, whereas no response was observed in 38 serum specimens from 38 healthy adults. This novel reovirus is a new etiologic agent of encephalitis.

Mammalian reoviruses, members of the genus *Orthoreovirus*, are nonenveloped double-stranded RNA viruses with a genome composed of 10 segments. These viruses have 3 major serotypes: type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D), which can be differentiated by neutralization and hemagglutination inhibition tests (*1*). A fundamental characteristic of these viruses, because of their segmented genome, is that 2 distinct viruses can infect the same cell and combine their genomes, thus generating novel viruses (*2*). The acronym reovirus (respiratory enteric orphan virus) is used to designate viruses isolated from the respiratory and enteric tracts of persons with mild respiratory or gastrointestinal symptoms (*3*). Although reovirus infection of humans usually induces mild symptoms, infection of newborn mice leads to severe pathologic conditions, such as lethal encephalitis, depending on the inoculation route and strain (*4*,*5*). Previous studies have described the isolation of 3 reovirus strains after cell culture of cerebrospinal fluid (CSF) from patients with meningitis: serotype 1 (*6*), serotype 3 (T3C96) (*7*), and serotype 2 (T2W) (*8*,*9*). The etiologic role of the T2W strain in meningitis could not be ascertained because the patient was co-infected with other agents (*8*). Similarly, new mammalian reoviruses, such as BYD1, JP, and BYL, were isolated from throat swab specimens of patients with severe acute respiratory syndrome (*10*). Similarly, Melaka virus (*11*), Kampar virus (*12*), and HK23629/07 virus (*13*) were isolated from adults with acute respiratory infection. Here, we report the isolation of a novel human type 2 reovirus (named MRV2Tou05) from 2 children hospitalized with acute necrotizing encephalopathy (ANE). Virologic, molecular, and serologic methods were used to detect the MRV2Tou05 strain.

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Patients and Methods

Patients

A 6-year-old boy (patient 1) and his 22-month-old cousin (patient 2) were hospitalized with the same ANE-specific symptoms a few days apart in March–April 2005 in Centre Hospitalier Universitaire, Clocheville (Tours, France). Serum, urine, CSF, and throat swab specimens were collected from both children. An influenza-like syndrome developed simultaneously in the mother of patient 2.

Laboratory Procedures

To detect herpesviruses and enteroviruses, we used PCR or reverse transcription PCR (RT-PCR), respectively, with commercially available reagents (herpes consensus generic detection kit and enterovirus consensus detection kit [Argene, Verniolle, France]). Nasopharyngeal aspirates were tested by indirect immunofluorescence, by using specific monoclonal antibodies (Argene), for influenza viruses A and B, respiratory syncytial virus, parainfluenza viruses 1–3, and adenoviruses. Serologic assays (ELISA) with commercially available reagents (Behring, Paris, France) were conducted to detect immunoglobulin (Ig) G and IgM against herpes simplex viruses, Epstein-Barr virus, measles virus, and mumps virus. Antibodies to influenza viruses were tested by complement fixation assays with antigens derived from influenza viruses A and B. Serologic assays were also conducted to detect IgG and IgM against hantavirus, tick-borne encephalitis virus, dengue virus, and chikungunya virus (National Reference Center for Arboviruses, Institut Pasteur, Paris). In addition, because the 2 patients had been in contact with an uncle who had returned from Asia (Indonesia), the presence of Hendra virus and Nipah virus nucleic acid sequences in CSF was investigated.

Virus Isolation, Propagation, and Identification

Urine and throat swab specimens from each patient were added to MRC5, MDCK, and Vero cells. Early viral stocks were made from urine and throat specimens added to MRC5 cells as soon as cytopathic effects (CPEs) were observed. Late viral stocks were obtained after inoculation of BGM cells. Cells were harvested as soon as CPEs were observed and fixed by incubation for 48 h in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.2, as described (*14*). Cell pellets were embedded in Epon resin (Sigma-Aldrich, Saint-Quentin Fallavier, France), which was allowed to polymerize for 48 h at 60°C. Ultrathin sections were cut, stained with 5% uranyl acetate and 5% lead citrate, and deposited on electron microscopy grids that were coated with collodion membrane for examination under a Jeol 1010 transmission electron microscope.

Genome Virus Amplification and Sequencing

Specific primers of each reovirus segment were constructed in the highly conserved regions (Table 1). The PCR products corresponding to large (L), medium (M), and small (S) segments were cloned and sequenced

Table 1. Primers used for amplification of MRV2TOU05, a novel human type 2 reovirus, and complete sequencing and reovirus detection*

Primer	Sequence, 5′ → 3′	Position (strain)	RT-PCR product size, bp	GenBank accession no.
For genome amplification of MRV2Tou05				
L1 forward	GCTACACGTTCCACGACAAT	1–20 (SC-A)	3,852	GU196306
L1 reverse	TGAGTTGACGCACCACGACCCA	3852–3831 (SC-A)		
L2 forward	ATGGCGAACGTTTGGGGAGT	13–32 (SC-A)	3,903	GU196307
L2 reverse	GATGAATTAGGCACGCTCACG	3915–3895 (SC-A)		
L3 forward	TAATCGTCAGGATGAAGCGGA	3–23 (SC-A)	3,897	GU196308
L3 reverse	TGAATCGGCCCAACTAGCAT	3899–3880 (SC-A)		
M1 forward	ATGGCTTACATCGCAGTTCCT	14–34 (SC-A)	2,278	GU196309
M1 reverse	CGTAGTCTTAGCCCGCCCC	2291–2273 (SC-A)		
M2 forward	TAATCTGCTGACCGTCACTC	3–22 (SC-A)	2,195	GU196310
M2 reverse	GTGCCTGCATCCCTTAACC	2197–2179 (SC-A)		
M3 forward	CGTGGTCATGGCTTCATTC	12–30 (SC-A)	2,230	GU196311
M3 reverse	GATGAATAGGGGTCGGGAA	2241–2223 (SC-A)		
S2 forward	CTATTCGCTGGTCAGTTATG	2–21 (SC-A)	1,330	GU196312
S2 reverse	GATGAATGTGTGGTCAGTCG	1331–1312 (SC-A)		
S3 forward	TAAAGTCACGCCTGTTGTCG	3–22 (SC-A)	1,178	GU196313
S3 reverse	ACCACCAAGACATCGGCAC	1180–1162 (SC-A)		
S4 forward	GTTGTCGCAATGGAGGTGTG	24–43 (SC-A)	1,158	GU196314
S4 reverse	TCCCACGTCACACCAGGTT	1181–1163 (SC-A)		
S1 forward	CCGATGTCCGAACTTCAACA	1–17 (MRV2Tou05)	1,423	GU196315
S1 reverse	ATGAATTGCCGTCGTGCCG	1423–1405 (MRV2Tou05)		
For reovirus detection test				
L3-2 reverse	GGATGATTCTGCCATGAGCT	705–686 (BYD1)	696	ND
L3-1 forward	CAGGATGAAGCGGATTCCAA	10–29 (T3D, T1L, T2J, SC-A, BYD1)		ND
L3-5 reverse	CCAACACGCGCAGGATGTTT	522–503 (T3D, BYD1, T1L)	512	ND
L3-1 forward	CAGGATGAAGCGGATTCCAA	10–29 (T3D, T1L, T2J, SC-A, BYD1)		ND

*RT-PCR, reverse transcription PCR; L, large segment; M, medium segment; S, small segment; ND, not determined.

in both directions. Sequence analysis was conducted by using Vector NTI (Invitrogen, Carlsbad, CA, USA). To obtain the S1 segment, we extracted total RNA from infected BGM cells with TRIZOL reagent (Invitrogen). The double-stranded RNA was obtained as previously described (*15*) and separated through migration on a 6% precast Poly(NAT) gel (Elchrom Scientific AG, Cham, Switzerland). A ≈1,400-bp band was recovered from the gel and directly used for RT-PCR with random primers (Invitrogen and Roche [La Rochelle, France]). The PCR products were purified from the gel and cloned, and several clones were sequenced. To complete the S1 sequencing, a specific S1 primer was constructed and used for the RT step. The amplification was done by using the specific S1 primer and a random primer (Roche). The band corresponding to the expected size (684 bp) was purified from the gel, cloned, and sequenced. The new sequences enabled construction of specific primers at each extremity (Table 1) to amplify the entire S1 segment and sequence in both directions.

Phylogenetic Analysis

Sequence alignments (nucleic acid and amino acid) were constructed using ClustalW 1.74 (*16*) and refined by visual inspection with SEAVIEW; distance matrix and phylogenetic trees were computed with PHYLO_WIN (*17*). Distances between sequences were computed by observed divergence. Trees were built by using the neighbor-joining method, and tree topologies were tested with 1,000 bootstrap sampling replicates.

Antireovirus IgG Detection

BGM cells infected with the MRV2Tou05 strain and noninfected cells were used for Western blot analysis with a 1:100 dilution of the 2 patients' serum specimens. Thirty-eight serum specimens (supplied by the Établissement Français du Sang, Lyon, France) obtained from 38 healthy blood donors were tested by Western blot for antibodies against the MRV2Tou05, Lang (T1L), Jones (T2J), and Dearing (T3D) strains.

Reovirus RNA Detection

Viral RNA was extracted from infected cells and supernatant and from patients' samples by using the Nuclisens EasyMAG Kit (bioMérieux). Mock cells and supernatant from uninfected cells were used for RNA extraction as negative controls. A molecular test was set up with the L3 segment as target with primers enabling detection of the 3 reovirus serotypes (Table 1).

Results

Patient Histories

Patient 1, a previously healthy 6-year-old boy, was admitted to Centre Hospitalier Universitaire (Tours) on March 29, 2005, with fever, vomiting, and impaired consciousness. Results of his neurologic examination were normal, except for palsy of the left facial nerve. The Glasgow Coma Scale score was 7. He could respond to simple verbal commands, such as requests to open eyes or make some other movements, but he was lethargic (no articulated language) and had epilepsy-like abnormal movements of the face or distal muscles. For 2 days previously, he had experienced a prodromal illness with high temperature (40°) and headache. Results of an analysis of CSF on the first day of hospitalization were within normal limits and showed a protein level of 0.81 g/L and 4 leukocytes/mm^3 on day 5. An electroencephalogram showed generalized slow-wave activity. Computed tomography of the brain showed a low-density change in the thalami. Brain magnetic resonance imaging (MRI) performed on the first day of hospitalization showed multiple symmetric lesions, with high signal intensity on T2-weighted images and low signal intensity on T1-weighted images, mostly involving the thalami bilaterally but also the brainstem tegmentum (Figure 1, panel A) and cerebral white matter in external capsules. He received treatment in the intensive care unit and was given acyclovir, methylprednisolone, and clonazepam intravenously. Beginning 2 days after he arrived at the hospital, he showed improvement, and he recovered completely in a few weeks. He was discharged on the 25th day of illness. A follow-up brain MRI, 1 year later, showed no abnormal findings. Three years later, his development and neurologic examination results were within normal limits.

Patient 2, a 22-month-old girl, was the cousin of patient 1. She was admitted 4 days later, on April 3, 2005, with drowsiness and fever after 1 day of high fever, asthenia, and rhinorrhea. A neurologic examination showed brisk deep tendon reflexes and extensor plantar responses; Glasgow Coma Score was 8. CSF initially showed a slightly elevated protein level (0.55 g/L, reference <0.30 g/L) with no cells, and findings were within normal limits on day 4 of illness (0.38 g/L protein, no cells). Radiologic examinations of the brain showed similar lesions to those of her cousin (Figure 1, panel B), and she received the same treatment. She experienced mild hepatomegaly on day 11 of illness. Her condition began to improve on day 7 of illness, and she was discharged from the hospital on day 18, April 20, with marked hypotonia, responsiveness but absence of language, and pyramidal tract signs. One year later, she remained easily tired, with uncoordinated movements. MRI of the brain found that the damage was less visible and the signal was much less marked.

Two other family members (the mother of patient 2 and the grandmother of both children) also had influenza-like symptoms (headache, fever, vomiting) at the same

period for a few days without neurologic signs. The past family history and medical history did not appear relevant.

Initial Virologic Investigation

Results of molecular tests for herpesviruses and enteroviruses performed initially on the CSF specimens from both children were negative. The results were also negative for infection of respiratory viruses (influenza viruses A and B, respiratory syncytial virus, parainfluenza viruses 1–3, and adenoviruses). Serologic test results for HIV were negative. Serologic assays for IgG and IgM against herpes simplex virus, Epstein-Barr virus, measles virus, and mumps virus did not show IgM. Serologic assays were negative for both IgG and IgM against hantavirus, tick-borne encephalitis virus, dengue virus, and chikungunya virus. The results were also negative for nucleic acid sequences of Hendra virus and Nipah virus (F. Wild, National Reference Center for Measles and Other Paramyxoviruses, pers. comm.). All serum specimens collected during the acute and convalescent phases were negative for antibodies against influenza viruses.

Virus Isolation and Propagation

Nonoriented virus isolation was attempted by inoculation of MRC5, MCDK, BGM, and Vero cell cultures with urine collected from each patient on April 7 and with a throat swab specimen from patient 2 on April 11. A CPE was observed on day 7 in MRC5 cells inoculated with urine from both patients, and on day 7 in MCR5 cells and day 10 in MDCK cells inoculated with a throat swab specimen from patient 2. A discrepant result for enterovirus was obtained when identification was attempted. Indeed, indirect immunofluorescence on fixed cells was positive for enterovirus (Pan-Enterovirus Blend, Light Diagnostics [Millipore, Molsheim, France]), but results of RT-PCR for detection of enteroviruses, performed on the cell culture supernatant, were negative. The virus isolates were then identified as reovirus type 2 by seroneutralization at the National Reference Center for Enteroviruses (Lyon, France). The discrepancy initially observed between immunofluoresence and RT-PCR results may be attributed to the cross-reactivity of the Pan-Entero Blend reagent toward reoviruses, as mentioned in the manufacturer's description. Microscopic examination after staining of infected cells showed voluminous cytoplasmic inclusions characteristic of CPEs induced by reoviruses; electron microscopy showed the accumulation of virions in formation (Figure 2).

Reovirus Detection in Patients' Specimens

Reovirus sequences were searched for retrospectively by RT-PCR in the patients' available specimens. A specific 512-bp fragment corresponding to the L3 expected region was obtained from urine specimens from both patients and from 1 serum specimen that was obtained 21 days after the onset of symptoms from patient 1 (data not shown). All amplified fragments were sequenced and showed identical profiles.

Molecular and Phylogenetic Characterization

The complete sequence of the MRV2Tou05 genome was determined in both directions. Nucleotide and deduced amino acid sequences obtained for each segment were analyzed in

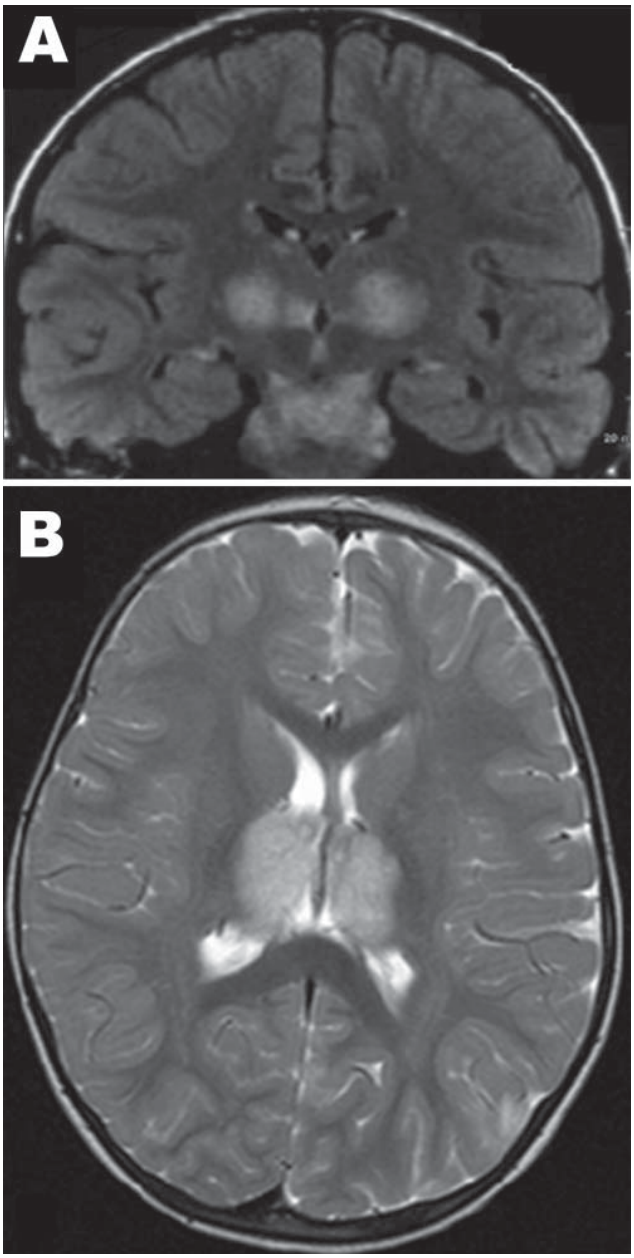

Figure 1. A) Magnetic resonance image of brain corona of patient 1, a 6-year-old boy with acute necrotizing encephalopathy (ANE). B). Axial-weighted images of brain thalami of patient 2, a 22-month-old girl with ANE, the cousin of patient 1.

the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov/Database/) to determine the percentage of identity with mammalian reoviruses. Sequence analysis of the 3 large segments, the 3 medium segments, and the S2, S3, and S4 segments indicated overall a close genetic relationship between MRV2Tou05 and swine SC-A viruses and a relationship with the 2 reovirus prototypes 1 and 3 (Table 2). The sequences of the MRV2Tou05 S1 gene showed great diversity at the nucleotide and amino acid levels, compared with the S1 gene from mammalian reoviruses and SC-A strains (Tables 2, 3). Otherwise, the S3 gene showed high identity with type 2 human reovirus strains (Table 3). The highest identities (83% and 99%) were with type-2 human isolates 302I and 302II from the People's Republic of China (*18*) and human isolates from the Netherlands (*18*), respectively, for S1 and S3 genes (Table 3). Nucleotide sequences of the prototype T2J and the novel MRV2Tou05 S1 genes shared 62% of positional identity, which provided sequence confirmation that this new isolate was a type 2 strain.

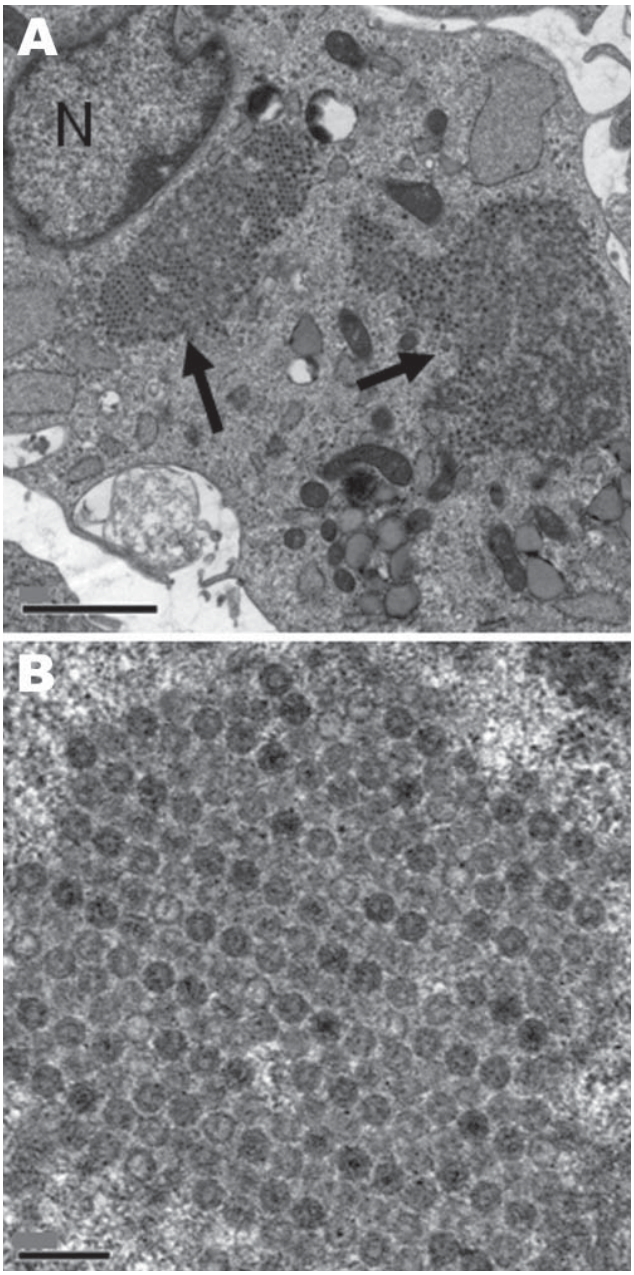

Figure 2. Electron microscopic images of the cytopathic effect induced in MRC5 cells by a reovirus isolate from throat specimens of patient 2, a 22-month-old girl with acute necrotizing ancephalopathy. N, nucleus; arrows indicate viral intracytoplasmic inclusions. Scale bars indicate 2 μm (A) or 0.2 μm (B).

To establish the evolutionary relationship of MRV2Tou05 with the known mammalian reoviruses, we constructed phylogenetic trees on the basis of the nucleotide sequences of the S1 and S3 segments (Figure 3). The S1 phylogenetic tree shows that the MRV2Tou05 S1 sequence is more closely related to the S1 genes of other type 2 reovirus strains and most divergent from the type 3 reovirus strains, including the SC-A strain. In contrast to the great diversity of the MRV2Tou05 S1 gene sequence compared with the other reovirus strains, similar trees obtained with sequences of the MRV2Tou05 S2, S3, and S4 genes showed a randomized genetic clustering between the different reovirus types for these genes (data not shown).

The 4 S segments, S1–S4, of MRV2Tou05, isolated from the throat swab specimen of patient 2 and the urine specimen of patient 1, were entirely sequenced. The nucleotide sequence was identical for 62 of 81 clones, and <3 point mutations were observed among the whole RNA segments for the remaining 19 clones, indicating that the 2 children had been exposed to the same novel isolate.

Serologic Analysis

Specific antibodies against MRV2Tou05 were detected in serum specimens from patient 2; reactivity was higher in the 2 specimens collected in the convalescent phase, i.e., 13 and 19 days after symptom onset (Figure 4, panels B, C) than in specimens collected in the acute phase (6 days after symptom onset) (Figure 4, panel A). Similarly, antibodies against MRV2Tou05 were detected in the serum specimens from patient 1 and the mother of patient 2 (data not shown). The molecular mass of 145 kDa corresponded to λ proteins encoded by the large segments, with a size of 142–145 kDa. None of the 38 serum specimens from healthy blood donors was positive for MRV2Tou05 reovirus (Figure 4, panel D) but 20 of 38 serum specimens from donors were positive for the reovirus prototype strains, with the highest prevalence for T1L and T3D (data not shown). In the donors' blood, we observed a signal directed against the λ proteins as well as 2 additional bands corresponding to the μ proteins (75 kDa and 100 kDa) from the human reovirus

Table 2. Nucleotide and amino acid identities for segments of MRV2Tou05, a novel human type 2 reovirus, isolated from patient 2, with reovirus prototypes and SC-A strain*

MRV2Tou05 RNA segment	Reovirus prototype strain, %						Swine reovirus strain, SC-A, %	
	T1L		T2J		T3D			
	Nucleotide	Protein	Nucleotide	Protein	Nucleotide	Protein	Nucleotide	Protein
L1	90	98	75	92	90	98	97	99
L2	86	97	73	87	77	93	97	98
L3	84	98	77	95	84	98	97	99
M1	92	97	70	80	92	96	90	95
M2	85	98	76	97	90	98	97	99
M3	85	95	71	82	85	95	97	98
S1	58	46	62	62	42	26	42	25
S2	85	98	76	94	85	98	88	98
S3	91	97	74	86	85	97	91	99
S4	87	97	79	91	87	96	95	99

*T1L, type 1 Lang; TJ2, type 2 Jones ; T3D, type 3 Dearing; L, large segment; M, medium segment; S, small segment.

prototypes. Antibodies against T1L and T3D strains were also found in serum specimens from patient 2, but not in specimens from patient 1 and from the mother of patient 2 (data not shown).

Discussion

For many cases of encephalitis (32%–75%), the etiologic agent remains unknown (*19*–*21*). In this study, a reovirus strain was isolated from 2 children who exhibited unexplained neurologic symptoms of encephalitis. On the basis of the neuroimaging findings, clinical features, and laboratory data, ANE was diagnosed (*22*). The viruses commonly involved in encephalitic syndromes were not detected in CSF specimens from both patients. Results of broad virologic investigations for other viruses were also negative. The reovirus isolation and the molecular detection of the specific sequences in patients' specimens demonstrated that the 2 hospitalized children with ANE had been exposed to a novel reovirus strain. Tyler et al. (*7*) recently described a novel type 3 reovirus isolated from a child with meningitis and provided direct evidence that reoviruses can be neurovirulent in humans.

Table 3. Comparison of S1 and S3 genes of MRV2Tou05, a novel human type 2 reovirus, with the most representative mammalian reovirus strains*

Strain	GenBank accession no.		% Similarity to MRV2Tou05 S1		% Similarity to MRV2Tou05 S3	
	S1	S3	Nucleotide	Protein	Nucleotide	Protein
T1C50	AY862133	NA	57	50	NA	NA
T1N84	AY862136	NA	58	53	NA	NA
T1N85	AY862135	U35346	57	51	98	99
T1C11	NA	U35359	NA	NA	91	98
T1C62	NA	U35356	NA	NA	91	99
T1C23	AY862134	NA	57	50	NA	NA
T2N73	AY862137	U35350	67	66	99	99
T2N84	AY862138	U35347	67	66	98	99
T2W	DQ220017	DQ220018	66	62	75	88
T2302II	EU049604	NA	83	89	NA	NA
T2302I	EU049603	NA	83	89	NA	NA
BYD1	DQ312301	DQ664191	66	67	84	95
SC-A	DQ911244	DQ411553	42	25	91	97
T3Co96	AY302467	NA	43	28	NA	NA
T3A	L37677	NA	48	25	98	97
T3C18	L37684	NA	42	26	NA	NA
T3C8	L37679	U35355	42	24	85	97
T3C31	L37683	NA	41	25	NA	NA
T3C9	L37676	U35352	40	25	82	97
T3C93	L37675	NA	42	25	NA	NA
T3C44	L37681	NA	42	26	NA	NA
T3C45	L37680	NA	42	25	NA	NA
T3C43	L37682	NA	42	25	NA	NA
T3C84	L37678	U35354	42	25	85	97
T3N83	NA	U35349	NA	NA	85	98

*S, small segment; NA, not available.

Sequence analysis and phylogenetic trees showed that most reovirus segments (L1–L3, M2–M3, S2, and S4) were closely related to the swine reovirus strain (91%–97% identity), except for the S1 and S3 segments. The S1 segment determines the reovirus serotype and encodes the nonstructural protein sigma 1s and the viral cell attachment protein sigma 1. The S1 gene showed a high identity score (83%) with human type 2 strains isolated from fecal specimens of 2 children in China in 1982 (*18*,*23*). This finding was compatible with the results of the neutralization assay performed with the initial infected cell culture supernatant. The S1 segment from reoviruses is highly diverse in size and sequence. This diversity may explain why, even within the same serotype, the S1 segment could not be amplified with a different set of primers initially defined by alignment of reovirus serotype 2 strains. The S3 segment is genetically similar to that of the human reovirus strains T2Neth/73 and T2Neth/84 (98% identity) isolated in the Netherlands in 1973 and 1984 (*24*). The S3 gene is known to encode the nonstructural protein σNS (*25*,*26*), which plays a notable role in the replication of reovirus gene segments and assortment (*27*,*28*). On the basis of these data, we conclude that the novel type 2 human reovirus, designated MRV2Tou05, might have originated from a reassortment between a human isolate and a swine reovirus, both probably first identified in China.

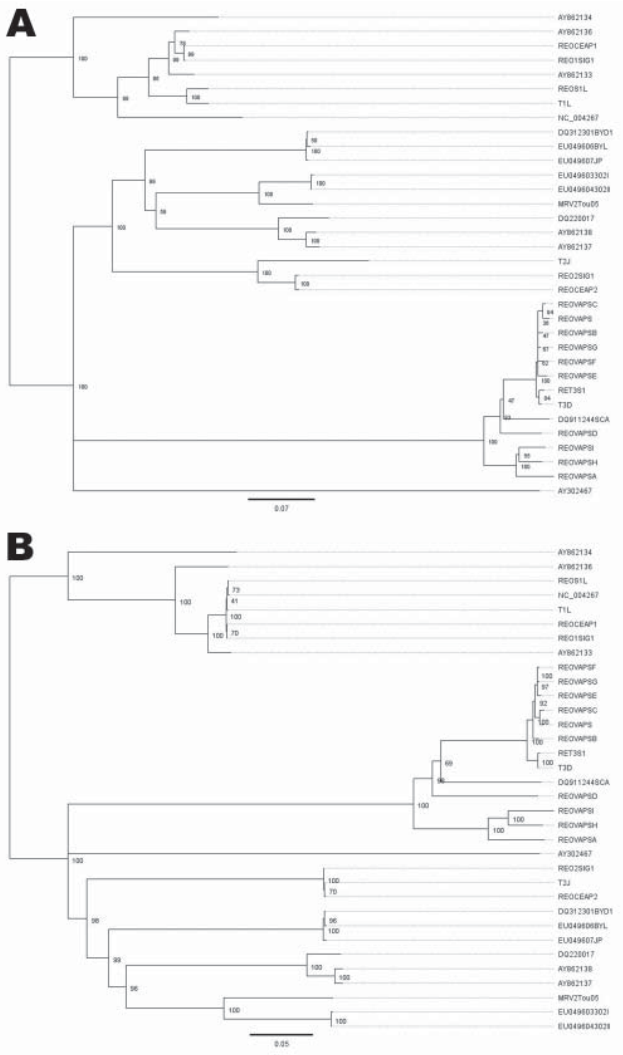

Figure 3. Phylogenetic trees of the small segment 1 of reoviruses. A) Nucleotide sequences; B) amino acid sequences. Scale bars indicate nucleotide (A) and amino acid (B) substitutions per site.

A specific antibody response against the MRV2Tou05 strain developed in the 2 patients and in the mother of patient 2, whereas no antibody response against MRV2Tou05 was detected in any of the 38 healthy blood donors. However, serum samples from 52% of these healthy adults contained antibodies directed against at least 1 of the 3 human reovirus prototype strains. In a study in Germany, similar seroprevalence was observed for reovirus type 3 antibodies in a healthy population (*29*). Although the studied population is limited, our data suggest that isolates related to the reovirus prototype strains are spread widely in the human population, in contrast to this novel type 2 MRV2Tou05 reovirus.

ANE predominantly affects infants and young children in eastern Asia, but sporadic cases are regularly diagnosed in other parts of the world. The most frequent pathogens involved in ANE are viruses, most commonly influenza A and B (*22*,*30*). The outcome of ANE has been reported to be generally poor, but the 2 patients described in this study recovered and had only mild sequelae or none (*31*,*32*). Influenza-associated encephalopathy has been mostly reported in children in Japan and Taiwan, which suggests a possible association with genetic or epigenetic factors in these countries (*30*). A similar disorder has been described as autosomal dominant ANE with incomplete penetrance in only 1 large family. It affects children, and clinical and radiologic findings seem identical to ANE, with sudden-onset encephalopathy triggered by viral infection including influenza. The outcome, however, seems worse, with death, mild to severe developmental regression, and recurrence in half of the survivors (*33*). The simultaneous occurrence of the cases described in this study and the familial clustering may suggest either a causative role for MRV2Tou05, the patients' genetic predisposition to such an agent, or both (*34*). Investigations are in progress to determine genetic susceptibility in the family.

The origin of several genome segments of this reassortant in swine in Asia and the relatedness of other segments to human serotype 2 reoviruses described in Asia are surprising. Possibly the MRV2Tou05 was imported by the uncle, who had just returned from Indonesia a few days before the onset of symptoms in the children and the mother. Searching for antibodies against MRV2Tou05

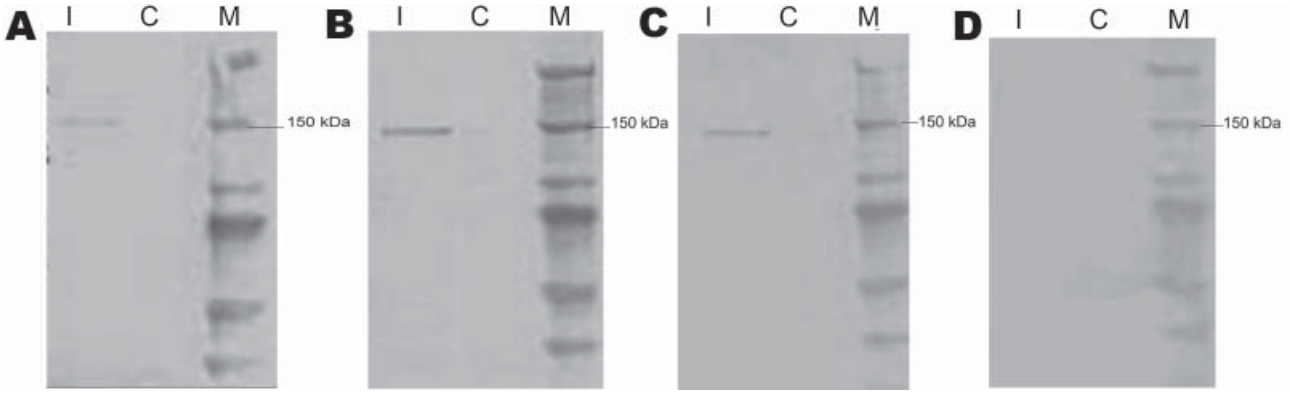

Figure 4. Results of serologic analysis by Western blot of serum specimens from patient 2, a 22-month-old girl with acute necrotizing ancephalopathy. Three serum specimens from patient 2, harvested at 6 (A), 13 (B), and 19 (C) days after onset of symptoms, and a serum specimen from a healthy donor (D) were incubated with reovirus MRV2Tou05–infected and –noninfected BGM cells. I, infected; C, noninfected; M, molecular weight markers (Precision Plus protein standards).

in serum specimens from the uncle would have been informative but no blood samples were available.

The role of reoviruses as etiologic agents for symptomatic human diseases remains controversial. They are designated as orphan viruses, and more than half of the adult population possess antibodies directed against reoviruses, which suggests that infection occurs frequently without any specific effect on human health. However, reovirus strains have been isolated from persons with serious human diseases (*35*). Indeed, an unknown reovirus strain in 1996, the T3C/96 strain in 2004, and the T2W strain in 2006 were isolated and reported from human meningitis patients, which shows that reoviruses can also cause central nervous systemic disease in humans. Melala virus, Kampar virus. and HK2369/07 virus were isolated from patients with acute respiratory infections, and BYD1, JP, and BYL strains of serotype 2 were isolated from patients with severe acute respiratory syndrome. In all these of cases, a reovirus strain with novel molecular characteristics was described, except for the 1996 case, but the characterization of the isolated strain was not reported.

Conclusion

This study describes the entire molecular characterization of a new reovirus strain isolated from 2 familial ANE patients. Its isolation and molecular detection from patients' samples and the specific immune response toward this type 2 strain suggest an etiologic role for this reovirus in these unexplained ANE cases. The reproduction of symptoms in an animal model and in vitro studies of the cellular interactions and apoptosis of MRV2Tou05 are needed to help clarify the exact role of this novel reovirus strain. Identifying the MRV2Tou05 reovirus sequence could contribute to the improvement of ANE diagnosis and treatment, for example, by confirming susceptibility to viral infection and clarifying the possible role of other common viruses in its pathogenicity.

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Dr Ouattara is a PhD student working at the Emerging Pathogens Laboratory of Fondation Mérieux. Her research activities are focused on the identification and characterization of new pathogens involved in encephalitis.

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Early Warning System for West Nile Virus Risk Areas, California, USA

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The Dynamic Continuous-Area Space-Time (DYCAST) system is a biologically based spatiotemporal model that uses public reports of dead birds to identify areas at high risk for West Nile virus (WNV) transmission to humans. In 2005, during a statewide epidemic of WNV (880 cases), the California Department of Public Health prospectively implemented DYCAST over 32,517 km^2 in California. Daily risk maps were made available online and used by local agencies to target public education campaigns, surveillance, and mosquito control. DYCAST had 80.8% sensitivity and 90.6% specificity for predicting human cases, and κ analysis indicated moderate strength of chance-adjusted agreement for >4 weeks. High-risk grid cells (populations) were identified an average of 37.2 days before onset of human illness; relative risk for disease was >39× higher than for low-risk cells. Although prediction rates declined in subsequent years, results indicate DYCAST was a timely and effective early warning system during the severe 2005 epidemic.

West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus*) is a mosquito-borne pathogen that has led to ≈30,000 reported (>325,000 estimated) human cases and 1,172 reported deaths in the United States since it was first detected in New York, New York, in 1999 (*1*). The virus was first detected in California in a pool of *Culex tarsalis* mosquitoes in July 2003 (*2*), and in 2004 and 2005 the state had the highest number of reported human cases (779 and 880, respectively) and deaths (29 and 19, respectively) in the United States (*3*). Humans are incidental, dead-end hosts of WNV and generally become infected after intense viral amplification and spillover from local avian populations (*4*). Birds are the natural reservoir and amplification hosts of WNV and infections can cause death rates up to 100% among avian species (*5*,*6*). Beginning in 2000, bird carcasses in California were submitted by local agencies to the WNV Dead Bird Surveillance Program (DBSP) at the California Department of Public Health (CDPH; previously known as the California Department of Health Services) as part of the California Mosquito-Borne Virus Surveillance and Response Plan (*7*,*8*). A toll-free telephone hotline and website for recording public reports of dead birds was established in 2002.

Previous efforts for the early detection and monitoring of WNV activity have used dead bird density or spatial scan statistic as a proxy for transmission risk for humans (*9*–*13*). However, aggregation of reports over nonuniform spatial units (i.e., counties and census tracts) may fail to detect WNV amplification clusters that span regional boundaries or that are contained within large areas. In addition, temporal aspects of the WNV transmission cycle should be considered to avoid false-positive identifications in circumstances in which sustained but slow transmission leads to an accumulation of dead bird reports above the designated risk threshold but does not result in spillover to the human population.

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Another approach is the DYCAST system (*14*,*15*), implemented in New York, New York, in 2001 and Chicago, Illinois, in 2002. This system detects statistically significant spatiotemporal clustering of dead bird reports by modeling the WNV amplification cycle using biological parameters; it also includes a statistical method for evaluating effectiveness of human case predictions in space and time. Results indicated that clusters of dead bird reports and human cases of WNV were significantly associated in space and time (*15*). This association suggests that this procedure may be useful for predicting areas at high risk for WNV transmission to humans. Because there is no drug prophylaxis, human vaccine, or treatment available for WNV, integrated pest management and personal mosquito protection remain the only options for reducing human illness and death, and early warning of high-risk areas allows for these measures to be implemented in a timely and effective manner. The objective of the present study was to evaluate implementation of DYCAST as an early warning system in California to target public education campaigns, surveillance, and mosquito control efforts during an anticipated statewide outbreak of WNV.

Methods

Data

Public reports of dead birds were obtained from the DBSP. Through press releases and various types of media campaigns at state and local levels, citizens were encouraged to use the hotline (1-877-WNV-BIRD) and website (www.westnile.ca.gov) to report dead birds (*7*). Information regarding location, date found, and species was collected for each dead bird reported to the hotline; multiple dead birds included in a single report were treated as multiple reports. Hotline staff screened and entered these data into an Access database (Microsoft Corporation, Redlands, WA, USA); data were subsequently geocoded by using ArcMap version 9.1 and associated 2005 StreetMap USA Plus AltNames street dataset (Environmental Systems Research Institute, Inc., Redlands, CA, USA). WNV became a reportable disease in California in 2005, and human data were collected by local health departments by standardized case history forms. Data were subsequently stripped of personal identifiers, and addresses were geocoded by using a CDPH batch geocoding service that used multiple reference databases (www.ehib.org). Use of human data was approved by the institutional review board at the California Health and Human Services Agency (project no. 05-06-51).

Procedure

The DYCAST procedure was implemented by using GIS software, Smallworld 3.2.1, and Magik programming language (General Electric Company, Fairfield, CT, USA). Regions comprising 32,517 km^2 among 16 participating agencies in 17 counties were superimposed by grids consisting of ≈0.44 km^2 (≈0.17 mi^2) cells (Figure 1). Clustering of dead bird reports was quantified by using a Knox test (*16*,*17*) implemented from the center of individual cells; spatial and temporal parameters were defined by using biologically relevant values (Figure 2). The 2.4-km (1.5-mi) radius of the spatial domain represents 2× the daily feeding distance (*14*) of *Culex* spp. mosquitoes in California (*18*). The effective flight range of these mosquitoes is also 2.4 km (*19*), which corresponds to the maximum distance from breeding sites over which a sufficient number of vectors are able to disperse a mosquito-borne disease (*20*). The temporal domain of 21 days was based on a 7-day extrinsic incubation period of WNV, which ranges from 5 to 8 days for *Culex* spp. mosquitoes at 28ºC in California (*21*), plus 2 avian infection cycles of 7 days each (approximate maximum time from infection to death; *5*,*14*,*22*). Candidate values for defining proximity of dead birds in space (0.40, 0.56, and 0.64 km) and time (3, 4, and 5 days) were based on the limited mobility (caused by lethargy, ataxia, and reluctance to fly) and lifespan of infected amplification hosts (*5*,*14*,*23*).

Figure 1. California counties with 2005 Dynamic Continuous-Area Space-Time (DYCAST) analysis regions (32,517 km^2), shown in black. Data were mapped by using ArcMap version 9.3.1 (Environmental Systems Research Institute, Inc., Redlands, CA, USA) and North American Datum of 1983, High Accuracy Reference Network (NAD83 HARN) California II State Plane coordinate system (Lambert Conformal Conic Projection).

During model calibration, locations of human cases were compared with DYCAST risk maps generated by using various combinations of candidate values; 0.40 km (0.25 mi) and 3 days were selected as the optimal combination for the final model. For this calibration, the daily DYCAST procedure was run retrospectively (once during May 2005) by using dead bird and human case data from May 1 through September 30, 2004 within Los Angeles, Orange, Riverside, and San Bernardino Counties, which contained 664 (85.2%) of 779 statewide cases in 2004.

The DYCAST procedure was run at the center of every cell for which a minimum of 15 birds (the analysis threshold) was reported within the spatiotemporal domain, to minimize statistical instability that otherwise occurs at lower numbers of birds (*14*). Clustering was evaluated by comparing the observed number of pairs of dead birds that were close in both space and time (based on aforementioned values of proximity), with the expected number of pairs given a random spatiotemporal distribution of these reports (*15*). The resulting p values were assigned to individual cells, which were considered to indicate high risk for WNV transmission to humans at $p \leq 0.1$ (*15*).

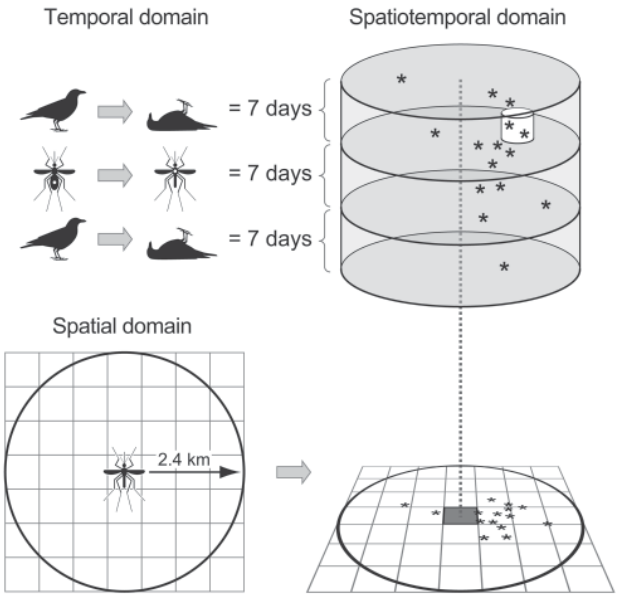

Figure 2. Schematic of the Dynamic Continuous-Area Space-Time (DYCAST) procedure, illustrating domains of Knox test (*16,17*) implemented at the center of an individual ≈0.44 km^2 grid cell. The 2.4-km (1.5-mi) radius of the spatial domain represents twice the daily feeding distance (*14*) of *Culex* spp. mosquitoes in California (*18*) and is equivalent to the effective flight range of these vectors (*19,20*). The 21-day temporal domain accounts for the extrinsic incubation period of West Nile virus (*21*) and 2 avian infection cycles of 7 days each (*5,14,22*). These bounds define the spatiotemporal domain, within which reports of dead birds (asterisks) are evaluated for proximity in space (0.40 km) and time (3 days) (small white cylinder). Statistical significance of dead bird report pairing is assessed by using random simulations ($p \leq 0.1$) (*15*). Procedure is repeated at other cell centers to create a continuous surface of risk.

Evaluation

Model evaluation was conducted by analyzing the relationship between the location of human cases and the ability of DYCAST to predict their occurrence in both space and time. Prediction was defined as the identification of a cell as high risk before or on the date of illness onset (*15*) of the earliest case located within a cell. Sensitivity was calculated as the number of high-risk cells classified as predicted (true positives) divided by the total number of cells in which a human case occurred. Specificity was calculated as the proportion of low-risk cells without cases (true negatives) to the total number of cells without cases. Because agreement between model predictions and cases can occur by chance, a spatiotemporal implementation of the κ statistic was used to provide a measure of chance-adjusted agreement (*15*,*24*).

Implementation

An initial pilot phase and subsequent prospective implementation occurred through a cooperative agreement with the Center for Advanced Research of Spatial Information at Hunter College, City University of New York. The Center for Vectorborne Diseases (CVEC) at the University of California Davis provided server infrastructure (Microsoft SQL Server, Microsoft Corporation; ArcIMS, Environmental Systems Research Institute, Inc.) for data exchange and implementation of interactive online risk maps, in collaboration with CDPH and the Mosquito and Vector Control Association of California. The Center for Advanced Research of Spatial Information calibrated and ran the DYCAST procedure and exported data to the CVEC map server. During the pilot phase, animations of daily risk from June 1 through June 23, 2005, were retrospectively generated for 3 study areas that were selected based on high numbers of dead bird reports: the south Sacramento Valley region (Sacramento, Placer, and Yolo Counties), the central San Joaquin Valley region (Fresno, Kings, and Tulare Counties), and the greater Los Angeles area.

Prospective modeling began on June 17, and on July 1 the system was fully implemented and integrated into the CDPH WNV Surveillance Program. This implementation involved running the DYCAST procedure for analysis regions every weekday through November 1, 2005; daily risk maps (Figure 3) were generated and made available in real time to mosquito control agencies via the CVEC password-protected website, the California Vectorborne Disease Surveillance Gateway (www.calsurv.org). These interactive maps were overlaid with county boundaries,

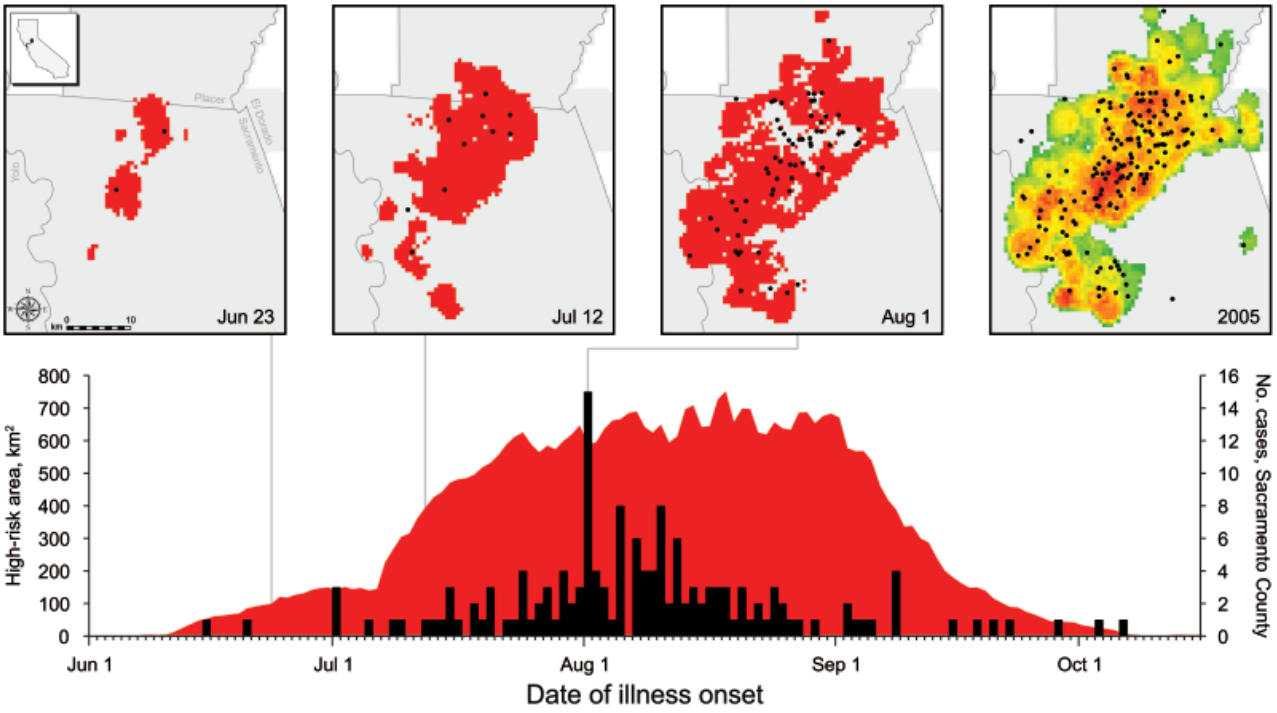

Figure 3. Dynamic Continuous-Area Space-Time (DYCAST) risk maps (top) and timeline (bottom) of West Nile virus epidemic in Sacramento County, California, 2005. Within timeline, black bars represent reported human cases within Sacramento County by date of onset of illness (n = 152; 11/163 cases were missing spatial and/or temporal data), and red region represents total area in Sacramento County designated by DYCAST as high risk by date of analysis. Maps illustrate areas of high-risk (red) cells during the last day of pilot-phase analysis (Jun 23), which detected the 2 emerging clusters seen above in the Arden-Arcade and Citrus Heights regions, as well as during rapid expansion of high-risk areas (Jul 12) and the peak of the epidemic (Aug 1). Map at far right displays cells color coded by number of days designated by DYCAST as high risk during 2005, from green (low) to red (high) (range 1–94 days, mean 47.7 days, median 51 days, SD 19.9 days). Human cases with onset of illness before or including respective date of analysis are shown as black circles; gray area represents DYCAST analysis regions. Inset at top left illustrates location (black square) in California corresponding to the risk maps. Data were mapped by using ArcMap version 9.3.1 (Environmental Systems Research Institute, Inc., Redlands, CA, USA) and North American Datum of 1983, High Accuracy Reference Network (NAD83 HARN) California II State Plane coordinate system (Lambert Conformal Conic Projection).

streets, and locations of reported and WNV-positive dead birds.

Implementation 2006–2009

Beginning in 2006, DYCAST was implemented for the entire state of California and adopted as a formal component of the California Mosquito-Borne Virus Surveillance and Response Plan (*8*). Addresses of where dead birds were found were automatically geocoded in real time by using the Yahoo Maps application programming interface (Yahoo! Inc., Sunnyvale, CA, USA), which allowed hotline staff to validate location data while callers remained on the line; birds not automatically geocoded were omitted from DYCAST analysis. Interactive DYCAST risk maps were made available online to local mosquito control agencies and integrated with dead bird, mosquito, and sentinel chicken surveillance data from May 1 through October 31, 2006, May 1 through August 31, 2007 and 2008, and March 1 through August 31, 2009. Statewide reports of DYCAST activity, including maps and animations of high-risk areas over time, were sent to local agencies on a routine basis. A real-time alert system was also introduced in 2006 to provide custom DYCAST reports and interpretations for counties experiencing rapidly increasing or elevated levels of high-risk areas (*8*).

In December of 2006 and 2007, links to web-based surveys regarding the DBSP were provided by email to 64 local mosquito control agencies in 47 counties, in part to assess which agencies used DYCAST to assist mosquito larviciding or adulticiding activities each year. For agencies that participated in the 2005 DYCAST program, the 2006 survey also asked if DYCAST results were used "to assist public education or to promote dead bird reporting" in 2005 (control activities were not surveyed for this year). Rate ratios (RRs) were used to compare annual DYCAST prediction rates of reported human WNV cases between

Table 1. Reported dead bird and human West Nile virus surveillance data, California, USA, 2003–2009*

Surveillance	2003	2004	2005	2006	2007	2008	2009
Dead birds							
Reported	8,650	93,053	109,358	46,345	27,611	33,594	15,472
Tested	1,765	5,723	9,227	6,535	6,000	6,124	2,805
Positive	96	3,232	3,046	1,446	1,396	2,568	515
Humans							
Cases	3	779	880	278	380	445	112
Fatalities	0	29	19	7	21	15	4

*Includes entire state of California. Data sources: California Department of Public Health, Vector-Borne Disease Section (http://westnile.ca.gov) (*2*,*3*).

agencies that did and did not use DYCAST to assist each mosquito control activity (*25*).

Results

During 2005, a total of 124,876 calls were placed to the DBSP hotline, >3 million hits were made to the website, and 109,358 dead birds were reported in California (Table 1) (*26*). DYCAST identified high risk in 9.7% of the analysis regions (7,160/73,767 cells; 3,139/32,517 km^2), with cells identified as high risk for a mean total of 39.0 days (range 1–117, median 39, SD 22.8 days). Relative risk of a WNV case in high-risk cells compared with low-risk cells was 39.10 (95% confidence interval [CI] 29.80–51.30; p<0.0001). Sensitivity and specificity of the DYCAST system were 80.8% (269/333 cells) and 90.6% (66,543/73,434 cells), respectively (Table 2). Prevalence of cells containing cases was 0.45% (333/73,767 cells), which resulted in low positive predictive value (3.8%; 269/7,160 cells) and high negative predictive value (99.9%; 66,543/66,607 cells). κ values maintained a moderate strength of chance-adjusted agreement (0.40<κ<0.60; *27*) for >4 weeks before onset of illness (Figure 4). Overall, 289/354 (81.6%) of cases were predicted (Table 3), with cells identified as high risk before onset of illness by a mean of 37.2 days (range 0–126, median 34, SD 20.9 days). A total of 252/354 (71.2%) of cases were predicted 15 days before onset of illness, and >50% of cases (179/354, 50.6%) were predicted 30 days before onset of illness.

According to the 2006 survey, 10/14 (71.4%) of responding local mosquito control agencies within the analysis regions used DYCAST results to assist public education or to promote dead bird reporting in 2005. DYCAST risk maps were also used to direct WNV surveillance (and ultimately control) efforts as early as the pilot phase, when 2 emerging clusters of high-risk cells were identified around the Arden-Arcade and Citrus Heights regions of Sacramento County on June 24, 2005 (Figure 3, June 23, 2005). These results were immediately shared with the Sacramento-Yolo Mosquito & Vector Control District (SYMVCD), which used the risk maps to deploy mosquito traps within the 2 high-risk clusters on June 28 (D. Brown, SYMVCD, pers. comm.). On June 29, SYMVCD collected and detected 4 WNV-positive *Cx. pipiens* mosquito pools from these traps in both areas, which represented the first positive mosquito pools in Sacramento County that year (*29*). Additionally, both human cases from Sacramento County with onset of illness before June 23 were located within cells identified by DYCAST as high risk, 12 and 2 days before onset of illness (Figure 3, June 23, 2005). Within Sacramento County, DYCAST predicted 142/152 (93.4%) of cases (11/163 cases were missing spatial or temporal data); 122/152 (80.3%) and 84/152 (55.3%) of cases were predicted 15 and 30 days, respectively, before onset of illness (Figure 4).

After 2005, the number of reported dead birds generally decreased (Table 1); the percentage of successfully geocoded dead bird reports ranged from 98.8% to 100% each year. The statewide DYCAST prediction rates for reported human cases during 2006–2009 were 26.3% (67/255), 33.0% (110/333), 16.3% (64/392), and 3.2% (3/93), respectively (Table 3). Responses to the 2006 and 2007 surveys were received from 47 agencies in 36 counties and 18 agencies in 19 counties, respectively. Results indicated that most of the agencies that responded each year used DYCAST to assist larviciding or adulticiding activities (Table 4). DYCAST prediction rates were significantly higher for agencies that answered "yes" to questions regarding larviciding (RR 10.06, 95% CI 2.45–41.32) and adulticiding (RR 10.91, 95% CI 2.65–44.88) in 2006 and larviciding (RR 10.16, 95% CI 1.41–73.00) in 2007 (Table 4). Conversely, the prediction rate was significantly lower for agencies answering "yes"

Table 2. Comparison of number of cells that contained reported human West Nile virus cases and number of cells identified as high risk, California, USA, 2005*

	Contained case		
High risk	Yes	No	Total
Yes	269	6,891	7,160
No	64	66,543	66,607
Total	333†	73,434	73,767

*True positive (yes/yes) designates cell identified by Dynamic Continuous-Area Space-Time (DYCAST) model as high risk before or on the date of onset of illness of earliest case located within cell. If cell was identified as high risk after date of onset of illness, or cell was never identified as high risk and a case occurred within it, it was designated false negative (yes/no).
†Number of cells that contained cases is less than the number of cases (354) because of 14 cells that contained 2 predicted cases, 3 cells that contained 3 predicted cases, and 1 cell that contained 2 missed cases.

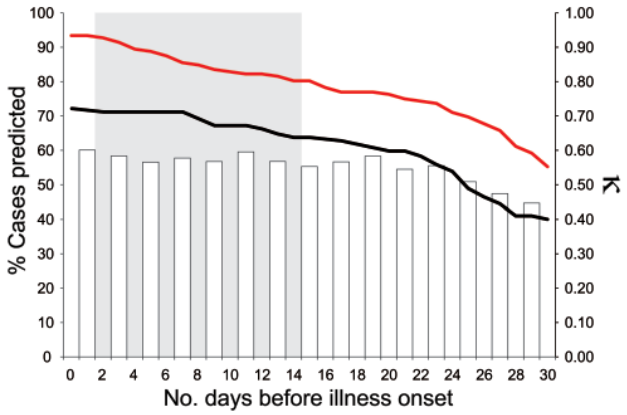

Figure 4. Percentages of reported human West Nile virus cases within Sacramento County (red line) and remainder of analysis regions (black line) predicted by Dynamic Continuous-Area Space-Time (DYCAST) in 2005, as well as κ values (white bars; calculated every other day with 1-day temporal window; *15*,*24*) illustrating chance-adjusted agreement between DYCAST results and cases in all analysis regions, by number of days before onset of illness. The wide gray vertical band represents the 2–14 day range of the human West Nile virus incubation period (*28*).

to the question regarding adulticiding (RR 0.37, 95% CI 0.15–0.91) in 2007. However, excluding the most extreme outlier with respect to total number of cases, an agency in Kern County whose jurisdiction included 43 and 124 cases in 2006 and 2007, respectively, RRs were not significant in 2007 and were incalculable for 2006 (Table 4).

Discussion

Results from prospective implementation of the DYCAST system in California indicate that the risk model provided accurate and early identification of areas at high risk for WNV transmission to humans during a statewide epidemic in 2005, and was used by local agencies to assist public education campaigns, surveillance, and mosquito control programs. Our findings indicate that DYCAST yielded high levels of sensitivity and specificity for predicting human cases and that relative risk for a WNV case was >39× higher in high-risk cells than in low-risk cells (this value should be considered somewhat inflated, however, because not all low-risk cells contained populated areas). Given the low prevalence of cells containing cases (0.45%), the dynamic nature of DYCAST, and the (>1 cell) spatial scale of WNV transmission and mosquito control (*8*), positive predictive value is considered inferior to other metrics such as κ for evaluating model predictions. κ values >0.50 indicate that DYCAST correctly identified >50% of cells expected to be misidentified by chance alone, which is considered high because WNV causes symptoms in only ≈20% of infections (*28*). Values maintained a moderate strength of chance-adjusted agreement for >4 weeks before onset of illness, which indicates temporal robustness of model predictions.

Cells containing predicted cases were identified as high risk before onset of illness by a mean of 37.2 days; given the 2–14 day range of the human WNV incubation period (*28*), this identification preceded transmission to humans and provided sufficient time to respond and potentially reduce the number of infections (Figures 4, 5). Indeed, 252/354 (71.2%) of cases were predicted 15 days before onset of illness, before the maximum range of the incubation period. Additionally, because the DYCAST procedure only analyzes dead bird reports, it provided for more timely results than did active systems relying on the collection and testing of bird carcasses.

Results from Sacramento County in 2005 demonstrate the practical application of DYCAST for conserving and directing public health resources, such as targeting surveillance efforts that detected the county's first positive mosquito pools that year. During subsequent months, Sacramento County was the location of the largest WNV epidemic in the United States, with 163 reported human cases (*30*) and an incidence rate of 14.5 infections per 100,000 population (*31*). DYCAST results played a key role in SYMVCD's decisions for implementing and targeting emergency aerial mosquito control in the county (D. Brown, pers. comm.; *31*), which ultimately reduced human illness and potential death from WNV infection (*32*).

Notably, prediction rates during 2006–2009 were substantially lower than in 2005, which has implications for the robustness of the model in nonepidemic years or regions. The fairly prevalent use of DYCAST results to assist mosquito control activities in 2006 and 2007 may have played a role in reducing the model's prediction rates in circumstances in which WNV transmission was successfully interrupted before human infection occurred (*31*,*32*). However, while DYCAST could have helped to reduce the absolute number of cases, relatively higher prediction rates were generally observed for agencies that used DYCAST results compared with agencies that did not (Table 4). One explanation is that these areas may have had higher rates of WNV transmission initially, which in turn may have increased agencies' likelihood of utilizing DYCAST for directing control activities or of simply conducting control activities in general. Furthermore, higher rates of WNV transmission may yield greater numbers of subsequent cases within high-risk cells or clusters, thereby increasing the prediction rate. This phenomenon could have also contributed to the higher prediction rates observed during the more severe epidemic in 2005, as could have the self-selecting nature of agencies that participated in the DYCAST program that year, which may have included

areas with higher rates of WNV transmission compared with the rest of the state or to subsequent years.

Efficacy and sustainability of the DYCAST system may be compromised by declines in dead bird reporting, which could be caused by public fatigue or apathy, reductions in reporting infrastructure, or declines in bird deaths caused by herd immunity (*33*). Potential approaches for ameliorating these effects could include recalibration of DYCAST parameters (e.g., lowering the analysis threshold), strategic timing and targeting of press releases and media campaigns, and technologic solutions such as mobile phone application software and text messaging to disseminate information and facilitate the reporting of dead birds. Furthermore, it is uncertain how DYCAST

Table 3. Reported human West Nile virus cases predicted by DYCAST system by county, California, 2005–2009*

	No./total cases (%)				
County	2005†	2006	2007	2008	2009
Alameda	NR	0/1 (0)	NR	0/1 (0)	NR
Amador	0/1 (0)	NR	NR	NR	NR
Butte	0/1 (0)	4/29 (13.8)	2/14 (14.3)	0/4 (0)	0/2 (0)
Calaveras	–	NR	NR	0/1 (0)	NR
Colusa	–	0/3 (0)	0/2 (0)	0/1 (0)	NR
Contra Costa	–	4/8 (50.0)	3/3 (100.0)	3/3 (100.0)	0/5 (0)
El Dorado	–	0/2 (0)	NR	0/1 (0)	0/1 (0)
Fresno	–	6/9 (66.7)	9/17 (52.9)	0/2 (0)	2/11(18.2)
Glenn	–	2/10 (20.0)	0/1 (0)	0/1 (0)	NR
Imperial	NR	0/1 (0)	0/2 (0)	NR	NR
Kern	–	2/45 (4.4)	74/126 (58.7)	2/2 (100.0)	0/15 (0)
Kings	–	0/1 (0)	0/6 (0)	0/1 (0)	0/3 (0)
Lake	NR	0/1 (0)	NR	NR	NR
Los Angeles	–	0/12 (0)	2/31 (6.5)	18/147 (12.2)	1/16 (7.1)
Madera	–	NR	0/2 (0)	NR	NR
Marin	NR	0/1 (0)	NR	NR	NR
Mendocino	NR	NR	0/2 (0)	NR	NR
Merced	15/24 (62.5)	0/4 (0)	0/3 (0)	0/1 (0)	0/4 (0)
Mono	NR	0/1 (0)	NR	NR	NR
Monterey	NR	NR	NR	NR	0/1 (0)
Napa	NR	0/1 (0)	0/1 (0)	NR	NR
Nevada	–	0/1 (0)	NR	NR	NR
Orange	–	0/5 (0)	0/9 (0)	18/60 (30)	0/2 (0)
Placer	29/32 (90.6)	3/8 (37.5)	1/4 (25.0)	1/5 (20.0)	NR
Riverside	0/10 (0)	0/4 (0)	0/16 (0)	0/55 (0)	0/2 (0)
Sacramento	142/152 (93.4)	9/15 (60.0)	7/22 (31.8)	4/12 (33.3)	NR
San Bernardino	0/6 (0)	0/3 (0)	0/3 (0)	5/29 (17.2)	0/2 (0)
San Diego	–	NR	0/12 (0)	1/30 (3.3)	0/4 (0)
San Joaquin	25/34 (73.5)	4/8 (50.0)	3/8 (37.5)	3/9 (33.3)	0/8 (0)
San Luis Obispo	NR	0/1 (0)	NR	NR	NR
San Mateo	0/1 (0)	NR	NR	NR	NR
Santa Clara	–	3/5 (60.0)	1/3 (33.3)	0/1 (0)	NR
Shasta	–	2/4 (50.0)	1/9 (11.1)	0/1 (0)	NR
Solano	4/5 (80.0)	6/8 (75.0)	NR	1/1 (100.0)	NR
Sonoma	0/1 (0)	NR	0/1 (0)	NR	NR
Stanislaus	67/79 (84.8)	3/10 (30.0)	7/20 (35.0)	6/16 (37.5)	0/12 (0)
Sutter	–	0/12 (0)	0/2 (0)	NR	NR
Tehama	–	0/6 (0)	0/3 (0)	0/4 (0)	NR
Tulare	–	0/5 (0)	0/9 (0)	2/3 (66.7)	0/3 (0)
Tuolumne	–	NR	NR	NR	NR
Ventura	NR	1/2 (50.0)	0/1 (0)	NR	NR
Yolo	7/8 (87.5)	18/26 (69.2)	0/1 (0)	0/1 (0)	0/2 (0)
Yuba	–	0 (0/3)	NR	NR	NR
Total	289/354 (81.6)	67/255 (26.3)	110/333 (33.0)	64/392 (16.3)	3/93 (3.2)

*DYCAST, Dynamic Continuous-Area Space-Time; NR, no reported human West Nile virus cases; –, nonparticipating counties with reported cases in 2005. Records without a geocodable address or onset of illness cannot be included in the DYCAST model and are therefore not included in these totals. Counties with no reported cases from 2005–2009 are not shown.
†Analysis regions (Figure 1) consisted of the 16 agencies that participated in the 2005 DYCAST program. The participating region within El Dorado County had no reported cases in 2005.

Table 4. DYCAST prediction rates of reported human West Nile virus cases, by survey answer, California, 2006–2007*

Year and activity	% (No.) agencies	Prediction rate			Prediction rate (excluding outlier)†		
		% Cases (no./total)	Rate ratio (95% CI)	p value	% Cases (no./total)	Rate ratio (95% CI)	p value
2006							
Larviciding			10.06 (2.45–41.32)	**0.001**		NA	NA
Yes	85.0 (34)	41.9 (52/124)			41.9 (52/124)		
No	15.0 (6)	4.2 (2/48)			0 (0/5)		
Adulticiding			10.91 (2.65–44.88)	**0.001**		NA	NA
Yes	74.4 (29)	39.7 (48/121)			39.7 (48/121)		
No	25.6 (10)	3.6 (2/55)			0 (0/12)		
2007							
Larviciding			10.16 (1.41–73.00)	**0.021**		5.63 (0.66–48.15)	0.115
Yes	72.2 (13)	56.4 (79/140)			31.3 (5/16)		
No	27.8 (5)	5.6 (1/18)			5.6 (1/18)		
Adulticiding			0.37 (0.15–0.91)	**0.031**		1.88 (0.22–16.05)	0.566
Yes	47.1 (8)	20.8 (5/24)			20.8 (5/24)		
No	52.9 (9)	56.4 (75/133)			11.1 (1/9)		

*DYCAST, Dynamic Continuous-Area Space-Time; CI, confidence interval; NA, not applicable. Agencies were asked whether they used DYCAST to assist larviciding, adulticiding; agencies that did not respond to survey, as well as answers of "Don't know" (2006: larviciding: n = 2, adulticiding: n = 4) and missing data (2006: larviciding: n = 5, adulticiding: n = 4; 2007: adulticiding: n = 1), were omitted from analysis. Number of analyzed agencies with reported human cases: 2006: larviciding: "Yes": n = 21, "No": n = 4; adulticiding: "Yes": n = 19, "No": n = 7; 2007: larviciding: "Yes": n = 6, "No": n = 4; adulticiding: "Yes": n = 5, "No": n = 4. p values are 2 tailed; statistically significant associations (p<0.05) are in **boldface**.
†Outlier, with respect to total number of cases, was a single agency with 43 and 124 cases in 2006 and 2007, respectively.

results are affected by spatial and temporal heterogeneities of WNV transmission, including inter- and intraspecies variability in the competence (*21*,*34*), pathology (*6*), and distribution of vector and host populations (*35*,*36*). Other confounding factors may include demographic and socioeconomic composition of human populations (*37*) as well as environmental (*38*) and meteorologic variation.

Regardless, DYCAST proved to be a timely and effective early warning system during a severe WNV epidemic. The use of such prospective measures enable the conservation and focus of valuable human and financial resources, which in some cases could be the difference in making an otherwise chaotic epidemic situation tractable. More responsive and efficient surveillance and control can prevent additional human disease, decrease reliance on more substantial control activities later in the season, and reduce indirect costs from medical expenses and productivity loss. The total cost of the 2005 WNV epidemic in Sacramento County alone has been estimated at ≈$3 million (*39*). Furthermore, dynamic monitoring of risk throughout the season may inform decisions for redirecting and triaging resources and may also provide a means for evaluating efficacy of mosquito control efforts. Ultimately, the DYCAST system illustrates the utility of establishing a biologically relevant, spatiotemporal framework for disease surveillance, and adaptation of the DYCAST method may be useful for detecting other infectious diseases and clustering phenomena.

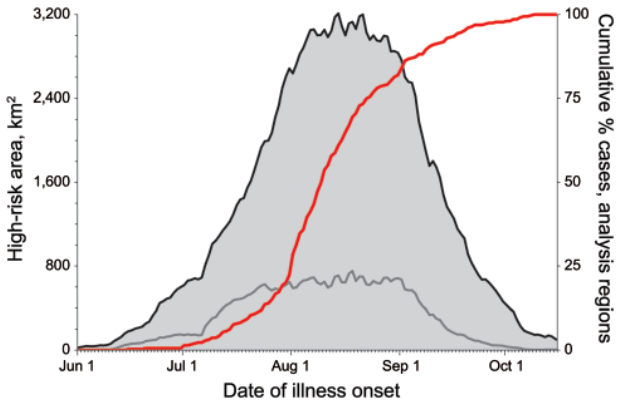

Figure 5. Analysis of West Nile virus cases, California, USA, 2005. Gray region represents area within all analysis regions (black line) and Sacramento County (gray line, for scale) designated by Dynamic Continuous-Area Space-Time as high risk by date of analysis. Red line represents cumulative percentage of reported human West Nile virus cases by date of onset of illness. Time between expansion of high-risk areas and subsequent increase in number of cases may provide an opportunity to respond before epidemic transmission occurs.

This study also highlights the benefits of interdisciplinary and interagency collaboration; synergies between 2 academic institutions and a governmental public health agency shortened the time from research to implementation, and engagement with local mosquito control agencies enabled the practical application of results in real time. Furthermore, our findings demonstrate the potential of harnessing the public's ability to provide timely and useful surveillance data through telephone and internet communications. The leveraging of similar sociotechnologic infrastructure, from mobile phones to internet search queries and social networks, may play a major role in the success, scalability, and cost-effectiveness of predicting and preventing emerging diseases in the future.

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Risk Factors for Pandemic (H1N1) 2009 Seroconversion among Adults, Singapore, 2009

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Release date: July 23, 2011; Expiration date: July 23, 2012

Learning Objectives

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

- Analyze the demographic risk factors for seroconversion for pandemic (H1N1) 2009
- Evaluate the environmental risk factors for seroconversion for pandemic (H1N1) 2009
- Design public health interventions to reduce the spread of pandemic (H1N1) 2009
- Identify laboratory markers indicating a lower risk for seroconversion for pandemic (H1N1) 2009

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A total of 828 community-dwelling adults were studied during the course of the pandemic (H1N1) 2009 outbreak in Singapore during June–September 2009. Baseline blood samples were obtained before the outbreak, and 2 additional samples were obtained during follow-up. Seroconversion was defined as a ≥4-fold increase in antibody titers to pandemic (H1N1) 2009, determined by using hemagglutination inhibition. Men were more likely than women to seroconvert (mean adjusted hazards ratio [HR] 2.23, mean 95% confidence interval [CI] 1.26–3.93);

Malays were more likely than Chinese to seroconvert (HR 2.67, 95% CI 1.04–6.91). Travel outside Singapore during the study period was associated with seroconversion (HR 1.76, 95% CI 1.11–2.78) as was use of public transport (HR 1.81, 95% CI 1.05–3.09). High baseline antibody titers were associated with reduced seroconversion. This study suggests possible areas for intervention to reduce transmission during future influenza outbreaks.

Each year, influenza causes large numbers of deaths (*1*,*2*) and billions of dollars in direct medical costs and indirect costs from declines in productivity and worker absenteeism (*3*). Influenza vaccines help prevent some costs, but the uptake of vaccination for influenza varies widely (*4*), and effective vaccines may not be sufficient during influenza pandemics (*5*). Use of antiviral drugs to mitigate the effects of seasonal and pandemic influenza is also subject to such limitations as cost considerations (*6*), the need for adequate and timely delivery of antiviral drugs (*7*), and concerns about the emergence of resistance (*8*).

Nonpharmaceutical measures have been proposed as adjuncts for reducing the risk for influenza infection during pandemics and seasonal epidemics (*9*). Studies suggest that physical interventions, such as handwashing, use of protective equipment (e.g., face masks), and social distancing measures, can effectively reduce transmission of respiratory viruses, including influenza (*10*,*11*).

A novel influenza A virus, pandemic (H1N1) 2009 virus, emerged in Mexico and the United States during 2009 and spread worldwide within months (*12*,*13*). A few studies have investigated transmission of pandemic (H1N) 2009 virus (*14*,*15*) and the public health interventions that could be used to mitigate its spread (*16*). However, although some data are available from studies conducted in institutions and households (*17*,*18*), little is known about population-level risk factors for pandemic (H1N1) 2009 virus infection.

We investigated risk factors for serologically detected pandemic (H1N1) 2009 virus infection during the first wave of the epidemic in Singapore in 2009. Our study population was a prospective community-dwelling cohort of adults. Singapore is a tropical city-state and global travel hub in Southeast Asia with a population of 5.0 million persons. Singapore detected its first imported cases of pandemic (H1N1) 2009 in late May 2009 and subsequently experienced an epidemic wave lasting ≈12 weeks starting in late June, peaking during the first week of August, and subsiding by early September (*19*–*21*).

Methods

Study Design and Recruitment

This prospective community cohort study was part of a larger study to determine serologic conversion to pandemic (H1N1) 2009 virus in different populations (*22*). Community-dwelling adults 21–75 years of age were recruited from an existing cohort study. The Singapore Consortium of Cohort Studies is a long-term study conducted by the National University of Singapore to study gene–environment interactions in chronic diseases. In June 2009, ≈9,000 persons were participating in the Multi-ethnic Cohort, a subcohort of the Singapore Consortium of Cohort Studies. Participants for the Multi-ethnic Cohort were recruited through public outreach and referrals from existing cohort members. From among these 9,000 participants, we contacted 1,296 randomly selected participants, of whom 894 (69%) agreed to participate. All participants provided written consent, and the study received ethics review and approval from the Institutional Review Board of the National University of Singapore.

We obtained up to 3 blood samples from each participant. Banked blood samples were used for the baseline sample (sample 1); these samples were obtained during June 29, 2005–June 27, 2009, before widespread community transmission of pandemic (H1N1) 2009 virus in Singapore. Two additional blood samples were obtained: an intra-epidemic sample (sample 2) collected ≈4 weeks after the epidemic peaked (August 20–29, 2009) and a postepidemic sample (sample 3) collected ≈4 weeks after epidemic activity had subsided (October 6–11, 2009).

A baseline phone interview with a standardized questionnaire was conducted at recruitment, followed by interviews every 2 weeks throughout the epidemic period. We obtained the following information: sociodemographic and personal behavior, such as sex and ethnicity, history of vaccination for seasonal influenza, and smoking; measures of social interaction, including frequency of use of public transport, travel overseas, and extent of social mixing (e.g., visits to mass entertainment and sports venues); information about household size, ages of household members, and whether other persons in the household and workplace had symptoms of acute respiratory infections (ARIs); and new-onset respiratory and constitutional symptoms.

Laboratory and Statistical Methods

Venous blood was obtained from participants, and serum was extracted on the same day. The hemagglutination-inhibition assay was performed according to standard protocols at the World Health Organization Collaborating Center for Reference and Research on Influenza in Melbourne, Victoria, Australia (*22*). We defined seroconversion as a ≥4-fold increase in antibody titers between any successive pairs of blood samples (between sample 1 and sample 2 for participants who provided only 1 other sample in addition to baseline; and between samples 1 and 2 or between samples 2 and 3 for participants who

provided 2 samples in addition to baseline). We considered participants to have seroconverted in the few instances in which titers increased ≥4-fold between samples 1 and 2 but not between samples 1 and 3.

The primary outcome of interest was seroconversion, and we evaluated other variables as independent predictors for seroconversion. Sociodemographic and biologic factors analyzed were age (calculated from date of birth obtained from the National Registration Identity Card [NRIC]), sex (obtained from NRIC), ethnicity (the 3 major ethnic groups in Singapore—Chinese, Malay, and Indian—and a category for other minorities, obtained from NRIC), self-reported dwelling type, self-reported vaccination ≥1× previously with the seasonal influenza vaccine (ever vs. never vaccinated), baseline antibody titer to pandemic (H1N1) 2009, and smoking behavior (current, former, and never). Measures of social mixing were self-reported overseas travel, self-reported frequency of use of public transport (bus and the Mass Rapid Transit metro system), and self-reported frequency of visits to the following places: mass entertainment venues (e.g., cinemas, stadiums, and theaters; shopping centers, markets, and supermarkets; restaurants, bars, clubs, and other eating or drinking establishments; places of worship; and other social gatherings with ≥10 persons). Except for overseas travel, these measures reflected lifestyle routine, and the behavior reported in the baseline interview was used to reduce any effect from reverse causality (e.g., avoiding particular activities or locations because of influenza symptoms). For the overseas travel variable, participants were classified as having ever traveled during the entire study period. We also examined the effects of the number of contacts in various age groups living in the same household and of exposure to contacts who were sick with ARI either at home or at work.

Bivariate analyses were performed for all variables, with a final multivariate model constructed with the key sociodemographic variables of age, ethnicity, sex, dwelling type (as a proxy for socioeconomic status), and variables of interest to our study (smoking, previous influenza vaccination, baseline antibody titer, travel and public transport use, working outside the home, contact with other persons at home or at work who had ARI, and number of household members), by using a Weibull proportional hazards model. We chose to work with a parametric survival analysis model rather than with the more familiar Cox semiparametric proportional hazards model because 1) event (seroconversion) times were all interval- or right-censored (rather than the more common combination of uncensored and right-censored observations) and 2) as a result of the short period of blood sampling, there were many tied interval endpoints, making exact methods (such as that of Kalbfleisch and Prentice [*23*]) too exacting and approximations (such as those of Breslow [*24*] or Efron [*25*]) too approximate.

The study comprised participants from separate households and some from households where at least 1 other household member was also part of the study. Because influenza infection is transmissible, independence of outcomes cannot be assumed for persons from the same household. To reduce estimation errors, we randomly selected 1 person from each household for each regression analysis. We then repeated this selection process and analysis 1,000× to reduce the effect of chance variation resulting from the selection process. Reported hazard rates (HRs) refer to the mean HRs of all 1,000 iterations. Similarly, reported 95% confidence intervals (CIs) refer to the mean upper and lower 95% bounds from all 1,000 iterations. All p values reported are 2-tailed, with the significance level set at 0.05. We calculated goodness-of fit by using a modified version of the Hosmer-Lemeshow test (*26*). The statistical package R version 2.9.2 (*27*) was used for all analyses.

Results

Of the 894 persons who agreed to participate in the study, 828 (93%) completed the baseline questionnaire. Baseline blood samples were available for all participants, sample 2 for 621 (69.5%) participants, and sample 3 for 689 (77.1%) participants. Three blood samples were available for 584 (65.3%) participants, and at least 2 samples were available for 727 (81.3%) persons. Figure 1 shows the pandemic (H1N1) 2009 epidemic curve in Singapore and the recruitment process for this study. When we compared characteristics of the 727 participants who completed the study with the 828 who were enrolled at the start of the study (Table), we found them to be broadly similar. All subsequent analyses were performed on these 727 persons. Of these, 494 came from separate households and 233 came from 117 households where at least 1 other household member was also part of this study; selection for 1 member per household resulted in a sample size of 611 persons.

In bivariate analyses, seroconverters were younger, more likely to smoke, and more likely to have traveled outside Singapore than were nonseroconverters (online Appendix Table, www.cdc.gov/EID/content/17/8/101270-appT.htm). Seroconverters also were more likely to be of Malay ethnicity and to have more household members 5–19 years of age; however, p values for these 2 variables were just >0.05.

We assessed the frequency of activities and visits to a variety of public places, including use of public transport, during a 14-day period at baseline among the 727 participants and the proportion in each frequency group who seroconverted by the end of the study (Figure 2; online Technical Appendix, Table 1, www.cdc.gov/EID/

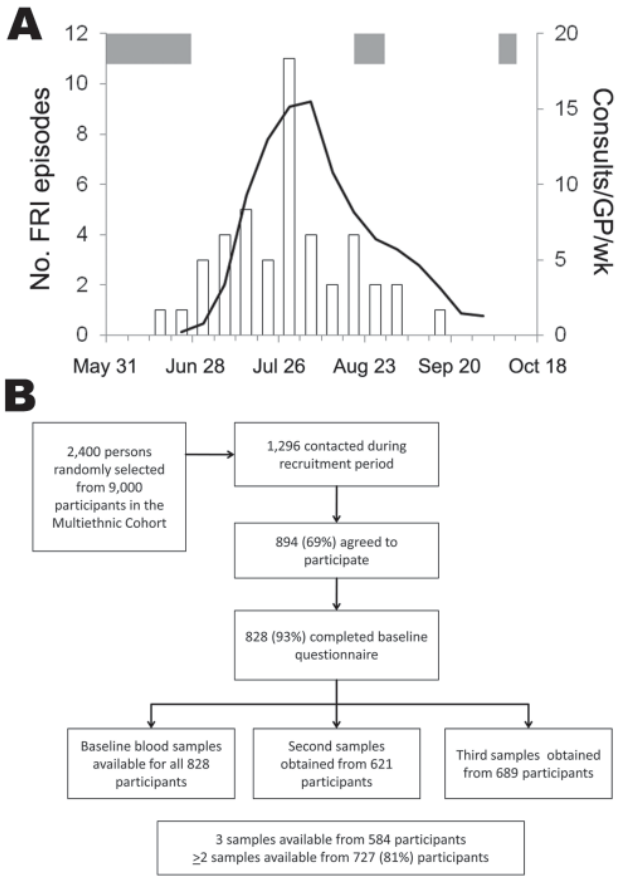

Figure 1. A) Estimated pandemic (H1N1) 2009 curve, Singapore, June–September 2009. Data obtained from general practice sentinel data. Blue bars indicate number of influenza-like illness episodes per general practice per week. Red curve indicates estimated number of consults for pandemic (H1N1) 2009 per general practice per week. Orange bars indicate period of blood sample collection in the adult cohort (*23*). B) Summary of recruitment numbers and dates of blood collection in the adult community cohort.

content/17/8/101270-Techapp.pdf). The frequencies of activities and visits to public venues were not associated with seroconversion. More frequent use of public transport appeared to be associated with higher seroconversion rate. The proportion of seroconverters in the "never" and "once/twice" categories were similar, as were those in the "several times" and "daily/almost daily" categories. We dichotomized this variable into frequent use (i.e., participants reporting several times or daily/almost daily use of public transport during the preceding 14 days at baseline) and seldom use (participants reporting no use or use once or twice during the preceding 14 days. Frequent users of public transport were more likely to seroconvert; however, this finding was not statistically significant.

In multivariate analysis (online Appendix Table), men were more likely than women to seroconvert (mean HR 2.23, 95% CI 1.26–3.93). Malays were more likely than Chinese to seroconvert (mean HR 2.67, 95% CI 1.04–6.91). Working outside the home and not having a work contact with ARI symptoms was associated with lower likelihood of seroconversion (mean HR 0.39, 95% CI 0.21–0.7); however, this decreased risk was not seen in workers who reported a work contact with ARI symptoms. Frequent use of public transport (mean HR 1.81, 95% CI 1.05–3.09) and travel out of the country (mean HR 1.76, 95% CI 1.11–2.78) also were associated with a higher risk for seroconversion, whereas high baseline antibody titers to pandemic (H1N1) 2009 virus were associated with lower risk for seroconversion (mean HR 0.5, 95% CI 0.27–0.94). The number of household members 5–19 years of age was marginally associated with increased risk for seroconversion (mean HR 1.17, 95% CI 0.95–1.43 for each additional member, compared with not having a household member in that age group), although the p value was not significant. There was good fit for the multivariate model (p = 0.625).

We repeated analyses (online Technical Appendix Tables 2, 3) by using a logistic regression model for all 727 participants. Odds ratios (ORs) were broadly similar to the corresponding HRs when Weibull regression was used, with significant associations obtained for foreign travel, public transport use, high baseline antibody titers, sex, ethnicity and employment outside the home (online Technical Appendix Tables 2, 3). These findings suggested that results are robust to the choice of modeling framework.

We investigated whether the association between seroconversion and foreign travel was greater in the earlier than in the later part of the outbreak by considering the subset of participants who provided 3 blood samples. We defined early seroconverters as participants who seroconverted between samples 1 and 2 and late seroconverters as those who converted between samples 2 and 3, and we investigated the estimated risk associated with foreign travel in logistic regression. After adjustment, the OR for foreign travel among early seroconverters was 2.06 (95% CI 1.25–3.55), whereas that for late seroconverters was 1.71 (95% CI 0.63–4.68).

Discussion

Understanding the factors that influence the risk for influenza infection will permit development of rational response strategies to reduce transmission. Although studies have been conducted of household and institutional risk factors (*14*,*17*,*18*,*28*) for pandemic (H1N1) 2009 virus transmission, few data are available on risk factors in the general population. Through the use of a population-based cohort design, our study suggests that several factors are associated with pandemic (H1N1) 2009 infection.

A history of travel abroad was associated with increased risk for seroconversion. Self-reports obtained throughout

the outbreak were used, and this result could have been subject to reverse causation; i.e., symptomatic persons may have reduced their frequency of travel. However, this bias, if present, would have resulted in a lower estimate of the true risk and therefore cannot explain the association observed. Whether the increased risk for seroconversion associated with overseas travel results from prolonged close proximity with infected travelers in airports and on airplanes or to transmission within other countries is unclear. Compared with the OR for early seroconverters, the OR for foreign travel in late seroconverters was not attenuated. If the risks associated with foreign travel resulted primarily from increased likelihood of transmission overseas, then we would expect the risk to be high during the start of an epidemic and drop substantially as local transmission increased. Our results imply that other explanations may be relevant, such as increased infection from prolonged close proximity with other infected persons during travel (e.g., on an airplane) or to increased use of public transport, such as buses or subway systems, during travel. A recent publication based on a retrospective cohort study of confirmed pandemic (H1N1) 2009 infection in persons returning from Mexico to New Zealand on a commercial airliner suggested a measureable risk associated with being on an airliner with infected persons (*29*). Further research is needed to understand the association between foreign travel and seroconversion. Although total restriction on global travel will not be feasible and is unlikely to prevent importation of infectious cases (*30*), such measures as travel advisories that promote personal hygiene and advise passengers to seek medical assistance promptly if symptoms develop might mitigate the effect of international travel on influenza transmission (*9*,*31*,*32*).

The local use of public transport was significantly associated with increased risk for seroconversion, even after adjustment. Confounding is unlikely to explain this association. Public transport in the local context involves close contact with a large number of persons, often for prolonged periods. Mathematical modeling studies have suggested that social distancing and reduction of mass gatherings are critical for preventing influenza spread (*9*). Although shutting down public transport systems is not possible without severe socioeconomic impact, other measures, such as reduction of crowding in public transport systems; better air circulation; and advice on personal hygiene etiquette and measures, including face mask use, may help to reduce transmission. Further studies are needed to characterize the mechanisms of influenza and respiratory virus transmission within public transport systems.

Compared with participants who did not have a job or worked from home, persons who worked away from home had a lower risk for seroconversion, but this risk was observed only among those who reported that no one in their workplace had symptoms of ARI. These findings suggest that transmission within the household may be more relevant than in the workplace. One possible explanation is that persons who do not work may be more involved in childcare activities at home. One study found that spending long periods exposed to a sick index patient is a risk

Table. Comparison of participants who provided follow-up blood samples with cohort at start of study of risk factors for pandemic (H1N1) 2009 seroconversion, Singapore, 2009*

Characteristic	Total, n = 828	Respondents, n = 727
Age, y, mean (SD)	43.4 (12.0)	43.6 (11.8)
Age group, y		
20–29	149 (18.0)	124 (17.1)
30–39	131 (15.8)	113 (15.5)
40–49	285 (34.4)	260 (35.8)
50–59	175 (21.1)	155 (21.3)
≥60	88 (10.6)	75 (10.3)
Sex		
F	482 (58.2)	432 (59.4)
M	346 (41.8)	295 (40.6)
Ethnicity		
Chinese	94 (11.4)	90 (12.4)
Malay	374 (45.2)	331 (45.5)
Indian	353 (42.6)	299 (41.1)
Other	7 (0.9)	7 (1.0)
Dwelling type		
≤3-room public housing	202 (24.4)	177 (24.3)
4-room public housing	360 (43.5)	310 (42.6)
5-room public housing or private housing	266 (32.1)	240 (33.0)
Smoking		
Current smoker	181 (21.9)	149 (20.5)
Nonsmoker/former smoker	647 (78.1)	578 (79.5)
No. household members		
1	580 (70.1)	496 (68.2)
2	208 (25.1)	195 (26.8)
3	36 (4.4)	33 (4.5)
4	4 (0.5)	3 (0.4)
Self-reported previous influenza vaccination		
No	751 (90.7)	662 (91.1)
Yes	77 (9.3)	65 (8.9)
Employment outside the home		
No	308 (37.2)	277 (38.0)
Yes	520 (62.8)	450 (62.0)
Baseline antibody titer		
Mean (SD)	0.22 (0.71)	0.25 (0.75)
By age group, y		
20–29		0.48 (1.06)
30–39		0.19 (0.58)
40–49		0.17 (0.59)
50–59		0.28 (0.84)
≥60		0.12 (0.52)
Public transport		
Seldom	294 (35.5)	249 (34.3)
Frequent	534 (64.5)	478 (65.7)

*Values are no. (%) except as indicated. Respondents are participants who provided at least 1 blood sample in addition to that obtained at baseline.

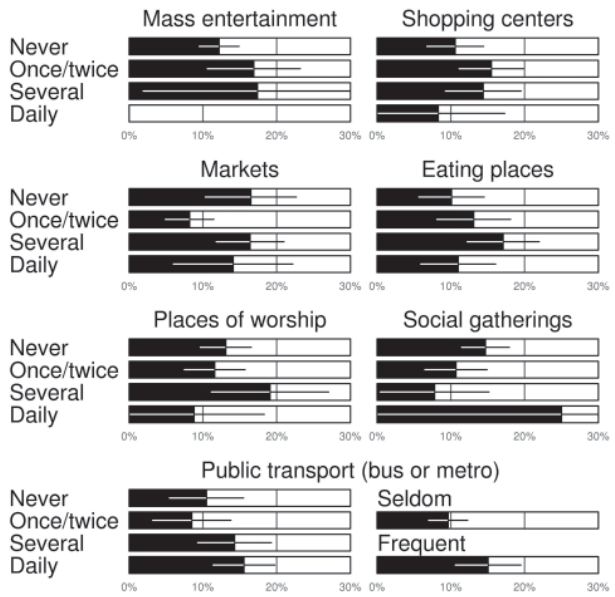

Figure 2. Study of seroconversion for pandemic (H1N1) 2009 virus, Singapore, June–September 2009. Public places were as follows: mass entertainment venues (e.g., cinemas, stadiums, and theaters; shopping centers, markets, and supermarkets; restaurants, bars, clubs, and other eating or drinking establishments; places of worship; and other social gatherings with ≥10 persons. Horizontal lines denote error bars for the estimates.

factor for infection (*17*), and other studies have implicated children, especially those that are attending school, as a source of influenza transmission (*33*,*34*). Our study suggests that having a school-aged household member (5–19 years of age) increased risk for seroconversion (however, in multivariate analyses, the effect was attenuated and was not statistically significant). Interventions that target schoolchildren, such as vaccination and school closures, may reduce infection among other family members.

Household studies, such as that by Cauchemez et al. (*14*), have shown that increasing household size reduced the probability of transmission by each index patient. The amount of time spent by any 1 person in close contact with an index patient is most likely to be lower in larger households. However, such studies do not assess the aggregate risk when >1 household members become infected during an outbreak. Our study suggests that having more household members in the 5–19-year age group increases overall risk for seroconversion during a pandemic, a finding that is biologically plausible because every household member is at risk for infection (and subsequent transmission) during an outbreak.

Having a contact with ARI at home was not associated with increased risk for seroconversion. This finding might result from the low specificity and sensitivity of ARI as a marker for exposure to pandemic (H1N1) 2009 because other viral causes of ARI (including other influenza strains) were cocirculating in substantial proportion in Singapore during the pandemic (*19*). Infections also could have been transmitted by asymptomatic infected persons—indeed, 27% of persons in our study who seroconverted did not have any ARI symptoms throughout the study period.

Baseline antibody titers were associated with lower seroconversion rates. Older participants, especially those ≥60 years of age, appeared to have a lower risk for seroconversion (however, this finding was not statistically significant after multivariate adjustment). Other studies also have reported lower rates of infection among older persons (*35*). Some groups (*36*) have proposed that previously acquired immunity might explain the protection, although in our study, older participants did not have higher levels of measureable antibodies to pandemic (H1N1) 2009 virus. Other factors, such as lower levels of social contact among elderly persons, might explain the observation.

Men were more likely than women, and Malays were more likely than Chinese, to seroconvert. Although the association with male sex has not previously been reported, a study of the 1957 influenza pandemic in Singapore also suggested higher infection rates in Malays (*37*). Unmeasured sociocultural and behavioral factors might explain these observations, and further studies are needed to confirm these observations and to understand the basis for the association.

Our study did not find any effect of the frequency of visits to public venues on the risk for seroconversion (results not shown). We could not obtain accurate estimates of the length of time spent at each venue, the number of persons with whom each participant had contact during the visit, and the proximity and intensity of contact. Studies that capture such detail would be useful to quantify the relative risks for various activities and visits to different public venues during pandemics. Such a study would have public health policy implications because one option for social distancing is to close public venues, such as cinemas and theaters, an option taken by the authorities in Mexico during the early stages of the pandemic (*38*).

Our study had other limitations. We used data obtained at baseline because these would not be subject to reverse causation. However, behavior recorded at baseline might not reflect actual behavior during the outbreak. We were unable to study the effect of personal hygiene measures, such as use of face masks and handwashing, because of the complexity of measuring these factors by using questionnaires every 2 weeks.

Our prospective cohort study suggests avenues for further research into public health interventions for influenza epidemics. We showed that overseas travel and use of public transport were significantly associated

with increased risk for seroconversion, and these offer potential areas for public health intervention, such as travel restriction, social distancing, and personal protection measures. Additional research is required to understand the reasons behind the increased risks associated with demographic factors of sex and ethnicity.

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Use of Medical Care during Pandemic (H1N1) 2009, Navarre, Spain

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Of 233 households with laboratory-confirmed pandemic (H1N1) 2009 in Navarre, Spain, only 64% (107/166) of contacts with influenza-like illness had sought medical care. This value was lower for adults (53%, 39/74) than for children <15 years of age (74%, 68/92), as well as for those with cases secondary to another household case (58%, 64/111).

Every year, influenza affects 5%–10% of the population, and most persons have various episodes of this disease during their lifetime (*1*,*2*). Influenza is typically characterized by respiratory symptoms and general symptoms such as fever, malaise, and myalgia (*2*,*3*). Most cases resolve without complications within 1 week (*4*). The epidemiologic context facilitates diagnosis of influenza because it usually occurs in seasonal waves (*5*). For these reasons, some persons with influenza do not seek medical care.

This proportion of influenza cases is hidden from the health system and epidemiologic surveillance, leading to underestimation of the true extent of the affected population. The purpose of this study was to estimate the proportion of persons with influenza who sought medical care in Navarre, Spain, during the 2009–10 influenza season and identify characteristics that differentiate persons who sought or did not seek medical care.

The Study

The study protocol was reviewed and approved by the Ethical Committee of the Barcelona Health Institute. During the 2009–10 influenza season, the Sentinel Network of Primary Care Physicians and Pediatricians of Navarre obtained nasopharyngeal swab specimens from patients with influenza-like illness (ILI) for virologic confirmation of influenza by using standard real-time reverse transcription PCR or cell culture (*6*).

In early 2010, a trained nurse telephoned households of persons who had had laboratory-confirmed pandemic (H1N1) 2009 in October and November 2009 while pandemic influenza was active in the area (index cases). When no response was received, the calls were repeated 5 times on different days and at different times. The interview was conducted by using a structured questionnaire. For each household, an attempt was made to talk to the adult who was primarily responsible for the health issues of those who lived there, usually the mother or father. When possible, other adults in the household were also interviewed.

Detailed information was obtained about index case-patients and all other persons living in the same household (contacts) with regard to sociodemographic data, medical history, influenza symptoms, and whether he or she had sought medical care. A question was asked about each of the following signs and symptoms: fever, cough, sore throat, headache, muscular or joint pain, nasal congestion, and vomiting.

This analysis included all household residents who had ILI for <7 days with respect to the onset of ILI in the index case-patient. ILI was defined as fever and either cough or sore throat in the absence of other diagnoses. The first person with ILI in the household was considered the primary case-patient, and others, if any, were considered secondary case-patients. Persons with index cases who led us to contact the household were excluded from analysis because, given the study design, they had all sought medical care.

Of 252 households that met the inclusion criteria, 233 (92%) were successfully contacted, and all persons agreed to participate in the study. Of 668 household contacts of index cases, 188 (28%) persons had ≥1 influenza symptoms and 166 (25%) met the criteria for having ILI. The proportion of symptomatic cases that met the criteria for ILI was higher among children (94%, 92/98) than among adolescents and adults (82%, 74/90; p = 0.021).

Of 166 persons with ILI, only 107 (64%, 95% confidence interval [CI] 57%–72%) had sought medical care. The percentage of those who sought medical care was lower among adults and adolescents (53%, 39/74) than among children <15 years of age (74%, 68/92; p = 0.006). This percentage was also lower among persons with cases secondary to a previous household case (58%, 64/111) than among primary case-patients (78%, 43/65; p = 0.010) (Table 1).

No differences were detected by sex, rural/urban residence, country of origin, vaccination against seasonal influenza, smoking status, or presence of major chronic

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Table 1. Persons with ILI who sought medical care, by sociodemographic and health-related characteristics, Navarre, Spain, 2009–10*

Characteristic	No. persons with ILI	No. (%) who sought medical care	Crude OR (95% CI)	p value†
Sex				
M	86	55 (64)	1	
F	80	52 (65)	1.0 (0.6–2.0)	1.000
Age, y				
<15	92	68 (74)	2.5 (1.3–5.2)	0.006
≥15	74	39 (53)	1	
Residence				
Rural	45	28 (62)	1	
Urban	121	79 (65)	1.1 (0.6–2.3)	0.719
Country of origin				
Spain	146	95 (65)	1	
Other	20	12 (60)	0.8 (0.3–2.1)	0.804
Major chronic conditions				
No	146	93 (64)	1	
Yes	20	14 (70)	1.3 (0.5–3.7)	0.629
Seasonal influenza vaccine				
No	149	94 (63)	1	
Yes	17	13 (76)	1.9 (0.6–6.1)	0.423
Smoked				
No	147	98 (67)	1	
Yes	19	9 (47)	0.5 (0.2–1.2)	0.127
Relation to other cases in household				
Secondary case	111	64 (58)	1	
Primary case	55	43 (78)	2.6 (1.3–5.5)	0.010
Antiviral treatment for primary case-patient				
No	75	45 (60)	1	
Yes	36	19 (53)	0.7 (0.3–1.7)	0.540
Total	166	120 (64)		

*ILI, influenza-like illness; OR, odds ratio; CI, confidence interval.
†By Fisher exact test.

conditions. The percentage of secondary case-patients who sought medical care was the same regardless of whether the primary case-patient received antiviral treatment. Persons who had sought medical care had a mean ± SD of 4.0 ± 0.8 symptoms, which was similar to those who had not sought medical care (4.0 ± 0.7 symptoms; p = 0.688). None of the contacts had received antiviral prophylaxis or vaccine for pandemic (H1N1) 2009.

The frequency with which persons with ILI sought medical care, by age group and symptoms, is shown in Table 2. Frequency of symptoms did not differ between persons who had sought medical care and those who had not sought medical care.

Logistic regression analysis showed that seeking medical care was more frequent among children than among adolescents and adults (adjusted odds ratio 2.2, 95% CI 1.1–4.3; p = 0.019) and among primary case-patients than among secondary case-patients (adjusted odds ratio 2.2, 95% CI 1.0–4.8; p = 0.038). A similar proportion of contacts with any symptom but who did not meet ILI criteria sought medical care (59%, 13/22) as did those who met ILI criteria (64%, 107/166; p = 0.642).

Conclusions

We studied persons who had ILI and were household contacts of persons with laboratory-confirmed influenza. It is likely that the cause of symptoms among persons with ILI was also infection with influenza virus. Approximately two thirds of these persons sought medical care. Care seeking was less frequent among adults and when there had already been another case within the household.

Epidemiologic surveillance systems and studies of influenza are typically based on cases of medically diagnosed ILI. Therefore, the proportion of persons not seeking medical care is usually unknown and not taken into account. Ideally, epidemiologic surveillance should consider these persons to avoid underestimating the actual magnitude of the disease (*7*). These persons may increase spread of the disease (*8*) and may also be more likely to self-medicate, resulting in possible risks to their health (*9*,*10*). Persons who do not seek medical care do not contribute to the cost of influenza from the health system perspective, but they do from the point of view of society because they can result in lost work hours, more medications used, and increased need for care (*11*).

Table 2. Persons with ILI who sought medical care, by age and signs or symptoms, Navarre, Spain, 2009–10*

	Children <15 y of age			Adolescents and adults		
Sign or symptom	No. persons with ILI	No. (%) who sought medical care	p value†	No. persons with ILI	No. (%) who sought medical care	p value†
Fever			ND			ND
No	0	0		0	0	
Yes	92	68 (74)		74	39 (53)	
Cough			1.000			1.000
No	4	3 (75)		7	4 (57)	
Yes	88	66 (75)		67	35 (52)	
Sore throat			0.464			0.815
No	35	24 (69)		42	23 (55)	
Yes	57	44 (77)		32	16 (50)	
Headache			0.342			0.759
No	44	35 (80)		12	7 (58)	
Yes	48	33 (69)		62	32 (52)	
Muscle pain			0.180			0.241
No	86	65 (76)		32	14 (44)	
Yes	6	3 (50)		42	25 (60)	
Rhinorrhea			0.804			0.479
No	29	21 (72)		44	25 (57)	
Yes	63	47 (75)		30	14 (47)	
Vomiting			0.277			0.473
No	81	58 (72)		73	39 (53)	
Yes	11	10 (91)		1	0	

*ILI, influenza-like illness; ND, not determined.
†By Fisher exact test.

When influenza symptoms were present, adults and adolescents sought medical care less often than children. This finding may explain in part why the incidence of medically diagnosed ILI is usually much higher among children (*4*,*6*,*12*).

This study was conducted during the pandemic influenza (H1N1) 2009 season. Therefore, its results may not be generalizable to other influenza seasons (*4*). Nevertheless, the study was conducted when pandemic (H1N1) 2009 virus had already been circulating in the population for several months, the initial alarm had abated, and the level of medical care had returned to levels similar to that for seasonal influenza.

We included symptoms of influenza that were reported by concerned persons or concerned parents. Thus, persons with mild symptoms may be underrepresented in our analysis. The probability of making telephone contact may have been higher in households with more members. However, only 8% (19/252) of households were not contacted. Families in whom none of the members had sought medical care were not included in the study, which may overestimate the proportion of persons who sought medical care.

Persons with influenza who did not seek medical care should be taken into account in estimations of the actual incidence of influenza and its effect on the general population. These persons may have a major effect on transmission and should be considered in planning prevention and control measures and in evaluations of the effects of this disease.

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Risk Factors for Death from Pandemic (H1N1) 2009, Southern Brazil

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To identify risk factors for death from pandemic (H1N1) 2009, we obtained data for 157 hospitalized patients with confirmed cases of this disease. Multivariate analysis showed that diabetes and class III obesity were associated with death. These findings helped define priority vaccination groups in Brazil.

In May 2009, pandemic (H1N1) 2009 was identified in Brazil (*1*). In June 2009, the first confirmed death from this disease was reported in southern Brazil. On July 16, 2009, Brazil declared sustained transmission of pandemic (H1N1) 2009, and the case definition for mandatory notification was limited to suspected influenza cases with fever >38°C, cough, and dyspnea or death, i.e., severe acute respiratory infection (*1*). During July 19, 2009–January 2, 2010, a total of 44,544 pandemic influenza cases were confirmed and 2,051 deaths were reported in Brazil, corresponding to notification and death rates of 23.3 cases and 1.1 deaths per 100,000 population. In southern Brazil, notification and death rates reached 110 cases and 3.0 deaths per 100,000 population, and a 4.6% case-fatality rate was observed among reported patients (*2*). To identify risk factors for death caused by pandemic (H1N1) 2009, we analyzed data for patients hospitalized with confirmed pandemic (H1N1) 2009 at the beginning of the pandemic in southern Brazil.

The Study

This study was conducted in 11 hospitals in 4 cities (Passo Fundo, Caxias do Sul, Santa Maria, and Uruguaiana) in Rio Grande do Sul (population 10,914,128 in 2009), the southernmost state in Brazil (*3*). At the time of this study, these 4 cities accounted for 52% of reported deaths from pandemic (H1N1) 2009 in this state.

All laboratory-confirmed (real-time reverse transcription PCR–positive) pandemic (H1N1) 2009 case-patients hospitalized in July 2009 who had shortness of breath or radiologic evidence of pneumonia and either died (case-patients) or were discharged (controls) were included. A standardized form was used that included data reported by patients who survived or their families (patients who died and patients <18 years of age) and information from medical chart review.

We analyzed factors associated with death by calculating odds ratios (ORs) and 95% confidence intervals (CIs). Variables with a p value <0.10 calculated by bivariate analysis were included in a multivariate unconditional logistic regression model adjusted for age and sex. All statistical analyses were conducted by using Epi Info for Windows version 3.5.1 (Centers for Disease Control and Prevention, Atlanta, GA, USA). A p value <0.05 was considered significant.

The number of confirmed pandemic (H1N1) 2009 case-patients enrolled in each city is shown in Figure 1. The study included 52 patients who died and 105 who survived (Figure 2). Characteristics and clinical findings of case-patients are shown in Table 1. A total of 136 (87%) of the 157 case-patients sought treatment before hospitalization (median 2 health care visits, range 0–5 visits). Obesity was the most frequent underlying medical condition (38%). Among obese case-patients, 19 (36%) had other risk factors for influenza complications. Diabetes was the most frequent underlying medical condition (21%) in obese case-patients. Thirty-four (49%) of 70 case-patients who did not have risk factors for influenza complications were obese (body mass index ≥30 kg/m^2), 6 (9%) had class III obesity (body mass index ≥40 kg/m^2), and 20 (29%) had hypertension.

Taking medication was reported by 107 (68%) case-patients, but none received oseltamivir before hospitalization. Hospitalization occurred a median of 5 days (range 0–15 days) after symptom onset. Most case-patients (94%) received antimicrobial drugs during hospitalization, and most (81%) began antimicrobial drug therapy on the day of hospitalization. Steroids were administered to 83 (53%) case-patients a median of 1 day (range 0–11 days) after admission.

Three deaths occurred during the first 24 hours of hospitalization. The case-fatality rate was higher among patients admitted to the intensive care unit (47 [59%] of 80 died). No difference was observed between patients who died and those who survived for median number of days between symptom onset and hospitalization (case-patients

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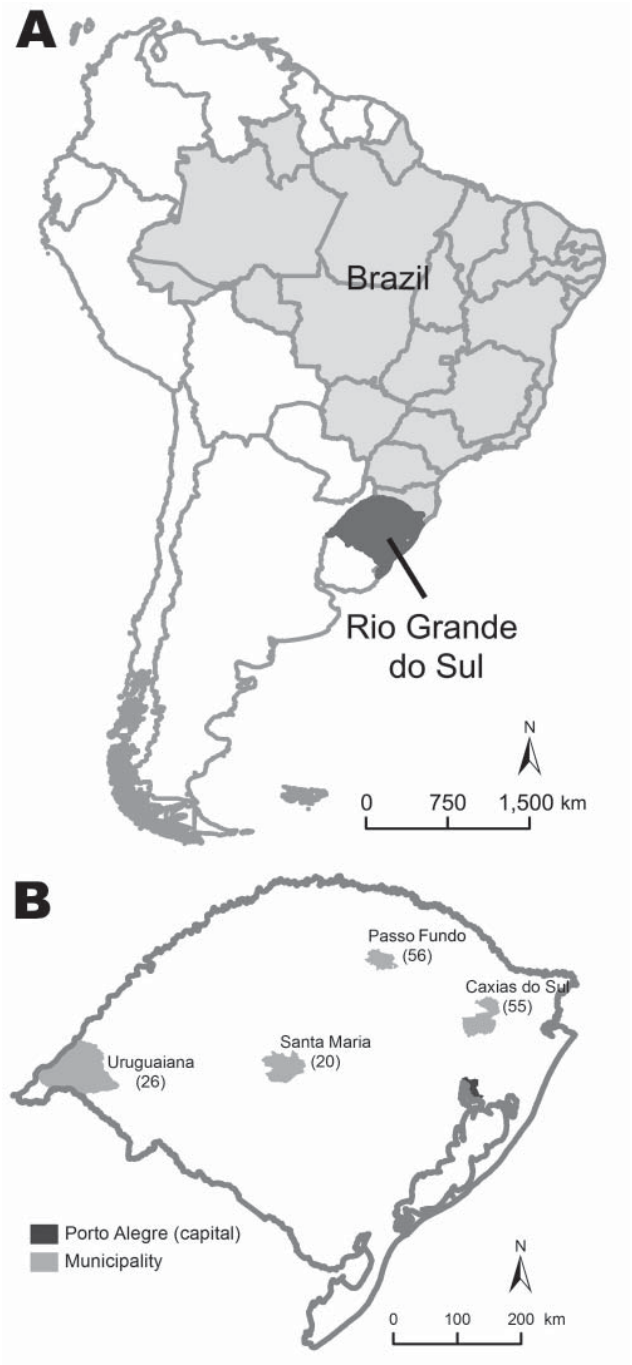

Figure 1. Location of Rio Grande do Sul, Brazil (A) and distribution of 157 patients with pandemic (H1N1) 2009 in 4 cities in this state (B). Values in parentheses are numbers of patients.

6 days, range 0–6 days; controls 5 days, range 0–15 days; p = 0.25) or initiation of oseltamivir treatment (case-patients 6 days, range 1–16 days; controls 5 days, range 0–19 days; p = 0.10). After we adjusted for age and sex, diabetes (OR 4.4, 95% CI 1.5–12.8) and class III obesity (OR 6.2, 95% CI 1.3–29.2) were independently associated with death from pandemic (H1N1) 2009. No association was found between oseltamivir treatment within 48 hours of symptom onset and death (Table 2).

Conclusions

This study confirmed findings from other countries suggesting that at the beginning of the epidemic, pandemic (H1N1) 2009 virus showed a pattern similar to that in the Northern Hemisphere. Consequently, vaccine recommendations in Brazil were made on the basis of epidemiology of pandemic (H1N1) 2009 in Brazil and other countries.

Identification of diabetes and class III obesity as independent risk factors for death caused by pandemic (H1N1) 2009 among hospitalized patients in Brazil was also consistent with findings from other regions (*4*–*8*). Prevalence of obesity ranged from 26% to 74% in critically ill pandemic (H1N1) 2009 patients worldwide (*5*). Diabetes is also considered a risk factor for seasonal influenza complications in nonelderly persons (*9*). Class III obesity might increase illness and death from influenza because it impedes pulmonary function and contributes to extended mechanical ventilation and hospitalization for these patients compared with nonobese patients (*10*). Also, class III obesity is frequently associated with other underlying illnesses, such as cardiovascular diseases and diabetes (*5*).

Diabetes and obesity were overrepresented among case-patients in this study compared with the general population of Rio Grande do Sul. A telephone survey conducted in Porto Alegre (capital of Rio Grande do Sul) found a 14.3% prevalence of self-reported obesity and 6.2% prevalence of self-reported diabetes in 2009 (*11*). Although we found a low frequency (8%) of class III obesity among patients who died, this frequency was 12.5× the estimate prevalence of class III obesity among adults in Brazil in 2003 (0.64%) (*12*).

Our study had several limitations. Data were collected retrospectively (median 54 days, range 1–93 days after

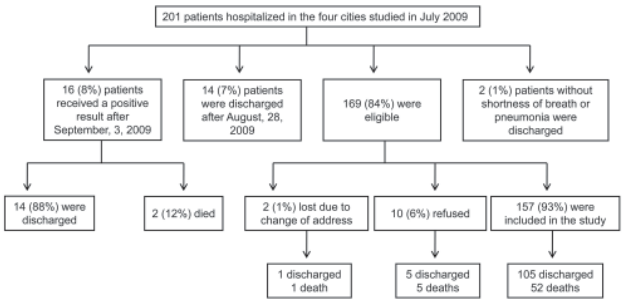

Figure 2. Sample selection process for 201 patients with pandemic (H1N1) 2009, Rio Grande do Sul, Brazil, 2009.

symptom onset) and by proxy interview for patients who died and pediatric patients and were therefore subject to recall bias. Data for analysis, including underlying illnesses and patient weight and height, were not systematically recorded in medical charts. Therefore, these data could not be used to validate questionnaire responses. Furthermore, hospitalized patients from whom nasopharyngeal aspirates or swab samples were not

Table 1. Characteristics of confirmed pandemic (H1N1) 2009 patients, Rio Grande do Sul, Brazil, 2009*

Characteristic	Value	No. patients
Demographic		
Male sex	78 (50)	157
Age, y	33 (0–73)	157
Family income, US$	678 (0–6,780)	146
Education level, y†	8 (0–19)	129
Residence city different from hospitalization city	46 (29)	157
Smoking habits†		
Current smoker	34 (24)	142
Years exposed to tobacco	14 (1–47)	33
Cigarettes/day	12 (1–60)	33
Pack-years	8 (0–93)	33
Former smoker	19 (13)	142
Years exposed to tobacco	11 (1–54)	18
Cigarettes/day	20 (3–60)	18
Pack-years	6.5 (0–162)	18
Signs and symptoms		
Cough	155 (99)	157
Fever	152 (97)	157
Shortness of breath	152 (97)	157
Myalgia	110 (70)	157
Chills	110 (70)	157
Arthralgia	73 (47)	157
Sore throat	74 (47)	157
Hemoptysis	18 (11)	157
Diarrhea	52 (33)	157
Vomiting	69 (44)	157
Conjunctivitis	9 (6)	157
Headache	56 (36)	157
Seasonal influenza vaccination in the previous year‡	13 (13)	101
Pneumonia vaccination in the previous year‡	5 (5)	101
Health care treatment before hospitalization	136 (87)	157
Risk factor for influenza complication§	87 (55)	157
Diabetes	23 (18)	125
Chronic lung disease	23 (18)	125
Immunosuppression	11 (9)	125
Chronic cardiovascular disease	8 (6)	125
Chronic renal disease	6 (5)	125
Pregnancy trimester¶	15 (25)	59
Second	5 (33)	15
Third	10 (67)	15
Age ≤5 y	16 (10)	157
Age ≥60 y	7 (5)	157
Obesity#	53 (38)	138
Class III obesity**	10 (7)	138
Hospitalization		
Admitted to intensive care unit	80 (51)	157
Mechanical ventilation	61 (39)	157
Invasive procedures	69 (44)	157
Clinical complications	54 (34)	157

*Values are no. (%) or median (range).
†Children <8 years of age were excluded from the denominator.
‡Influenza and pneumonia vaccination were checked on the vaccination card.
§Obesity was not included.
¶Percentage of pregnancy among women of reproductive age (15–49 years) was included.
#Body mass index (BMI) data were available for 138 patients. Obesity in adults was BMI ≥30 kg/m^2; in children and adolescents ≤19 years of age, obesity was BMI for age >+2 z scores. Pregnant women were excluded.
**Class III obesity was BMI ≥40 kg/m^2. Patients ≤19 years of age and pregnant women were excluded.

Table 2. Characteristics of hospitalized pandemic (H1N1) 2009 case-patients, Rio Grande do Sul, Brazil, 2009*

	Outcome, no. (%) case-patients		Unadjusted		Adjusted†	
Characteristic	Died	Survived	OR (95% CI)	p value	OR (95% CI)	p value
Demographic						
Male sex	30 (58)	48 (46)	1.6 (0.8–3.2)	0.16	NC	NC
Current smoker	15 (30)	19 (21)	1.6 (0.7–3.6)	0.21	NC	NC
Former smoker	4 (8)	15 (18)	0.4 (0.1–1.3)	0.13	NC	NC
Underlying medical condition‡	30 (58)	57 (54)	1.1 (0.6–2.2)	0.69	NC	NC
Diabetes	14 (27)	9 (9)	3.9 (1.6–9.8)	0.01	4.4 (1.5–12.8)	<0.01
Chronic lung disease	9 (19)	14 (18)	1.1 (0.4–2.7)	0.87	NC	NC
Immunosuppression	2 (4)	9 (12)	0.3 (0.1–1.7)	0.14	NC	NC
Chronic cardiovascular disease	5 (11)	3 (4)	3.0 (0.7–13.1)	0.13	NC	NC
Chronic renal disease	1 (2)	5 (6)	0.3 (0.1–2.8)	0.27	NC	NC
Pregnancy§	5 (28)	10 (24)	1.2 (0.3–4.2)	0.51	NC	NC
Age ≤5 y	2 (4)	14 (13)	0.3 (0.1–1.2)	0.06	NC	NC
Age ≥60 y	4 (8)	3 (3)	2.8 (0.6–13.2)	0.17	NC	NC
Class III obesity¶	26 (57)	27 (29)	5.3 (1.3–21.7)	<0.01	6.2 (1.3–29.2)	0.02
Oseltamivir treatment	25 (48)	64 (61)	0.6 (0.3– 1.2)	0.12	NC	NC
Oseltamivir ≤48 h after symptom onset	2 (12)	12 (19)	0.4 (0.1–1.8)	0.18	NC	NC
Steroid treatment	32 (71)	51 (57)	0.5 (0.3–1.2)	0.12	NC	NC
Antimicrobial drug treatment	99 (94)	49 (94)	1.0 (0.2–4.1)	0.99	NC	NC

*OR, odds ratio; CI, confidence interval; NC, not calculated.
†Adjusted for sex and age group (reference age 0–5 years).
‡Obesity excluded.
§Women of reproductive age included (15–49 years).
¶Class III obesity was a body mass index ≥40 kg/m^2. Patients ≤19 years of age and pregnant women were excluded.

obtained were excluded from the study. Thus, the sample analyzed might not be representative of all hospitalized case-patients with severe pandemic (H1N1) 2009 during the study. However, demographic characteristics of study patients were similar to those of reported hospitalized case-patients with suspected pandemic (H1N1) 2009. Conclusions from small case series are limited, and results from this study should be considered in the context of studies in different populations. Quality of hospital care is likely to have a major role in survival rates but is difficult to compare between settings.

To reduce incidence of illness and death, the Brazilian Ministry of Health obtained 110 million doses of monovalent pandemic (H1N1) vaccine for distribution in the first 3 months of 2010. Persons with chronic medical conditions, including diabetes and obesity, received priority for vaccination on the basis of international recommendations (*13*,*14*) and those of the Brazilian Ministry of Health (*15*). In 2010 in Brazil, >89 million persons were vaccinated against pandemic (H1N1) 2009.

Our study characterized hospitalized case-patients in southern Brazil at the beginning of the pandemic. In addition, we confirmed that class III obesity and diabetes were independent risk factors for death in hospitalized case-patients with pandemic (H1N1) 2009, reinforcing the need for obtaining body mass index data for suspected case-patients during hospitalization. Furthermore, our results contributed to identification of priority groups for pandemic (H1N1) 2009 vaccination in Brazil.

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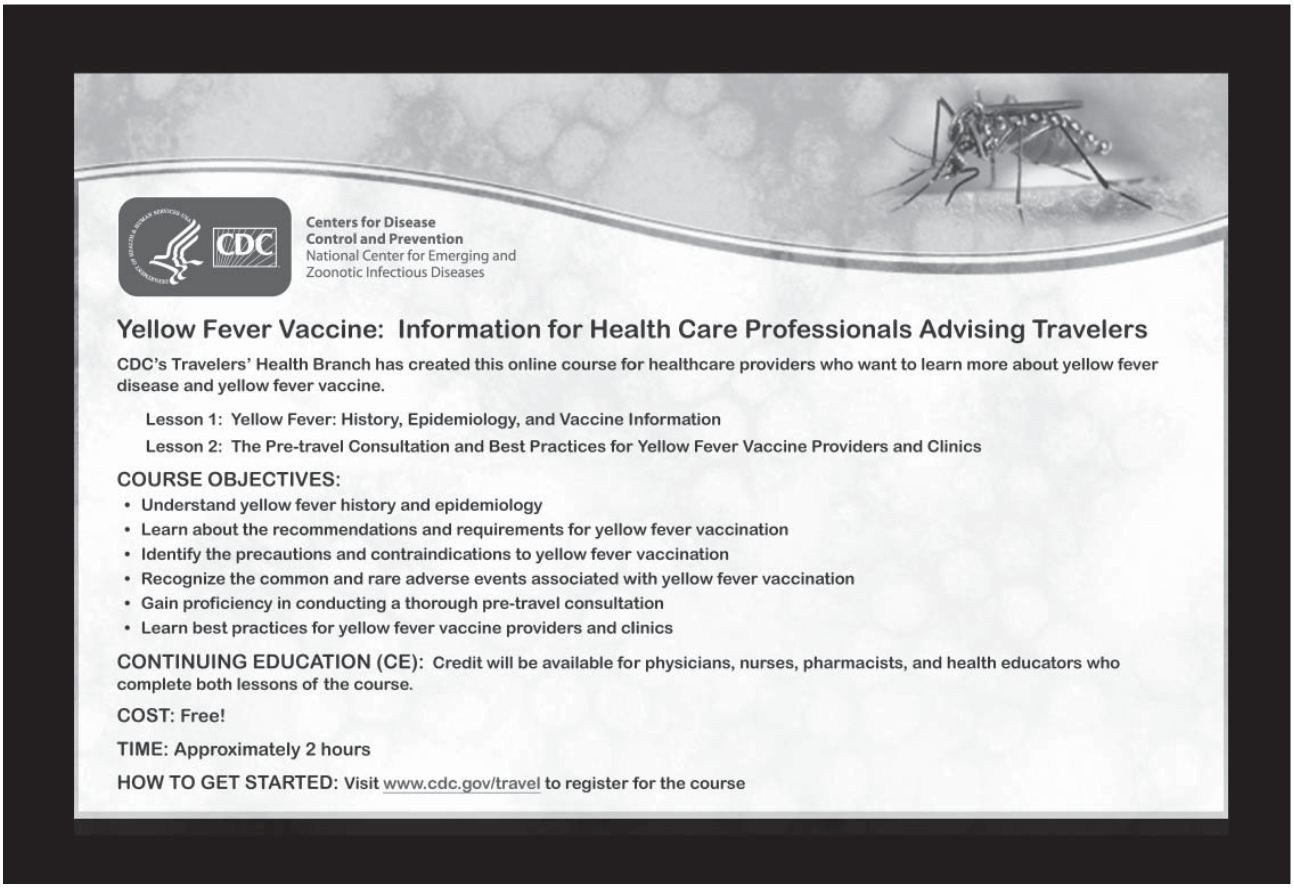

Multidrug-Resistant Pandemic (H1N1) 2009 Infection in Immunocompetent Child

AliReza Eshaghi, Samir N. Patel, Alicia Sarabia, Rachel R. Higgins, Alexei Savchenko, Peter J. Stojios, Yan Li, Nathalie Bastien, David C. Alexander, Donald E. Low, and Jonathan B. Gubbay

Recent case reports describe multidrug-resistant influenza A pandemic (H1N1) 2009 virus infection in immunocompromised patients exposed to neuraminidase inhibitors because of an I223R neuraminidase mutation. We report a case of multidrug-resistant pandemic (H1N1) 2009 bearing the I223R mutation in an ambulatory child with no previous exposure to neuraminidase inhibitors.

The neuraminidase inhibitors (NAIs) oseltamivir and zanamivir are antiviral agents approved for treatment of infections caused by pandemic (H1N1) 2009 influenza virus. Since the 2008–09 influenza season, almost all seasonal influenza (H1N1) viruses have been oseltamivir resistant because of an H275Y (histidine to tyrosine NA mutation, N1 NA numbering) mutation. Despite widespread use of oseltamivir during the 2009 pandemic, NAI resistance is rare in pandemic (H1N1) 2009 viruses (*1*). Zanamivir resistance is also rare in influenza viruses. A Q136K (glutamine to lysine mutation, N2 NA numbering) mutation conferring zanamivir resistance in influenza (H1N1) viruses has been described in an in vitro study but has not been detected in clinical specimens from patients (*2*). An influenza B strain carrying a R152K (arginine to lysine) mutation and resistant to oseltamivir and zanamivir has been reported (*3*). Recent case reports described multidrug-resistant pandemic (H1N1) 2009 infection in immunocompromised patients exposed to oseltamivir and zanamivir because of an I223R (isoleucine to arginine) mutation in NA (*4–6*). We report a case of infection by multidrug-resistant pandemic (H1N1) 2009 virus bearing the I223R mutation in an ambulatory child with no previous exposure to NAI.

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The Study

On October 30, 2009, a 15-year-old girl with a history of asthma sought treatment at an emergency department in the Greater Toronto area after 3 days of cough and rhinorrhea and 1 day of chest pain. Several children at her school also had respiratory symptoms. On arrival, she was febrile to 39.6°C and mildly dehydrated; physical examination was otherwise unremarkable. Blood count and chest radiograph showed no abnormalities. The child received intravenous rehydration in the emergency department, was discharged home with a prescription for oseltamivir therapy, and recovered uneventfully. A nasopharyngeal swab was forwarded to Ontario Agency for Health Protection and Promotion (OAHPP) for influenza testing. Pandemic (H1N1) 2009 was detected by real-time reverse transcription PCR (*7*). Subsequently, the specimen was screened by a single-nucleotide polymorphism assay distributed by Canada's National Microbiology Laboratory and the World Health Organization pyrosequencing protocol for the presence of the H275Y mutation (*8*). Both assays confirmed the isolate was wild type (histidine) at aa 275 of NA.

As part of pandemic surveillance, the specimen was cultured in rhesus monkey kidney cells and whole genome sequencing was performed by using a modified World Health Organization protocol (*9*). Sequences were deposited into GenBank under accession nos. CY060619–CY060626. In comparison with A/California/7/2009 (H1N1), several nonsynonymous mutations were identified: I201V and E538K in polymerase; S220T, D239E, and K465R in hemagglutinin; V100I and M316I in nucleoprotein; S99P and I123V in nonstructural protein; T16I, V106I, I223R, N248D, and N369K in NA. Apart from I201V, which is of unknown significance and has not been previously documented in pandemic (H1N1) 2009, these mutations were detected in 22% to 72% of pandemic (H1N1) 2009 strains circulating in Ontario at the same time that underwent whole genome sequencing. The I223R mutation results from a 1 nucleotide substitution at codon 223 of NA. To rule out the possibility of acquisition of I223R during culture in rhesus monkey kidney cells, the NA gene of the primary sample and its first passage were sequenced. Both had 100% identical nucleotide composition.

The 50% inhibitory concentration (IC_{50}) values for oseltamivir carboxylate and zanamivir, determined by chemiluminescent NAI assay (NA-Star; Applied

Table 1. Susceptibility of I223R mutant and control pandemic (H1N1) 2009 strains to oseltamivir carboxylate in the chemiluminescent NA inhibition assay, Canada, 2010*

		Susceptibility			
		OAHPP testing		NML testing	
Virus strain	NA mutation†	Mean IC_{50} ± SD, nmol	-fold increase	Mean IC_{50} ± SD, nmol	-fold increase
A/Ontario/313762/2009	I223R	9.49 ± 2.19‡	28	10.95 ± 2.5‡	22
A/California/07/2009-like control	Wild type	0.34 ± 0.14§		0.49 ± 0.31§	
Oseltamivir-resistant control	H275Y	57.1 ± 21.48¶	168	81.42 ± 24.1¶	166

*NA, neuraminidase; OAHPP, Ontario Agency for Health Protection and Promotion; NML, National Microbiology Laboratory; IC_{50}, 50% inhibitory concentration.
†Mutations presented in N1 numbering.
‡For 7 and 4 experiments done by OAHPP and NML, respectively.
§For 17 and 1,446 experiments done by OAHPP and NML, respectively.
¶For 13 and 14 experiments done by OAHPP and NML, respectively.

Biosystems Ltd., Foster City, California, USA) at OAHPP, were 9.49 (SD ± 2.19) nmol and 2.46 (SD ± 0.30) nmol, respectively (Table 1, 2) (oseltamivir carboxylate and zanamivir supplied by Hoffmann-La Roche Ltd [Basel, Switzerland] and GlaxoSmithKline [Brentford, UK], respectively). Compared with a wild-type control, the I223R mutant exhibited 28- and 12-fold increases in IC_{50}s for oseltamivir and zanamivir, respectively. The oseltamivir IC_{50} of the I223R strain was elevated, but not as much as observed in an H275Y control, which had a 168-fold IC_{50} elevation compared to the wild-type strain and was 6× higher than that of the I223R strain when tested in parallel. Similar results were obtained when the sample was retested at the National Microbiology Laboratory (Tables 1, 2).

The clinical significance of the I223R mutation is poorly understood because the IC_{50}s for oseltamivir and zanamivir are well below achievable serum levels when administered at recommended doses. Oral oseltamivir at a dose of 75 mg 2×/d resulted in a maximum serum concentration (C_{max}) of 348 ng/mL (1,115 nmol). Repeated inhalation of 10 mg of the dried powder formulation of zanamivir produced a C_{max} of 39 to 54 ng/mL (117.5–162.7 nmol) at 1 to 2 postdose, with an elimination half life of 4–5 (*10*). Intravenous zanamivir at a dose of 600 mg resulted in a C_{max} of 32,000–39,000 ng/mL (96,300–117,360 nmol).

I223 is recognized as one of the framework residues responsible for stabilizing the NAI active site; type-specific mutations at these residues have resulted in reduced susceptibility to NAIs (*11*,*12*). Although the exact mechanism by which mutations at the framework residue alter susceptibility to particular NAIs is not clear, simulation studies suggest that the NA electrostatic potential plays a major role in the interaction and stabilization of NAIs within the NA cavity (*13*). Nonhomologous substitution of a nonpolar hydrophobic amino acid, isoleucine, with the positively charged (polar) hydrophilic amino acid, arginine (I223R), seems to be a key point in alteration of the NA cavity. These changes most likely result in active site endpoint interactions affecting drug binding affinity and could disturb the proposed electrostatic binding funnel instrumental in directing NAIs into and out of binding sites on NA (*14*).

Three independent case reports described infections caused by multidrug-resistant pandemic (H1N1) 2009 in immunocompromised patients who received prolonged treatment with oseltamivir followed by zanamivir; 2 of the infections were fatal. In 2 patients, infection developed (H275Y followed by I223R alone with simultaneous reversion to wild type at position 275) (*4*,*5*); dual H275Y/I223R mutations developed in the third patient (*6*). Our patient is unique because she was immunocompetent, had no prior exposure to NAIs, and had an uneventful recovery. A similar resistance profile was seen in the published case exhibiting I223R alone, where IC_{50}s for oseltamivir, zanamivir, and peramivir were elevated by 45-, 10-, and 7-fold, respectively (*4*). The origin of the multiresistant isolate in this patient's case could not be established. The I223R mutation may have occurred spontaneously

Table 2. Susceptibility of I223R mutant and control pandemic (H1N1) 2009 strains to zanamivir in the chemiluminescent NA inhibition assay, 2010*

		Susceptibility			
		OAHPP testing		NML testing	
Virus strain	NA mutation	Mean IC_{50} ± SD, nmol	-fold increase	Mean IC_{50} ± SD, nmol	-fold increase
A/Ontario/313762/2009	I223R	2.46 ± 0.30†	12	6.84 ± 1.3†	9
A/California/07/2009-like control	Wild type	0.20 ± 0.11‡		0.79 ± 0.45‡	
Oseltamivir-resistant control	H275Y	0.20 ± 0.05§		0.76 ± 0.35§	

*NA, neuraminidase; OAHPP, Ontario Agency for Health Protection and Promotion; NML, National Microbiology Laboratoy; IC_{50}, 50% inhibitory concentration.
†For 7 and 4 experiments done by OAHPP and NML, respectively.
‡For 15 and 1,446 experiments done by OAHPP and NML, respectively.
§For 9 and 14 experiments done by OAHPP and NML, respectively.

in our patient. Alternatively, she acquired infection in the ambulatory setting, possibly as part of a school outbreak. Resistance may have evolved following random mutation, or during NAI therapy in another patient. We could not investigate this further because no samples were submitted from contacts. Using reverse genetics, it has been recently shown that an I223V NA change increased oseltamivir and peramivir resistance in pandemic (H1N1) 2009 and also restored NA substrate affinity and replication fitness in vitro (*15*).

Conclusions

Although the I223 residue is highly conserved across pandemic (H1N1) 2009 strains, the global distribution of pandemic (H1N1) 2009 was made possible by the virus adapting for stable circulation through genetic changes contributing to fitness and facilitating transmissibility from person to person. This report of community acquisition of a multidrug-resistant strain of pandemic (H1N1) 2009 reinforces the need to continue close monitoring for the emergence of resistant viruses and incorporation of screening for newly discovered resistance mutations into clinical diagnostics.

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Predictors of Pneumococcal Co-infection for Patients with Pandemic (H1N1) 2009

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We conducted a systematic investigation of pneumococcal co-infection in patients with a diagnosis of pandemic (H1N1) 2009 and any risk factor for complications or with severity criteria. We found 14% prevalence, with one third of patients having nonpneumonic infections. A severity assessment score >1 and high C-reactive protein levels were predictors of pneumococcal co-infection.

Influenza virus and *Streptococcus pneumoniae* are 2 of the most frequently implicated pathogens in respiratory tract infections in humans. Although an interaction between these microorganisms has been suggested (*1*–*8*), few data are available addressing the prevalence, clinical spectrum, or predictive factors of pneumococcal co-infection for patients with influenza.

We investigated the prevalence and clinical characteristics of pneumococcal infection in patients infected with pandemic (H1N1) 2009 virus. We compared those patients with patients in whom only influenza or pneumococcal infection was diagnosed during the pandemic to identify potential predictors of co-infection.

The Study

This prospective study was conducted in Spain from July 2009 through March 2010 during the outbreak of pandemic (H1N1) 2009. All adult (≥18 years of age) patients with an influenza-like illness who sought medical attention and had ≥1 risk factor for contracting influenza-related complications, according to Centers for Disease Control and Prevention recommendations (www.cdc.gov/flu/about/disease/high_risk.htm) or any other severity criteria, were sent to an outpatient infectious diseases clinic and were included in a protocol described below.

The protocol included the collection of oropharyngeal and nasopharyngeal swab samples, tested as previously described (*9*). All patients were asked for a urine sample for pneumococcal antigen detection. A sputum sample was obtained if spontaneously expectorated, and 2 blood cultures were collected when low systolic blood pressure, hypo- or hyperthermia, signs of sepsis, or a score >2 obtained by using the British Thoracic Society's CURB-65 assessment tool (*10*) were present. Patients had chest radiographs taken, C-reactive protein (CRP) measured, and the severity of illness calculated by using the CURB-65 score.

The CDC real-time reverse transcription PCR (rRT-PCR) protocol for detection and characterization of the pandemic (H1N1) 2009 virus was used for the diagnosis of influenza cases. The BinaxNOW *S. pneumoniae* urinary antigen test (Inverness Medical Diagnostics, Princeton, NJ, USA) was performed and read as previously described (*11*). Criteria for diagnosis of *S. pneumoniae* infection required isolation of the microorganism from blood, isolation of the predominant organism from a qualified sputum sample (*11*), or a positive urinary antigen test result.

A total of 418 patients with an influenza-like illness were evaluated, of whom 179 were confirmed as having cases of pandemic (H1N1) 2009 virus by rRT-PCR. Of these, 99 (55.3%) patients provided a urinary sample for pneumococcal antigen detection, 37 (20.7%) a sputum sample, and 48 (26.8%) blood cultures. There were no significant differences in demographic or clinical data among patients with or without a valid urinary sample for diagnostic testing, with the exception of pneumonia, which was more frequent among patients with an available sample (32.3% vs. 3.8%, $p<0.001$). Of 239 patients with rRT-PCR negative for influenza, pneumococcal infection was investigated in 171, of whom 43 (25.1%) had pneumococcal disease.

Of 100 patients who had influenza, a test available for pneumococcal detection, and no other bacterial pathogens identified, 14 had pneumococcal infection diagnosed (14%, 95% confidence interval 7.2–20.8; $p = 0.03$, compared with patients without influenza). Of these, 13 had a positive urinary antigen test result; 8 (57.1%) of the 14 had infection diagnosed only on the basis of this result. Of the remaining 6 patients, *S. pneumoniae* was isolated from blood in 2 and in sputum from 4. Demographic characteristics did not differ between pneumococcal–co-infected and non–co-infected patients (Table). Compared with patients with influenza infection only, those with pneumococcal co-infection more frequently had pneumonia ($p<0.001$), were more frequently admitted to hospital ($p<0.001$) and to the intensive care unit ($p = 0.034$), had lower O2 saturation ($p = 0.006$) and higher

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Table. Demographic and clinical data of patients with pandemic (H1N1) 2009 virus, by pneumococcal co-infection status, Spain, July 2009–March 2010*

Variable	Influenza plus pneumococcal disease, n = 14	Influenza without pneumococcal disease, n = 86	p value†	Influenza plus pneumococcal pneumonia, n = 9	Influenza plus nonpneumococcal pneumonia, n = 15	p value†
Female sex	8 (57.1)	41 (47.7)	0.511	6 (66.7)	7 (46.7)	0.341
Age, y	42.1 (35.7–57.0)	39.2 (27.3–52.1)	0.371	39.7 (31.1–63.1)	43.6 (31.7–51.3)	0.788
Concurrent conditions‡	10 (71.4)	71 (82.6)	0.325	7 (77.8)	11 (73.3)	0.808
Pregnancy	1 (7.1)	1 (1.1)	0.188	1 (11.1)	0	0.261
Decompensated concurrent condition	3 (21.4)	15 (17.4)	0.628	2 (22.2)	5 (33.3)	0.604
HIV infection	0 (0)	8 (9.3)	0.303	0	2 (13.3)	0.280
Smoker	3 (21.4)	21 (24.4)	0.908	2 (22.2)	3 (20.0)	0.782
Alcoholism	2 (14.2)	6 (6.9)	0.293	1 (11.1)	1 (6.66)	0.674
Hospital admission	12 (85.7)	21 (24.4)	0.000	9 (100)	11 (73.3)	0.090
ICU admission	2 (14.3)	2 (2.3)	0.034	2 (22.2)	1 (6.7)	0.265
Pneumonia	9 (64.3)	15 (17.4)	0.000	9 (100)	15 (100)	NA
Pneumococcal bacteremia	2 (14.2)	NA	NA	1 (11.1)	0	0.303
Oliguria/anuria	0 (0)	1 (1.1)	0.694	0	0	NA
O_2 saturation <94%	3 (21.4)	12 (13.9)	0.403	2 (22.2)	7 (46.7)	0.311
Axillary temperature, °C	38.1 (37.5–38.9)	36.8 (36.4–37.8)	0.009	37.7 (37.5–38.8)	36.5 (35.9–37.8)	0.679
O_2 saturation, %	95 (85.2–95.5)	96.3 (94–98)	0.006	93 (72–95)	93 (78–110)	0.308
CURB-65 score	0 (0–2)	0 (0–1)	0.201	2 (0–2.5)	0 (0–1)	0.156
Confusion	4 (28.6)	1 (1.2)	0.000	4 (44.4)	0	0.005
BUN level >20 mg/dL	2 (14.3)	4 (4.7)	0.628	2 (22.2)	4 (26.7)	0.808
Respiratory rate ≥30 breaths/min	3 (21.4)	3 (3.5)	0.009	3 (33.3)	1 (6.7)	0.090
BP <90/60 mm Hg	2 (14.3)	2 (2.3)	0.034	2 (22.2)	2 (13.3)	0.572
Age ≥65 y	2 (14.3)	4 (4.7)	0.159	2 (22.2)	1 (6.7)	0.265
CURB-65 score >1	9 (64.3)	62 (72.1)	0.550	5 (55.5)	7 (46.7)	0.673
CURB-65 score ≥2	5 (35.7)	3 (3.5)	0.000	5 (55.5)	1 (6.7)	0.007
C-reactive protein, mg/L	190.7 (74.0–190.7)	26.6 (11.80–79.35)	0.000	255 (134–320)	89 (60–162)	0.008
Procalcitonin, ng/mL§	1.25 (0.12–26.00), n = 6	0.5 (0.1–0.5), n = 19	0.198	13.5 (0.59–27.70), n = 4	0.5 (0.08–6.03), n = 3	0.289
Death	0	0	NA	0	0	NA

*All values are number (%) for categorical variables and median (interquartile range) for continuous variables. NA, not applicable; ICU, intensive care unit; CURB-65, confusion of new onset, urea greater than 7 mmol/L (blood urea nitrogen [BUN] >19), respiratory rate ≥30 breaths/min, systolic blood pressure (BP) ≤90 mm Hg or diastolic blood pressure ≤60 mm Hg, age ≥65 (*10*).
†The χ^2 or Fisher exact test was used for categorical variables and the Mann-Whitney test for continuous variables.
‡Asthma, chronic lung disease, heart disease, neurologic and neurodevelopmental conditions, blood disorders, endocrine disorders (such as diabetes mellitus), kidney disorders, liver disorders, metabolic disorders, weakened immune system, people <19 y of age who are receiving long-term aspirin therapy, persons who are morbidly obese (body mass index ≥40).
§Measured in patients with signs of sepsis; n values as shown.

axillary temperature (p = 0.009), and more frequently had the following CURB-65 score criteria: confusion (p<0.001), respiratory rate >30 breaths/min (p = 0.009), and systolic blood pressure <90 mm Hg (p = 0.03) (Table). CURB-65 score was >1 for 35.7% of patients with pneumococcal co-infection but only 3.5% of those with influenza infection only (p<0.001). Levels of CRP were significantly higher in patients with influenza plus pneumococcal disease (190.7 mg/L vs. 26.6 mg/L; p <0.001).

When only influenza cases with pneumonia were analyzed and those with pneumococcal co-infection (n = 9) were compared with patients in whom only influenza was identified (n = 15), patients with pneumococcal co-infection more frequently had confusion (p = 0.005), a CURB-65 score >1 (p = 0.007), higher CRP levels (255 mg/L vs. 89 mg/L, p = 0.008), and a statistical trend to tachypnea >30 (p = 0.09) and to higher hospital admission (p = 0.09) (Table).

Pneumococcal infection characteristics were also compared between patients with and without (n = 43) pandemic (H1N1) 2009 infection included in the study. Confusion according to CURB-65 criteria was more frequent among patients with both infections (p = 0.003), while other clinical data did not differ between groups.

Conclusions

We found that the prevalence of concurrent pneumococcal infection was 14% in patients who had pandemic (H1N1) 2009 virus infection and any risk factor for influenza-related complications or who met severity

criteria. Of note, infection in more than half these patients would not have been diagnosed if a pneumococcal urinary antigen test had not been performed. We evaluated the frequency of pneumococcal disease in patients with the pandemic (H1N1) 2009 virus through a systematic investigation by using validated diagnostic methods. Although a recent study identified a high frequency of *S. pneumoniae* and *Haemophilus influenzae* in nasopharyngeal swabs from patients with influenza A (H1N1) tested by using molecular techniques (*12*), their clinical significance and positive predictive value remain undetermined.

We characterized the clinical spectrum of pneumococcal infection accompanying influenza pandemic. Moreover, the systematic calculation of the CURB-65 score provided us a simple, objective, and useful tool for categorization of severity and comparison between patients. Although the prevalence of pneumococcal infection in patients with influenza might have been overestimated by more frequent urinary sampling among those with pneumonia, we found that more than one third of the patients had nonpneumonic pneumococcal infections. Compared with patients with influenza only, pneumococcal co-infected patients showed a higher severity of disease as defined by a higher frequency of CURB-65 criteria, lower O_2 saturation, and more frequent admission to the intensive care unit. Furthermore, a CURB-65 score >1 was found to be a predictive factor of pneumococcal co-infection. Additionally, levels of CRP were also much higher in patients with pneumococcal infection. Although many co-infected patients also had pneumonia, a fact which might have explained the above-mentioned findings, the same predictive factors distinguished between influenza pneumonia and influenza plus pneumococcal pneumonia.

Pneumococcal infection was more prevalent among patients with a negative test result for influenza. This prevalence could be explained because, presumably, many of the noninfluenza patients met the influenza-like illness definition due to bacterial infection. However, it is unknown whether some of the influenza-attributable pneumococcal infection might have been underestimated, because *S. pneumoniae* might follow influenza after a lag period, and a delay in the request for medical attention might diminish the sensitivity of diagnostic tests for influenza. Nonetheless, data from treated patients have shown a median duration of viral shedding of 5 to 9 days and slower viral clearance with delayed antiviral drug administration (*13*).

In summary, the prevalence of pneumococcal co-infection during the influenza A (H1N1) 2009 pandemic was noteworthy, and it was associated with a higher severity of disease. In one third of the cases the clinical signs and symptoms did not indicate pneumonia, and more than one half could only be diagnosed with the urinary antigen test. A CURB-65 score >1 and CRP levels proved to be useful tools to identify patients at higher risk for pneumococcal co-infection for whom physicians should adopt additional diagnostic and therapeutic measures.

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Pandemic (H1N1) 2009–associated Deaths Detected by Unexplained Death and Medical Examiner Surveillance

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During the pandemic (H1N1) 2009 outbreak, Minnesota, New Mexico, and Oregon used several surveillance methods to detect associated deaths. Surveillance using unexplained death and medical examiner data allowed for detection of 34 (18%) pandemic (H1N1) 2009–associated deaths that were not detected by hospital-based surveillance.

The emergence of pandemic (H1N1) 2009 influenza illustrated the need for improved surveillance to identify deaths resulting from emerging pathogens. Common methods for identifying infectious cause–related deaths include reports by health care providers and review of death certificates. These methods have limitations for identifying deaths caused by emerging pathogens because the disease may not be fully defined or death certificates may not indicate an infectious cause. During an emerging pathogen epidemic, it is important to investigate deaths occurring outside of traditional settings to determine if sudden deaths occurring in the community are a result of the novel pathogen.

In 1995, the Centers for Disease Control and Prevention (CDC) Emerging Infections Program (EIP) Unexplained Deaths Program (UNEX) began in 4 states (*1*). Under UNEX, deaths likely resulting from an infection, but for which routine testing did not identify a pathogen, are investigated. State and CDC Infectious Diseases Pathology Branch researchers partner with medical examiners and hospital pathologists to review cases and autopsy reports. Expanded resources for specimen testing are provided, which increases the likelihood of a pathogen-specific diagnosis.

The Medical Examiner Infectious Disease Death Surveillance Program (Med-X) was developed in 1999 by the New Mexico Office of the Medical Investigator, New Mexico Department of Health, and Infectious Diseases Pathology Branch to review deaths for infectious causes on the basis of preestablished sets of symptoms and pathologic syndromes (*2*,*3*). If there is evidence of an infectious process, specimens are tested to achieve an organism-specific diagnosis. Both UNEX and Med-X have been shown to be useful for bioterrorism and infectious death surveillance (*4*–*6*).

The EIP has also established population-based active surveillance for all laboratory-confirmed influenza-related hospitalizations and deaths. Minnesota, New Mexico, and Oregon participate in the UNEX, Med-X, and EIP Influenza Surveillance programs to identify all potential influenza-associated deaths.

The Study

During the spread of pandemic (H1N1) 2009, UNEX cases were reported to the Minnesota and Oregon health departments by physicians, infection preventionists, and hospital pathologists (Figure 1). Both states also conducted statewide surveillance by using Med-X. New Mexico detected cases through the New Mexico Office of the Medical Investigator and its Med-X system. Medical examiners investigated all decedents for influenza-like

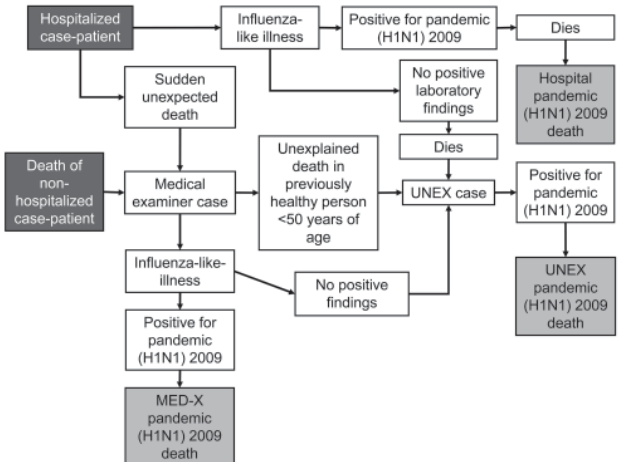

Figure 1. Procedure for evaluating pandemic (H1N1) 2009–associated deaths in Minnesota, New Mexico, and Oregon, April–December 2009. UNEX, Centers for Disease Control and Prevention Emerging Infections Program Unexplained Deaths Program; Med-X, Medical Examiner infectious Disease Death Surveillance Program.

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Table 1. Descriptive characteristics of pandemic (H1N1) 2009–associated deaths, by surveillance program, Minnesota, New Mexico, and Oregon, April–December 2009*

Characteristic	Hospital surveillance decedents, n = 160	UNEX/Med-X decedents, n = 34	p value†
State			
Minnesota	50 (76)	16 (24)	
New Mexico	42 (81)	10 (19)	
Oregon	68 (89)	8 (11)	0.09‡
Influenza type§			
Pandemic (H1N1) 2009	82 (89)	25 (96)	
Influenza A, not subtyped	10 (11)	1 (4)	0.45‡
Age, y			
Median	51.0	37.5	<0.001¶
Mean	50.4	33.4	<0.001#
Male gender	94 (59)	17 (50)	0.35
Race/ethnicity			
White	123 (77)	18 (53)	
Black	7 (4)	0 (0)	
American Indian/Alaska Native	7 (4)	7 (21)	
Asian/Pacific Islander	2 (1)	2 (6)	
Hispanic	21 (13)	7 (21)	0.001‡
Autopsy performed	27 (17)	29 (85)	<0.001
Place of death			
Hospital/emergency department	146 (91)	15 (44)	
Residence	12 (8)	18 (53)	
Other	1 (1)	0 (0)	
Unknown	1 (1)	1 (3)	<0.001‡

*Values are no. (%) except as indicated. UNEX, Centers for Disease Control and Prevention Emerging Infections Program Unexplained Deaths Program; Med-X, Medical Examiner Infectious Disease Death Surveillance Program.
†By χ^2 or Fisher exact test.
‡By χ^2 test among all categories.
§Data available from Minnesota and New Mexico only; n = 92 for hospital surveillance and n = 26 for UNEX/Med-X.
¶By Wilcoxon Mann-Whitney test.
#By analysis of variance F-test.

illness (ILI) based on pre- or postmortem findings as well as sudden deaths in previously healthy persons <50 years of age. Each state expanded its EIP Influenza Surveillance

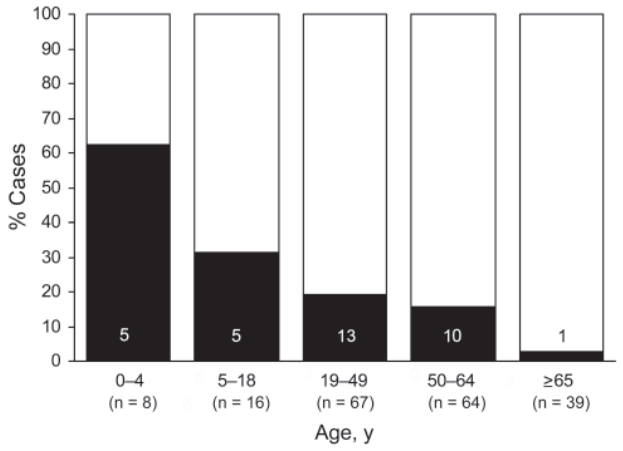

Figure 2. Pandemic (H1N1) 2009–associated deaths, by age group and surveillance program, Minnesota, New Mexico, and Oregon, April–December 2009. White bar sections, deaths detected through hospital surveillance; black bar sections, deaths detected through Centers for Disease Control and Prevention Emerging Infections Program Unexplained Deaths Program and Medical Examiner Infectious Disease Death Surveillance Program.

statewide during the pandemic (H1N1) 2009 pandemic. In addition, hospitalized persons with ILI, including decedents, were reported to the state health department by physicians, infection preventionists, and hospital pathologists.

Pre- and/or postmortem specimens, including nasopharyngeal, nasal, or throat swabs; nasal or endotracheal aspirates; bronchial alveolar lavage specimens; sputum; frozen and fixed respiratory tissue; and serum specimens, were tested at state laboratories or at CDC for pandemic (H1N1) 2009 virus. Tests included PCR, virologic culture, immunohistochemistry, serology, and influenza antigen detection. In a few instances, it was not possible to characterize the virus beyond influenza type A because of limited specimen availability; these cases were assumed to be pandemic (H1N1) 2009. Because UNEX and Med-X are not mutually exclusive, all pandemic (H1N1) 2009–associated deaths were determined to be UNEX/Med-X cases if they were captured through either of those programs (Figure 1).

Data were collected on underlying medical conditions, symptoms, and clinical outcomes from medical records, case investigations, and autopsy reports. In Minnesota and New Mexico, all decedents with positive laboratory

findings were reviewed to determine if their deaths were due entirely or in part to pandemic (H1N1) 2009. If influenza was determined not to be related to the death, it was not included as a pandemic (H1N1) 2009–associated death; 7 decedents in Minnesota and 2 in New Mexico were thus excluded. Oregon included all hospital surveillance deaths with positive influenza (H1N1) test results as subtype H1N1 associated without further review, but UNEX/Med-X cases were reviewed for a causal relationship to pandemic (H1N1) 2009. Deaths occurring during April–December 2009 were included in this analysis.

Characteristics of UNEX/Med-X cases versus hospital surveillance cases were compared by using the χ^2 or Fisher exact test. The Wilcoxon Mann-Whitney test was used to compare medians. SAS version 9.1 software (SAS Institute Inc., Cary, NC, USA) was used for all analyses.

A total of 194 pandemic (H1N1) 2009–associated deaths were detected in this analysis, 160 (82%) through hospital surveillance and 34 (18%) through UNEX/Med-X. The additional surveillance resulted in the detection of 21% more total cases than hospital surveillance alone. Minnesota had the highest proportion of UNEX/Med-X–detected cases with 24% (16/66); Oregon had the lowest with 11% (8/76) (Table 1). Decedents detected by using UNEX/Med-X were more frequently of a nonwhite race (47% vs. 23%); an increased percentage of deaths of American Indians/Alaska Natives was detected through UNEX/Med-X versus hospital surveillance (21% vs. 4%).

UNEX/Med-X decedents were more likely to have had an autopsy performed (85% vs. 17%) and were more likely to have died in their residences (53% vs. 8%) than decedents detected by hospital surveillance. The median age of UNEX/Med-X decedents was 37.5 years, compared with 51.0 years for hospital surveillance decedents ($p<0.001$) (Table 1). The percentage of UNEX/Med-X decedents among age groups decreased with increasing age (62.5% among those 0–4 years of age compared with 2.6% among those ≥65 years of age; Figure 2).

More hospital surveillance than UNEX/Med-X decedents (89% vs. 68%) were determined to have ≥1

Table 2. Clinical description of patients whose deaths were associated with pandemic (H1N1) 2009, by surveillance program, Minnesota, New Mexico, and Oregon, April–December 2009*

Underlying conditions	Hospital surveillance decedents, n = 160	UNEX/Med-X decedents, n = 34	p value†
≥1 conditions	142 (89)	23 (68)	0.002
Asthma	24 (15)	3 (9)	0.43
Chronic lung disease	51 (32)	7 (21)	0.19
Cardiovascular disease	59 (37)	8 (24)	0.14
Chronic metabolic disease	55 (34)	9 (26)	0.37
Renal disease	17 (11)	1 (3)	0.21
Neuromuscular disorder	19 (12)	2 (6)	0.54
Cancer, past 12 months	10 (6)	0 (0)	0.21
Lymphoma/leukemia	9 (6)	0 (0)	0.36
Immunosuppressive conditions	30 (19)	2 (6)	0.08
Pregnancy	0	0	
Obesity‡	29 (18)	9 (27)	0.27
Morbidly obese‡	23 (14)	4 (12)	1.0
Body mass index‡			
Median	29.2	31.8	0.95§
Mean	32.6	30.7	
Clinical outcomes¶			
Pneumonia	72 (78)	15 (58)	0.04
Viral	12 (17)	6 (40)	
Bacterial#	8 (11)	2 (13)	
Both	8 (11)	1 (7)	
Unknown	44 (61)	6 (40)	
Acute respiratory distress syndrome	34 (37)	4 (15)	0.06
Myocarditis	0 (0)	2 (8)	0.05

*Values are no. (%) except as indicated. UNEX, Centers for Disease Control and Prevention Emerging Infections Program Unexplained Deaths Program; Med-X, Medical Examiner Infectious Disease Death Surveillance Program.
†By χ^2 or Fisher exact test.
‡Obese was defined as either documentation in the medical record of "obese" or a body mass index (BMI) ≥30 kg/m^2 and <40 kg/m^2. Morbidly obese was defined as either documentation in the medical record of "morbidly obese" or a BMI ≥40 kg/m^2. If there was a discrepancy, BMI was used. BMI data were available for 80 case-patients and were calculated for adults by using National Institute of Health BMI calculation tables and for children 2–19 years of age by using the Centers for Disease Control and Prevention pediatric BMI calculation.
§By Wilcoxon Mann-Whitney test.
¶Data available from Minnesota and New Mexico only; n = 92 for hospital surveillance and n = 26 for UNEX/Med-X.
#Bacterial species included 4 *Streptococcus pneumoniae*, 4 methicillin-resistant *Staphylococcus aureus*, 3 methicillin-susceptible *S. aureus*, 2 group A *Streptococcus* spp., and 2 others. Some case-patients had >1 species identified.

underlying condition. Specific underlying conditions were more frequently identified among hospital surveillance than UNEX/Med-X decedents, except for obesity (Table 2). Pneumonia, including viral pneumonia, was frequently reported among decedents. Acute respiratory distress syndrome was documented for 37% of hospital and 15% of UNEX/Med-X decedents. Two previously healthy children with nasopharyngeal swabs positive for influenza had evidence at autopsy of viral myocarditis.

Conclusions

UNEX/Med-X surveillance captured 11%–24% of pandemic (H1N1) 2009–associated deaths in the 3 states. Other estimates of deaths resulting from pandemic (H1N1) 2009 may be increased with better data on nonhospitalized and sudden unexplained deaths (*7*,*8*). Estimates from surveillance in New York, New York, which included medical examiner and unexplained respiratory cause–related death surveillance, indicate 17% of decedents died at home and 6% had not sought any prior medical care (*9*–*11*).

UNEX/Med-X decedents were younger and more often previously healthy than hospital surveillance decedents, a finding that would change the estimated impact of pandemic (H1N1) 2009 among those populations in particular. Consistent with other studies (*12*), larger racial/ethnic disparities, particularly among Native American/Alaska Native populations, may be detected by UNEX/Med-X than have been detected through other surveillance methods. Although we were unable to determine the cause of these disparities, the findings warrant further study and attention to these populations regarding public health resources.

Even with an emphasis on deaths among those <50 years of age, UNEX and Med-X programs are critical for detecting severe illnesses that rapidly progress to death and could otherwise go undetected. Partnering with medical examiners and pathologists to identify infectious cause–related deaths among persons who were previously healthy is important to give a clear picture of the entire mortality spectrum.

Although it is important to accurately measure the impact of a disease, it is perhaps more important to quickly identify new serious disease threats. Approximately one tenth to one quarter of the influenza deaths detected in this study, and particularly those in younger, healthier persons, were not detected by hospital surveillance when influenza awareness was at its peak. This finding argues for surveillance systems like UNEX and Med-X as a means of quickly detecting emerging, severe infectious disease threats. Because pathogens are likely to emerge over broad geographic areas, we recommend a standardized approach to death investigations to fully understand the epidemiologic and clinical features of illness caused by a particular pathogen.

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Human Parvovirus 4 as Potential Cause of Encephalitis in Children, India

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To investigate whether uncharacterized infectious agents were associated with neurologic disease, we analyzed cerebrospinal fluid specimens from 12 children with acute central nervous system infection. A high-throughput pyrosequencing screen detected human parvovirus 4 DNA in cerebrospinal fluid of 2 children with encephalitis of unknown etiology.

Encephalitis is a major cause of death and disability globally. In Asia, Japanese encephalitis virus is the most commonly recognized cause, with 30,000–50,000 cases and ≈10,000 deaths annually (*1*). However, for most encephalitis cases, the cause is unknown, even with comprehensive screening for recognized agents (*1*).

The advent of high-throughput sequencing technologies enabled the direct characterization of microbial nucleic acid from clinical samples and revolutionized the process for identifying potential etiologic agents of disease. A key feature is that the method is unbiased and can detect any nonhost nucleic acid sequences present in a sample. It has the potential to detect expected agents, known but unexpected agents, and novel agents (*2*).

Human parvovirus 4 (PARV4) is a single-stranded DNA virus first detected in 2005 (*3*). Infections with PARV4 are accompanied by acute viremia for several weeks (C. Sharp et al., unpub. data), followed by seroconversion for antibody and virus clearance. As observed with another human parvovirus, parvovirus B19, there is long term persistence of viral DNA sequences in several tissues but not the brain (*4*). We describe 2 children in southern India with suspected encephalitis and high PARV4 levels in their cerebrospinal fluid (CSF).

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The Study

We obtained CSF from a cohort of children (<16 years of age) hospitalized with a suspected acute central nervous system (CNS) infection at the Vijayanagar Institute of Medical Sciences, Bellary, India, October 2005–October 2007, as previously described (*5*). Suspected CNS infection was defined as a febrile illness (for <2 weeks) and ≥1 of the following signs or symptoms: severe headache, altered mental status, seizures, or focal neurologic signs (*6*).

To investigate whether uncharacterized infectious agents were associated with neurologic disease, we obtained CSF specimens from 12 patients with acute CNS infection (i.e., febrile illness with CSF leukocyte count ≥5 cells/mm^3 or protein >45 mg/dL). These patients had negative diagnostic test results for pathogens known to cause CNS infection in this region of India at the time of investigation (e.g., Japanese encephalitis virus, chikungunya virus, dengue fever virus, and *Plasmodium falciparum*); in addition, CSF culture was performed and no bacterial organisms were found (*5*). Total nucleic acid was extracted from whole CSF and randomly amplified as previously described with the modification that 6-nt barcodes were added to the 5′ end of the primers used for the amplification (*7*). The amplified materials were pooled together and processed by using a high-throughput pyrosequencing technique on a GS FLX Titanium Platform (454 Life Sciences/Roche, Branford, CT, USA).

The raw sequence reads were deconvoluted on the basis of the barcode and then processed through a standardized bioinformatic pipeline (*2*). The sequences of interest were then categorized into taxonomy groups based on the best BLAST (www.ncbi.nlm.nih.gov/BLAST/) hit. For 2 of the 12 patients, patients VES085 and VES065, viral sequences were detected in the CSF.

We identified 17 (92.6%–98.2% sequence identity) distinct sequence reads in the CSF of patient VES085 and 6 (95.6%–98.9% sequence identity) distinct reads from patient VES065 with pairwise identities based on BLASTn alignment of each read to the reference genome (GenBank accession no. EU175855.1). To verify the results, we used PARV4-specific PCR primers to screen all 12 original CSF samples as described (*8*). Only samples from VES085 and VES065 were positive; all other samples were negative by PCR.

PARV4 viral loads in the 2 CSF samples and the corresponding serum sample from 1 patient collected contemporaneously were semiquantified by limiting dilution PCR by using primers on 5 replicate samples of each dilution. The PCR conditions demonstrated single copy sensitivity (data not shown), and endpoint titers

Table 1. Clinical and laboratory characteristics of children with CSF positive for human parvovirus 4, India*

ID, date of illness	Age, y	Clinical course	Outcome	CSF				Serum		
				PCR	WCC†	Protein, mg/dL	Glucose, mg/dL	IgM	IgG	PCR
VES085, 2006 Jan	2	Prodomal illness for 12 days; febrile with frequent generalized convulsions during first week of admission; CSF examination on day 12 of illness	Discharged against medical advice on day 18 after admission	+ 1.5×10^7 copies/mL	4	59	32	+	–	+ 5.6×10^9 copies/mL
VES065 2005 Nov	3	Prodomal illness for 9 days; febrile, poor appetite, and a generalized convulsion on day 9; CSF examination on day 9 of illness	Recovered on day 14 with no residual neurologic deficit	+ 3.2×10^5 copies/mL	8	15	60	NA	NA	NA

*CSF, cerebrospinal fluid; ID, patient identification code; WCC, leukocyte count; Ig, immunoglobulin; +, positive; –, negative; NA, not available.
†Leukocyte count differential 100% lymphocytes for each patient.

of 50% positivity were calculated by using the Reed-Muench formula (*8*,*9*). Both the CSF and 1 serum sample demonstrated high endpoint titers, indicating acute infection and substantial virus spread into the CNS of the 2 patients (Table 1).

To genetically characterize PARV4 variants infecting the patients, we generated overlapping sets of amplicons spanning the complete genome of PARV4 from the 3 samples. No variation was seen between the CSF and serum-derived sequences from VES085. The fully assembled coding regions of the variants generated from both patients were compared to previously described PARV4 variants (n = 18 viral proteins 2/1 and n = 19 nonstructural sequences) (Table 2). Phylogenetic analysis demonstrated that both variants belonged to genotype 2 (Figure). All sequences have been submitted to GenBank (accession nos. HQ593530–HQ593532).

The serum sample was screened for immunoglobulin (Ig) G and IgM PARV4-specific antibodies by using a previously developed serologic assay for PARV4 antibodies (*10*). The serum of VES085 was IgM positive and IgG negative, consistent with an acute infection (Table 1).

Through serologic screening of age-matched controls, we were able to largely rule out the possibility that detection of PARV4 DNA in samples from 2 of the 12 patients was simply incidental to a high background incidence of PARV4 infection in this age group. Although published data on the duration of viremia in patients with acute PARV4 infections has not been directly determined, viral loads as found in the study patients are of relatively short duration in other parvovirus infections (16 days for viral loads >5,000 DNA copies/mL in the case of B19) (*11*). Furthermore, through testing sequential samples from persons exposed to PARV4, we have found no persons with viremia duration of >6 weeks (C. Sharp et al., unpub. data). Using the 6-week (maximum) estimate and a background incidence of infection of 2.2% per year (based on the detection of 4/41 seropositive children with a mean age of 4.5 years), the likelihood of viremia detection would be at most 0.25%, a figure consistent with the actual measured absence of viremia detection in the control cohort.

Conclusions

PARV4 is a recently identified virus found in human blood and in a variety of tissues but with no known disease association. In this study we detected PARV4 DNA in 2 of 12 CSF samples from patients with suspected encephalitis of unknown cause in southern India. Unfortunately, no samples were available from the wider cohort for further testing. The presence of PARV4 DNA and IgM against PARV4 in acute-phase serum from the 1 child with this

Table 2. Percent divergence of the nonstructural coding region of human PARV4–positive encephalitis patients VES065 and VES085, compared with previously described PARV4 variants*

Patient or genotype	Genotype 3, aa (nt), n = 1	Genotype 2, aa (nt), n = 7	Genotype 1, aa (nt), n = 11	VES085 serum, aa (nt), n = 1	VES085 CSF, aa (nt), n = 1	VES065 CSF, aa (nt), n = 1
VES065 CSF, n = 1	9.5 (3.5)	3.7 (1.5)	10.3 (3.7)	0.2 (0.0)	0.2 (0.0)	
VES085 CSF, n = 1	9.4 (3.5)	3.6 (1.5)	10.2 (3.7)	0.0 (0.0)		
VES085 serum, n = 1	9.4 (3.5)	3.6 (1.5)	10.2 (3.7)			
Genotype 1, n = 11	8.0 (2.6)	9.3 (2.9)	0.9 (0.6)			
Genotype 2, n = 7	8.6 (2.7)	1.8 (0.7)				
Genotype 3, n = 1						

*PARV4, human parvovirus 4; CSF, cerebrospinal fluid.

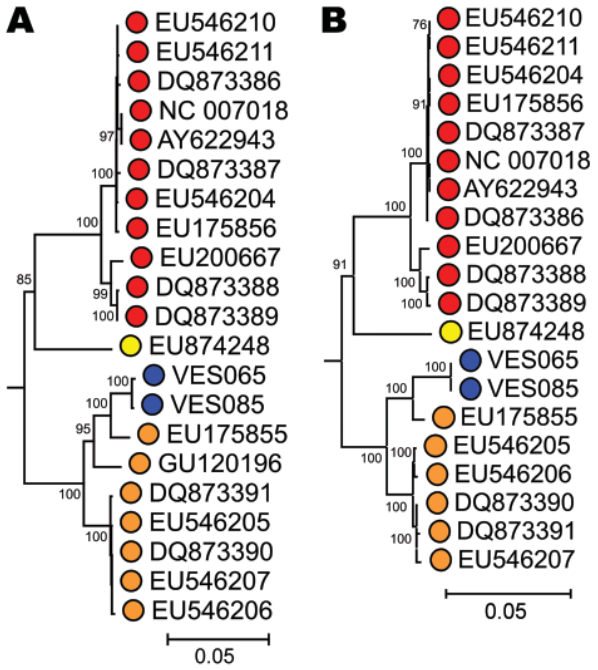

Figure. Phylogenetic analysis of (A) complete nonstructural (NS) and (B) viral protein (VP) 1/VP 2 gene sequences of human parvovirus 4 (PARV4) variants isolated from patients with encephalitis of unknown etiology using 167 available sequences from human PARV4 variants (genotypes 1–3). Blue, study sample; red, genotype 1; orange, genotype 2; yellow, genotype 3. The porcine hokovirus sequence (GenBank accession no. EU200671) was used as an outgroup (not shown). The trees were constructed by neighbor-joining of pairwise maximum-composite likelihood corrected distances between nucleotide sequences; bootstrap values ≈70% are shown. Scale bars represent an evolutionary distance of 0.05.

sample available further supports the contention that this was an acute infection.

Brain biopsy, the definitive investigation, was not available at the time. More often than not, clinicians have to rely on surrogate markers to diagnose encephalitis (e.g., CSF pleocytosis, febrile illness, and focal neurologic signs in the right clinical context). It is more common to have CSF pleocytosis than not, though of course there are well characterized cases of CNS infection (e.g., dengue) with no pleocytosis (*6*).

CSF erythrocyte count was not routinely measured in this resource-limited setting. Although it is theoretically possible the parvovirus detected in the CSF could have reflected spillover from a blood-contaminated spinal tap, there was no overt evidence of hemorrhagic CSF. The fact that patient VES085 was febrile with a neurologic syndrome and positive serum IgM, and patient VES065 had similar clinical signs with high PARV4 viral CSF levels suggests that they did indeed have a CNS infection.

PARV4 is primarily found in injection drug users and persons who have received blood products; PARV4 is therefore presumed to be transmitted parenterally. However, the risk for such transmission in the children in our study was low, which supports the possibility of an alternative route of transmission (*8*,*12*). HIV testing was not available.

The use of high-throughput sequencing for identifying an unexpected possible cause of CNS infection (PARV4) is cutting edge; this technique's utility also extends to a recent identification of astrovirus in a case of encephalitis (*13*). With the high seroprevalence of PARV4 infection highlighted in recent reports (5%–37%), there is a clear need to further assess the role of PARV4 in CNS infection across the globe (*14*,*15*).

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Hepatitis E Virus Genotype 3 in Humans and Swine, Bolivia

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We determined the seroprevalence of hepatitis E virus (HEV) in persons in 2 rural communities in southeastern Bolivia and the presence of HEV in human and swine fecal samples. HEV seroprevalence was 6.3%, and HEV genotype 3 strains with high sequence homology were detected.

Hepatitis E virus (HEV) is the causative agent of epidemic and sporadic acute hepatitis in areas with poor sanitary conditions. The infection is endemic to southeast and central Asia, the Middle East, northern and western parts of Africa, and North America (*1*). Sporadic cases of HEV infection have also been reported in industrialized countries, usually associated with traveling in disease-endemic areas. Transmission of HEV occurs predominantly by the fecal–oral route, mostly through contaminated drinking water (*1*). Several animal sources of HEV have been identified (*2*). The first strain of animal origin was identified in 1997 in swine in the United States (*3*). Subsequently, several studies documented that swine are the largest reservoir of HEV (*4*–*7*).

Little data are available on HEV seroprevalence in countries in South America (*8*). The first study of HEV infection in Bolivia was a seroprevalence survey conducted in 1997 in rural southeastern Bolivia (*9*). The purposes of this study were to reassess HEV seroprevalence in humans in the same area and identify the virus in humans and swine.

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The Study

The survey was conducted in November–December 2006. We surveyed 2 rural communities in southeastern Bolivia: Bartolo in Hernando Siles Province, Department of Chuquisaca; and Casas Viejas in Vallegrande Province, Department of Santa Cruz.

The local economy is based on agriculture and animal farming. Persons live in close contact with animals, mainly swine, in the absence of adequate housing or fencing. Informed consent to obtain samples was obtained from all adults and from parents of minors participating in the survey. The study design, including its ethical aspects, was approved by the Bolivian Ministry of Health and the local health authorities.

Blood samples were taken from 236 persons (172 in Bartolo and 64 in Casas Viejas, age range 1–87 years). Individual fecal samples were obtained from 122 persons (90 in Bartolo and 32 in Casas Viejas, age range 3–62 years). Feces samples were combined into 22 pools (16 in Bartolo and 6 in Casas Viejas). Each pool consisted of 4–10 individual fecal samples from persons of the same age range. Individual fecal samples were taken also from 121 swine (67 in Bartolo and 54 in Casas Viejas, age range 2–12 months) and combined into 22 pools (13 in Bartolo and 9 in Casas Viejas).

Serum samples were tested for immunoglobulin (Ig) G against HEV. Samples from persons with fecal samples positive for HEV RNA were also tested for IgM against HEV by using commercial ELISA kits (HEV IgG/IgM; DIA.PRO Srl, Milan, Italy).

HEV RNA was detected by using reverse transcription PCR (RT-PCR) with 22 human and 22 swine fecal pools. Three grams of feces from each pool were homogenized in phosphate-buffered saline and centrifuged for 1 hour at 4°C. RNA was extracted by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). RT-PCR was performed in reduced reaction volumes of 25 μL instead of 50 μL (*10*). For human pools positive for HEV RNA, PCR was conducted with individual fecal samples that had been used in the pool.

For phylogenetic analysis, internal primer sequences were used to amplify isolates of human and swine HEV. The 348-nt sequence in open reading frame 2 of HEV isolates was analyzed and compared with corresponding regions of other known human and swine HEV strains available in GenBank. Sequences were aligned by using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2). Phylogenetic analysis was conducted by using MEGA5 (www.megasoftware.net). Geographic origin, identification code, and GenBank accession numbers of nucleotide sequences of the HEV strains used in the phylogenetic and sequence analyses are Japan, JJT-Kan (AB091394); Japan, JSN-Sap-FHo2C (AB200239); Japan, JSN-Sap (AB091395); Japan,

Table. Prevalence of antibodies against hepatitis E virus in 236 human serum samples, by patient age, Bartolo and Casas Viejas, Bolivia, 2006

Patient age, y	No. positive/no. tested (% positive)			95% Confidence interval for total % positive
	Bartolo	Casas Viejas	Total	
1–5	2/18 (11)	0/5 (0)	2/23 (8.7)	1.1–28
6–10	0/26 (0)	0/12 (0)	0/38 (0)	0–9.2
11–20	1/35 (2.8)	0/7 (0)	1/42 (2.4)	0–12.6
21–30	1/25 (4)	1/6 (16.7)	2/31 (6.4)	0.8–21.4
31–40	1/19 (5.2)	0/6 (0)	1/25 (4.0)	0.1–20.3
41–50	5/18 (27.8)	0/3 (0)	5/21 (24.0)	8.2–47.1
51–60	1/19 (5.3)	0/10 (0)	1/29 (3.4)	0–17.8
>60	1/12 (8.3)	2/15 (13.3)	3/27 (11.0)	2.3–29.1
Total	12/172 (7)	3/64 (4.7)	15/236 (6.3)	3.6–10.3

JKK-Sap (AB074917); People's Republic of China, swDQ (DQ279091); Japan, JRA1 (AP003430); Japan, HEJI4 (AB080575); United States, avian-HEV (EF206691); Japan, swJ8–5 (AB248521); People's Republic of China, swCH31 (DQ450072); Japan, swJ12–4 (AB248522); India, Ind-sw01 (AY723745); Japan, HE-JA37 (AB220978); Japan, HE-JA04–1911 (AB248520); Japan, JMNG-Oki02C (AB236320); Japan, HE-JA41 (AB220979); Japan, swJ13–1 (AB097811); India, Yam67 (AF459438); India, Hyderabad (AF076239); and Mexico, Mexican strain (M74506).

A total of 15 (6.3%) of 236 serum samples were positive for HEV by IgG ELISA (Table). The prevalence of IgG against HEV (7%) was higher in persons in Bartolo than in persons in Casas Viejas (4.7%) (p = 0.5). Seroprevalence did not show a linear trend associated with age; the highest seroprevalence was in found for persons 41–50 years of age in both communities (median age of 15 HEV seropositive persons 45 years, range 2–87 years). None of the persons in these 2 groups had a history of jaundice. No sex-related differences in seroprevalence were observed in these communities (male participants 47%, female participants 53%).

HEV-RNA was detected in 5 (22.7%) of 22 human fecal pools. All 31 fecal samples in the 5 HEV-positive pools were evaluated for HEV RNA. Four samples obtained from persons with an age range of 10–48 years were positive. All 4 amplification products were sequenced. HEV RNA was detected in 7 (31.8%) of 22 swine fecal pools (6 [46%] in Bartolo and 1 [11%] in Casas Viejas). Fecal samples from all 4 persons positive for HEV by PCR were negative for IgM and IgG against HEV by ELISA.

Phylogenetic analysis was performed on 11 amplification products obtained from 4 human and 7 swine fecal samples. Swine HEV sequences were closely related to human HEV sequences (76% nt and 92% aa homologies), and all belonged to HEV genotype 3. The amino acid homology among human HEV sequences was 96%. The phylogenetic tree produced from alignment of the 348-nt open reading frame 2 sequences is shown in the Figure.

Conclusions

In a survey performed in 1997 in southeastern Bolivia, we found a seroprevalence of 7% for antibodies against HEV (*9*), a relatively high seroprevalence among young

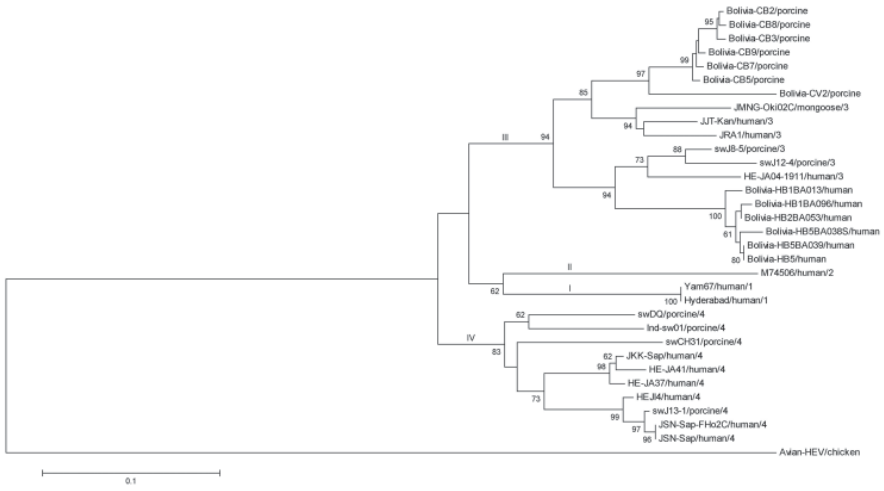

Figure. Phylogenetic tree with alignments of 348-bp open reading frame 2 sequences from human and swine samples of hepatitis E virus (HEV), Bartolo and Casas Viejas, Bolivia, compared with sequences of various HEV isolates. The tree was constructed by using the neighbor-joining method and evaluated by using the interior branch test method with MEGA5 software (www.megasoftware.net). Percentage of bootstrap support is shown by values at the branch nodes of the tree. Only nodes with a bootstrap value >60% are labeled; these values are the result of resampling the data 1,000 times. Isolate names are followed by species of origin. Roman numerals indicate genotype. Avian HEV was used as the outgroup. Scale bar indicates nucleotide substitutions per site.

adults, and a low seroprevalence among children. This study, conducted 9 years later in the same area, showed a similar seroprevalence (6%) and age-dependent distribution of antibodies against HEV and identified HEV RNA in fecal samples from human and swine populations.

Sequence comparisons and phylogenetic analyses showed that swine HEV strains were closely related to human strains; all belonged to genotype 3. HEV was also detected on swine farms in South America (*11*,*12*). The high degree of nucleotide sequence homology observed suggests that swine could also be a major source of HEV in the area of our study, but we are uncertain whether these findings can be extrapolated to other areas of Bolivia. Absence of clinical signs in the swine studied is not an unexpected finding because swine naturally infected with HEV are usually asymptomatic (*13*).

With regard to humans, no history of jaundice in seropositive persons was reported. The 4 persons with positive HEV RNA results by RT-PCR but negative IgM and IgG results by ELISAs were asymptomatic at sample collection and had no signs or symptoms of hepatitis 3 months after testing. We cannot explain the negative HEV serologic results for these persons. This finding could be attributed to early infection, transient intestinal virus passage, or low accuracy of assays used (*14*). Apparent limited illness associated with HEV infection in humans may be caused by attenuated virulence of genotype 3 strains (*15*). Additional studies are needed to define illness associated with HEV infection in humans and determine whether HEV infection is present in other animal species, particularly rodents, and their role in transmitting this virus (*13*).

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Porcine Rotavirus Closely Related to Novel Group of Human Rotaviruses

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We determined nucleotide sequences and inferred amino acid sequences of viral protein (VP) 4, VP6, VP7, and nonstructural protein 4 genes of a porcine rotavirus strain (SKA-1) from Japan. The strain was closely related to a novel group of human rotavirus strains (B219 and J19).

Rotaviruses, a member of family *Reoviridae*, are a major etiologic agent of acute gastroenteritis in humans and animals worldwide. Rotaviruses are classified into 7 groups designated A–G (*1*–*3*). Group A rotaviruses cause severe diarrhea in infants and children, and is estimated to be associated with 527,000 childhood deaths annually. They are also responsible for diarrhea in young mammals and birds of various species. Group B rotaviruses were first detected in a large water-borne outbreak of diarrhea among adults in the People's Republic of China, and were recently found in Bangladesh, India, and Myanmar. They have also been detected in cows, pigs, and rats. Group C rotaviruses cause sporadic and epidemic gastroenteritis in children and adults. They have also been detected in pigs, cows, and other animals. Group E rotaviruses were detected in pigs, and group D, F, and G rotaviruses were detected in chickens.

The complete nucleotide sequence of the genome of an avian group D rotavirus strain has been reported (*4*). However, little information on rotavirus groups E–G has been reported (*1*–*3*,*5*,*6*).

Human rotavirus strains J19 and B219, which are not classified into group A, B, or C, have been detected in China and Bangladesh (*7*–*11*). Strain J19, which was detected during a large epidemic of diarrhea in adults in China in 1997, has been propagated in human embryo kidney cells (*7*,*8*). Complete nucleotide sequences of all 11 RNA segments of strain J19 have been determined (*9*). In addition, the complete nucleotide sequence of the genome of Bangladesh strain B219 has been determined (*10*,*11*). Comparative sequence analysis showed that these 2 rotavirus strains are part of a novel group of rotaviruses.

We determined complete nucleotide sequences of the 4 RNA segments encoding viral protein 4 (VP4), VP6, VP7, and nonstructural protein 4 (NSP4) of a porcine rotavirus strain (SKA-1) from Japan (*12*) by using cDNA products obtained by a single-primer amplification method (*13*,*14*). Sequence data showed that SKA-1 is closely related to the novel group of human rotaviruses (J19 and B219).

The Study

A fecal specimen was obtained from a piglet experimentally infected with strain SKA-1, which was first isolated from a pig with diarrhea in Tottori Prefecture, Japan. RNA was extracted from a 20% fecal suspension in phosphate-buffered saline by using the ISOGEN-LS Kit (Nippon Gene Ltd., Toyama, Japan). Cloning was performed according to the method described by Lambden et al. (*13*) with some modifications (*14*). During single-primer amplification, several DNA bands were detected that appeared to correspond to RNA segments of a rotavirus.

DNA was purified by using a Wizard SV Gel and a PCR Clean-Up System (Promega, Madison, WI, USA) and cloned into the PCR-TOPO vector by using a TOPO TA Cloning Kit (Invitrogen, Carslbad, CA, USA). Six clones for each of the segments were selected and used for sequencing. Sequence analysis and comparisons were performed by using GENETYX-WIN software (GENETYX, Tokyo, Japan) and MEGA software (www.megasoftware.net/).

We selected 4 genes encoding VP4, VP6, VP7, and NSP4 for sequence analysis. Sequence data were obtained for comparative sequence analysis among group A and nongroup A rotaviruses. There was little identity for any of the 4 genes between SKA-1 and group A or C rotaviruses. In contrast, nucleotide and amino acid sequences of the 4 genes of SKA-1 showed relatively high identities with those of a novel group of rotavirus strains (J19 and B219). The VP6 gene, which is associated with group specificity, of SKA-1 showed highest identities among the 4 genes with those of the novel group human rotaviruses: 72%–73% at the nucleotide level and 76%–77% at the amino acid level. VP6 also showed relatively high identities with those of group B rotaviruses: 52%–53% at the nucleotide level and 36%–40% at the amino acid level (Table). The other 3 genes (VP4, VP7, and NSP4), also showed high identities with those of the novel group of human rotaviruses, although identity values were lower than those for the VP6 gene (Table).

Lengths and nucleotide sequences of the 5′ and 3′ noncoding regions of the 4 genes of SKA-1 were similar to those of the novel group of rotaviruses. Using the VP7 gene as a reference, we identified sequences of the 5′ noncoding

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Table. Identities of nucleotide and amino acid sequences of VP4, VP6, VP7, and NSP4 of porcine rotavirus strain SKA-1 with those of group B and a novel group of rotaviruses*

Strain	Species	Group	% Identity of nucleotide (amino acid) sequences			
			VP4	VP6	VP7	NSP4
J19	Human	Novel	59.3 (52.0)	72.9 (76.8)	64.4 (56.3)	62.1 (36.1)
B219	Human	Novel	58.3 (51.7)	72.2 (76.5)	64.6 (55.9)	62.3 (35.2)
Bang 373	Human	B	48.6 (29.5)	52.5 (37.3)	48.4 (21.8)	48.4 (17.3)
WH-1	Human	B	48.8 (29.0)	52.7 (37.9)	48.9 (21.8)	49.3 (16.8)
ADRV	Human	B	49.1 (28.6)	52.7 (36.9)	49.1 (21.8)	49.4 (16.8)
RUBV226	Bovine	B	47.8 (29.8)	52.4 (39.2)	NA	NA
DB176	Bovine	B	47.3 (29.9)	52.4 (39.4)	50.7 (21.4)	NA
Nemuro	Bovine	B	NA	52.6 (38.2)	49.6 (20.2)	NA
Po/PB-F18	Porcine	B	NA	NA	(21.4)†	NA

*VP, viral protein; NS, nonstructural protein; NA, not available. GenBank accession nos.: SKA-1 VP4, AB576625; SKA-1 VP6, AB576626; SKA-1 VP7 AB576627; SKA-1 NSP4; AB576628.
†Nucleotide sequence of the noncoding region of this strain was not available.

region of VP7 as 5′-GGAACTTTAAAGCC-3′ for strain SKA-1, 5′-GGCAATTTGAAGCC-3′ for the novel group of human rotaviruses, and 5′-GGCAATAAA-3′ for group B rotaviruses. Phylogenetic analysis of the 4 genes also showed that SKA-1 is closely related to the novel group of human rotavirus strains (J19 and B219) (Figure).

Conclusions

Although group A rotaviruses have been extensively studied, nongroup A rotaviruses have not been extensively studied. In particular, there is little information on group D, E, F, and G rotaviruses. A novel group of human rotaviruses (J19 and B219) are not related to any other groups of rotaviruses.

Rotavirus strain SKA-1 was detected in a fecal specimen from a piglet with diarrhea in Japan, and was isolated in MA-104 cells in 1999. Its group specificity was suggested to be group B on the basis of reverse transcription PCR results with a group B–specific primer. However, our sequence analysis showed that the porcine SKA-1 strain is different from group B human and porcine rotaviruses.

Nucleotide and amino acid sequences of SKA-1 did not show identities with those of group A or C rotaviruses, although they showed some relatedness to those of group

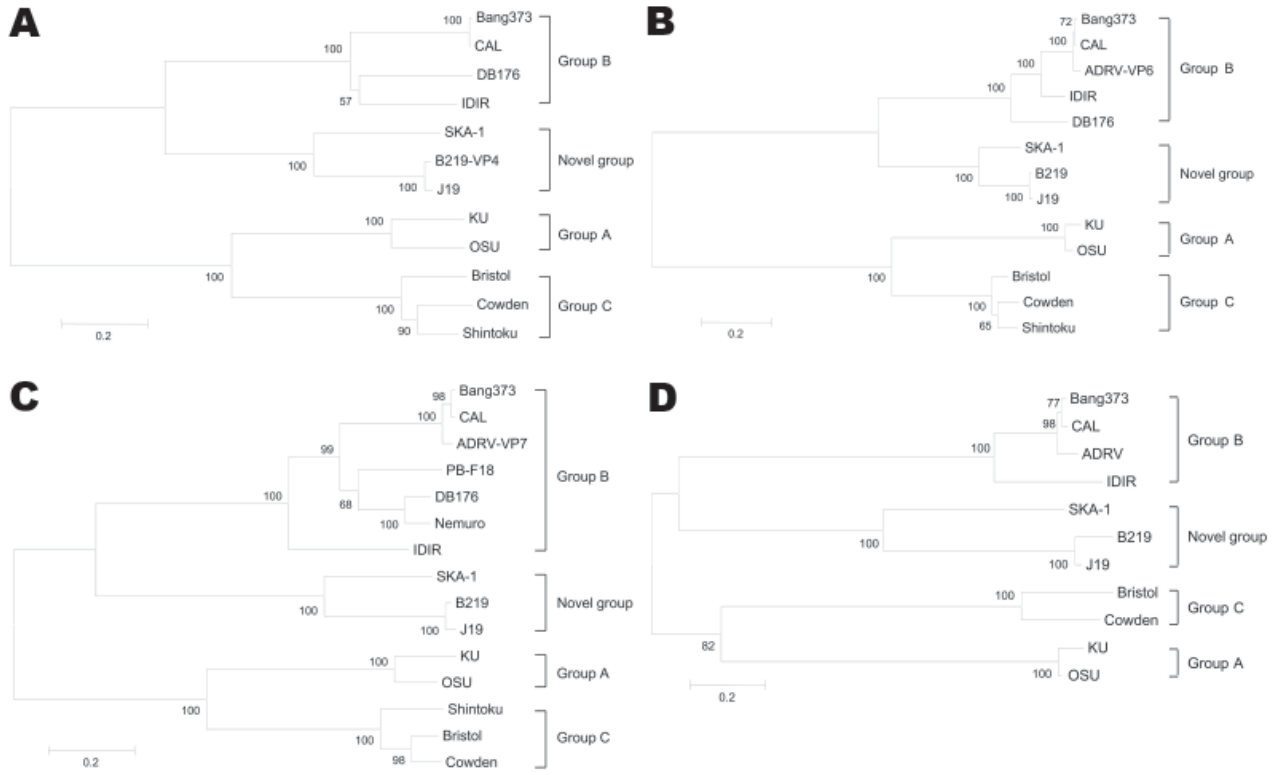

Figure. Phylogenetic trees for A) viral protein (VP) 4, B) VP6, C) VP7, and D) nonstructural protein 4 genes of group A, B, and C rotaviruses, a novel group of human rotaviruses, and porcine rotavirus strain SKA-1. Scale bars indicate nucleotide substitutions per site.

B rotaviruses. However, high identities were observed between SKA-1 and a novel group of human rotaviruses. In addition, there are similarities in the 4 genes analyzed in this study between SKA-1 and the novel group of human rotaviruses: 1) nucleotide sequences and nucleotide numbers of noncoding regions at the 5′ and 3′ ends are similar to each other; 2) lengths of nucleotide and deduced amino acid sequences are similar to each other; and 3) phylogenetic analysis showed that these viruses are in the same cluster.

Except for porcine strain SKA-1, there has been no report of animal rotaviruses being classified into a novel group of human rotaviruses. A survey of the prevalence of antibodies against SKA-1 among humans and animals, including pigs, would be useful. A classification system for nongroup A rotaviruses has not been established because 1) information on nucleotide sequences of group D, E, F, and G rotaviruses is lacking; 2) no expressed reference VP6 proteins are available; 3) reference strains of group D–G rotaviruses have not been adapted to cell culture; and 4) it is not known whether fecal samples or RNA or extracted RNA of group E–G strains are available in any laboratories. On the basis of serologic characterization and sequence analysis, a classification system for nongroup A rotaviruses, which includes the novel group of human and porcine rotaviruses such as J19, B219, and SKA-1, should be established.

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Enterovirus 68 in Children with Acute Respiratory Tract Infections, Osaka, Japan

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Enterovirus 68 strains were detected in 14 specimens from children with respiratory tract infections and 1 specimen from a child with febrile convulsions during 2010 in Osaka, Japan. These strains had deletions in the 5′ untranslated region and were genetically different from reported strains. This virus is associated with respiratory tract infections in Japan.

Enterovirus 68 (EV68) belongs to the family *Picornaviridae*, genus *Enterovirus*, and species *Human enterovirus D* (*1*). EV68 was isolated from 4 children with pneumonia and bronchiolitis in the United States in 1962 (prototype Fermon strain) (*2*,*3*) and is associated with respiratory tract infections (RTIs) (*3*–*5*). The most common age group for infection with EV68 is 1–4 years of age, but ≈25% of EV68 cases occur in adults ≥20 years of age (*5*).

Because of its acid sensitivity and low optimum growth temperature (33°C), EV68 shares characteristics with human rhinovirus (HRV) (*3*,*6*) and is genetically and antigenically similar to HRV 87 (*6*,*7*). During 1970–2005, only 26 EV68 strains were detected in the United States (*5*). Fourteen detections of EV68 were reported during 2006–2009 in Japan: 2 in 2006, 8 in 2007, and 4 in 2009 (*8*). EV68 is rarely detected in Japan, and no epidemics have been reported. We report deletions in genomes of EV68 strains detected in Japan.

The Study

During October 2009–October 2010, a total of 448 respiratory specimens were obtained from 448 patients (258 male patients and 190 female patients) with RTIs and fevers in a virus surveillance system in Osaka, Japan (*9*). The mean ± SD age of the patients was 41.4 ± 53.7 months (range <1–404 months), and 351 (78.3%) were <5 years of age.

Procedures for viral nucleic acid extraction and cDNA synthesis have been reported (*9*). PCR for detecting HRV and enterovirus was conducted by using EVP4 and OL68-1 primers, which detected HRV and human enterovirus, respectively, in amplicons of ≈530 and 650 bp, respectively (*7*).

Results showed 178 positive specimens (140 for HRV, 16 for human enterovirus, 7 for HRV and human enterovirus, and 15 for an unexpected amplicon of ≈600 bp). To identify the 600-bp amplicon, we sequenced viral protein 4 (VP4) and VP1 genes. BLAST analysis (www.ncbi.nlm.nih.gov/) showed that these isolates had high identity with EV68 VP4 (98.5%–99.5% with the Pav254–26868 strain [GenBank accession no. HM370293]) and VP1 (96.7%–97.5% with the MD02–1 strain [GenBank accession no. AY426491]). Therefore, these isolates were EV68 positive.

EV68 was not detected in virus isolation tests with Vero and RD-18S cells. EV68 was detected during June–September 2010 (Figure 1). Characteristics of 15 EV68-positive patients are shown in Table 1. Phylogenetic analysis using VP1 sequences (14 of 15 Osaka strains were sequenced) demonstrated that Osaka strains were clustered in 1 group and differed from previously reported strains (Figure 2).

Nucleotide and amino acid identities among 4 Osaka EV68 (JPOC10-290, 378, 396, and 404 strains; nt 501–7265 corresponding to the 37–99 strain), Fermon, and 37–99 strains were determined. To determine sequences, we synthesized cDNA by using specific primers and amplified 4 segments (nt 160–1153, 543–3391, 3132–4032, and 3747–7333 corresponding to the 37–99 strain). The Fermon and 37–99 strains are the only EV68 strains for which complete genome sequences are available. The 37–99 strain was isolated from a 6-year-old girl with pneumonia in 1998

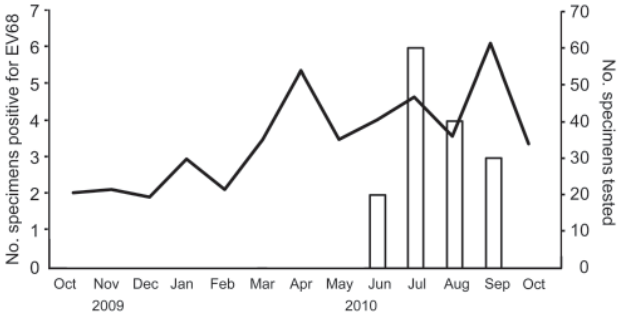

Figure 1. Monthly distribution of enterovirus 68 (EV68) in Osaka, Japan, October 2009–October 2010, Bars indicate no. specimens positive for EV68 and line indicates no. specimens tested.

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Table 1. Characteristics of 15 patients in whom enterovirus 68 was detected, Osaka, Japan, 2010

Patient no.	Age/sex	Sample no.	Month sampled	Clinical samples	Diagnosis or signs
1	4 y 9 mo/M	JPOC10–200	Jun	Nasal mucus	Bronchitis, fever (38.7°C), wheezing, coughing
2	0 y 7 mo/F	JPOC10–290	Jun	Nasal mucus	Asthmatic bronchitis, fever (39.3°C)
3	3 y 6 mo/M	JPOC10–373	Jul	Nasal mucus	Asthmatic bronchitis, fever (37.4°C)
4	2 y 10 mo/F	JPOC10–378	Jul	Nasal mucus	Pneumonia, fever (40°C), pharyngitis
5	4 y 6 mo/F	JPOC10–396	Jul	Nasal mucus	Pneumonia, wheezing, dyspnea
6	5 y 0 mo/F	JPOC10–402	Jul	Nasal mucus	Asthma, respiratory failure
7	1 y 8 mo/M	JPOC10–404	Jul	Nasal mucus	Febrile convulsion, fever (39°C)
8	3 y 9 mo/M	JPOC10–412	Jul	Sputum	Lower respiratory tract infection, fever (39°C)
9	1 y 3 mo/F	JPOC10–441	Aug	Nasal mucus	Asthmatic bronchitis, fever (38.2°C), wheezing
10	4 y 1 mo/M	JPOC10–445	Aug	Sputum	Asthmatic bronchitis
11	1 y 6 mo/M	JPOC10–471	Aug	Nasal mucus	Bronchopneumonia, fever (39°C)
12	0 y 3 mo/M	JPOC10–515	Aug	Throat swab	Pharyngitis, fever (38°C)
13	3 y 5 mo/M	JPOC10–573	Sep	Throat swab	Asthmatic bronchitis, fever
14	0 y 7 mo/M	JPOC10–616	Sep	Nasal mucus	Asthmatic bronchitis
15	1 y 5 mo/M	JPOC10–618	Sep	Nasal mucus	Pneumonia, fever (39°C)

(H. Norder, pers. comm.). The sequenced region coded partial 5′ untranslated regions (UTRs) and all structural and nonstructural viral proteins (VP4–3D). Identities between strains were calculated by using BioEdit version 7.09 (www.mbio.ncsu.edu/bioedit/bioedit.html) (Table 2).

Among Osaka strains, nucleotide and amino acid sequences were highly conserved (nt identity 96.5%–100% and aa identity 98.6%–100%). In contrast, Osaka strains had lower similarities with the Fermon strain (nt identity 83.5%–91.7% and aa identity 90.6%–100%) than with the 37–99 strain (nt identity 91.9%–98.4% and aa identity 95.4%–100%). When we compared individual viral proteins in Osaka strains with those in the Fermon strain, no gene except for VP4 showed >90% nt sequence identity; gene 2B showed the lowest identity (83.5%–85.8%). In contrast, the 37–99 strain had >91.9% nt identity with Osaka strains.

Regarding amino acids, <95% identity was observed in VP1, VP2, and VP3 in the Fermon strain, and no genes with <95% aa identity were found in the 37–99 strain in contrast with Osaka strains. Moreover, no integration or deletion of nucleotides was observed in VP4–3D sequences among Osaka, Fermon, and 37–99 strains.

To clarify why EV68 Osaka strain genomes were smaller than those of other enteroviruses and the EV68 Fermon strain (*7*), we aligned the partial 5′ UTR sequences (nt 541–820 corresponding to the Fermon strain) of 4 Osaka, Fermon, and 37–99 strains (online Appendix Figure, www.cdc.gov/EID/content/17/8/110028-appF.htm). Results showed that the Osaka and 37–99 strains had deletions at nt 681–704 and 717–727 in contrast with the Fermon strain. Moreover, a 1-nt deletion in Osaka strains was identified at nt 641 in contrast with the Fermon and 37–99 strains. Only the JPOC10–378 strain had a 1-nt deletion at nt 670.

Conclusions

Because 14 patients with EV68 were detected during 2006–2009 (*8*), detection of 15 patients with EV68 during a 4-month period suggests an EV68 epidemic in the summer of 2010 in Japan. Phylogenetic analysis with VP1 sequences showed that Osaka strains differed genetically from previously reported strains.

For precise analysis of Osaka, Fermon, and 37–99 strains, nucleotide and amino acid sequences were

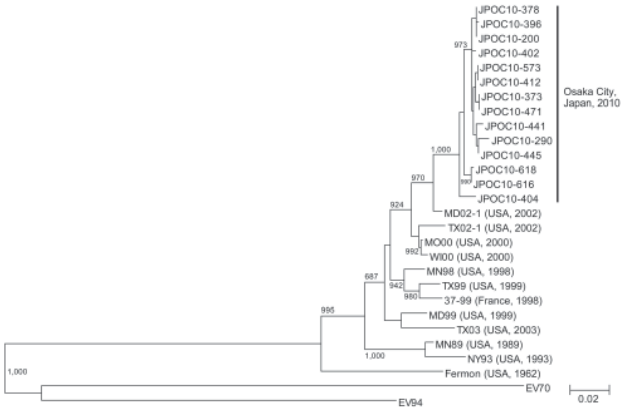

Figure 2. Phylogenetic tree of enterovirus 68 viral protein 1 gene sequences constructed by using a 927-nt sequence corresponding to nt sequence 2355–3281 in strain 37–99, Osaka, Japan, October 2009–October 2010. Tree was constructed by using the neighbor-joining method. Sequences were aligned by using Clustal X version 1.81. (www.clustal.org/). Genetic distances between sequences were calculated by using the Kimura 2-parameter method. Bootstrap values from 1,000 replicates are shown at the nodes. Location and year of collection are shown in parentheses. GenBank accession numbers for strains used in this analysis were Fermon, AY426531; 37–99, EF107098; JPOC10–200, AB601872; JPOC10–290, AB601882; JPOC10–373, AB601873; JPOC10–378, AB601883; JPOC10–396, AB601884; JPOC10–402, AB601874; JPOC10–404, AB601885; JPOC10–412, AB601875; JPOC10–441, AB601876; JPOC10–445, AB601877; JPOC10–471, AB601878; JPOC10–573, AB601879; JPOC10–616, AB601880; JPOC10–618, AB601881; MD02–1, AY426491; TX02–1, AY426495; MO00, AY426493; WI00, AY426494; MN98, AY426497; TX99, AY426498; MD99, AY426499; TX03, AY426500; MN89, AY426489; NY93, AY426490; EV70, D00820; and EV94, DQ916376. Scale bar indicates nucleotide substitutions per site.

Table 2. Comparison of nucleotide and amino acid sequences of Osaka enterovirus 68 strains detected in Osaka, Japan, October 2009–October 2010, with Fermon and 37–99 strains*

	Nucleotide identity, %			Amino acid identity, %		
Region	Osaka strains	Fermon	37–99	Osaka strains	Fermon	37–99
VP4–3D	98.4–99.6	87.8–87.9	93.9–94.1	99.4–99.8	95.8–96.0	98.4–98.7
VP4	97.5–100	90.8–91.7	94.6–95.6	100	100	100
VP2	97.5–99.7	87.3–87.7	94.4–94.6	100	94.3	99.1
VP3	97.7–99.7	85.5–85.8	93.9–94.4	99.5–100	94.0–94.4	98.7–99.1
VP1	97.7–99.8	87.5–87.8	92.5–93.2	99.0–100	90.6–90.9	95.4–96.1
2A	96.5–99.7	86.6–87.5	93.1–93.6	98.6–100	97.2–97.9	99.3–100
2B	96.6–99.6	83.5–85.8	91.9–92.5	98.9–100	96.9–97.9	97.9–98.9
2C	98.7–99.6	89.0–89.2	94.5–94.7	100	97.8	99
3A	99.6–100	89.5–89.8	94.0–94.3	98.8–100	96.6–97.7	98.8–100
3B	98.4–100	87.8–89.3	96.9–98.4	100	100	100
3C	98.2–99.3	87.0–87.7	92.4–93.2	99.0–100	96.2–96.7	98.1–98.5
3D	99.0–99.6	88.6–88.9	94.6–95.0	99.0–100	97.4–97.8	98.3–99.0

*Osaka strains, JPOC10-290, JPOC10-378, JPOC10-396, and JPOC10-404; VP, viral protein.

compared in all viral proteins. Results showed that Osaka strains more closely resembled the 37–99 strain than the Fermon strain. Alignment of partial 5′ UTR sequences showed that Osaka and 37–99 strains had deletions in 2 regions in contrast with the Fermon strain, and the amplicon was shorter than expected. Moreover, Osaka strains had 1-nt deletions in contrast with the 37–99 strain.

The 5′ UTR of enterovirus contains an internal ribosome entry site (*10*) that is associated with translational efficiency and virulence of the enterovirus (*11*,*12*). Deleted regions of Osaka strains appear to be in the flanking region between the internal ribosome entry site and an open reading frame (*1*). Detection of EV68 in numerous patients was reported in France during 2008 (*4*) and Italy during 2008–2009 (*13*). Because this deletion was found in the 37–99 strain in 1998, recent detection of EV68 in Japan might be associated with this change in the viral genome. Smura et al. reported that serum samples from 281 pregnant women in Finland in 1983, 1993, and 2002 had high titers of neutralizing antibody against EV68 (*14*). This result indicates that EV68 has been in Finland since 1983.

All EV68-positive patients in this study were <5 years of age and had lower respiratory tract inflammation. Seroepidemiologic studies in Finland showed that most adults might have been previously infected with EV68 and therefore might have neutralizing antibodies (*14*). Increased detection of EV68, especially in infants and children, will provide useful epidemiologic data.

Recent studies showed that EV68-infected human leukocytes produced infectious progeny virus (*14*). This result indicates that EV68 can replicate in blood and may damage the central nervous system. EV68 was detected in cerebrospinal fluid of a young adult patient with acute flaccid paralysis (*5*). Epidemiologic data for EV68 are lacking, and little information is available regarding virologic characteristics. If one considers results of phylogenetic analyses and nucleotide and amino acid identities, evolutionary changes might have occurred in EV68. Our results show the potential role of EV68 infection in infants and children with RTIs.

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Bagaza Virus in Partridges and Pheasants, Spain, 2010

Montserrat Agüero, Jovita Fernández-Pinero, Dolores Buitrago, Azucena Sánchez, Maia Elizalde, Elena San Miguel, Ruben Villalba, Francisco Llorente, and Miguel Ángel Jiménez-Clavero

In September 2010, an unusually high number of wild birds (partridges and pheasants) died in Cádiz in southwestern Spain. Reverse transcription PCR and virus isolation detected flavivirus infections. Complete nucleotide sequence analysis identified Bagaza virus, a flavivirus with a known distribution that includes sub-Saharan Africa and India, as the causative agent.

An essential feature of certain emerging pathogens is their ability to expand their geographic ranges. Several arboviral diseases, particularly those caused by flaviviruses, have been found in new areas beyond their usual ranges. The best example of this phenomenon was introduction of West Nile virus into the Americas in 1999 (*1*). Other recent expansions of flaviviruses were the introductions of Japanese encephalitis virus into Australia in 1995–1998 (*2*) and Usutu virus into Europe in 2001 (*3*).

We report an outbreak of disease in wild birds (partridges and pheasants) in Spain that was caused by a flavivirus, Bagaza virus (BAGV). This virus was first isolated in Bagaza, Central African Republic, in 1966, from a pool of mixed-species female *Culex* spp. mosquitoes (*4*). It has subsequently been found in mosquitoes in other countries in western Africa (*5*,*6*) and in India, where serologic evidence suggests that this virus may infect humans (*7*), although its pathogenicity in humans is uncertain. BAGV has been shown to be synonymous with Israel turkey meningoencephalitis virus, a pathogen affecting poultry (turkeys) and reported only in Israel and South Africa (*8*).

The Study

In September, 2010, an unusually high number of red-legged partridges (*Alectoris rufa*) died on several hunting properties in southwestern Cádiz, the southernmost province in Andalusia, Spain. Clinical signs included weakness, prostration, lack of motor coordination, weight loss, and white diarrhea. Some common pheasants (*Phasianus colchicus*) were also affected. These findings coincided in time with the first cases of West Nile virus (WNV) infection in horses detected in Spain (*9*).

Because red-legged partridges have recently been shown to be susceptible to WNV disease (*10*), it immediately raised suspicions that WNV could be responsible for these deaths. Therefore, samples were obtained from dead partridges (n = 11) and pheasants (n = 2) and sent to the Spanish Central Veterinary Laboratory in Algete for diagnostic analysis. The carcass of 1 of the affected partridges was also subjected to necroscopic analysis of different tissues, including heart, intestine, lung, liver, kidney, brain, and feathers (Table).

Analysis by real-time reverse transcription PCR (RT-PCR) specific for lineage 1 and lineage 2 WNV (*11*) showed negative results for all samples tested. However, a heminested RT-PCR specific for a broad range of flaviviruses (*12*) showed positive results for all samples (Table), indicating that a non-WNV flavivirus was present in samples from different tissues of diseased birds.

cDNA obtained after real-time RT-PCR (252-bp fragment) or heminested RT-PCR (214-bp fragment) amplification of samples was subjected to nucleotide sequencing. Resulting nucleotide sequences were compared with those in GenBank by using BLAST analysis (www.ncbi.nlm.nih.gov). Our isolates had >90% homology with 2 BAGV strains in GenBank (strain DakAr B209 from Africa, GenBank accession no. AY632545, and strain 96363 from India, GenBank accession no. EU684972).

Virus isolation was performed by infection of embyonated chicken eggs with tissue homogenates from an infected partridge and confirmed by pan-flaviviral RT-PCR and nucleotide sequencing (Table). Virus was detected more frequently in allantoic fluid of infected eggs than in other egg tissues (Table). Further propagation was accomplished in BSR cells, a clone of baby hamster kidney-21 cells.

Genomic characterization of virus was performed by bidirectional sequencing of real-time RT-PCR fragments amplified from heart and brain samples from 1 of the affected partridges. We used 27 primer sets designed for this study on the basis of sequences for BAGV in GenBank. Full-length genome sequences were obtained by assembling overlapping nucleotide sequences and using the SeqScape program (Applied Biosystems, Foster City, CA, USA). Two full-length genome sequences obtained from heart (BAGV Spain H/2010, GenBank accession no. HQ644143) and brain (BAGV Spain B/2010, accession no. HQ644144), were identical.

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Table. Analysis of red-legged partridges (*Alectoris rufa*) and common pheasants (*Phasianus colchicus*) for WNV and BAGV, Cádiz, Spain, 2010*

Species	No. animals analyzed	Sample type	No. positive/no. tested		Virus isolation in ECE, no. samples positive/no. tested†
			WNV	BAGV	
A. rufa	11	Brain	0/11	11/11	1/3 (AF)
		Cloacal swab	0/2	1/2	NA
		Oral swab	0/2	1/2	NA
		Gut	0/1	1/1	NA
		Heart‡	0/1	1/1	1/3 (AF)
		Kidney‡	0/1	1/1	2/3 (AF, CM, and VS)
		Lung	0/1	1/1	NA
		Liver	0/1	1/1	NA
		Blood	0/1	1/1	NA
		Feathers§	0/3	3/3	NA
P. colchicus	2	Brain	0/2	2/2	NA

*West Nile virus (WNV) or Bagaza virus (BAGV) nucleic acids were identified by using real-time reverse transcription PCR (RT-PCR) or panflaviviral heminested RT-PCR, respectively, and amplicon (214 bp) sequencing. ECE, embryonated chicken egg; AF, allantoic fluid; NA, not analyzed; CM, chorioallantoic fluid; VS, viscera.
†ECEs were infected with homogenates of various samples.
‡Samples from which full-length sequence of BAGV was obtained.
§Tail, breast, and wing feathers from 1 partridge were analyzed.

To assess phylogenetic relationships between these new BAGVs and other flaviviruses, the complete BAGV sequence obtained in this study (accession no. HQ644143) or a partial envelope (E) protein–coding gene subregion were aligned with other complete flaviviral genomes in GenBank by using the ClustalW algorithm in MEGA5 (*13*). Phylogenetic trees were constructed by using the complete BAGV genomic sequence or the partial E region (the E region is a well-known variable region in flaviviruses, and additional relevant nucleotide sequences are available in GenBank). Maximum-likelihood, neighbor-joining, or maximum-parsimony algorithms were used, and bootstrap tests with 500 replicates were performed to support each tree grouping. All generated trees showed similar topology and clustered the genomic sequence obtained in this study within the BAGV branch. Maximum likelihood trees are shown in Figures 1 and 2.

Sequence of BAGV from Spain was closely related to the 2 unique full-length BAGV sequences available in GenBank and showed greater similarity with the strain from Africa (94.1% nt identity) than with the strain from India (92.8% nt identity). Identity between the isolates from Africa and India was 95.0%, indicating that they are more related to each other than to BAGV from Spain. A total of 742 nt and 637 nt differences (70 nt and 47 aa differences) were observed between BAGV from Spain and the isolates

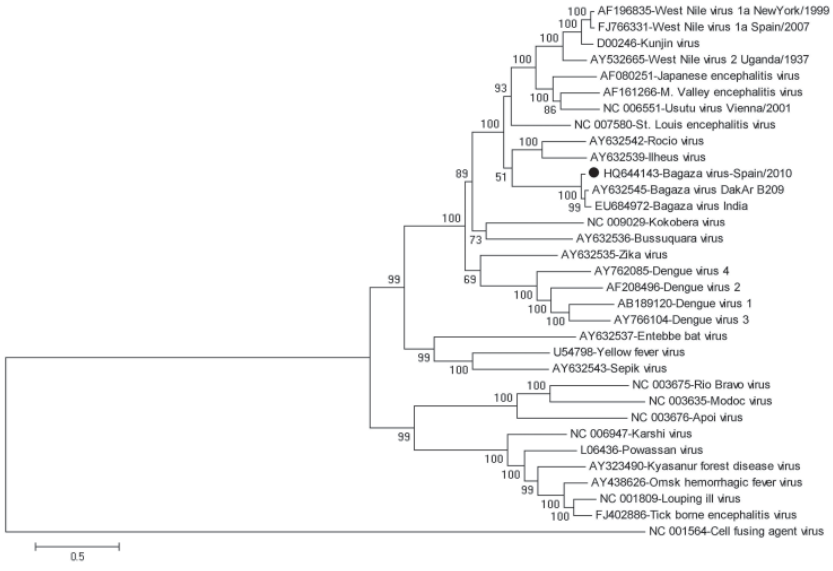

Figure 1. Phylogenetic relationships between a full-length genomic sequence for Bagaza virus identified in Cádiz, Spain, 2010 (solid circle) and 32 full-length flavivirus sequences, including 2 Bagaza virus isolates from GenBank. The phylogenetic tree was inferred by using the maximum-likelihood method. Percentage of 500 successful bootstrap replicates is indicated at the nodes. Evolutionary distances were computed by using the optimal general time reversible + Γ + proportion invariant model. A discrete Γ distribution was used to model evolutionary rate differences among sites (5 categories, G parameter = 2.0552). The rate variation model enabled some sites to be evolutionarily invariable (+I, 10.1524% sites). The tree is drawn to scale, and branch lengths are indicated as number of nucleotide substitutions per site. There were 9,803 positions in the final dataset. Phylogenetic analyses were conducted by using MEGA5 (www.megasoftware.net). GenBank accession numbers are indicated beside each isolate/strain name.

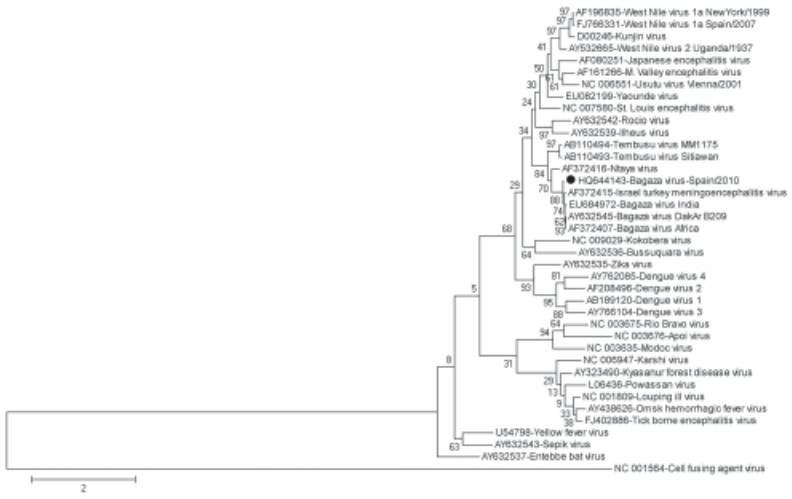

Figure 2. Phylogenetic relationships between partial envelope protein–coding gene sequence for Bagaza virus identified in Cádiz, Spain, 2010 (solid circle) and 38 equivalent flavivirus nucleotide sequences, including those for 3 Bagaza virus isolates, 1 Israel turkey meningoencephalitis virus, 1 Ntaya virus, and 2 Tembusu viruses from GenBank. The phylogenetic tree was inferred by using the maximum-likelihood method. Percentage of 500 successful bootstrap replicates is indicated at the nodes. The optimal Tamura-Nei with Γ distribution model was selected to compute evolutionary distances. A discrete Γ distribution was used to model evolutionary rate differences among sites (5 categories, G parameter = 1.8944). The tree is drawn to scale, and branch lengths are indicated as number of nucleotide substitutions per site. There were 941 positions in the final dataset. Phylogenetic analyses were conducted by using MEGA5 (www.megasoftware.net). GenBank accession numbers are indicated beside each isolate/strain name.

from India and Africa, respectively. Genetic relatedness between all 3 viruses was high (>92%), which indicates that they belong to the same *Flavivirus* species (*Bagaza virus*). The tree based on the E region grouped Israel turkey meningoencephalitis virus and BAGV within the same cluster and showed that both viruses are closely related to Ntaya virus (Figure 2).

Conclusions

BAGV has been detected and isolated in Cádiz, Spain. It appeared to seriously affect partridges and, to a lesser extent, pheasants, and caused an unusually high number of deaths in these birds. No signs of infection and no deaths were observed for other bird species. However, whether other bird species are susceptible to disease caused by BAGV should be determined because this virus is similar to Israel turkey meningoencephalitis virus, a relevant pathogen for turkeys. Also, other vertebrates could be at risk for infection with this virus. Thus, experimental studies on the pathogenicity of this virus in specific vertebrates should be conducted.

Transcontinental spread of flaviviruses has been often associated with bird migrations (*14,15*). Thus, infected birds migrating between Africa and Europe could have introduced BAGV into Spain. However, there is no evidence of transmission of this virus by migratory birds, and alternative explanations (poultry industry or trading of exotic birds for commercial or hunting purposes) should not be overlooked.

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etymologia

Rotavirus

[ro′tə-vi″rəs]

From the Latin *rota,* wheel, plus *virus*. After viewing the virus through an electron microscope in 1974, Flewett et al. suggested the name rotavirus on the basis of the pathogen's shape. The International Committee on Taxonomy of Viruses approved the name 4 years later.

Source: Dorland's illustrated medical dictionary. 31st edition. Philadelphia: Saunders, 2007; Flewett TH, Bryden AS, Davies H, Woode GN, Bridger JC, Derrick JM. Relation between viruses from acute gastroenteritis of children and newborn calves. Lancet. 1974;304:61–3. doi:10.1016/S0140-6736(74)91631-6; Matthews RE. Third report of the International Committee on Taxonomy of Viruses. Classification and nomenclature of viruses. Intervirology. 1979;12:129–296. doi:10.1159/000149081

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Crimean-Congo Hemorrhagic Fever Virus in Hyalommid Ticks, Northeastern Kenya

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As part of ongoing arbovirus surveillance, we screened ticks obtained from livestock in northeastern Kenya in 2008 to assess the risk for human exposure to tick-borne viruses. Of 1,144 pools of 8,600 *Hyalomma* spp. ticks screened for Congo-Crimean hemorrhagic fever virus by reverse transcription PCR, 23 pools were infected, demonstrating a potential for human exposure.

Crimean-Congo hemorrhagic fever virus (CCHFV), a member of the genus *Nairovirus*, family *Bunyaviridae*, causes hemorrhagic disease in humans with a >30% case-fatality rate. The virus was first described in 1944 in the Crimea in the former Soviet Union and later was found to be similar to a virus isolated in 1956 in the Belgian Congo (*1*).

Domestic ruminants are infected through tick bites and are able to infect more ticks to perpetuate the virus (*2*,*3*). The virus may be transmitted to humans by the bite of an infected tick or by contact with body fluids from an infected animal or person (*4*). The main vectors of CCHFV are ticks in the genus *Hyalomma*, family *Ixodidae*, with other ixodid ticks and ticks from the family *Argasidae* also contributing to transmission (*2*). The virus is transovarially transmitted among ticks (*2*,*3*); consequently, ticks are also reservoirs of CCHFV.

In Kenya, CCHFV has been detected on only 2 occasions: in *Rhipicephalus pulchellus* ticks collected in the 1970s from a dying sheep in a veterinary laboratory in the town of Kabete outside Nairobi (*2*) and from a person with Crimean-Congo hemorrhagic fever in western Kenya in October 2000 (*5*). Evidence of CCHFV activity in Kenya is limited, and although tick-borne arbovirus surveillance in Kenya has demonstrated circulation of a range of viruses, to our knowledge, detection of CCHFV has not been reported (*6*,*7*).

Crimean-Congo hemorrhagic fever is a substantial public health threat because of the associated high mortality rate (30%–60%), the potential for person-to-person transmission, the unavailability of a licensed vaccine, and the limited treatment options for infected persons (*3*,*4*). Entomologic surveillance is valuable for assessing the risk for human exposure and for identifying so-called hot spots for focused preventive action to minimize the effects of virus outbreaks. As part of ongoing entomologic arbovirus surveillance conducted by the United States Army Medical Research Unit in Kenya and the Kenya Medical Research Institute, ticks were collected from livestock in the semi-arid areas of Kenya, where intense pastoralist farming is practiced, to assess the risk to the community for tick-borne arbovirus exposure.

The Study

Ticks were sampled in the villages of Diiso and El-Humow and at the livestock market and abattoirs in Garissa District, North Eastern Province of Kenya, during April–May 2008 (Figure). Garissa District is in a semi-arid to arid ecologic zone that receives sporadic rainfall from March to May; vegetation consists primarily of *Acacia-Commiphora* bushes. Its population is largely composed of nomadic herders who travel between districts in northern Kenya in search of water and pasture (*8*).

Ticks were picked by hand from infested livestock, stored in labeled sterile vials, and transported in liquid nitrogen to the Kenya Medical Research Institute laboratory. In the laboratory, ticks were washed in sterile water, rinsed first with 70% ethanol, and then rinsed with minimum essential medium containing antimicrobial agents (100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μL/mL amphotericin B). They were identified to species by using taxonomic keys (*9*,*10*) and pooled in groups of 2 to 10 by species, sex, collection date and site, and host. The tick pools were homogenized by using 90-mesh alundum sand in a prechilled, sterile mortar and pestle with 1.6 mL–2 mL ice-cold bovine albumin 1 medium (1× medium 199 with Earle salts, 1% bovine albumin, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μL/mL amphotericin B) under high containment. The homogenates were clarified by centrifugation at 1,500 rpm for 15 min at 4°C, and supernatants were stored at –80°C.

Viral RNA was extracted from tick homogenates by using Trizol-LS (Invitrogen, Carlsbad, CA, USA)

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reagent, according to the manufacturer's instructions. RNA was screened by reverse transcription PCR (*11*) to amplify a 536-bp fragment of the gene encoding for the nucleocapsid protein in the small (S) segment of the CCHFV genome by using the following primers (*12*): CCHF F2 (5′-TGGACACCTTCACAAACTC-3′) and R3 (5′-GACAAATTCCCTGCACCA-3′), positions 135–153 and 653- 670, respectively, on the reference strain CCHFV 10200.

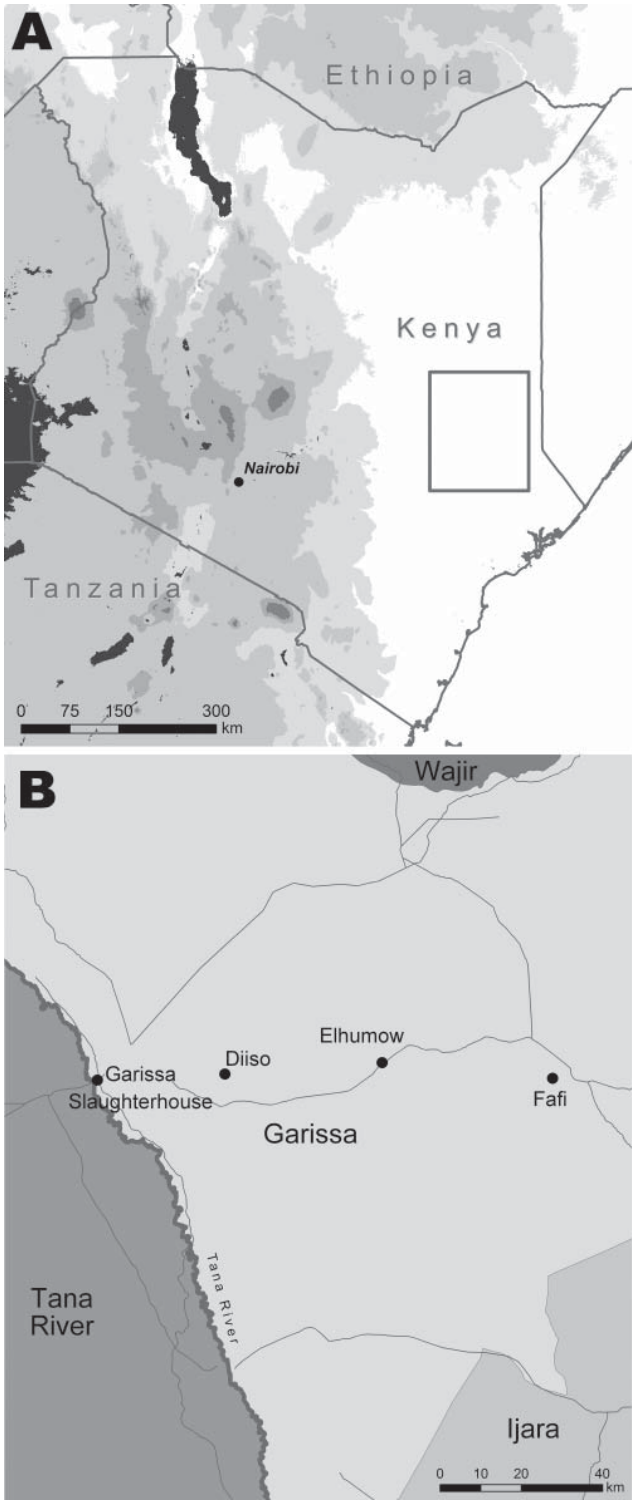

Figure. Location of Garissa District (A, box) in North Eastern Province, Kenya, and tick collection sites (B).

Electrophoresis of the PCR products was performed by using 1% agarose gels in Tris-acetate-EDTA buffer containing ethidium bromide; product bands were visualized and documented with the Canon UVP PhotoDoc-It gel imaging system (UVP, LLC, Upland, CA, USA) mounted with a digital camera. The PCR products of a subset of 4 of the CCHFV-positive homogenates were purified by using the QIAquick PCR Purification Kit (QIAGEN Sciences, Germantown, MD, USA), according to the manufacturer's instructions, and sequenced by using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI 3730 and automated 3130xl Genetic Analyzer (Applied Biosystems). The sequences were analyzed by using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the GenBank database to confirm the identity of the virus. Data (including tick species, collection site, animal host, and virologic test results) were entered into an Excel database (Microsoft Corp., Redmond, WA, USA) and analyzed by using pivot tables. A total of 8,600 ticks, of 3 genera and 8 species, were sampled primarily from camels, cattle, goats, and sheep, principally *Hyalomma rufipes* and *Hy. truncatum*. Ticks of the genus *Hyalomma* were sampled 3× more frequently in Diiso than in El-Humow (Table). CCHFV was detected in 23 *Hyalomma* spp. tick pools from Diiso and the Garissa slaughterhouse, including 4 pools of *Hy. rufipes* (3 from cattle and 1 from a camel), 18 pools of *Hy. truncatum* (14 from cattle and 4 from camels), and 1 unidentified *Hyalomma* species (Table) in which single DNA bands corresponding to the predicted 536-bp PCR product were detected.

Conclusions

The detection of CCHFV in pools of *Hyalomma* spp. ticks from Diiso village and the Garissa District slaughterhouse provides strong evidence of CCHFV presence in northeastern Kenya and indicates that CCHFV circulation in Kenya is underestimated. CCHFV was detected only in ticks collected from cattle and a camel. Livestock play a role in the amplification of the virus because the animals become viremic for 7 days (*2*,*3*), during which time they can infect more ticks. Our findings indicate that CCHFV circulates in northeastern Kenya with substantial involvement of camels and cattle. The detection of CCHFV in ticks from camels at the slaughterhouse also suggests the potential of exposure for abattoir workers. The presence of CCHFV among hyalommid ticks in northern

Table. Ticks species sampled from different livestock animals from 4 sites within Garissa District, northeastern Kenya, and CCHFV infection detected from tick pools, April–May 2008*

Site and animal	Tick species	No. ticks	No. pools tested	No. CCHFV-positive pools
El-Humow village				
Goat	*Amblyomma variegatum*	64	16	0
Goat	*Am. gemma*	47	6	0
Cattle	*Am. lepidum*	48	6	0
Camel	*Rhipicephalus appendiculatus*	94	12	0
Camel	*Hyalomma rufipes*	17	3	0
Cattle	*Hy. rufipes*	124	16	0
Sheep	*Hy. rufipes*	50	7	0
Camel	*Hy. truncatum*	22	3	0
Cattle	*Hy. truncatum*	623	81	0
Sheep	*Hy. truncatum*	24	3	0
Cattle	*Hyaloma* sp.	73	10	0
Cattle	*Rh. pulchellus*	748	94	0
Sheep	*Rh. pulchellus*	162	21	0
Diiso village				
Cattle	*Hy. rufipes*	160	20	3
Camel	*Hy. truncatum*	1,034	132	4
Cattle	*Hy. truncatum*	1,513	191	14
Camel	*Hyalomma* sp.	191	24	0
Cattle	*Hyalomma* sp.	192	59	1
Camel	*Rh. pulchellus*	168	21	0
Cattle	*Rh. pulchellus*	1,297	163	0
Goat	*Rh. pulchellus*	420	53	0
Livestock market				
Cattle	*Hy. truncatum*	266	34	0
Cattle	*Hyalomma* sp.	7	1	0
Slaughterhouse				
Camel	*Hy. rufipes*	90	12	1
Camel	*Hy. truncatum*	642	81	0
Camel	*Hyalomma* sp.	76	19	0
Camel	*Rh. pulchellus*	448	56	0
Totals		8,600	1,144	23

*CCHFV, Congo-Crimean hemorrhagic fever virus.

Kenya highlights the risk to the resident population and requires the assessment of human exposure. Health care workers must therefore help create awareness among the population and take steps to prepare for and prevent outbreaks.

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Poliomyelitis Outbreak, Pointe-Noire, Republic of the Congo, September 2010–February 2011

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On November 4, 2010, the Republic of the Congo declared a poliomyelitis outbreak. A cross-sectional survey in Pointe-Noire showed poor sanitary conditions and low vaccination coverage (55.5%), particularly among young adults. Supplementary vaccination should focus on older age groups in countries with evidence of immunity gaps.

On November 4, 2010, the Ministry of Health of the Republic of the Congo officially declared a poliomyelitis outbreak centered in Pointe-Noire following the laboratory identification of poliovirus type 1 in a patient with acute flaccid paralysis (AFP) (*1*,*2*). A provisional total of 554 AFP case-patients that included a high proportion (68%) of male patients was identified nationally, with paralysis onset from September 20, 2010, through February 27, 2011 (*3*). During this same period, 451 cases (81.4%), which included 184 deaths, were reported in Pointe-Noire. Most cases were found in the young adult population (57.4% in patients 15–24 years of age), which had a higher case-fatality ratio (CFR) of 40.8% compared with the general population of the country (*3*).

In most developing countries, prevention and control of poliomyelitis relies on oral polio vaccine (OPV). Routine vaccination focuses on infants <11 months of age with trivalent OPV, and supplementary immunization activities (SIAs) provide additional opportunities for vaccination for children <5 years of age (*4*,*5*). In response to the epidemic, the Ministry of Health, in collaboration with the World Health Organization, launched 4 rounds of national SIAs that used monovalent type 1 and bivalent types 1 and 3 OPV during November 12–16, 2010, December 3–7, 2010, January 11–15, 2011, and February 22–26, 2011 (*3*). To assess risk factors for infection and estimate vaccination coverage, Epicentre and Médecins Sans Frontières implemented a cross-sectional survey in 1 affected neighborhood of Pointe-Noire.

The Study

This survey was a single-community rapid assessment of epidemiologic conditions potentially contributing to the outbreak, including vaccination coverage. The survey was done in Mbota, a neighborhood composed of 4 sectors, comprising 9.5% of the city population. This neighborhood was selected from Loandjili district (Figure 1) on the

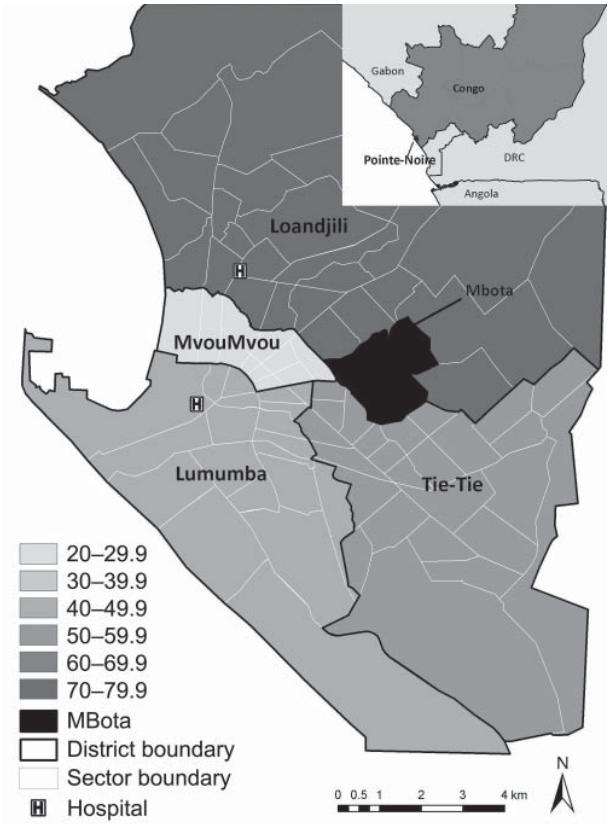

Figure 1. Pointe-Noire, Republic of the Congo, representing the 4 districts, Lumuba, MvouMvou, Tie-Tie, and Loandjili. The gray scale represents the cumulative acute flaccid paralysis (AFP) rate per 100,000 inhabitants September 2010–February 2011. Black area represents the 4 sectors of Mbota where the cross-sectional survey took place. The last census provided by local health authorities occurred in 2007, with a growth rate applied to estimate the 2010 official population size in Pointe-Noire, aggregated by gender and age. We used a 2005 population survey to disaggregate population figures by gender and 5-year age groups and calculate specific acute flaccid paralysis rate. DRC, Democratic Republic of the Congo.

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basis of local expert consultation to ensure socioeconomic diversity and representativeness. To estimate vaccination coverage of 80% among children with 5% precision at a 95% confidence level, we aimed to sample at least 246 households. We assumed each household had on average 1 child <5 years of age. We also assumed 5 persons per household on average, which corresponds to ≈1,230 persons overall. Households were selected in each sector of the neighborhood. From a starting point on the border of the sector, interviews focused on every fifth household, following a predefined direction toward the center of the sector, subsequently covering the entire neighborhood. Data were collected through face-to-face interviews December 9–10, 2010, by using a structured questionnaire. The questions included household demographics, water supply, and sanitary conditions. In addition, information for each person in the household was collected about vaccination history (routine and SIAs), any previous illness, and access to health care during the past 3 months. Vaccination coverage was estimated on the basis of reported personal history or documentary evidence of vaccination: a fingernail marked with ink indicating vaccination during SIAs or a vaccination card indicating routine vaccination.

Data were eventually collected from 1,849 persons in 317 households of Mbota with all ages and both genders represented. Following the 2 first rounds of SIAs, most surveyed persons (1,812 [98%], 95% confidence interval [CI] 97.3%–98.6%) reported having been vaccinated with monovalent OPV and 1,769 (95.7%, 95% CI 94.7%–96.6%) received 2 doses in November and December 2010. Among vaccinated persons, 627 (33.9%, 95% CI 31.7%–36.1%) had fingernails marked with ink; the rest were absent during the interview or ink had already worn off. There were no significant differences in SIA vaccine coverage by gender or age. Routine vaccination coverage was estimated as 55.5% (95% CI 53.3%–57.8%) among the surveyed persons (Table 1). Among reported vaccinated persons, 21.3% (95% CI 18.8%–23.8%)

Table 1. Persons vaccinated against poliomyelitis (routine vaccination), by age and gender, Mbota, Pointe-Noire, Republic of the Congo, December 2010

Age, y, and gender	No. (%) persons*					Total no. persons
	Vaccinated, card	Vaccinated, recall	Total vaccinated	Nonvaccinated	Unknown	
<5						
M	43 (35.5)	67 (55.4)	110 (90.9)	5 (4.1)	6 (5)	121
F	54 (36.2)	72 (48.3)	126 (84.5)	9 (6)	14 (9.4)	149
Total	97 (35.9)	139 (51.5)	236 (87.4)	14 (5.2)	20 (7.4)	270
5–9						
M	26 (25.5)	53 (52)	79 (77.5)	10 (9.8)	13 (12.8)	102
F	23 (20.9)	66 (60)	89 (80.9)	7 (6.4)	14 (12.7)	110
Total	49 (23.1)	119 (56.1)	168 (79.2)	17 (8)	27 (12.7)	212
10–14						
M	7(7.2)	62 (63.9)	69 (71.1)	9 (9.3)	19 (19.6)	97
F	16 (16)	51 (51)	67 (67)	7 (7)	26 (26)	100
Total	23 (11.7)	113 (57.4)	136 (69.1)	16 (8.1)	45 (22.8)	197
15–19						
M	4 (4.4)	39 (42.4)	43 (46.8)	9 (9.8)	40 (43.5)	92
F	8 (7.3)	49 (45)	57 (52.3)	12 (11)	40 (36.7)	109
Total	12 (6)	88 (43.8)	100 (49.8)	21 (10.4)	80 (39.8)	201
20–24						
M	2 (3)	25 (37.3)	27 (40.3)	7 (10.5)	33 (49.3)	67
F	4 (3.2)	61 (48)	65 (51.2)	14 (11)	48 (37.8)	127
Total	6 (3.1)	86 (44.3)	92 (47.4)	21 (10.8)	81 (41.8)	194
25–29						
M	2 (2.3)	37 (43)	39 (45.3)	12 (14)	35 (40.7)	86
F	11 (10.4)	50 (47.2)	61 (57.6)	15 (14.2)	30 (28.3)	106
Total	13 (6.6)	87 (45.3)	100 (51.9)	27 (14.1)	65 (33.9)	192
>29						
M	4 (1.5)	80 (29.2)	84 (30.7)	33 (12)	157 (57.3)	274
F	11 (4.2)	84 (32.3)	95 (36.5)	30 (11.5)	135 (51.9)	260
Total	15 (2.8)	164 (30.7)	179 (33.5)	63 (11.8)	292 (54.7)	534
Totals						
M	88 (10.5)	363 (43.3)	451 (53.8)	85 (10.1)	303 (36.1)	839
F	127 (13.2)	433 (45.1)	560 (58.3)	94 (9.8)	307 (32)	961
Total	215 (11.9)	796 (44.2)	1,011 (56.1)	179 (10)	610 (33.9)	1,800

*Card, vaccination documented by card; recall, person remembers received vaccination; total, card + recall.

showed their vaccination card, and among those, 78.7% (95% CI 73.1%–84.4%) were vaccinated ≥3 times. Routine vaccination coverage did not vary significantly by gender but decreased with age (p <0.0001). For persons younger than 5 years, it was 87.5% (95% CI 83.6%–91.4%) and <52% for anyone >15 years of age. In the most affected age groups, 15–19 and 20–24 years, vaccination coverage was estimated to be respectively 49.3% (95% CI 42.4%–56.1%) and 46.2% (95% CI 39.3%–53.1%) (Figure 2). By comparison, administrative vaccination coverage varied since 1986 between a reported 21% in 1997 and 112% in 2009 (Table 2).

The number of persons per bedroom, an indicator of crowding, ranged between 1 and 9, and there were >4 persons per bedroom for 12.1% (95% CI 8.4%–15.7%) of all households with no statistical differences among sectors. Tap water either from a neighbor's house (41.9%, 95% CI 35.9%–47.8%) or within the house (30.7%, 95% CI 25.2%–36.3%) was the primary potable water source recorded. Wells were also common (23.3%, 95% CI 18.3%–28.4%). When the primary source of potable water was unavailable, wells were the most frequently mentioned alternative source (37.8%, 95% CI 32.0%–43.6%). Most households reported use of a latrine shared with neighbors (57.4%, 95% CI 51.8%–63.1%) or within their house (31.4%, 95% CI 26.1%–36.7%). Few surveyed persons (16.1%, 95% CI 14.4%–17.7%) reported illness during the recall period, but if sick, 73.3% (95% CI 67.4%–79.2%) they sought health care, which suggests little underreporting of AFP cases.

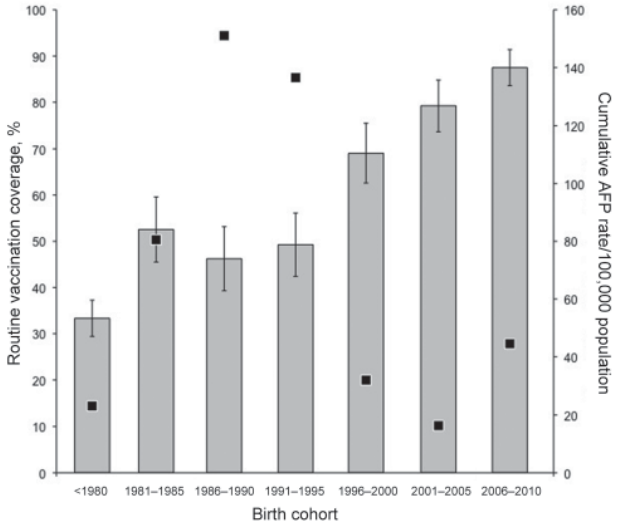

Figure 2. Routine vaccination coverage and acute flaccid paralysis (AFP) rate by birth cohort. Gray bars represent the vaccination coverage according to the survey implemented in Mbota, Pointe-Noire, Republic of the Congo, in December 2010. Error bars represent the 95% confidence interval. Black squares represent AFP rate per 100,000 inhabitants for each of the designated birth cohorts.

Conclusions

The cross-sectional survey confirmed administrative estimates of low routine vaccination decreasing with age. Specifically, in the surveyed young adult population (15–29 years of age) it varied between a reported 46.2% and 52.6%. According to administrative data, the reported vaccination coverage varies between 41.7% and 79% in young adults between 15 and 24 years of age. Civil conflicts spanning the 1990s have potentially undermined vaccination efforts, especially affecting those currently 10–20 years of age. A further method of protection for a person is past exposure to wildtype strains. To our knowledge, no polio transmission had been reported in Pointe-Noire since the last outbreak in the city in 1969 (*6*). Low vaccination coverage and no previous exposure to wild virus likely led to an accumulation of susceptible persons. Once introduced, the disease spread quickly through this highly susceptible, undervaccinated, and underexposed young adult population, with disease spread facilitated by frequent flooding and poor to medium household sanitation conditions, i.e., crowding sleeping areas, shared latrines between households, and common sources for water consumption.

Poliovirus infections lead to clinical disease more frequently in older age groups, (*7*), which partially explains the observed shift of AFP cases toward young adult populations. Moreover, children were better protected with higher vaccination coverage. Death is more frequent when infection occurs in older age groups (*8*,*9*), which explains part of the high observed CFR. Clinical manifestations of poliovirus infections are more common in boys and men than in girls and women (*10*), as observed, and even though males and females share similar vaccination coverage, women in the young adult age groups may have additionally benefitted from exposure to excreted OPV while caring for the young (*11*).

As seen in the Republic of the Congo, or previously in Namibia (*12*), Cape-Verde (*13*), any country with no recent wild-type polio transmission and a similarly low level of vaccination coverage may face similar outbreaks characterized by a large proportion of cases in older age groups and a high CFR (*14*). Avoiding outbreaks will rely on ensuring vaccination of the at-risk population. Routine vaccination activities should be reinforced by SIAs punctually focusing on older age groups (especially among persons 15–35 years of age) when evidence of immunity gaps are documented by serologic surveys or low historical vaccination coverage data. Lessons from the epidemic should benefit health authorities in better prevention and response to further outbreaks on the road to eradication.

Acknowledgments

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Table 2. Cumulative age-specific AFP rate and vaccination coverage from administrative data and a cross-sectional survey conducted in December 2010, by age group, Mbota, Pointe-Noire, Republic of the Congo*

Age group, y	AFP rate/100,000 persons (95% CI)	Survey vaccination coverage, % (95% CI)	Administrative vaccination coverage, %	5-y average administrative vaccination coverage, %
>29	23.1 (16.9–29.3)	33.3 (29.4–37.3)	†	†
25–29	80.5 (59.2–101.8)	52.6 (45.5–59.6)	†	†
20–24	151.1 (124.3–177.9)	46.2 (39.3–53.2)	41.7–79.0	67.0
15–19	136.5 (112.4–160.6)	49.3 (42.4–56.2)	47–77†	60.5†
10–14	31.9 (20.7–43.2)	69.0 (62.6–75.5)	21–75†	36.7†
5–9	16.3 (8.1–24.6)	79.3 (73.7–84.7)	38–67	53.1
0–4	44.5 (32.2–56.8)	87.5 (83.6–91.4)	81–112	93.9

*AFP, acute flaccid paralysis; CI, confidence interval.
†Administrative vaccination coverage provided by health authorities at the country level 1986–2000, at the regional level 2001–2006, and at the city level 2007–2009. Vaccination coverage was partially incomplete for 1992–2000 because of civil conflict. Thus, values for 1994, 1998, and 2000 consisted of interpolated values from years for which data are available.

and colleagues from World Health Organization and the Centers for Disease Control and Prevention for the harmonization of the database and stimulating technical discussions.

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Neurologic Disorders and Hepatitis E, France, 2010

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We report meningitis with diffuse neuralgic pain or polyradiculoneuropathy associated with PCR-documented acute hepatitis E in 2 adults. These observations suggest that diagnostic testing for hepatitis E virus should be conducted for patients who have neurologic symptoms and liver cytolysis.

Hepatitis E virus (HEV), a major cause of acute hepatitis in tropical and subtropical countries, is emerging in industrialized countries, where an increasing number of autochthonous hepatitis E cases have been reported (*1*–*5*). Nonetheless, the clinical spectrum of HEV infection still might be incompletely characterized. During the past decade, 12 cases of hepatitis E with neurologic signs and symptoms have been reported (*1*–*4*,*6*–*13*). We report 2 cases of meningitis or polyradiculoneuropathy in association with PCR-documented acute hepatitis E in France during 2010.

The Cases

Case 1 occurred in a 54-year-old woman hospitalized in August 2010 for subacute diffuse paresthesia and pain of the limbs (with ulnar localization on upper limbs and proximal pain in legs), headaches, cervicalgia, photophonophobia, transient fever, and nausea. Reflexes were all present, without pyramidal signs. No abnormalities were found by medullar magnetic resonance imaging. Nerve conduction study was not done because of rapid clinical improvement beginning <1 week after onset. The patient had no notable medical history. At admission, alanine aminotransferase level was 566 IU/L (reference values 8–34 IU/L), γ-glutamyl transferase level was 184 IU/L (reference 9–38 IU/L), alkaline phosphatase level was 125 IU/L (reference 42–98 IU/L), and bilirubin was 11 μmol/L (reference 5–34 μmol/L). Cerebrospinal fluid (CSF) was clear; protein and glucose levels were 1.03 g/L (reference 0.15–0.45) and 3.4 mmol/L (reference 3.0–4.5 mmol/L), respectively; leukocyte count was 74 cells/mm^3, including 90% mononuclear cells; erythrocyte count was 1 cell/mm^3. Immunoelectrophoresis performed in CSF and serum indicated rupture of the blood–brain barrier. Antibiotherapy with ceftriaxone, amoxicillin, and acyclovir was introduced 2 days after admission and continued for 2 weeks. CSF culture remained sterile at day 5.

Several infectious causes for neurologic symptoms and acute hepatitis were excluded by nucleic acid and serologic assays performed on serum and CSF (online Appendix Table, www.cdc.gov/EID/content/17/8/102028-appT.htm). In contrast, HEV infection was diagnosed by detection of anti-HEV immunoglobulin (Ig) M (cutoff optical density ratio >10; threshold value 1 [Adaltis, Rome, Italy]) and by detection of HEV RNA in serum (*5*). Concurrently, anti-HEV IgM (Assure, MP Diagnostics, Illkirch, France) and HEV RNA were detected in CSF. HEV RNA levels measured by real-time PCR were ≈2 log greater in the serum (cycle threshold [C_t] 27) than in CSF (C_t 33). HEV RNA open reading frame 2 sequences were recovered from serum (GenBank accession nos. HQ702487 and HQ702490) and CSF (GenBank accession no. HQ702488) by using in-house protocols (*14*); they showed 100% nt identity with each other. Phylogenetic analysis identified genotype 3, which is commonly found in autochthonous cases in France (*4*,*5*). No source for HEV transmission was identified, including eating undercooked pork or drinking unsafe water; the patient had returned from Greece 3 weeks before her hospitalization. She recovered fully within 2 weeks after onset of hepatitis and neurologic disorders. Liver parameters returned to within normal limits within 2 weeks, and HEV RNA was cleared in serum after 3 weeks.

Case 2 occurred in a 49-year-old man hospitalized in July 2010 for acute proximal sensorimotor weakness of the limbs. Clinical examination showed proximal muscle weakness associated with paresthesia and pain of upper and lower limbs. Muscle stretch reflexes were diminished in the lower limbs. No pyramidal signs were evident. Nerve conduction study results were normal, and needle electromyography showed abnormal spontaneous activity and neurogenic recruitment in weak muscles. The diagnosis of acute inflammatory polyradiculoneuropathy was retained. Liver enzymes were elevated: alanine aminotransferase 78 IU/L (reference <60 IU/L) and γ-glutamyl transferase 81 IU/L (reference <60 IU/L); bilirubin levels were within reference range. CSF was macroscopically clear; protein and glucose levels were

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1.02 g/L (reference 0.15–0.45 g/L) and 3.6 mmol/L (reference 2.2–3.9 mmol/L), respectively. Leukocyte count was 10 cells/mm^3, including 87% lymphocytes; erythrocyte count was 3 cells/mm^3. CSF culture was sterile after 5 days. As in case 1, several infectious etiologies were excluded (online Appendix Table). Anti-HEV IgM was detected in serum (optical density ratio >10) but not in CSF. HEV RNA was detected by real-time PCR only in serum (C_t 34). HEV RNA was of genotype 3 (GenBank accession no. HQ702489); nucleotide identity with sequences from case 1 was 91.5%. No source for HEV transmission was identified, and the patient had not traveled abroad. Clinical outcome spontaneously improved within 2 weeks. HEV RNA test results 2 months postadmission were negative.

Conclusions

In these 2 cases, the temporal association between acute hepatitis and neurologic signs and symptoms, atypical neurologic features, and exclusion of a large panel of other potential etiologies are incentive to consider HEV as a possible cause of meningitis, diffuse neuralgic pain, and polyradiculoneuropathy. The association of neurologic signs and symptoms with acute hepatitis E was previously reported for several patients (online Appendix Table). Eight case-patients had Guillain-Barré syndrome (GBS) (*3*,*4*,*6*–*11*), which has been associated with a variety of bacterial or viral infections, including hepatitis viruses other than HEV (*10*,*11*,*15*). Four other reports described distinct clinical presentations of acute meningoencephalitis with seizure (*1*), Bell palsy (*2*), acute transverse myelitis (*12*), and neuralgic amyotrophy (*13*).

In most cases, only a few potential etiologies for GBS were tested for. In addition, in all but 2 reported cases (*3*,*4*), HEV diagnosis was based only on detection of anti-HEV IgM in serum. Dalton et al. detected HEV genotype 3 RNA in serum from a patient with acute hepatitis E in association with GBS, whereas HEV RNA testing of CSF was negative (*3*). Kamar et al. described polyradiculoneuropathy with central nervous system involvement in a kidney transplant recipient who had chronic hepatitis E 33 months after acute hepatitis E (*4*). In this case, HEV RNA was sequenced concurrently in serum and CSF at hospitalization for neurologic involvement. By contrast, the 2 cases in our report occurred concomitantly with acute hepatitis E. In the patient reported by Kamar et al. and in case-patient 1 in this report, HEV RNA load was ≈2 log lower in CSF than in serum (*4*). Rupture of the blood–brain barrier could have enabled passive entry of the virus into CSF. The negativity of HEV RNA testing in the CSF in case-patient 2 might be explained by lower titers in CSF than in serum because HEV RNA titer in serum was low. In the case reported by Kamar et al., phylogenetic analysis suggested that neurologic symptoms could be associated with emergence of neurotropic variants because clonal HEV RNA in CSF clustered apart from those found in serum. In case-patient 1 reported here, HEV sequences recovered from serum and CSF were 100% identical; nevertheless, they were compared on a short open reading frame 2 fragment (106 nt) and on samples collected at time of acute hepatitis E, whereas comparison was performed 33 months postacute hepatitis in the case reported by Kamar et al. Regarding the outcome of neurologic symptoms associated with acute hepatitis E, patients fully recovered within 1 and 24 weeks, except in the cases reported by Chalupa et al. (*10*) and Kamar et al. (*4*). In this latter case-patient, no improvement was noted after 4 months, and the patient died of decompensated cirrhosis.

Previous and current observations suggest that HEV diagnostic testing should be performed for patients who have concurrent neurologic symptoms and liver cytolysis. Further investigations are needed to assess the actual prevalence of HEV infections in cases of neurologic disorders, especially those of unknown etiology.

Addendum

Kamar et al. recently reported neurologic complications in 7 (5.5%) patients among 126 with locally acquired acute and chronic HEV genotype 3 infection diagnosed during 2004–2009 at 2 hospitals in the United Kingdom and France (Kamar N, Bendall RP, Peron JM, Cintas P, Prudhomme L, Mansuy JM, et al. Hepatitis E virus and neurologic disorders. Emerg Infect Dis. 2011;17:173–9).

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Cowpox Virus in Llama, Italy

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Cowpox virus (CPXV) was isolated from skin lesions of a llama on a farm in Italy. Transmission electron microscopy showed brick-shaped particles consistent with orthopoxviruses. CPXV-antibodies were detected in llama and human serum samples; a CPXV isolate had a hemagglutinin sequence identical to CPXV-MonKre08/1–2-3 strains isolated from banded mongooses in Germany.

The llama (*Lama glama*) is a South American camelid used as a pack and meat animal by Andean cultures since pre-Hispanic times. Today, llama breeding is spreading in North America where the animals are used for wool production and as livestock guards. In Italy, llamas are raised in the northern and central regions to produce meat and wool, but they are more commonly considered companion animals or used as pack animals for trekking tours in the mountains.

Viral diseases of llamas are becoming better known as a result of extensive research in North America (*1*) prompted by the recent growth in commercial breeding of New World camelids. Many of the viral diseases that affect camelids are related to bovine, equine, ovine, and swine virus infections. When examining skin lesions on llamas, viral diseases to consider as differential diagnoses include vesicular stomatitis, rabies, poxvirus (contagious ecthyma and cowpox virus [CPXV]) (*2*), foot-and-mouth disease, bluetongue, and mucocutaneous fibropapillomas (*3*).

In July 2009, five of 7 llamas at a farm near Calcata (Viterbo) in Northern Latium, Italy, had skin lesions at different sites (palpebral conjunctiva, auricles, teats, mouth, and anus) that evolved from nodules to crusts; some had a crater morphologic appearance typical of poxvirus lesions (Figure 1, panel A). Within 10 days, 2 males showed depression and lethargy, anorexia, and recumbency until death. A short time later, another llama, a 7-year-old female, became ill and was euthanized. Necropsy was conducted on the euthanized animal, and samples were collected for laboratory investigation. No other animals belonging to numerous species of birds (local and exotic) and mammals (goats, cattle, swine, donkeys, and horses) living at the farm showed any of the above-mentioned symptoms.

Because mice and rats are considered carriers of CPXV (*4*) and birds of prey at the farm were fed frozen

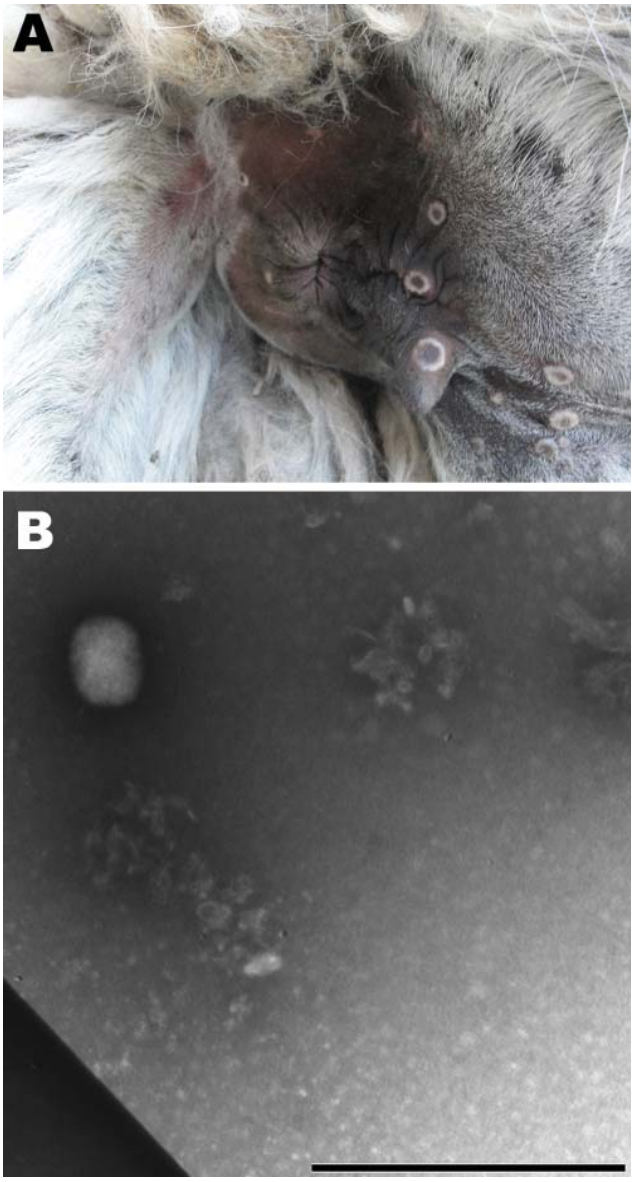

Figure 1. A) Skin lesions showing a crater morphologic appearance typical of poxvirus around the anus of a llama from a farm near Calcata (Viterbo) in Northern Latium, Italy. B) Electron micrography image of skin lesion sample showing negatively stained brick viral particle of ≈160–220 nm, consistent with orthopoxvirus. Scale bar = 1 μm.

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mice imported from a large farm in Germany, 2 lots of these mice were sampled (31 animals in total) along with 44 wild gray rats (*Rattus rattus*) captured on the farm for molecular analysis and virus isolation. Blood samples were collected for serologic analysis from 4 cattle, 25 goats, 18 donkeys, 3 horses, the 4 living llamas, and 4 humans (the farmer and 3 workers).

At necropsy, mandibular lymphoadenopathy; hemorrhagic gastroenteritis; and congestion of stomach, spleen, and kidney were observed in the euthanized female llama, signs consistent with a bacterial infection. In fact, a strain of *Salmonella enterica* serovar Typhimurium was isolated from lung, liver, spleen, kidney, and lymph node samples from the dead female llama and from chicken feces.

Samples of skin lesions were fixed in 10% buffered formalin, embedded in paraffin, cut 4 μm thick, and stained with hematoxylin and eosin. Histologically, the skin lesions showed eosinophilic granular intracytoplasmic inclusion bodies in basal and spinous layers of epidermis, compatible with poxvirus infection; superficial ulceration and extensive intraepidermal abscesses were also found.

Because we suspected a poxvirus, the skin lesions were processed (*5*) for electron microscopy techniques. Brick particles of ≈160–220 nm were observed, consistent with orthopoxvirus (Figure 1, panel B).

Two mammal cell lines (Vero and BHK_{21}) were used for virus isolation from skin lesions of the euthanized animal. During incubation of the cell cultures at 37°C, focal areas of cytopathogenic effect appeared within 3 days from injection, with complete lysis of the monostrate within 5 days. All the samples from mice and rats (pool of liver, spleen, and kidney) had negative results after 3 passages on both cell lines.

Total viral DNA was extracted from homogenized crusts and Vero cells supernatant by using conventional chemistry (QIAamp DNA Mini Kit; QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Five μL of DNA was used as template for a 20 μL total volume real-time PCR (*6*) targeting a region of the cytokine response–modifying protein B (*crmB*) gene, identifying an orthopoxvirus. A wider region of *crmB* (*7*) was amplified by conventional PCR and sequenced to confirm orthopoxvirus identity (data not shown). Phylogenetic analysis was performed by using a complete gene of the hemagglutinin sequence. The phylogenetic tree shows that the llama hemagglutinin sequence is identical to clusters with the cowpox virus strains isolated in Germany in 2008 (CPXV-MonKre08/1–2-3) (Figure 2).

A stock virus suspension of the viral strain isolated from the llama was produced and titrated on Vero cells to use as viral antigen in an in-house seroneutralization (SN) test. The blood of the 4 surviving llamas collected

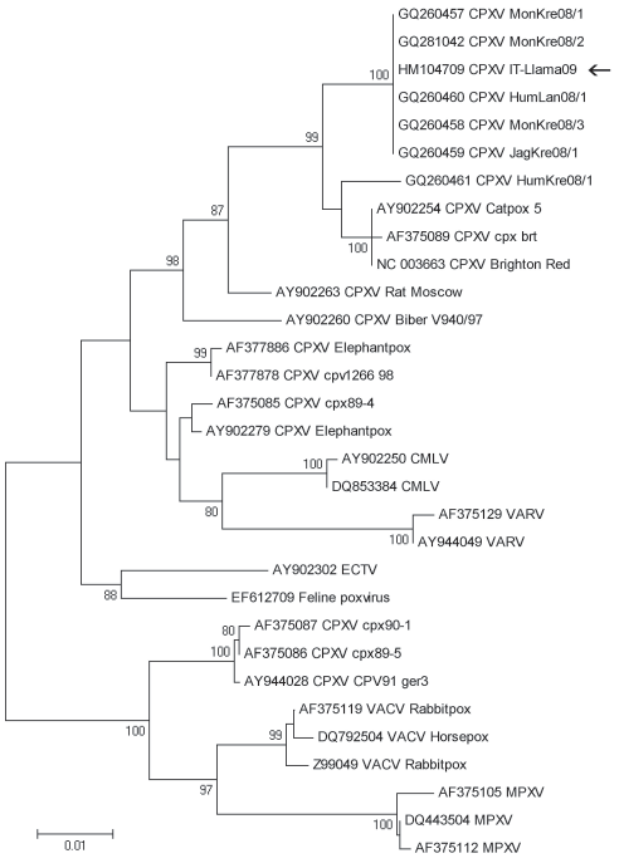

Figure 2. Phylogenetic tree based on nucleotide sequences of the complete hemagglutinin open reading frame (921 bp) from the llama orthopoxvirus isolate (arrow) and additional orthopoxvirus sequences available in GenBank. The tree has been constructed by using nucleotide alignment, the Kimura 2-parameter algorithm, and the neighbor-joining method implemented in MEGA4.1 software (www.megasoftware.net). Bootstrap values >75 are shown at nodes. CPXV, cowpox virus; CMLV, camelpox virus; VARV, variola virus; ECTV, ectromelia virus; VACV, vaccinia virus; MPXV, monkeypox virus. Scale bar indicates genetic diversity at the nucleotide level.

19 days after the female llama's euthanasia was assayed by CPXV SN test, as well as for antibodies against bluetongue virus (by ELISA), infectious rhinotracheitis virus (ELISA), *Brucella* (Rose Bengal plate test), and neosporidia (ELISA), all with negative findings. The SN test for detection of CPXV antibodies was also conducted in llama serum collected ≈3.5 months later (day 105), in parallel with samples collected from various animal species (bovine, equine, and caprine). Only the 4 llama samples collected at day 105 showed CPXV antibodies (Table). The antibody titers (immunoglobulin [Ig] G and IgM) in the farm personnel serum samples were determined by indirect immunofluorescence assay by using in-house

Table. Results of seroneutralization test for cowpox virus and IFA of serum samples from livestock and humans living on a farm, Italy*

Sample source	No. positive/no. tested		Serum titers, dilution
	Seroneutralization	IFA (IgG)	
Llamas†			
Day 19	0/4	ND	ND
Day 105	4/4	ND	64; 11; 4; 32
Cattle	0/4	ND	
Goats	0/25	ND	
Horses	0/3	ND	
Donkeys	0/18	ND	
Humans	ND	3/4	640; 20; 80

*IFA, immunofluorescence assay; Ig, immunoglobulin; ND, not done.
†Days shown are after a female llama at the farm was euthanized.

slides with Vero-E6 cells infected with a Lancy-Vaxina smallpox vaccine virus. Serum from a person vaccinated 4 years previously was used as a positive control. All serum samples had negative results for IgM (<20), while 3 persons had different IgG titers (Table). Only 1 of the 3 persons (titer 640) had been previously vaccinated against smallpox.

Conclusions

Following this outbreak, no other clinical signs have been described among livestock and humans at the farm and no other reports of CPXV have been recorded in Italy. The owner stated all the llamas were born on the farm and had had no contact with exotic mammals. Because the identified strain is apparently identical to the German 2008 mongoose isolates (CPXV-MonKre08/1–2-3) (*8*,*9*), it is tempting to hypothesize that the virus was introduced to the farm through frozen rats used as food for birds of prey. Such rats were sold by a German distributor throughout Europe, which also sold infected rats to the zoo where the identical mongoose CPXV isolate has been described (A. Kurth, pers. comm.).

Cowpox virus is distributed in Europe, western Russia, and adjacent areas of northern and central Asia, with an increasing number of reports in Europe (*10*). In Germany, CPXV has been found in animals in zoos. More recently, CPXV infections have been reported in mongooses (*8*,*9*). CPXV has been recently isolated from 2 cats and persons in contact with them in northern Italy (*6*). To date, no additional reports on the spread of this virus in Italy are available, although it is becoming increasingly popular to own wild and exotic pets and a wide range of recognized wild and domestic animal CPXV-hosts is increasing.

CPXV is responsible for human cowpox, a rare zoonotic infection, which is a self-limiting disease except in immunocompromised and eczematous patients, particularly children, in whom it can become severe. Furthermore, numerous reports of human cowpox affecting young people in Europe indicate that the lack of smallpox vaccination, stopped in 1977, may render people more susceptible to CPXV. For these reasons, CPXV is considered a pathogen of public health importance.

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Novel GII.12 Norovirus Strain, United States, 2009–2010

Everardo Vega and Jan Vinjé

In October 2009, a novel GII.12 norovirus strain emerged in the United States and caused 16% of all reported norovirus outbreaks during the winter season. Sequence analysis demonstrated a recombinant virus with a P2 region that was largely conserved compared with previously sequenced GII.12 strains.

Noroviruses are the leading cause of viral gastroenteritis outbreaks in the United States (*1*). Over the past decade most norovirus outbreaks have been caused by genogroup (G) II.4 noroviruses, while each of the other genotypes did not cause >7% of the outbreaks (*2*,*3*). However, previous studies have suggested that non-GII.4 noroviruses have been predominant in the past (*3*,*4*). For example, analysis of archived samples from 1974 through 1991 has shown that the frequency of GII.3 was 48% compared with 16% for GII.4 and 14% for GII.7 strains (*3*). Therefore, it is essential to study sudden increases of non-GII.4 strains to determine possible signatures that could be associated with increased transmissibility or population susceptibility. In this article, we describe the emergence of a novel GII.12 strain in the United States in the winter of 2009–10 that was associated with a large number of the norovirus outbreaks.

The Study

From October 2009 through June 2010, fecal specimens from patients affected by 194 outbreaks from 21 states were submitted to the Centers for Disease Control and Prevention (Atlanta, GA, USA); 39 (20%) of the viruses were typed as GII.12 by phylogenetic analysis by using region D sequences (*5*). During the same period, CaliciNet data confirmed an identical GII.12 strain that caused 67 (14%) of the 469 outbreaks reported by 12 states (*6*). To further study these new strains, we amplified the P2 region from 38 GII.12 outbreaks and 3 GII.12 strains reported to the Centers for Disease Control and Prevention from 2007 through March 2010 (online Appendix Figure, www.cdc.gov/EID/content/17/8/110025-appF.htm) using the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA). The final reaction mix consisted of 400 nM of oligonucleotide primers EVP2GII12F, 5′-ATC TAA TGG YTC TGG TGA TGA TG-3′ and EVP2GII12R, 5′-YGC CAC ACC TCC TTT AAG AG-3′. The primers annealed at positions 1132 and 1891 of the GII.12 strain Honolulu (GenBank accession no. AF414420) to yield a product of 759 bp. Cycling conditions included reverse transcription for 30 min at 48°C; denaturation for 2 min at 94°C; followed by 40 cycles of 94°C for 15 s, 48°C for 30 s, 68°C for 1 min; and a final extension step of 68°C for 5 min. A 2.5-kb region, including the complete open reading frame (ORF) 2 and partial ORF3 genes, was amplified by using GII conserved primers RING2-PCR (5′-TGG GAG GGC GAT CGC AAT CT-3′) and PanGIIR1 (5′-GTC CAG GAG TCC AAA A-3′). The primers annealed at positions 535 and 2888 of the GII.12 strain Honolulu (AF414420) to yield a

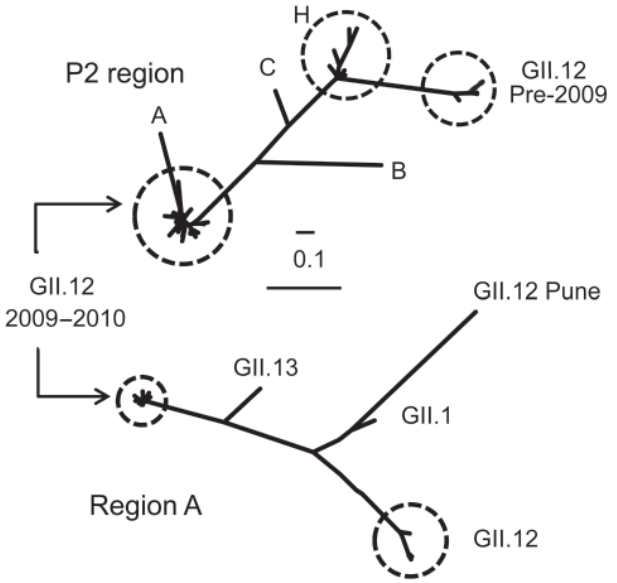

Figure 1. Phylogenetic trees of the P2 region in open reading frame (ORF) 2 and region A in ORF1 of noroviruses, United States. The P2 region and region A phylogenetic trees include GII.12 sequences from strains submitted to GenBank and GII.12 sequences reported in this study. In addition, region A analysis includes GII.12 Pune (GenBank accession no. EU921353), GII.1 (accession no. U07611), and GII.13 (accession no. DQ379714) sequences. Branch identifiers for the P2 region are as follows, with GenBank accession numbers in parentheses: A) Pune (EU921353); B) Pirna (AF427119); C) Wortley (AJ277618); H) Akabane (EF547403), Hiroshima (AB044366), Chitta virus (AB032758), Honolulu (AF414420), Gifu'96 (AB045603), U1GII (AB067536), U1 (AB039775), and Schwerin (AF397905). The GII.12 phylogenetic tree of the P2 region and region A region include GII.12 sequences StGeorge (accession no. GQ845370), Shelby (accession no. HQ688986), and all GII.12 noroviruses from 2009 through June 2010 (not distinguishable from GII.12 2009–10 cluster). The P2 region analysis also includes the Velence strain (accession no. HQ115742). Scale bars indicate nucleotide subsitutions per site.

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product of 2.3 kb. The GII.12 P2 sequences were similar to 2 GII.12 strains detected in sporadic cases in Australia (*7*) and Hungary in 2009 (Figure 1).

Phylogenetic analysis of P2 sequences from all GII.12 strains indicated a temporal pattern, with the new strains clustering separately from GII.12 strains detected before 2009 (online Appendix Figure). The new GII.12 strains clustered with GII.12 strains from Australia and Hungary in both the P2 and region A (*5*) (Figure 1). A single amino acid change in P2, at aa 392, occurred consistently in the new strains compared with archival GII.12 noroviruses (Figure 2). Additional amino acid substitutions were identified outside the P2 region at positions 22, 47, and 465 (Figure 2). Partial RNA-dependant RNA polymerase sequences confirmed that the new GII.12 strains were recombinant viruses, as reported previously (*7*) (Figure 1). Because different norovirus polymerases may have different nucleotide incorporation rates (*8*), and thus could play a role in enhanced replication efficiency of the new GII.12 strains, we amplified and analyzed a partial region of the polymerase gene but found no differences between the GII.12 strains pre- or post-2009.

Conclusions

A novel GII.12 norovirus strain emerged in winter 2009–10 and caused 16% of the norovirus outbreaks in the United States. Sequence comparison with archival GII.12 strains demonstrated that even though there were clear and distinct nucleotide changes in the P2 region, there were few amino acid changes in the complete viral protein (VP) 1 (Figure 2). The increase in the number of GII.12 outbreaks (online Appendix Figure) was confirmed by data from CaliciNet, which indicates that the increase was not caused by surveillance bias (*6*). Both P2 as well as polymerase sequence analysis demonstrated the emergence of a recombinant strain without novel amino acid substitutions in the P2 region, as has been reported for emerging GII.4 variants (*9*). All nucleotide substitutions throughout the P2 region of the new GII.12 strains, compared with pre-2009 GII.12 strains, were synonymous or unstable mutations (Figure 2).

Most norovirus evolution studies have focused on the VP1, more specifically the P2 region, where the antigenic and histo–blood group antigen attachment sites are located (*7*,*9*,*10*). The novel GII.12 virus had 1 unique amino acid substitution in the P2 domain but with similar biochemical properties, suggesting that the emergence of this virus may have been caused by virulence features coded by signatures outside of the P2 region or by changes in population susceptibility. In contract to a recent report (*11*), our results suggest that the nonstructural proteins or regions outside of the P2 region may play a role in the evolution and virulence of norovirus strains. Substitutions in some key amino acids in the N-terminal region of the capsid have been speculated to be involved in the formation of secondary structures for efficient initiation of translation of VP1 (*12*).

The fact that 16% of all reported outbreaks were caused by a rare genotype highlights the importance of norovirus strain typing, which may provide insights into identifying which viral and/or host factors enable the emergence of novel norovirus strains. CaliciNet, which demonstrated its usefulness in this study, will be an important tool for monitoring changing trends and emergence of novel strains. However, until a cell culture system, small animal model, or infectious clone for human norovirus is available, the role of structural or nonstructural genes on norovirus pathogenicity and transmissibility will be difficult to assess.

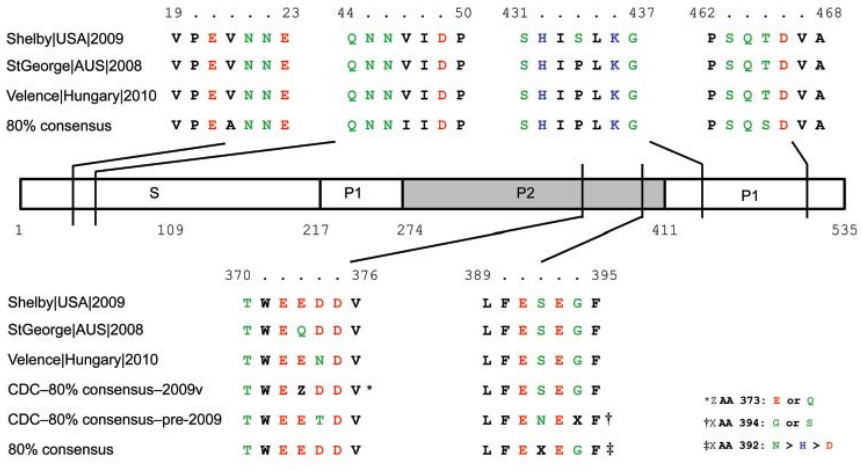

Figure 2. GII.12 norovirus viral protein (VP) 1 cartoon depicting amino acid similarities and locations between 2009–10 GII.12 strains and pre-2009 GII.12 strains. The S, P1, and P2 domains of VP1 are labeled accordingly. The VP1 amino acid numbering is based on the GII.12 prototype strain Wortley (GenBank accession no. AJ277618). Amino acid types are indicated by colors: green, polar; blue, basic; red, acidic; and black, hydrophobic. The 80% consensus sequence is based on a VP1 consensus sequence from the following GII.12 strains: GenBank accession nos. EU921353, AF427119, AJ277618, EF547403, AB044366, AB032758, AF414420, AB045603, AB067536, AB039775, and AF397905. Three recombinant 2009–2010 GII.12 strains—StGeorge (accession no. GQ845370), Velence (accession no. HQ115742), and Shelby (accession no. HQ688986)—were not included. The Centers for Disease Control and Prevention (CDC)–80% consensus–2009v and CDC–80% consensus–pre-2009 sequences are based on an 80% consensus P2 sequence from samples received from 2009–2010 (2009v) or from samples before 2009 (pre-2009), respectively.

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Novel Lyssavirus in Natterer's Bat, Germany

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A virus isolated from a Natterer's bat (*Myotis nattererii*) in Germany was differentiated from other lyssaviruses on the basis of the reaction pattern of a panel of monoclonal antibodies. Phylogenetic analysis supported the assumption that the isolated virus, Bokeloh bat lyssavirus, may represent a new member of the genus *Lyssavirus*.

Bats have been identified as carriers or reservoirs for a plethora of viruses, including human pathogens like severe acute respiratory syndrome coronavirus, henipaviruses, filoviruses, or lyssaviruses, which cause rabies (*1*). The genus *Lyssavirus* within the family *Rhabdoviridae* contains 11 viruses: rabies virus (RABV), Lagos bat virus, Mokola virus, Duvenhage virus, European bat lyssaviruses types 1 and 2 (EBLV-1 and EBLV-2), Australian bat lyssavirus, Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus, and West Caucasian bat virus (*2*). A proposed new species, Shimoni bat virus, has recently been isolated from *Hipposideros commersoni* leaf-nosed bats (*3*). Although RABV, which circulates in dogs, causes most of the ≈55,000 human deaths from rabies per year, most bat lyssaviruses have been demonstrated to cause human rabies (*4*).

From 1977 through 2009, a total of 928 cases of bat rabies (EBLV-1 and EBLV-2) were detected in Europe, but only 10 of the 45 known indigenous bat species tested positive for lyssavirus; most were serotine bats (*Eptesicus serotinus*) associated with EBLV-1 (*5*,*6*). In Germany, bat rabies has been known since the middle of the 20th century, and most isolated viruses were characterized as EBLV-1 (*6*). EBLV-2 is associated with *Myotis* spp. bats (*M. daubentonii* and *M. dascyneme*) and has only sporadically been found in Europe and in Germany (*7*). The transmission of EBLV-1 and EBLV-2 in bats in Europe is still only poorly understood (*8*). We report lyssavirus infection in a Natterer's bat.

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The Study

In November 2009, a bat was found on the ground in Bokeloh, Lower Saxony, Germany (52°25′13.99″N; 9°23′31.56″E). The bat was morphologically identified as a Natterer's bat (*M. nattererii*). It was given mealworms and water ad libitum supplemented with minerals and vitamins. In February 2010, the bat began to act aggressively, directly approaching any moving object, vigorously trying to bite, and screaming ferociously. This agitated stage lasted for 7 days and was followed by general weakness, lethargy, and paralysis. After the first 3 days of the clinical course, the bat stopped drinking and eating. Ten days after recognition of the first clinical signs, the animal died.

The bat was submitted for testing, and rabies diagnosis was performed by using immunohistochemical analysis. Lyssavirus antigen was detected in numerous neurons of the cerebral cortex, cerebellum, and especially the nucleus funiculi lateralis and the nucleus olivaris of the medulla (Figure 1). Organs other than the central nervous system, e.g., the salivary glands, did not contain lyssavirus antigen. After the first cell passage in rabies tissue culture infection test (*9*), virus was isolated from brain tissue. Antigenic typing performed with a panel of 10 antinucleocapsid monoclonal antibodies (*10*) clearly differentiated the isolated virus from all other tested lyssavirus species (Table). Thus, the virus isolate was tentatively named Bokeloh bat lyssavirus (BBLV).

Results of discriminatory reverse transcription PCR results for EBLV-1 and EBLV-2 (*11*,*12*) were negative, and only a generic reverse transcription PCR (*13*) yielded a 605-bp amplification product similar to that of the positive control. The nucleotide sequence was determined by using standard methods (primers and protocols are available upon request). Sequence analysis of the nucleoprotein gene performed with MEGA version 4.0 software (www.megasoftware.net/mega4/mega.html) showed that BBLV differed from all other published lyssavirus sequences with the highest nucleotide identity to KHUV (80%), followed by ARAV (79%), EBLV-2 (79%), Australia bat lyssavirus (77%), EBLV-1 (77%), Irkut virus (76%), Shimoni virus (76%), RABV (73-75%) and Duvenhage virus (75%). Lagos bat virus (72-74%), Mokola virus (72%), and West Caucasian bat virus CBV (72%) showed the highest divergence to BBLV. Also, phylogenetic analysis based on concatenated N-P-M-G-L nucleotide sequences showed that BBLV is most closely related to KHUV, followed by EBLV-2 (Figure 2).

Conclusions

We report the discovery of a lyssavirus (designated as BBLV) from a Natterer's bat that died with rabies-like clinical signs. Initially, a distinctive pattern in the reaction with a panel of antinucleocapsid monoclonal antibodies indicated the presence of an antigenically atypical isolate. The differentiation from other lyssavirus species was confirmed by phylogenetic analysis (Figure 2).

BBLV is pathogenic because it caused a fatal disease in the Natterer's bat that was similar to the clinical picture of rabies seen in other bats. Viral antigen was present in many locations of the brain (Figure 1) but surprisingly not in the salivary glands.

Since the exact date of infection is unknown, the incubation period can only be estimated as >4 months. Whether the Natterer's bat is the natural reservoir species of BBLV or whether it was a cross-species spillover remains a subject for further studies. However, closely related lyssavirus species were also isolated from *Myotis* spp. bats (EBLV-2 from *M. daubentonii*, *M. dasycneme*, KHUV from *M. mystacinus*, and ARAV from *M. blythii*) indicating that *Myotis* spp. bats play a key role in lyssavirus epidemiology. If one considers the history of bat rabies in Europe, it seems unlikely that BBLV had spread from a distant origin into central Europe or that the bat itself was translocated over long distances.

The fact that BBLV has been identified only in 1 bat is puzzling, considering the relatively high level of surveillance in Germany. Also, of 63 Natterer's bats tested during 1999–2010 in a retrospective study, none tested positive for rabies (T. Müller et al., unpub. data). Germany is the only country where several bat species other than serotine bats, i.e., *Pipistrellus nathusii, Pipistrellus pipistrellus*, and *Plecotus auritus*, have been found infected with EBLV-1 during this study (T. Müller et al., unpub. data), and this is the second discovery in recent years of a new lyssavirus species through routine passive bat rabies surveillance. Also, in 2007 a Daubenton's bat found on the ground was taken to a rehabilitation center, where it died and subsequently tested positive for EBLV-2 (*7*). In both cases, the person who took care of the animal had completed the full preexposure vaccination, as a required risk-mitigating measure.

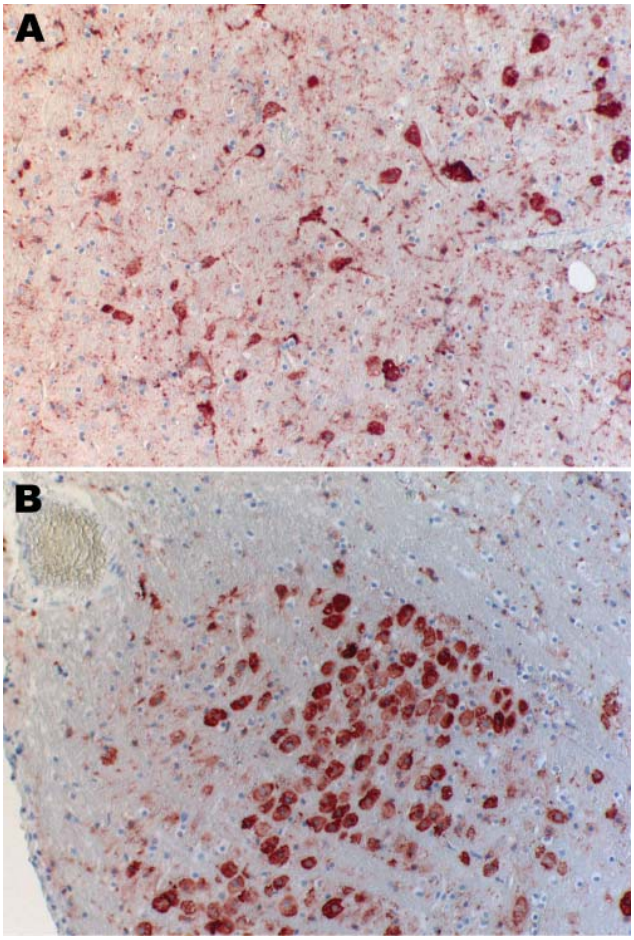

Figure 1. Immunohistochemical analysis of brain of Natterer's bat for lyssavirus antigen by using the avidin biotin complex method. A) Cerebrum showing a large number of neurons. Cytoplasmic granular-to-diffuse staining for rabies antigen is visible in the perikarya and neuronal processus. B) Medulla and neurons of the nucleus funiculi lateralis showing strong cytoplasmic staining for rabies antigen. Original magnifications ×20.

Table. Reactivity of 10 monoclonal antibodies against nucleocapsid protein of BBLV compared with 7 other lyssaviruses, Germany*

Antibody	RABV	LBV	MOKV	DUVV	EBLV-1	EBLV-2	ABLV	BBLV
W239.17	+++	+++	+++	+++	+++	+++	+++	+++
W187.5	+++	–	–	–	–	–	+++	–
W187.11.2	+++	–	–	–	–	–	+++	+++
MW187.6.1	+++	+++	+++	+++	–	–	+++	+++
MSA6.3	–	–	+++	–	+++	+++	–	+++
LBV7.36	–	+++	–	–	–	+++	–	–
DUV6.15.19	–	–	–	+++	+++	–	–	–
S62.1.2	–	–	–	–	+++	+++	–	–
P 41	–	–	–	–	–	–	–	–
Z144.88	–	–	–	–	–	–	–	–

*BBLV, Bokeloh bat lyssavirus; RABV, rabies virus; LBV, Lagos bat virus; MOKV, Mokola virus; DUVV, Duvenhage virus; EBLV, European bat lyssavirus; ABLV, Australian bat lyssavirus; +++, strongly positive; –, negative.

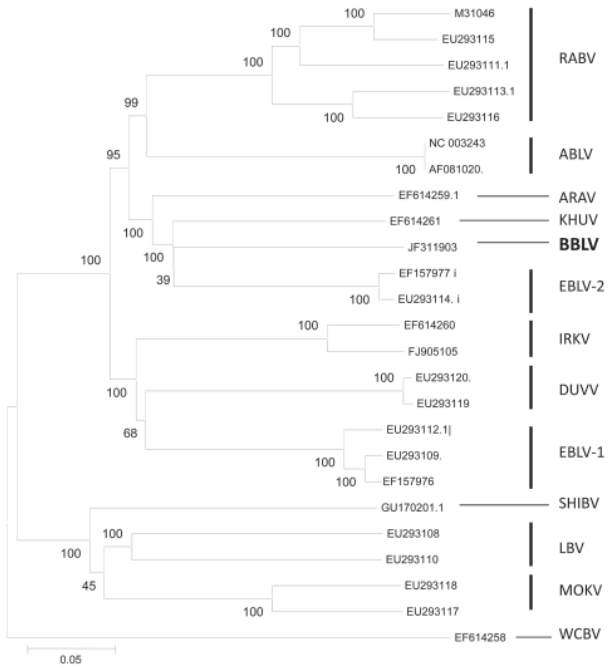

Figure 2. Phylogenetic tree inferred from concatenated N-P-M-G-L sequences of bat lyssaviruses. The neighbor-joining method (Kimura 2-parameter) was used as implemented in MEGA4 software (www.megasoftware.net). Bootstrap values (500 replicates) are shown next to branches. Scale bar indicates nucleotide substitutions per site. Virus isolated in this study is shown in **boldface**. RABV, rabies virus; ABLV, Australian bat lyssavirus; ARAV, Aravan virus; KHUV, Khujand virus; BBLV, Bokeloh bat lyssavirus; European bat lyssavirus; IRKV, Irkut virus; DUVV, Duvenhage virus; SHIBV, Shimoni bat virus; LBV, Lagos bat virus; MOKV, Mokola virus; WCBV, West Caucasian bat virus.

An encounter with a BBLV-infected Natterer's bat could lead to a fatal outcome because bat lyssaviruses have caused several human cases of infection (*4*). In Europe, species conservation and research require the handling of bats by bat workers. During 2000–2010, a total of 37,140 handlings were recorded for the Natterer's bat (Bat Marking Centre, Saxon State Office for Environment and Geology, Dresden, Germany), underlining the need for adequate prophylaxis for bat handlers. If one considers the close phylogenetic relationship between BBLV and EBLV-2, humans who receive rabies prophylaxis will likely be protected. However, recent studies of the antigenic relationships of lyssaviruses have shown the difficulty of interpreting antigenic differences by using sequences alone (*14*). Thus, in vitro and in vivo cross-neutralization and protection studies with current anti-RABV vaccines are urgently required for assessing the public health risk posed by this new lyssavirus.

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Imported Measles and Implications for Its Elimination in Taiwan

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During November 2008–May 2009, an outbreak of 53 measles cases occurred in Taiwan. Of these, 3 cases were sporadic, and the other 50 cases could be grouped into 8 clusters by genetic analysis. We determined 7 H1 genotypes linked to importation and 1 G3 genotype linked to an untraceable source.

The availability of a measles vaccine in the 1960s led to substantial reductions in the incidence of measles globally. Along with improvements in living conditions, nutrition, and the availability of antimicrobial drugs for secondary bacterial infections, the mortality rate was greatly reduced (*1*). A live-attenuated measles vaccine was introduced to Taiwan in 1968, and a routine vaccination policy was established in 1978 to provide measles vaccine to infants at 9 and 15 months of age. During 1992–1994 and 2001–2004, two catch-up campaigns were implemented, targeting birth cohorts September 1976 to September 1990 and September 1990 to September 1994, respectively. Starting in 2006, the measles, mumps, and rubella (MMR) vaccine targeted infants 12–15 months old and 6-year-old children. The coverage rates for the first dose of measles vaccine and the second dose of MMR were 91% and 95%, respectively, since 1996. In recent decades in Taiwan, 4 major measles outbreaks have occurred, with 2,219 (in 1985), 1,386 (1988), 1,060 (1989), and 303 (1992) reported cases. Beginning in 1993, the annual number of reported measles cases was <100; by the end of 2007, the annual number of confirmed measles cases was <10 (*2*). The ability to actively control measles has increased in Taiwan since 1991 because of a combined plan to eliminate polio, measles, congenital rubella syndrome and neonatal tetanus.

The key to controlling measles is achieving and sustaining high levels of vaccination coverage. Finding a way to prevent the transmission of measles is an important topic for any country in the elimination phase. Here, we report several measles outbreaks that occurred in Taiwan during 2008 and 2009.

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The Study

In total, 140 suspected measles cases were reported to the Taiwan Centers for Disease Control from November 2008 to May 2009, and 53 were confirmed (online Appendix Table, www.cdc.gov/EID/content/17/8/100800-appT.htm). The criteria for confirming cases included laboratory diagnosis and establishing an epidemiologic link to the laboratory-diagnosed case. The criteria for the laboratory diagnosis included the presence of measles immunoglobulin (Ig) M, isolation of measles virus, or identification of measles virus by reverse transcription PCR (*2*,*3*).

A timetable for occurrence of the 53 measles cases was constructed (Figure 1, panel A) on the basis of the week of onset of rash for each patient. The locations of the hospitals that reported these cases are shown in Figure 1, panel B.

Cluster 1 involved nosocomial infections in hospital A (case-patients 1–5) and hospital B (case-patients 6–8); the transmission between case-patient 5 and case-patient 6 was through household contact. The index case-patient (case 7) was discovered later. Cluster 2 (case-patients 9–21) caused nosocomial infections in hospitals C (case-patients 9–10) and D (case-patients 10–17 and 19). Case-patient 18 was reported by hospital F, case-patient 20 from clinic b and case-patient 21 from hospital O. With the exception of case-patient 20, who attended kindergarten with case-patient 19, there was no exposure linkage between case-patients 18 and 21; the phylogenic data (Figure 2) did, however, indicate the patients were infected with related strains. Cluster 3 involved case-patients 22–24; the index case-patient was the source of transmission and caused the nosocomial infection in hospital E. Cluster 4 (case-patients 25–33) caused nosocomial infections in hospital F (case-patients 25 and 27–33), and case-patients 25 and 26 attended the same school. Cluster 5 (case-patients 34–35) was a nosocomial infection in hospital G. Cluster 6 involved 10 cases: the index case-patient, case 37, was reported by hospital I. Case-patients 36–43 were from the same military base, and case-patients 37, 44, and 45 were relatives. Cluster 7 caused nosocomial infections in hospital H (case-patients 46 and 47) as well as household transmissions (case-patients 47 and 48). Cluster 8 (case-patients 49 and 50) was transmitted by contact at a dormitory. Case-patient 51 had a sporadic case from an unidentified source; case-patients 52 and 53 had sporadic cases linked to importation from Vietnam and India, respectively.

Of these 53 patients with confirmed measles, 36 (67.9%) were male. There were 15 children (28.3%) <12 months of age and 19 children (35.8%) from 12 months to 6 years of age. Adults accounted for 19 cases (35.8%). Only 1 patient (1.88%) had received 2 doses of a measles-containing vaccine; 4 patients (7.5%) had received 1 dose; 7 patients (13.2%) recalled having received vaccines; 9

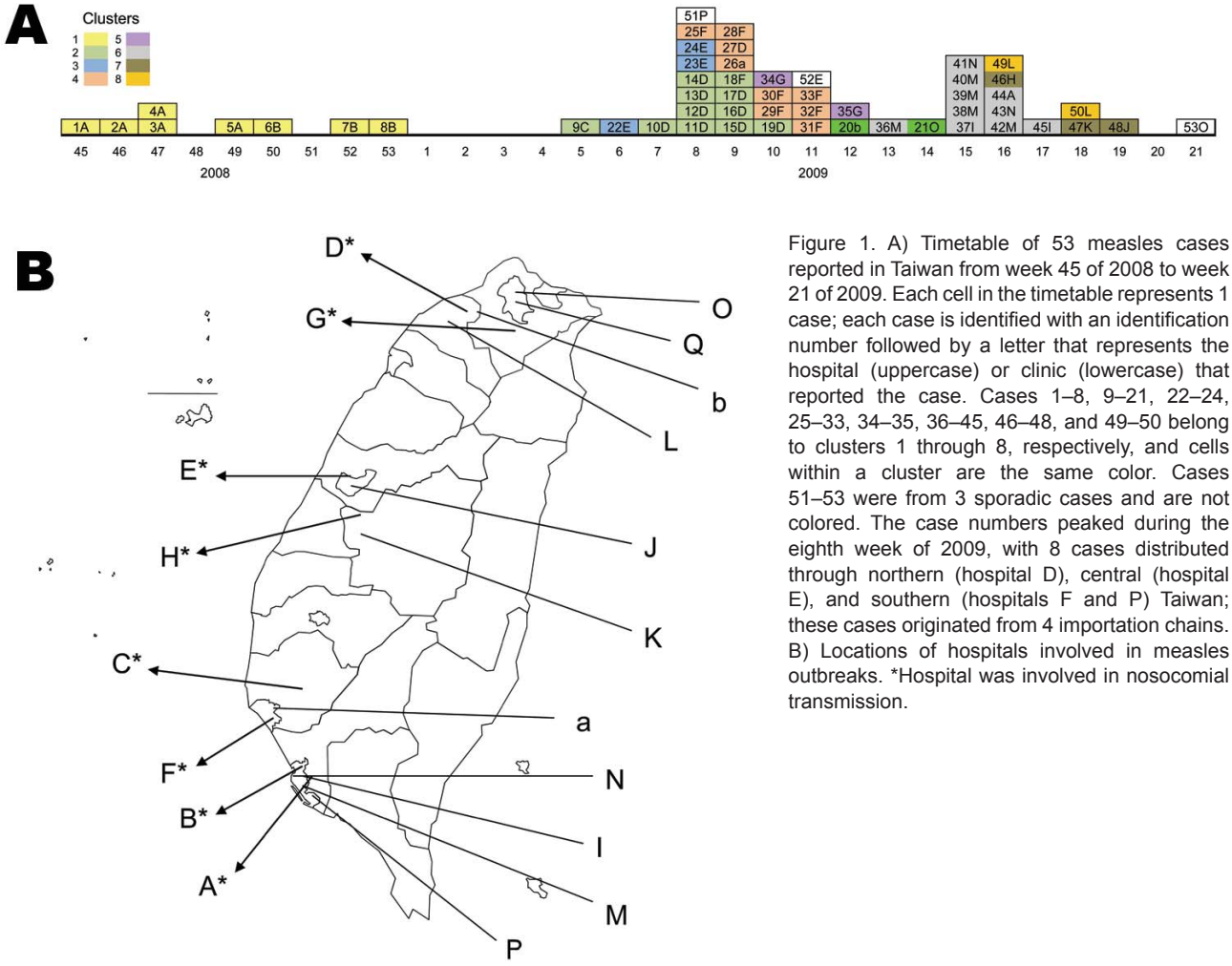

Figure 1. A) Timetable of 53 measles cases reported in Taiwan from week 45 of 2008 to week 21 of 2009. Each cell in the timetable represents 1 case; each case is identified with an identification number followed by a letter that represents the hospital (uppercase) or clinic (lowercase) that reported the case. Cases 1–8, 9–21, 22–24, 25–33, 34–35, 36–45, 46–48, and 49–50 belong to clusters 1 through 8, respectively, and cells within a cluster are the same color. Cases 51–53 were from 3 sporadic cases and are not colored. The case numbers peaked during the eighth week of 2009, with 8 cases distributed through northern (hospital D), central (hospital E), and southern (hospitals F and P) Taiwan; these cases originated from 4 importation chains. B) Locations of hospitals involved in measles outbreaks. *Hospital was involved in nosocomial transmission.

adults (17.0%) did not know their vaccination status; and the remaining 31 patients (58.4%) had not received any vaccines. Among the 6 nosocomial measles outbreaks, 2 infection sources (clusters 2 and 5) were children <12 months old and therefore not eligible for the first dose of the MMR vaccine, 4 clusters (clusters 1, 3, 4, and 7) were caused by children between 13–17 months old who were not vaccinated, and 1 cluster (cluster 8) was caused by an adult from Vietnam with an uncertain vaccination history. The outbreak on the military base was caused by an adult who was assumed to have been vaccinated.

Phylogenic analysis determined that the measles virus genotypes from these 8 outbreaks fell into 6 clades (Figure 2). Although the source was untraceable, the measles virus sequences from cluster 6 belonged to genotype G3; the sequence was 100% identical to a G3 strain (MVi/H Kajang.MYS/11.09) isolated from Malaysia (T. Tran, pers. comm.). The other sequences all belonged to genotype H1 and were grouped into 5 lineages. Clusters 1, 2, and 3 were linked to importation from the People's Republic of China and were grouped into different lineages. Clusters 4, 5, 7, and 8, which originated in Vietnam, were divided into 2 lineages, with clusters 4, 7, and 8 in the same lineage and cluster 5 in another lineage.

Conclusions

Among these 53 confirmed cases, 26 (49%) patients contracted measles from hospital exposure, and 3 patients (5.7%) were hospital staff. Vaccination policies for workers in health care institutes should be strengthened because these workers have the highest potential risk of contracting the disease (*4*,*5*). Many other febrile exanthematic diseases caused by pathogens other than measles virus, such as enterovirus, rubella virus, parvovirus B19, human herpesvirus-6, or Kawasaki syndrome, could have symptoms similar to measles and be misdiagnosed (*6*–*8*). It is therefore vital to ensure that clinicians are fully aware of the disease and can recognize it in a timely manner.

After import-associated measles outbreaks were recognized, the MMR vaccination was recommended for

persons planning to travel to measles-endemic countries. Successful immunization policies that include routinely vaccinating children with 2 doses of a measles-containing vaccine greatly reduce disease contraction among preschool and primary school children (*9–11*). Because measles outbreaks may occur in areas with high vaccine coverage (*12–14*), the higher proportion of measles cases in adults, especially in young adults, should be addressed further and evaluated with regard to the possible need for additional immunizations.

The major threats to measles control in Taiwan are similar to those faced by other countries in the elimination phase; the key questions are 1) how to detect the index case in real time, and 2) how to prevent import-associated transmission. Among these continuous outbreaks, 6 chains originated from children <18 months of age without adequate immunization who had recently traveled to measles-endemic countries. Clinicians being alert for patients being brought for treatment with a fever and rash at pediatric clinics will greatly improve the sensitivity of measles surveillance systems and prevent further nosocomial transmission. Special emphasis should be paid to pediatric patients without vaccination records and with a history of travel abroad. Of course, the ultimate goal of eliminating measles requires an agreement from all countries to contain the disease in their own territories.

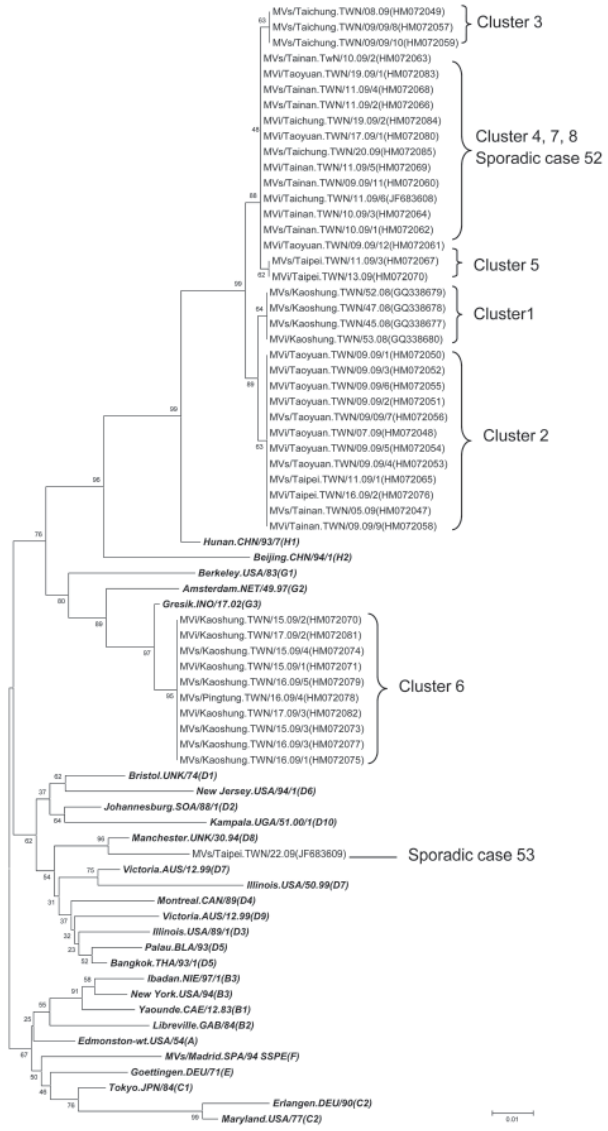

Figure 2. Phylogenetic analyses of the 456 carboxyl-terminal nucleotides of the N gene sequences of isolates obtained from 45 measles case-patients from November 2008 through May 2009, Taiwan. The respective accession number for each sequence is shown in parentheses following the strain name. ***Boldface italics*** indicate World Health Organization reference strains. The unrooted neighbor-joining consensus tree was generated by bootstrap analysis of 1,000 replicates by using MEGA4 software (www.megasoftware.net). Scale bar indicates nucleotide substitutions per site.

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Pygmy Rice Rat as Potential Host of Castelo dos Sonhos Hantavirus

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To study the dynamics of wild rodent populations and identify potential hosts for hantavirus, we conducted an eco-epidemiologic study in Campo Novo do Parecis, Mato Grosso State, Brazil. We detected and genetically characterized Castelo dos Sonhos virus found in a species of pygmy rice rat (*Oligoryzomys utiaritensis*).

Hantaviruses are RNA viruses (family *Bunyaviridae*, genus *Hantavirus*) distributed worldwide. In nature, these viruses are maintained in persistently infected rodents without disease manifestation. Hantaviruses are transmitted to humans through a respiratory route, mainly by inhalation of aerosolized, virus-infected particles in rodent excreta, such as feces, saliva, or urine. Hantavirus pulmonary syndrome (HPS) was first recognized in 1993 after an outbreak of acute respiratory distress syndrome associated with Sin Nombre virus occurred in the southwestern United States (*1*). In the same year, another hantavirus (Juquitiba virus) was identified in association with HPS cases in the state of São Paulo in southeastern Brazil (*2*).

Since 1993, molecular techniques have been used to identify New World hantaviruses in samples obtained from humans suspected of having hantavirus infection throughout the Americas and from captured rodents that test seropositive for hantavirus-specific immunoglobulin (Ig) G (*3*–*5*). Most known hantaviruses associated with rodent reservoir species have been identified in this way. However, for some hantaviruses, including Castelo dos Sonhos virus (CASV), the virus–host association remains unknown.

CASV was first identified in samples from a patient with HPS in 1995 and was the first hantavirus described in the Brazilian Amazon region (*3*). We report here data obtained during an eco-epidemiologic study conducted in the municipality of Campo Novo do Parecis, Mato Grosso State in central-western Brazil (Figure 1), including the identification of a possible rodent reservoir for CASV.

The Study

During 2005 through and 2007, research was conducted in the municipality of Campo Novo do Parecis (13°40′31″S 57°53′31″W) to study the dynamics of wild rodent populations and prevalence of hantavirus infection. Of 459 rodents captured during the project, 89 were classified as *Olygoryzomys utiaritensis* (a species of pygmy rice rat). Blood samples obtained from the rats were serologically screened by IgG-ELISA by using the Andes virus antigen as previously described (*5*).

DNA samples from the rats were isolated from liver preserved in ethanol. We amplified cytochrome *b* mitochondrial DNA (≈1,140 bp) with primers L14724 and Citb-rev by using standard PCR procedures and sequenced the samples with the same primers and an additional internal primer MVZ16 (*6*). Sequencing was performed with ABI 3130xl (Applied Biosystems, Foster City, CA, USA) automatic DNA sequencer. Kimura 2-parameter models were used for constructing neighbor-joining (NJ) dendrograms by using MEGA4 software (*7*). Confidence intervals for NJ trees were obtained by bootstrap analysis based on 2,000 replicates. Despite morphologic similarities between *O. nigripes* (black-footed pygmy rice rat) and *O. utiaritensis*, the NJ analysis showed that *O. utiaritensis* pygmy rice rats are more closely related to Moojen's pygmy rice rat (*O. moojeni*) (7.4% Kimura 2-parameter distance estimates) than to any other *Oligoryzomys* species (Figure 2, panel A). We deposited all animal carcasses in the National Museum, Rio de Janeiro, Brazil. The record of the 4 CASV-positive animals in the National Museum and other data are shown in Table 1.

For hantavirus detection, reverse transcription PCR was used to synthesize cDNA with generic hantavirus primers (*3*) as previously described (*8*). N gene partial nucleotide sequences were obtained by the Sanger method (*9*) by using the same primers. At least 3 amplicons per sample were sequenced in both directions to improve coverage and confidence on results. The obtained sequences

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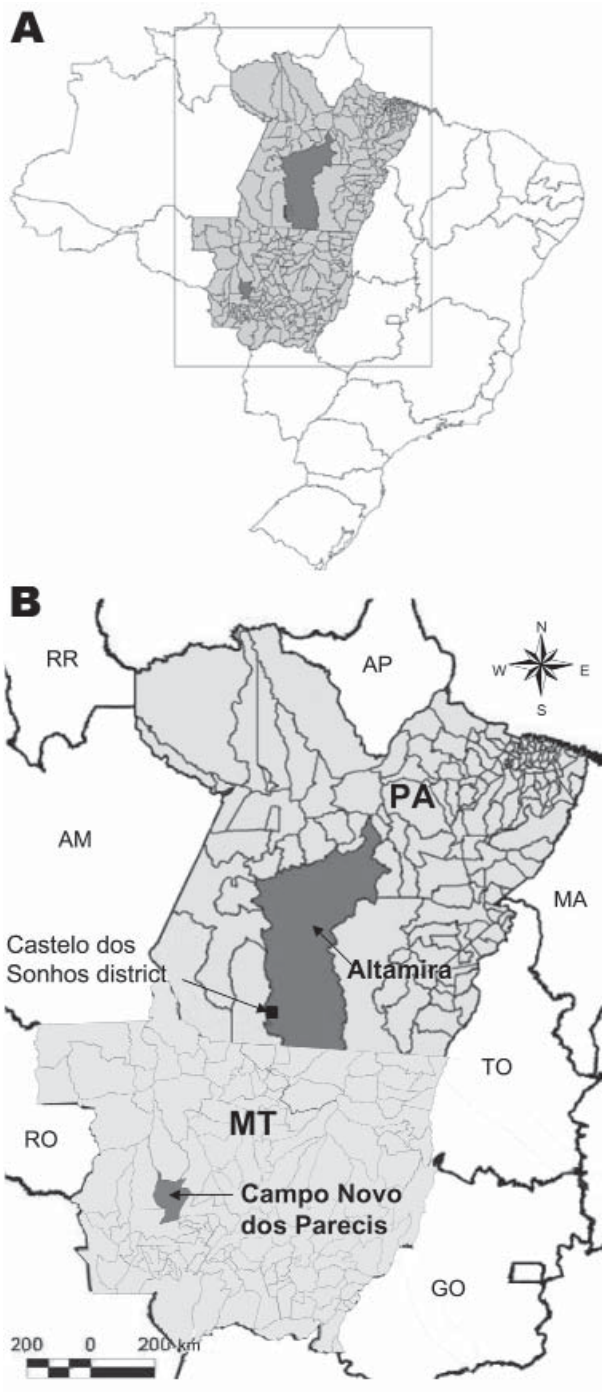

Figure 1. Location of Mato Grosso State, Brazil, showing the municipality of Campo Novo do Parecis where pygmy rice rats (*Oligoryzomys utiaritensis*) were found infected with Castelo dos Sonhos virus and the Castelo dos Sonhos district in the municipality of Altamira, Pará State, both locations where hantavirus pulmonary syndrome cases caused by Castelo dos Sonhos virus have been frequently found. MT, Mato Grosso State; PA, Pará State; AP, Amapá State; AM, Amazonas State; MA, Maranhão State; TO, Tocantins State; RO, Rondônia State; GO, Goiás State. Source: Laboratório de Geoprocessamento do Instituto Evandro Chagas, Secretaria de Vigilância em Saúde do Ministério da Saúde.

were aligned with other hantavirus sequences available in GenBank (www.ncbi.nlm.nih.gov) with ClustalW software (www.clustal.org) in BioEdit 5.0 (www.mbio.ncsu.edu/BioEdit/biodoc.pdf). The maximum-likelihood and Bayesian methods were implemented in PHYML and MRBAYES version 3, respectively, and used for phylogenetic reconstructions (*10*,*11*). Modeltest version 3.7 was used to determine the best nucleotide substitution model (*12*).

Four of 89 pygmy rice rat samples tested were IgG positive (AN711258, AN717313, AN717307, and AN729965) (Table 1). Partial N gene nucleotide sequences (≈400 nt; nucleotide position from 30–450 related to the CASV N gene sequence; GenBank accession no. AF307324) were obtained from lung fragments of the 4 IgG-positive rodents. Obtained sequences showed high nucleotide and amino acid homology (92.4% and 100%, respectively) with other CASV sequences in GenBank. The rodent-related strains showed 0.6% nt divergence among them, 2.1% nt sequence divergence with HPS-related strains, and 7.6% nt sequence divergence with the CASV prototype strain. These results were also confirmed by phylogenetic analysis that grouped the studied strains based on the N gene partial sequence analysis together with CASV prototype strain and local strains recovered from patients with HPS (Table 2) in the area (Figure 2, panel B).

After its isolation in 1995 from 1 patient with HPS in the Castelo dos Sonhos district of Altamira in southeast Pará State, CASV has only occasionally been detected in Pará and neighboring Mato Grosso states in Brazil's Amazon region. However, a recent study performed among residents of 4 municipalities along interstate highway BR-163, which runs between southeastern Pará and northern Mato Grosso, has suggested continuous CASV circulation with occurrence of small outbreaks, sporadic HPS cases, and silent infections (*13*). In fact, between 1995 and 2010, a total of 72 HPS cases in southeastern Pará State were reported to the Brazilian Ministry of Heath. Although eco-epidemiologic studies were conducted as part of case investigations, none of the captured rodent species were found to be reservoirs for CASV on the basis of molecular tests used to detect the hantavirus genome.

Among Brazilian states, Mato Grosso has the fourth highest number of reported HPS cases. Cases have been associated with agricultural activities, mainly cultivation of soybean and grains, and occasionally corn and other beans. Previous studies in Mato Grosso have demonstrated the circulation of the Laguna Negra virus in the region as associated with rodents belonging to the genus *Calomys* (*14*,*15*). However, the complexity of clinical outcomes observed for patients, as well as the high case-fatality rate reported in the state of Mato Grosso and Brazilian Amazon (43.3%), were not similar to data associated with

Laguna Negra infections in Paraguay (*3*), which suggests that 2 different hantaviruses related to HPS cases are co-circulating in this state.

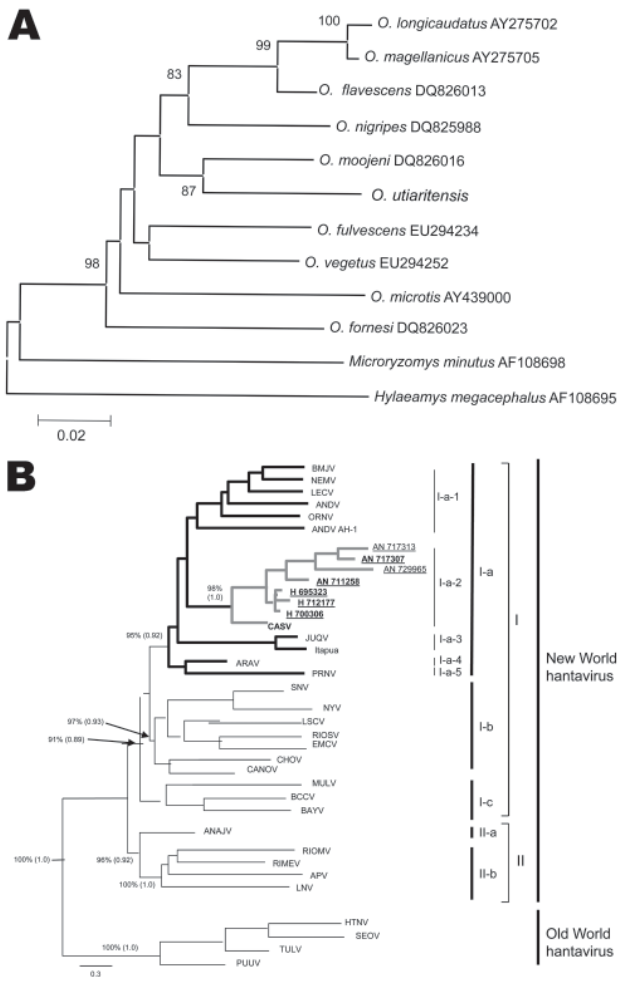

Figure 2. A) Phylogenetic tree constructed by using the neighbor-joining method for characterization of pygmy rice rats by using the cytochrome *b* DNA. Scale bar indicates nucleotide sequence divergence among the rodent species sequences. B) Phylogenetic tree using the maximum-likelihood (ML) and Bayesian methods based on partial nucleotide sequences of N gene obtained from pygmy rice rats captured in Campo Novo do Parecis, Mato Grosso State, Brazilian Amazon (New World major group, Group I, Clade Ia, Subclade Ia2, underlined strains and branches highlighted in **boldface**). Numbers at right indicate (left to right) subclades, clades, groups, and major groups. Green branch corresponds to Castelo dos Sonhos virus, including prototype strain, samples from pygmy rice rats, and hantavirus pulmonary syndrome–associated strains. Values over each main node placed outside and inside parentheses correspond to ML (bootstrap values) and Bayesian posterior probabilities (BPP), respectively. The arrows indicate the exact position for the bootstrap and BPP values over the main branch nodes. Scale bar indicates nucleotide sequence divergence among the hantavirus sequences. Complete definitions and GenBank accession numbers are available online (www.cdc.gov/EID/17/8/101547-F2.htm).

Table 1. Pygmy rice rat (*Oligoryzomys utiaritensis*) samples from which Castelo dos Sonhos virus genome was amplified, sequenced, and phylogenetically analyzed, Brazil*

IEC no.	SVS no.	MN no.	GenBank accession no.
AN 711258	328	MN 74939	HQ719472
AN 717307	33	MN 75063	HQ719471
AN 717313	39	MN 74965	HQ719470
AN 729965	183	MN 75064	HQ719469

*IEC, Instituto Evandro Chagas; SVS, Secretaria de Vigilância em Saúde; MN, Museu Nacional.

Table 2. Human samples from which Castelo dos Sonhos virus genome was amplified, sequenced, and phylogenetically analyzed, Brazil*

IEC number	Patient age, y/sex	Place of infection†	GenBank accession no.
H 695323	24/M	Castelo dos Sonhos	HQ719468
H 700306	30/M	Castelo dos Sonhos	HQ719466
H 712177	36/M	Cachoeira da Serra	HQ719467

*IEC, Instituto Evandro Chagas.
†All located in Pará State, Brazil.

Conclusions

The study of dynamics of rodent populations during the period identified the rodent population that naturally occurs in the rural zone of the municipality of Campo Novo do Parecis in Mato Grosso. Lung samples from 4 pygmy rice rats showed anti-hantavirus antibodies, yielding hantavirus RNA amplification. Sequencing and phylogenetic analysis indicated those rodents were infected by CASV, suggesting this species of pygmy rice rat as a potential host of CASV. Furthermore, this rodent species was previously unknown in the Amazon region. Pygmy rice rats may be a reservoir for CASV, and this hantavirus may be responsible for HPS cases in Campo Novo do Parecis and in neighboring municipalities located in the midwestern region of Mato Grosso state in central Brazil, near the border with Pará state, and also in the Castelo dos Sonhos district of Pará state.

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West Nile Virus Infection in Killer Whale, Texas, USA, 2007

Judy St. Leger, Guang Wu, Mark Anderson, Les Dalton, Erika Nilson, and David Wang

In 2007, nonsuppurative encephalitis was identified in a killer whale at a Texas, USA, marine park. Panviral DNA microarray of brain tissue suggested West Nile virus (WNV); WNV was confirmed by reverse transcription PCR and sequencing. Immunohistochemistry demonstrated WNV antigen within neurons. WNV should be considered in cases of encephalitis in cetaceans.

West Nile virus (WNV) is a single-stranded RNA virus of the genus *Flavivirus* that is transmitted by mosquitoes. In humans and animals, WNV has been associated with a spectrum of clinical conditions from asymptomatic infections to sudden death. These have been identified in a variety of animal species. Among marine mammals, WNV infection has been reported in a harbor seal (*Phoca vitulina*) (*1*). We describe WNV infection in a killer whale (*Orcinus orca*) and seroprevalence in conspecific cohort and noncohort groups.

The Study

In 2007, a 14-year-old male killer whale at a marine park in San Antonio, Texas, USA, died suddenly without notable premonitory signs. On gross examination, mild multifocal meningeal hyperemia and petechial parenchymal hemorrhage were noted in the right cerebrum and cerebellum. The left hemisphere of the brain appeared normal. Focally extensive tan discoloration and fibrosis were present in the right accessory lung lobe with associated hemorrhage and congestion. Both lung lobes were mildly and diffusely heavy and wet. All thoracic and abdominal lymph nodes were moderately enlarged and edematous. The second gastric chamber displayed numerous chronic and active ulcerations of 1.5–2 cm. Fresh and buffered 10% formalin-fixed specimens were collected. Fresh tissues were stored at –80°C. Tissues fixed in 10% buffered formalin were processed routinely and stained with hematoxylin and eosin for histologic examination.

Histologic review demonstrated moderate multifocal subacute vasculitis and nonsuppurative encephalitis. Inflammatory lesions of the central nervous system were focused in gray matter of the medulla oblongata, pons, mesencephalan, and cerebellum. Lesions were bilateral but more severe on the right side. Meninges demonstrated moderate focally extensive and multifocal areas of acute meningeal congestion and hemorrhage. Mild multifocal lymphocytic infiltrates expanded the leptomeninges. Blood vessels demonstrated mild to moderate acute necrosis and lymphocytic and contained plasmacytic and neutrophilic infiltrates within vascular walls. Encephalitis was characterized by perivascular lymphocytes and fewer plasma cells expanding the Virchow-Robbins spaces. Small, scattered, perivascular ring hemorrhages were noted. A few multifocal loosely arranged glial nodules were within cerebral white matter.

Predominant lesions in the lungs were areas of chronic and active abscessation amid a focally extensive area of mixed inflammation and fibrosis. There was moderate diffuse acute pulmonary edema and congestion. Gastric ulcerations were present in the first gastric chamber and were chronic and active. They were characterized by central ulcerations with necrosis and a mixed inflammatory infiltrate surrounded by variable fibrosis and a rim of epithelial hyperplasia. Changes in spleen, lymph node, and kidney included acute edema, congestion, and vascular dilation.

Conventional diagnostic assays were performed for aerobic, anaerobic, and fungal microbes in liver, lung, kidney, cerebrospinal fluid, and brain. All yielded minimal growth of *Escherichia coli*.

The final diagnosis was fulminant peracute bacteremia and septicemia secondary to a primary viral infection associated with nonsuppurative encephalitis. Published etiologic considerations for cetacean nonsuppurative encephalitis include morbillivirus and protozoal infections (*2*). A DNA microarray with highly conserved sequences from >1,000 viruses was selected to screen for known and novel viruses (*3*). Total RNA was extracted from brain tissue and hybridized to a microarray as described (*4*). Analysis of the resulting hybridization pattern demonstrated a strong hybridization signal to many oligonucleotide probes on the microarray from the family *Flaviviridae*, in particular to WNV. Consensus reverse transcription PCR primers (*5*) targeting WNV were used to confirm the microarray results. Sequencing of the 261-bp amplicon (GenBank accession no. HQ610502) yielded a sequence with 99% nt identity and 100% aa acid identity to WNV strain OK03 (GenBank accession no. EU155484.1), a strain originally identified in Oklahoma, USA.

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To further support a WNV diagnosis, we performed immunohistochemical staining on brain tissue. The immunoperoxidase stain used was a commercial rabbit polyclonal antibody (BioRelience Corp., Rockville, MD, USA) with peroxidase-tagged goat antirabbit immunoglobulin G (DakoCytomation, Carpinteria, CA, USA) bridge and 3-amino-9-ethylcarbazole (DakoCytomation) as the chromogen. This staining demonstrated abundant WNV antigen within the cytoplasm of a small number of neurons and glial cells and in fewer macrophages in the brain tissue (Figure).

We evaluated WNV exposure within the same cohort, as well as a geographically distant cohort of whales by using serologic testing. All testing was performed at the same laboratory by using a standard plaque-reduction neutralization test. In this assay, a 90% neutralization cutoff was used (*6*). A 90% plaque-reduction titer >10 was considered positive. Serum from the affected whale and 5 cohort killer whales from the same marine park in San Antonio as well as 5 whales housed at another facility in Orlando, Florida, USA, were evaluated. In each facility, the animals have regular contact with each other. The facilities are geographically separated so the animals do not have exposure to those in the other park. All 6 animals from Texas had 90% plaque-reduction titers >10, ranging from 40 to 80. The 5 whales housed together in Orlando had no measurable titer.

Conclusions

We demonstrate that WNV can infect and cause disease in killer whales. These findings broaden the known host tropism of WNV to include cetaceans in addition to previously known pinnipeds. Although we cannot definitively attribute the cause of death of this whale to WNV, the observed lesions are consistent with those caused by WNV in other animals. The serologic results demonstrate that subclinical infections can occur and that exposure can be variable. We did not determine specific dates of exposure for these populations. Both Bexar County, Texas, and Orange County, Florida, have had WNV in wildlife since 2002. We continue annual serology on previously negative animals to document seroconversion. Mosquito management practices are similar in both facilities and have been expanded since this diagnosis. Differences in WNV prevalence or mosquito numbers may have played a role in the different serologic results.

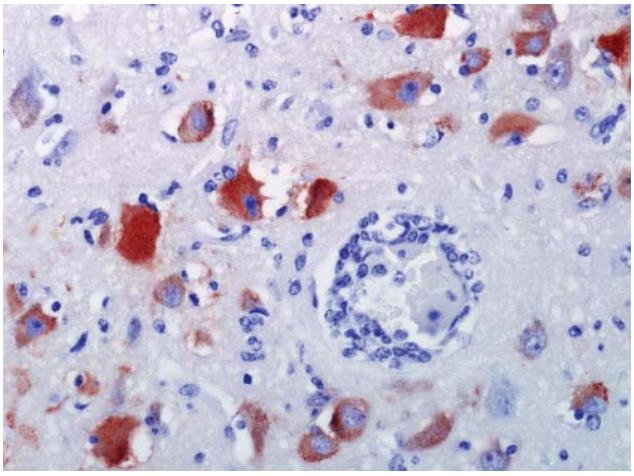

Figure. Brain specimen from killer whale (*Orcinus orca*) with West Nile virus infection that died at a marine park, San Antonio, Texas, USA, 2007. Neurons and glial cells demonstrate abundant intracytoplasmic West Nile viras antigen. Blood vessel demonstrates mild vasculitis and perivascular lymphocytic infiltrate. Original magnification ×200.

Health evaluations of free-ranging and captive cetaceans should include WNV serology to assess exposure rates. This report focuses on killer whales, but the "loafing" behavior (stationary positioning at the water's surface) is commonly seen in many coastal dolphins, thereby increasing the likelihood of mosquito bites and exposure to WNV. Serologic screening of bottlenose dolphins (*Tursiops truncatus*) from the Indian River Lagoon demonstrated WNV titers (*7*). WNV-associated disease in these animals has not been reported. Active screening for WNV may enhance diagnostic investigations.

As with many species of birds and mammals, WNV infection carries a risk for zoonotic transmission. Until the implications of this infection in marine mammals are better understood, biologists and veterinarians working with cetaceans should consider this possibility. Potential viral shedding can occur through the oropharygeal cavity and feces as well as through blood and organs during necropsies.

Finally, our study demonstrates the broad applicability of using panviral microarray-based diagnostics. Even though PCR diagnostics are well developed for WNV, the agent was not initially considered as a potential pathogen in this species. Panviral microarray can be used not only to identify novel viruses but also to detect unsuspected agents.

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Dr St. Leger is the director of pathology and research for SeaWorld Parks and Entertainment, serves on the editorial board of the journal Veterinary Pathology, and is a board member of the CL Davis Foundation. Her primary research interests are comparative pathology and infectious disease investigations of marine and aquatic animals.

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Fatal Neurologic Disease and Abortion in Mare Infected with Lineage 1 West Nile Virus, South Africa

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In 2010, lineage 1 West Nile virus was detected in South Africa in the brain of a pregnant mare that succumbed to neurologic disease and in her aborted fetus, suggesting an association with abortion in horses. All West Nile virus strains previously detected in horses and humans in South Africa were lineage 2.

West Nile virus (WNV), a mosquito-borne flavivirus, may cause outbreaks of febrile disease and encephalitis in humans and horses. Although <1% of human patients experience severe disease (*1*), up to 90% of symptomatic cases in horses result in neurologic disease with case-fatality rates of 30%–40% (*2*). In sheep, WNV infection may result in abortion, stillbirth, and neonatal death (*3*). In humans, transmission by transplacental route and breastfeeding has been described. Congenital WNV infection has been accompanied by bilateral chorioretinitis and severe malformation of the fetal central nervous system (*4*). We report a case of WNV with fatal neurologic disease and abortion in a horse.

Five genetic lineages of WNV exist, the major 2 being lineages 1 and 2 (*5*,*6*). Lineage 1 is distributed widely in North and South America, Europe, parts of Asia, North Africa, and Australia. Lineage 2 strains have been identified in humans and horses with febrile and neurologic disease in southern Africa and Madagascar (*7*) and recently emerged in central Europe causing encephalitis in birds, humans, and horses (*7*,*8*). WNV has become recognized as an important horse pathogen in South Africa with all cases positive by nucleic acid detection or virus isolation belonging to lineage 2 (*6*,*7*,).

Bird deaths due to WNV are rare in South Africa, probably because of the long-term endemic nature of the virus, which limits their use in sentinel surveillance (*9*). A positive correlation exists between occurrence of symptomatic equine and human cases, which suggests equine outbreaks might predict disease risk for humans (*3*).

The Study

Over the past 3 years, the zoonosis group, Department Medical Virology, University of Pretoria, has investigated horses as sentinels to detect WNV activity in South Africa. The study was approved by the University of Pretoria ethical committee.

Horses are economically important in South Africa, and diagnoses for animals with severe or fatal disease are frequently requested because of the presence of African horse sickness virus in the country, which is a notifiable disease (www.nda.agric.za/vetweb). As part of a WNV surveillance program, in an attempt to determine the contribution of WNV to neurologic infections in animals we invited veterinarians throughout the country to report cases of neurologic disease in horses for free diagnosis. Findings of the first year of this study were published in 2009 (*10*). WNV was identified for up to 21% of undiagnosed neurologic cases in horses. All cases detected by real-time reverse transcription PCR (RT-PCR) belonged to lineage 2, and 70% of cases were fatal or resulted in the horse being euthanized (*10*).

On May 24, 2010, a 7.5-month pregnant, 8-year-old thoroughbred mare was found recumbent on a farm outside Ceres in the Western Cape. Temperature was within normal limits, mucous membranes indicated mild toxicity, and tongue tone was normal with mild fasciculations. She was treated with intravenous fluids, dimethyl sulfoxide, and cortisone. When rolled over, she got up but had severe hindquarter incoordination and went down again after 1 minute. She was treated with penicillin twice a day, phenylbutazone, and vitamin B1 over the next 3 days. She rose a few times but went down again and was not able to get up on days 4 or 5. She aborted on day 6 and died on day 7. The brain from the fetus was sampled after abortion, and the brain from the mare was examined postmortem; samples were submitted to the WNV surveillance project. Half of the mare's brain was sent in formalin to the University of Pretoria, Onderstepoort, Faculty of Veterinary Sciences for histopathologic examination.

Viral RNA was extracted from 30-mg brain sections with the QIAGEN RNeasy Plus Mini Kit (QIAGEN, Valencia, CA, USA). WNV nonstructural protein 5–specific nested real-time RT-PCR that distinguishes lineages 1 and 2 by hybridization probe-melting curve analysis (*11*)

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DOI: 10.3201/eid1708.101794

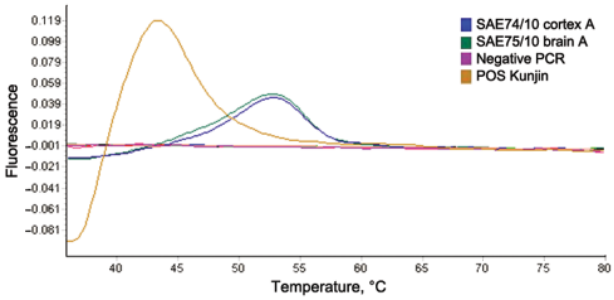

Figure 1. Dissociation curve analysis of the diagnostic nested real-time reverse transcription PCR of West Nile virus isolated from a mare and fetus with fatal neurologic disease, South Africa, 2010. Positive control (Kunjin L1b); SAE74/10 (fetus); SAE75/10 (mare). Expected melting peak of lineage 2 = lineage 1b+6°C; lineage 1a = lineage 1b+10°C.

detected WNV lineage 1 in the brains of the mare and foal (Figure 1) and confirmed by sequencing (results not shown, genome positions 9091–9191). A larger region of the NS5 gene (788 bp) of the mare could be amplified by a nested RT-PCR (*12*) as well as the E gene (*7*). Maximum-likelihood analysis clustered these sequences with lineage 1 strains from North Africa and Europe (Figure 2). Strain PAH001 from Tunisia isolated in 1997 from a person who died of neurologic disease (*13*) was most closely related (p-distance 2.2%; MEGA4, www.megasoftware.net) followed by strains from Russia (p-distance 2.7%–2.9%). Lineage 2 WNV strains were 17.8%–19.4% different from this strain, SAE75/10. E-protein analysis also grouped SAE75/10 with isolates from Russia (Ast02–2-692, Ast02–2-25) and Tunisia (PAH001) with p-distances of 2.3% and 2.7%, respectively. Lineage 2 WNV strains differed by 23.6%–23.9%.

Light microscopic examination of hematoxylin and eosin–stained formalin-fixed wax-embedded cerebellum and cerebrum sections of the mare showed mild to moderate nonsuppurative meningoencephalitis, with olfactory lobe and cortex showing the most pronounced lesions. Lesions included mononuclear perivascular cuffing, glial flares in the cerebellar molecular region, mulitfocal glial nodules, diffuse white matter gliosis, vascular congestion, and macroscopically visible intermittent vascular distention with blood, especially in the thalamus. Occasional Purkinje neurons had nuclear chromatolysis. Unfortunately, brain and other tissues of the aborted foal were not formalin fixed.

Continued surveillance over the past 3 years identified lineage 2 strains as the causative agent in all other acute WNV cases positive by RT-PCR and will be reported in more detail elsewhere. Routine use of real-time nested RT-PCR that distinguish lineage 1 and 2 by hybridization probe analysis (*12*) resulted in rapid detection of a lineage 1 strain as an emerging pathogen in the country. This technique is especially useful in surveillance studies where routine virus isolation is not attempted due to low viremia and suboptimum specimens from horses such as this.

This strain appears to have been associated with abortion of the fetus and death of the mare. To our knowledge, the only published report identified of natural

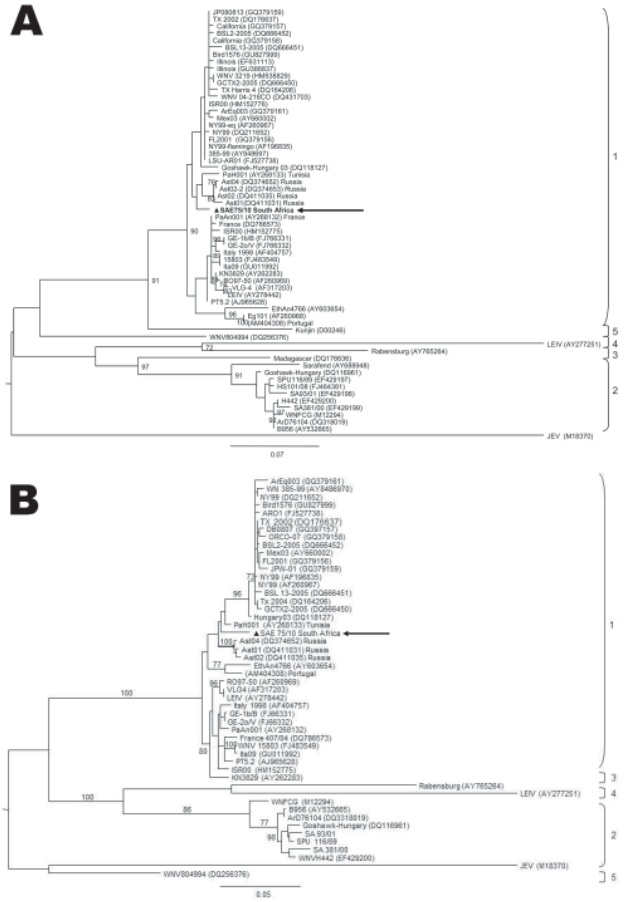

Figure 2. Maximum-likelihood comparison of a partial section of the E-protein gene (A) and NS5 gene (B) of West Nile virus (WNV) lineage 2 strains isolated in South Africa (SA) in 2010 from a mare with fatal neurologic disease and representative sequences of other WNV lineages from various regions of the world. The lineage 1 strain, SAE75/10, identified in South Africa is indicated by a triangle and arrow. Sequences were aligned with MAFFT version 6 (http://align.bmr.kyushu-u.ac.jp/mafft/software) and edited with BioEdit version 7.0.9.0 (www.mbio.ncsu.edu/bioedit). Maximum-likelihood midpoint rooted trees were drawn by using PHYML (www.atgc-montpellier.fr/phyml) under 100 bootstrap repetitions and the HKY codon position substitution model and drawn to scale with the bars indicating 0.07 nt substitutions. Only bootstrap values >70 are shown. Reference strains used from GenBank that were most closely related to SA L1: PAH001, Tunisia (AY268133); Ast04-2-824A, Russia (DQ374652); Ast02-2-25, Russia (DQ374653); Ast02-2-692, Russia (DQ411031); Ast01-187, Russia (DQ411035); Ge1b/B, Spain (FJ766331); GE-2o/V, Spain (FJ766332); WNV Italy-1998-equine, Italy (AF404757); WNV15803, Italy (FJ483549); and Ita09, Italy (GU011992).

WNV infection of pregnant mares was of 8 lineage 1 WNV–infected horses with neurologic signs from New York and New Jersey, USA, of which 3 were pregnant but none aborted (*14*). As in the case we report, 7/8 horses received dimethyl sulfoxide, flunixin meglumine, and phenylbutazone intravenously, and 2 dexamethasone intravenously for 1–3 days. Corticosteroids are regarded as ineffective in the induction of abortion in mares unless large repeated doses are used (*15*).

Conclusions

This report of a lineage 1 WNV strain emerging in South Africa confirms the sensitivity of horses as sentinels for detecting new strains and WNV activity in WNV-endemic countries. Continued surveillance will determine if lineage 1 is sustained in this region. In addition, transplacental transmission of WNV in the horse suggests a risk for abortion in pregnant mares with severe neurologic WNV disease.

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Circulating Coxsackievirus A16 Identified as Recombinant Type A Human Enterovirus, China

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To determine the relationship of coxsackievirus A16 (CA16) to prototype CA16-G10, we conducted a phylogenetic analysis of circulating CA16 strains in China. Complex recombinant forms of CA16-related viruses involving multiple human enteroviruses, subgroup A (CA4, CA16, and enterovirus 71), are prevalent among patients with hand, foot, and mouth disease.

Coxsackievirus A16 (CA16) is a member of the family *Picornaviridae*, genus *Human enterovirus* (HEV). These viruses can be further divided into 4 subgroups on the basis of molecular typing: HEV-A, HEV-B, HEV-C, and HEV-D. The first, and prototype, CA16 strain, CA16-G10, was isolated in South Africa almost 60 years ago (*1*) and was subsequently sequenced in 1994 (*2*). CA16, along with enterovirus71 (EV71), CA2, and CA4, is a member of the HEV-A subgroup. CA16 is commonly associated with hand, foot, and mouth disease (HFMD) in children and sometimes causes aseptic meningitis, encephalitis, myocarditis, and poliomyelitis-like paralysis (*3*).

Enteroviruses related to HFMD have been endemic to Southeast Asia and the Pacific region for decades (*4–7*). Recently, a dramatic increase in HFMD prevalence has been reported in the People's Republic of China (*8–10*). Partial viral sequencing (e.g., of the viral protein [VP] 1 region), serologic characterization, or both, have shown that 10%–50% of viruses from HFMD patients are related to prototype CA16-G10, and thus they have been classified as CA16 strains (*11*). The relationship of circulating CA16-related viruses to CA16-G10 has not been well studied. Therefore, we conducted a serial phylogenetic analysis of existing and new CA16 sequences from northern, central, and southern China to examine whether CA16–G10 truly is the parental strain of circulating CA16 strains. As a result, we found that current CA16 strains in China, although still related to CA16–G10, are recombinant HEV-A.

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The Study

Twenty-four enterovirus sequences, mainly from HEV-A group were retrieved from the National Center for Biotechnology Information website. Phylogenetic analysis, performed with the MEGA4 program (*12*), indicated that CA16 strains shzh00-1, shzh05-1, and GZ08 did not cluster with CA16-G10 (Figure 1, panel A), although they indeed belonged to HEV-A and clustered with EV71A (BrCr), EV71B (EV71/9/97/SHA89), and EV71C (S10862-SAR-98) (Table). Thus, the so-called CA16 full-length sequences (shzh00-1, shzh05-1, and GZ08) from China are distinct from CA16-G10 full-length sequences.

Because the shzh00-1, shzh05-1, and GZ08 sequences clustered within the HEV-A group and were determined to be CA16 on the basis of the VP1 region (data not shown), we examined these 3 sequences for evidence of recombination. Shzh00-1 was chosen as representative because it was isolated earlier. It has 7,410 nt in its genome, including the 5′ untranslated region (UTR) (1–745), structural protein (746–3331), and nonstructural proteins P2 (3332–5065) and P3 (5066–7327), with the rest of its genome as 3′ UTR (7328–7410). Bootscanning with a sliding window of 500 nt, overlapping by 20 nt, was performed with the SimPlot program (version 3.5.1) (*14*) to investigate the possibility of recombination within the shzh00-1 sequence. Various HEV-A sequences were used as reference sequences. The results indicated that the 5′ UTR of the shzh00-1 sequence had relatively high similarity to CA4 (Figure 1, panel B). The P1 (VP4, VP2, VP3, and VP1) region was more similar to that of CA16-G10. However, part of the P2/P3 region of the shzh00-1 sequence (2C, 3A, 3B, and 3C) had relatively high similarity to EV71A but not to CA16-G10 (Figure 1, panel B). Similar results were obtained for the shzh05-1 and GZ08 sequences (data not shown). Thus, shzh00-1, shzh05-1, and GZ08 are recombinant type A HEVs that contain CA4, CA16, and EV71.

To examine the circulation status of recombinant CA16 in China, we characterized 24 additional CA16-related sequences from HFMD patients from central (Hangzhou, Zhejiang Province) and northeastern (Changchun, Jilin Province) China (Table). The break point around position 3555 interested us most because it not only roughly separated the P1 region from P2/P3 but also divided the open reading frame (746–7327) into CA16-like and non–CA16-like fragments. Thus, we selected this region spanning the recombination break point between the CA16 sequence and the downstream sequence.

Bootscanning of these new sequences showed that the sequences from Changchun and Hangzhou have a

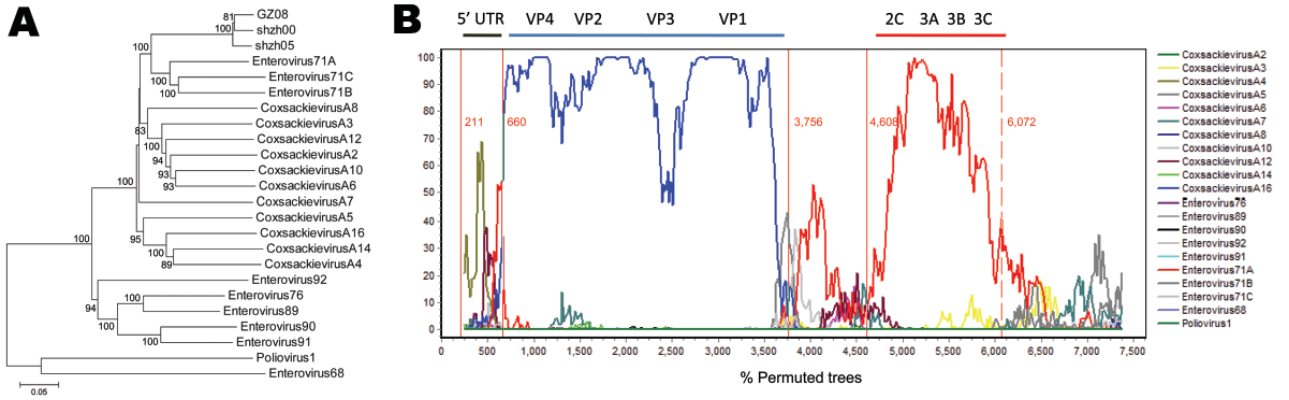

Figure 1. Classification of coxsackievirus 16A (CA16) sequences from the People's Republic of China into human enterovirus (HEV) groups. A) Phylogenetic analysis performed by using all HEV reference samples from China classified as HEV-A but not as CA16. All sequences were retrieved from GenBank. The sequences used corresponded to positions 2–7,407 bp in shzh00-1. MEGA4 software (*12*) was used as the analytic program and Kimura 2-parameter as the model. The phylogenetic tree was determined for 1,000 replicates with random seeds. Only strong bootstrap values (>70%) are shown. Scale bar shows nucleotide substitutions per site. B) Bootscanning analysis of shzh00-1. For all HEV-A sequences, together with sequences from 2 outgroups, shzh00-1 showed possible recombination with CA4 and enterovirus 71A. The vertical red lines with numbers show the possible recombination break points as determined by genetic algorithm recombination detection (*13*). The sequences used corresponded to positions 2–7,407 in shzh00-1. Bootscanning was performed with a window size of 500 nt and step of 20 nt. Because of gaps in alignment, break points 211, 660, 3,756, 4,608, and 6,072 correspond to positions 207, 647, 3,555, 4,406, and 5,854 in shzh00-1, respectively. UTR, untranslated region; VP, viral protein.

recombination pattern similar to that of shzh00–1 (Figure 2, panel A; data not shown). Most Changchun and Hangzhou sequences changed from a CA16-like fragment into a non–CA16-like fragment at the break point around position 3555. However, when shzh00-1 was used as the reference sequence, bootscanning of these Changchun and Hangzhou sequences showed a high similarity to shzh00-1 through the entire sequenced region (data not shown). Phylogenetic

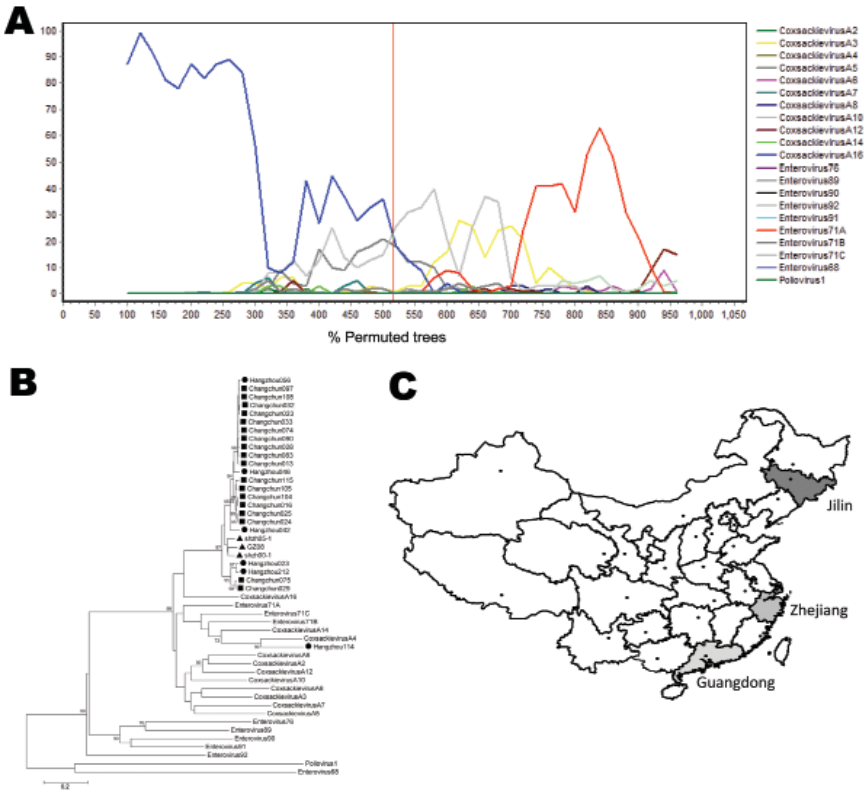

Figure 2. Phylogenetic analysis of Changchun and Hangzhou coxsackievirus 16A (CA16) sequences. A) Bootscanning results representing selected Changchun and Hangzhou sequences. changchun104 was shown for bootscanning analysis with human enterovirus A (HEV-A) sequences and shzh00-1 as references. The results suggested changchun104 was similar to shzh00-1. The red vertical line indicates position 3,555, which corresponded to shzh00-1. B) hangzhou212, hangzhou023, and all Changchun sequences clustered with shzh00-1, shzh05-1, and GZ08, with a very high bootstrap value (100%). Another Hangzhou sequence, hangzhou114, was most likely related to CA4. ● indicates Hangzhou sequences; ■ indicates Changchun sequences, and ▲ indicates shzh00-1, shzh05-1, and GZ08. Scale bar indicates nucleotide substitutions per site. C) Map of the People's Republic of China indicating the provinces where shzh00-1–like CA16 sequences were characterized.

Table. Origin of sequences used for phylogenetic analysis of coxsackievirus strains, China, 2010*

Strain	Type	Sample date	Country	City or state	GenBank accession no.
G-10	CA16	1951	South Africa		U05876
High point	CA4	1948	United States	North Carolina	AY421762
BrCr	EV71A	1970	United States	California	U22521
UH1/PM/1997	EV71B	1997	Malaysia		AM396587
S10862-SAR-98	EV71C	1998	Malaysia		DQ341359
shzh00-1	CA16	2000	China	Shenzhen	AY790926
shzh05-1	CA16	2005	China	Shenzhen	EU262658
GZ08	CA16	2008 Jun	China	Guangzhou	FJ198212
changchun013	CA16	2010 Jun	China	Changchun	HQ450582
changchun016	CA16	2010 Jun	China	Changchun	HQ450581
changchun023	CA16	2010 Jun	China	Changchun	HQ450580
changchun024	CA16	2010 Jun	China	Changchun	HQ450579
changchun025	CA16	2010 Jun	China	Changchun	HQ450578
changchun028	CA16	2010 Jun	China	Changchun	HQ450577
changchun029	CA16	2010 Jun	China	Changchun	HQ450576
changchun032	CA16	2010 Jun	China	Changchun	HQ450575
changchun033	CA16	2010 Jun	China	Changchun	HQ450574
changchun074	CA16	2010 Jun	China	Changchun	HQ450573
changchun075	CA16	2010 Jun	China	Changchun	HQ450572
changchun083	CA16	2010 Jun	China	Changchun	HQ450571
changchun090	CA16	2010 Jun	China	Changchun	HQ450570
changchun097	CA16	2010 Jun	China	Changchun	HQ450569
changchun104	CA16	2010 Jun	China	Changchun	HQ450568
changchun105	CA16	2010 Jun	China	Changchun	HQ450567
changchun108	CA16	2010 Jun	China	Changchun	HQ450566
changchun115	CA16	2010 Jun	China	Changchun	HQ450565
hangzhou023	CA16	2010 Jun	China	Hangzhou	HQ450561
hangzhou042	CA16	2010 Jun	China	Hangzhou	HQ450563
hangzhou046	CA16	2010 Jun	China	Hangzhou	HQ450560
hangzhou056	CA16	2010 Jun	China	Hangzhou	HQ450559
hangzhou114	CA4	2010 Jun	China	Hangzhou	HQ450564
hangzhou212	CA16	2010 Jun	China	Hangzhou	HQ450562

*CA, coxsackievirus A; EV, enterovirus.

analysis indicated that 23 of the Changchun and Hangzhou sequences formed a strong cluster with shzh00-1, shzh05-1, and GZ08 (Figure 2, panel B). Notably, 1 sequence clustered with the CA4 reference sequence, indicating that CA4-related viruses are also circulating among HFMD patients in China. These data suggest that shzh00-1 and these Changchun and Hangzhou sequences are likely derived from a common ancestor. New sequences identified in this study have been submitted to GenBank under accession nos. HQ450559–HQ450582.

Conclusions

As many as 40% of cases of HFMD in China have been attributed to CA16 infection on the basis of partial viral genome determination (*11*). In the current study, we demonstrated that circulating CA16 viruses in China are actually complex recombinant viruses involving multiple type A HEVs, including CA4, CA16, and EV71 (Figure 1). The 5′ UTR region (207–647 bp) of these viruses had the highest similarity to CA4. Most of the P1 region resembled that of the prototype CA16-G10 strain. The nonstructural protein domains (P2 and P3) had a 1.5-kb fragment (4,406–5,854 bp) that was most similar to EV71A. Several regions of shzh00-1 remain unclassified, including part of 2A, 2B, part of 2C, and 3D.

Over the past 30 years, numerous large outbreaks of CA16-associated HFMD, along with outbreaks caused by EV71, have been reported in China. However, full molecular characterization of circulating CA16 viruses in China had not been conducted. The current study provides evidence that circulating recombinant forms of the CA16-related viruses are prevalent among HFMD patients throughout China (Figure 2, panel C). The origin, including the place and date, of the current recombinant CA16 viruses is not clear. The exact parental viral strains involved in the generation of CA16 recombinant viruses are also unknown. Some of the parental virus strains of China CA16 could have become extinct or are yet to be discovered. Notably, CA4 was detected in 1 of our samples from Zhejiang Province (central China) and has also been reported in Gansu Province (northwestern China) (*15*). The identification of CA16-related HFMD,

based mainly on VP1 sequences, has been widely reported in many parts of China, including Guangdong, Fujian, Jiangsu, and Inner Mongolia Provinces, and Beijing and Shanghai. Unfortunately, the sequences of other parts of these viral genomes, especially the regions spanning the recombination site, have not been determined. Further detailed characterization of HEV sequences from HFMD patients is still needed, but the information obtained thus far has implications for addressing the future emergence of new pathogenic HEVs and for vaccine development to manage the increasing prevalence of HFMD.

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Canine Distemper Outbreak in Rhesus Monkeys, China

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Since 2006, canine distemper outbreaks have occurred in rhesus monkeys at a breeding farm in Guangxi, People's Republic of China. Approximately 10,000 animals were infected (25%–60% disease incidence); 5%–30% of infected animals died. The epidemic was controlled by vaccination. Amino acid sequence analysis of the virus indicated a unique strain.

Canine distemper is a highly contagious infectious disease of canine and feline species caused by canine distemper virus (CDV), a member of family *Paramyxoviridae* (*1*). Susceptible animals include dogs, wolves, jackals, foxes, mongooses, badgers, raccoon dogs, skunks, minks, and ferrets (*2*–*6*). Case-fatality rates for these animals has ranged from 30% to 80% and even to 100% of ferrets (*7*). Natural infection with CDV has occasionally been reported in bears, lesser pandas, and giant pandas (*8*–*10*). Monkeys are not generally considered susceptible but can be experimentally infected (*11*,*12*). In 1989, the first natural case of canine distemper in a monkey (*Macaca fuscata)* was reported (*13*). Recently, natural canine distemper infection was reported in a few monkeys in Beijing, People's Republic of China, with a description of the clinical signs and pathogenic changes (*14*). This outbreak most likely resulted from secondary transmission of CDV originating in a larger outbreak on a Guangxi breeding farm, where a similar disease had occurred 2–3 years earlier. Here we describe this larger outbreak and provide a more detailed epidemiologic analysis.

The Study

In 2006, an unidentified respiratory disease occurred in rhesus monkeys (*Macaca mulatta*) at a breeding farm in the Guangxi Zhuang Autonomous Region in southern China. The farm, the largest in China, comprised 31,260 monkeys, of which ≈1 in 5 was unweaned. Approximately 10,000 monkeys contracted the disease, and 4,250 died. The morbidity rate in young monkeys was 60%, with an ≈30% death rate (25% and 5%, respectively, for adults). In 2007, surviving monkeys were vaccinated with an inactivated suspension made from the livers and lungs of dead animals. After vaccination, the number of cases decreased during 2007 and 2008 to ≈100–200 per year.

Cases occurred throughout 2006. Because most authorized suppliers of monkeys to research laboratories in China obtain their breeding stock from this farm, the disease spread throughout China, particularly to experimental animal facilities in Wuhan, Kunming, and Beijing (*14*). The disease also was introduced into a few wildlife parks in China; however, perhaps because of the low population density of susceptible animals in these locations, further spread has not been reported.

Initially, CDV was not suspected as the causative agent of the monkeys' illness. In late 2008, however, tissue specimens from infected animals that had been stored in a freezer for 2 years were analyzed and found to contain CDV. Four serum samples from adult monkeys whose illnesses had naturally resolved had titers of 4–32 virus neutralizing antibodies against CDV, whereas virus neutralizing antibodies could not be detected in 3 serum samples from uninfected monkeys. After this identification, all experimental monkeys were vaccinated with attenuated CDV vaccine starting in early 2009. Whether the vaccines actually boosted immunity or whether they were simply given coincident with waning of the outbreak from increasing immunity, the number of cases has since remained low (≈130 in 2009 and 20–30 in 2010 [not all confirmed]). Additionally, anti-CDV serum seemed to help infected animals recover more rapidly from the infection.

Infected monkeys initially displayed measles-like signs, including respiratory signs; anorexia; fever; and red rashes over the entire body, with reddening and swelling of the footpads; conjunctivitis; and thick mucoid nasal discharge. Coma preceded death. Postmortem examination demonstrated discrete purple or rosy rashes on the body, with macules of 2–4 mm (Figure 1, panel A), which in some cases were confluent and had a dark rosy color. Rashes on the face were vesicular and ulcerated after suppuration, forming a scab. Other signs included congestion, redness and swelling of the pars oralis pharyngis, diffuse hemorrhagic spots on the papillae of the tongue, suppurative conjunctivitis (Figure 1, panel B), and rhinitis with copious thick mucous exudates. Blood stasis patches in the lungs and interstitial fibrosis were observed in most affected

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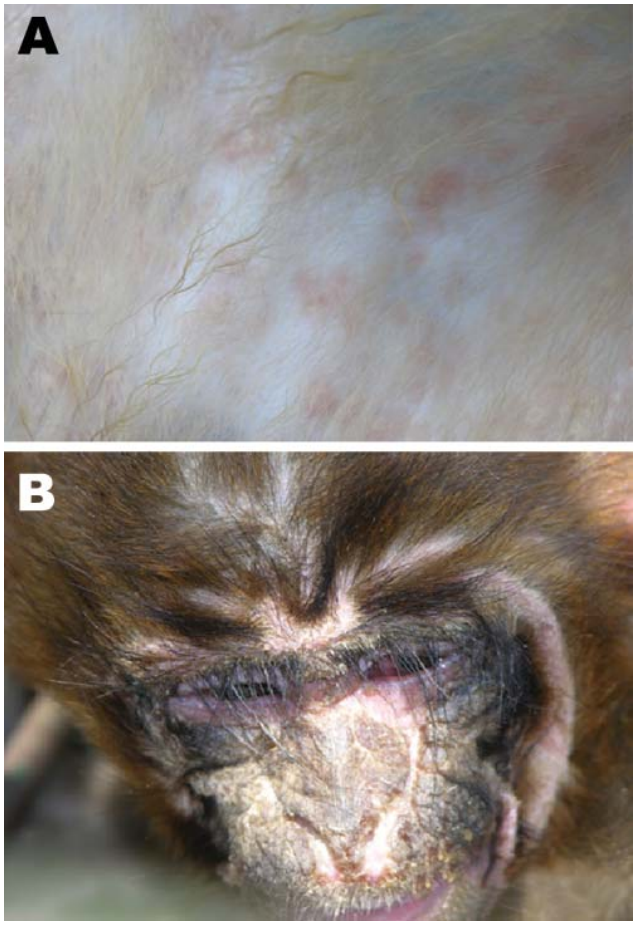

Figure 1. Canine distemper virus signs in rhesus monkeys at necropsy. A) Rash; B) suppurative conjunctivitis.

lungs. Infected monkeys also had blood stasis in parts of the liver, with tiny khaki-colored hemorrhagic spots on the surface.

Because of the signs of a measles-like infection, identification of the etiologic agent first focused on measles virus and later on CDV. Reverse transcription PCR amplification of lung specimens by using virus-specific primers was negative for measles virus but positive for CDV. The supernatant of ground liver samples also was positive for CDV by immunochromatographic analysis (BIT Rapid Color CDV cassette, Bioindist Co. Ltd., Yong-in City, Gyeonggi-do, South Korea). Lung specimens also infected tree shrews (1–2 months old) after subcutaneous injection, resulting in excitement and death from encephalitis and enterohemorrhage.

The full-length viral genome was amplified and sequenced (GenBank accession no. HM852904). We constructed phylogenetic trees by the neighbor-joining method, using the full sequence of this virus and others available in GenBank. The full genome of this isolate shared the highest homology with a ferret isolate (accession no. AY386316) from the United States, a raccoon isolate (accession no. AY649446) from the United States, and a dog isolate (accession no. AB474397) from Japan (Figure 2, panel A). The L gene showed the highest identity (95.6%–96.5%) with that of US ferret and raccoon isolates (accession nos. AY386316 and AY466011); phylogenetic analysis of the H gene indicated an eastern Asian source of this isolate, which shared high homology with isolates from different species of animals in China, Taiwan, and Japan (Figure 2, panel B). Overall, the amino acid homology of the Guangxi isolate with the others was 96.0%–97.3%, clustering it in a large clade that includes CDV isolates from Asia. Nevertheless, the isolate is unique in that it contains multiple amino acid changes in its viral structural proteins, none of which have been found in other isolates.

Reasons for the epidemic remain unclear. The first monkey to contract the infection was in the farm in Guangxi; however, the source of the infection is unknown because there were no dogs or other fur-bearing animals at the farm. Breeding facilities were self-contained, with no introduction of external animals and no other farms nearby. Food for the monkeys had no animal content except fish powder. One possible source of infection is contact by the monkeys at the farm with local wild monkeys. Another possibility is spillover of the virus from a stray dog carrying CDV that became adapted to the new host.

Large-scale breeding and caging of the monkeys might have contributed to increasing susceptibility to canine distemper infection. Reared in groups of 20–30 within fenced-off areas or in pens of several hundred, close contact between animals was inevitable. CDV appears to have been transmitted by droplets derived from feces or other body discharges, all of which contained CDV.

This canine distemper outbreak poses a threat to monkey populations. Because the Guangxi farm routinely supplies monkeys for animal facilities throughout China, monitoring for canine distemper in its monkeys, including those shipped to other animal facilities, as well as human handlers, is advisable to prevent possible secondary spread and interspecies transmission.

Conclusions

Although CDV spread has been largely controlled by inactivated and live canine distemper vaccines, sporadic cases still occur and the high number of mutations in the virus makes future transmission unpredictable. Therefore, surveillance for canine distemper should be considered among monkey populations and among humans who have close contact with them.

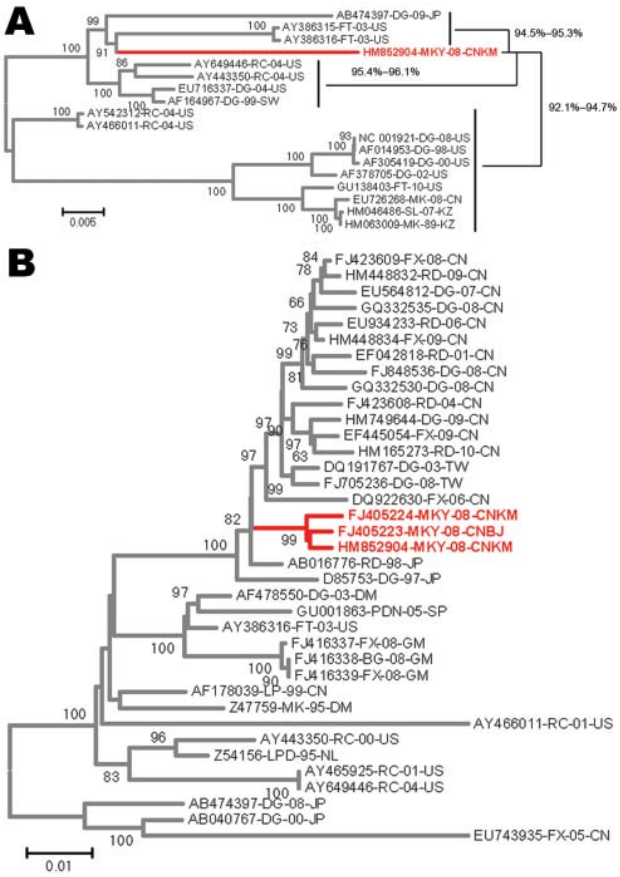

Figure 2. Phylogenetic analysis of the canine distemper virus by comparison of the genome or gene of the monkey isolate with other canine distemper virus isolates. A) Full genome. B) H gene. FX, fox; CN, People's Republic of China; RD, raccoon dog; DG, dog; TW, Taiwan; MKY, monkey; CNKM, Kunming, People's Republic of China; CNBJ, Beijing, People's Republic of China; JP, Japan; DM, Denmark; PDN, *Lynx pardinus*; SP, Spain; FT, ferret; US, United States; GM, Germany; BG, badger; LP, lesser panda; MK, mink; RC, raccoon; LPD, leopard; NL, the Netherlands. Scale bars indicate phylogenetic distance between isolates.

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Aichi Virus Shedding in High Concentrations in Patients with Acute Diarrhea

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We assessed Aichi virus shedding in patients with gastroenteritis and negative test results for other viral and bacterial infections. High concentrations of up to 1.32×10^{12} RNA copies/g stool were found in 10 (2.0%) of 499 outpatients sampled in northern Germany, 2004. These data substantiate Aichi virus pathogenicity in humans.

The family *Picornaviridae* includes 12 established genera, and representatives of 5 of these have been found in humans (*Enterovirus, Hepatovirus, Parechovirus, Cardiovirus,* and *Kobuvirus*). Among those, human pathogenicity has been proven consistently only for enteroviruses (including polioviruses), hepatitis A virus, and parechoviruses. Several as-yet-unclassified picornaviruses have been found over the past few years in humans, termed cosavirus, klassevirus, and salivirus (*1*–*3*). For gastrointestinal pathogens, data on virus quantity in stool can exclude ingestion from nutritional sources of viruses that may be detected but do not replicate in the human gut. Prevalence studies with appropriate control groups and proof of the absence of co-infections with other pathogens are required to provide evidence in favor of human pathogenicity. For most of the novel viruses, these data are still awaited.

A novel human picornavirus termed Aichi virus (AiV; genus *Kobuvirus*), was described initially in 1991 (*4*) and epidemiologically linked with spontaneous and food-associated diarrhea in humans (*5*,*6*). Recently, it was also detected in sewage-polluted water (*7*). However, no quantitative data of AiV shedding have become available so far, possibly because of technical peculiarities such as high genomic GC content (≈60%) and strong RNA secondary structures, which may have contributed to a lack of sequence information and prevented more precise molecular detection. In this study, we analyzed well-established cohorts of patients with gastroenteritis and an appropriate control group. Stool samples from patients who had negative test results for other common viruses and bacterial infections showed high AiV shedding by highly sensitive real-time reverse transcription PCR (RT-PCR), thereby substantiating AiV human pathogenicity.

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The Study

The picornavirus 5′ untranslated region (5′ UTR) has proven the most suitable genomic target for molecular detection (*8*,*9*). At the time of this study, only 5 AiV complete genomes were available in GenBank, complicating selection of reliable oligonucleotides for universal detection. A nested RT-PCR encompassing the AiV 5′ UTR was developed (Table 1) and used for screening of stool samples collected in northern Germany from outpatients with gastroenteritis. The first subcohort of this collection consisted of 499 patients with gastroenteritis; samples were collected evenly from January through December 2004 in a prospective study on acute community-acquired diarrhea by 47 general practitioners in Bremen, northern Germany (*10*). The second subcohort consisted of 39 control patients without symptoms of gastroenteritis seen by the same physicians. The third subcohort consisted of 118 patients with diarrhea linked to outbreak scenarios involving canteen food (n = 36), kindergartens (n = 54), or retirement homes (n = 28).

We purified viral RNA from stool samples by using the QIAGEN Viral RNA and DNA Stool Mini Kits (QIAGEN, Hilden, Germany) as described (*8*); 9 samples were positive for AiV by nested RT-PCR. The 5′ UTR sequences of these viruses were determined and deposited in GenBank (accession nos. GQ927704–GQ927712). With additionally available sequence data, real-time RT-PCR targeting conserved regions of the viral 5′ UTR was developed (Table 1). Assay sensitivity was determined to be ≈1.5 copies per reaction by using photometrically quantified in vitro cRNA transcripts, as described (*9*).

Retesting of all samples with this highly sensitive assay increased the AiV detection rate, yielding 10 positive samples. All case-patients were part of the subcohort of symptomatic outpatients seen by general practitioners (Figure 1). In this cohort, AiV was detected at a 2.0% rate (10/499 patients). No patients from the control group (n = 39) or from foodborne outbreaks (n = 118) had positive test results for AiV. However, this difference was not statistically significant for any group comparison (Fisher exact test, 2-tailed $p>0.05$ for all). As

Table 1. PCR oligonucleotides used for AiV amplification and quantification, Germany, 2004*

ID no.	Sequence, 5′ → 3′	Position†	Genome location	Orientation	RT-PCR type	Usage
AiV-F65	CACCGTTACTCCATTCAGCTTCTTC	65–89	5′ UTR	+	Nested, 1st round‡	Determination of suitable genomic target region for quantitative real-time RT-PCR
AiV-F69	GTTACTCCATTCAGCTTCTTCGGAAC	69–94	5′ UTR	+	Nested, 2nd round§	
AiV-R1039	CAGGATTGGACATCAGAATCATAGAG	1039–1064	Leader	–	Nested, 2nd round§	
AiV-R1049	GGATAGAACCAGGATTGGACATCAG	1049–1073	Leader	–	Nested, 1st round‡	
AiV-F274	CCAGCCTGACGTATCACAGG	274–293	5′ UTR	+	Real-time¶	Viral RNA quantification
AiV-R313	AAGCTGCTCACGTGGCAATTGTG	313–335	5′ UTR	–	Real-time¶	
AiV-P294	FAM-CTGTGTGAAGYCC-MGBNFQ	294–306	5′ UTR	+ (probe)	Real-time¶	
AiV-F2984	CAGGCATTCATCTCYGCAGGTGAA	2984–3007	VP1	+	Nested, 1st round‡	Determination of viral genotype
AiV-F2995	CTCYGCAGGTGAATCCTTCAACGT	2995–3018	VP1	+	Nested, 2nd round§	
AiV-R3881	TTGCGGATGGCCCAGTGGACGTA	3884–3906	VP1	–	Nested, 1st round‡	
AiV-R3884	GATGGCCCAGTGGACGTAGGT	3881–3901	VP1	–	Nested, 2nd round§	

*AiV, Aichi virus; ID, identification; RT-PCR, reverse transcription PCR; UTR, untranslated region.
†Relative to AiV GenBank accession no. AB040749.
‡25-μL QIAGEN OneStep RT-PCR reactions as described by the manufacturer (QIAGEN, Hilden, Germany) used 400 nmol/L each of 1st-round primers, 1 μg bovine serum albumin, and 5 μL RNA extract. Amplification involved 30 min at 50°C; 15 min at 95°C; 10 cycles of 20 s at 94°C, 30 s starting at 60°C with a decrease of 1°C per cycle, and 50 s at 72°C; and 40 cycles of 20 s at 95°C, 30 s at 54°C, and 50 s at 72°C; and a final elongation step of 5 min at 72°C.
§50-μL Platinum Taq reactions as described by the manufacturer (Invitrogen, Karlsruhe, Germany) used 1 μL of 1st-round PCR product, 2.5 mmol/L $MgCl_2$, and 400 nmol/L each of 2nd-round primers. Amplification involved 3 min at 94°C and 45 cycles of 20 s at 94°C, 30 s at 60°C, and 40 s at 72°C.
¶25-μL QIAGEN OneStep RT-PCR reactions used 3 μL of RNA extract, 600 nmol/L of each primer, and 320 nmol/L of the probe. Cycling in an Applied Biosystems (Darmstadt, German) 7700 SDS instrument involved the following steps: 55°C for 15 min, 95°C for 15 min, and 45 cycles of 95°C for 15 s and 58°C for 30 s (fluorescence measured).

shown in Table 2, all AiV-positive patients had abdominal pain, diarrhea, or nausea. No other virus commonly associated with diarrhea was detected in any patient, including norovirus, rotavirus, adenovirus, astrovirus, parechovirus, or enterovirus. A bacterial cause of disease was ruled out by using standard culture methods (*10*). No clear association with a foodborne etiology, sociologic risk factor (including travel history), or contact with animals was observed. As shown in Table 2, high RNA copy numbers of up to 1.32×10^{12} per gram of stool (mean 1.32×10^{11}, median = 1.82×10^{7}) were found in patients with positive test results.

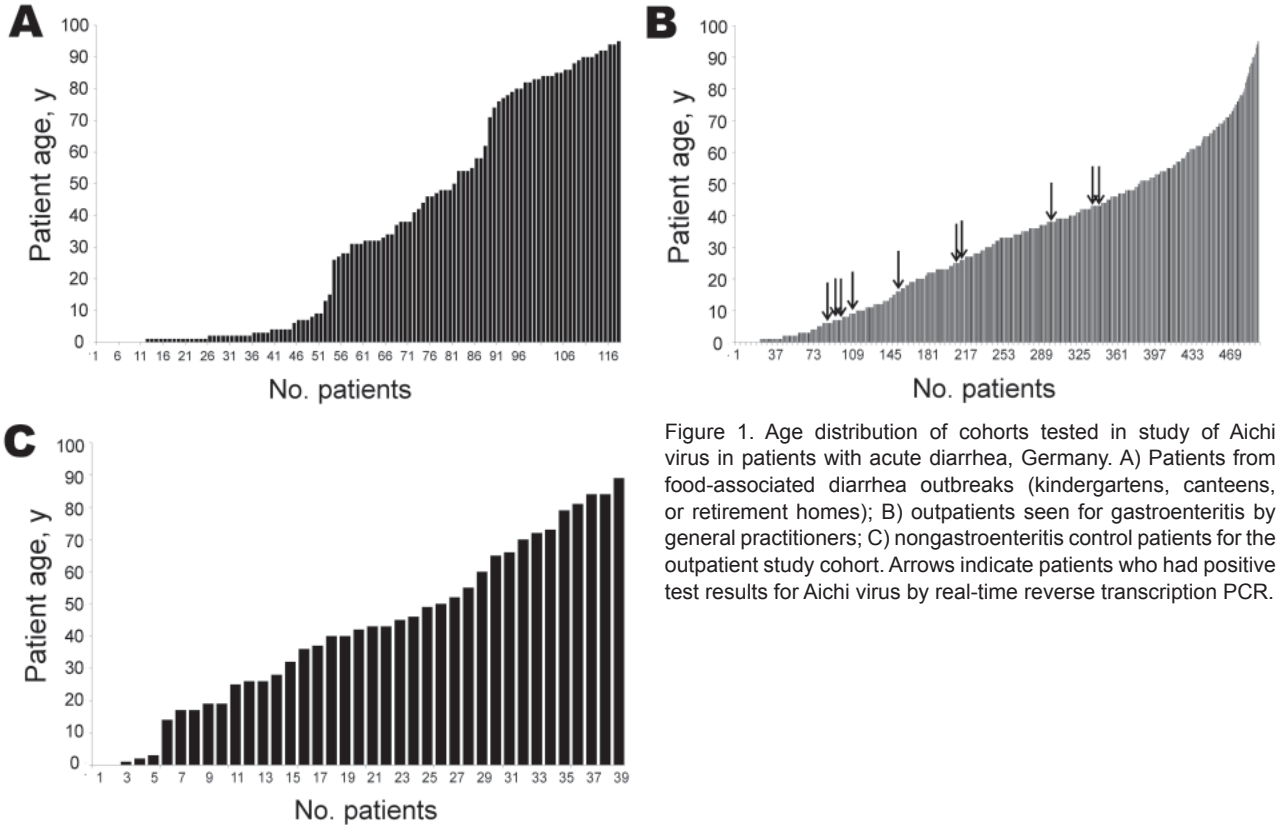

Figure 1. Age distribution of cohorts tested in study of Aichi virus in patients with acute diarrhea, Germany. A) Patients from food-associated diarrhea outbreaks (kindergartens, canteens, or retirement homes); B) outpatients seen for gastroenteritis by general practitioners; C) nongastroenteritis control patients for the outpatient study cohort. Arrows indicate patients who had positive test results for Aichi virus by real-time reverse transcription PCR.

Table 2. Characteristics of patients positive for Aichi virus, Germany, 2004*

Sample ID no.	Sampling date	Patient age, y/sex	Animal contact	Diarrhea	Emesis	Symptomatic contact person	Recent travel history	Other symptoms	Suspicious food ingested	Virus concentration†
D/VI 2169	Oct 19	7/M	No	Yes	Yes	Mother	No	Abdominal pain	None	1.30×10^{12}
D/VI 2244	Nov 2	9/M	No	Yes	No	None	No	Abdominal pain	None	1.37×10^{7}
D/VI 2287	Nov 8	27/M	No	Yes	No	None	No	None	None	6.77×10^{7}
D/VI 2321	Nov 10	26/F	No	No	No	None	No	Abdominal pain	Fast-food chicken nuggets	6.09×10^{9}
D/VI 2359	Nov 16	16/M	2 birds	Yes	No	None	No	Abdominal pain	Ground pork	2.79×10^{6}
D/VI 2524	Dec 4	7/M	No	Yes	No	None	Italy	Abdominal pain	Chicken	4.21×10^{7}
D/VI 2528	Dec 2	43/F	No	No	Yes	Daughter	No	Abdominal pain	None	4.42×10^{6}
D/VI 2535	Dec 2	6/F	No	No	Yes	Mother	No	Throat pain	None	2.27×10^{7}
D/VI 2582	Dec 6	38/F	Cat	Yes	Yes	None	No	Abdominal pain	Minced meat	9.99×10^{6}
D/VI 2591	Dec 7	43/F	Dog	Yes	Yes	None	No	None	None	1.08×10^{2}

*No patients had fever (>38.5°C) or co-infections. All samples were tested for norovirus, rotavirus, adenovirus, astrovirus, parechovirus, enterovirus, and common bacterial pathogens. ID, identification.
†RNA copies/g stool.

Although samples had been collected throughout 2004, all AiV-positive cases occurred during 8 weeks from October to December and originated from a geographically restricted area within the city of Bremen. To verify if this temporal and geographic accumulation of cases represented a point-source outbreak, we amplified and sequenced the entire viral protein (VP) 1 gene, which is commonly used for picornavirus typing, from 9/10 samples (GenBank accession nos. GQ927704–GQ927712). Failure of VP1 amplification in sample D/VI2591 was probably caused by low virus concentration. As shown in Figure 2, a total of 8 samples formed a distinct phylogenetic cluster within AiV genotype B. The first 3 strains, sampled from October 19 through November 10 (Table 2), were almost identical in VP1, with only 1 strain (D/VI2244) diverging by 2 synonymous substitutions. All other samples (November 16–December 7) showed a VP1 nucleotide diversity of up to 0.8% (2–7/864 nt) and an amino acid diversity of up to 1.4% (1–4/288 residues) in comparison to the 3 initially sampled specimens and to each other. Strain D/VI2287, sampled November 8, belonged to AiV genotype A, with nucleotide and amino acid differences of up to 13.2% and 5.4% from the genotype B strains.

Conclusions

For many of the recently described picornaviruses, human pathogenicity is still under study. With the advent of metagenomics, the description of multiple novel viruses can be expected. Although it is generally difficult to generate sufficiently large and appropriately sampled control groups in studies on respiratory and enteric diseases, quantitative data on virus shedding and proof of monocausality can contribute to confirm the link of novel viruses to human disease. For AiV, the high concentrations found in several samples in this study provide support for viral replication in humans. However, virus shedding appeared unrelated to the severity of symptoms, and clinical presentations were generally mild. Contrary to the findings of previous studies from France and Tunisia (*5*,*6*), no food association could be observed in this study. In agreement with some, but not all, published reports (*5*,*11*), no case of AiV-associated gastroenteritis from this study had apparent co-infections, further supporting AiV pathogenicity in humans. The overall 2.0% detection rate of AiV in stool samples from outpatients with gastroenteritis is compatible with detection rates in recent studies from several European and Asian countries (*5*,*12*). Surprisingly, AiV infection affected all age groups. This was in sharp contrast to parechoviruses and cardioviruses detected predominantly in patients <6 years of age in the same study cohorts (*8*,*9*) and indicated different modes of transmission and maintenance of these genetically related picornaviruses at the population level. Similarly, the lower AiV infection rate described here was consistent with the 51.0% seroprevalence rate described in German infants <2 years of age (*13*) compared with >75.0% described for cardioviruses and parechoviruses (*14*,*15*).

The geographic and temporal accumulation of cases, together with the observed sequence variation, supports locally and temporally restricted circulation of AiV with human-to-human transmission, rather than a point-source epidemic pattern as observed in foodborne infections. Fecal–oral human-to-human transmission would be

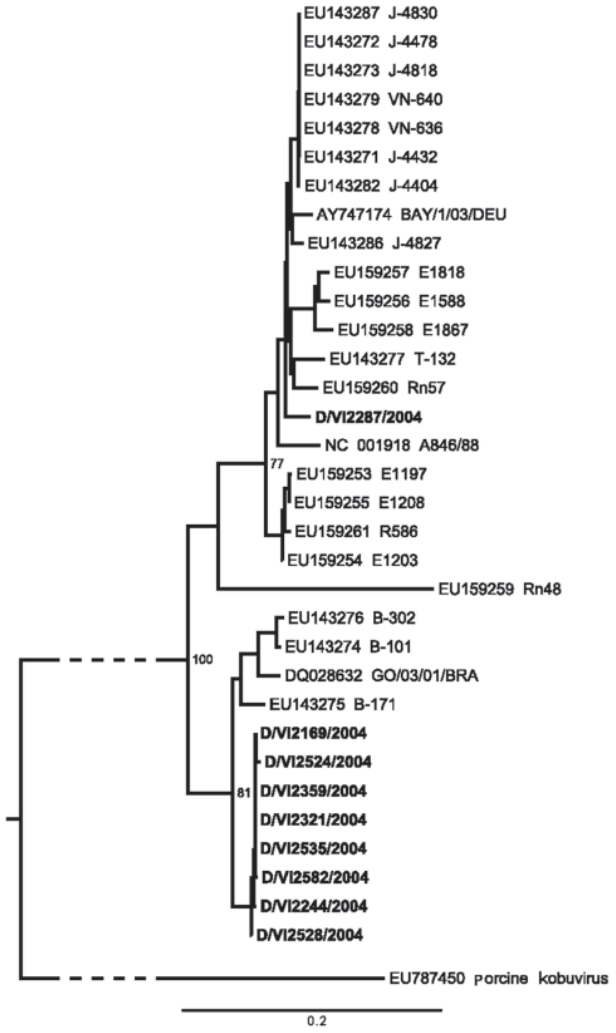

Figure 2. Neighbor-joining phylogeny of Aichi virus (AiV) viral protein 1 gene of strains from study of AiV in patients with acute diarrhea (**boldface**), Germany, compared with strains from GenBank. The tree was generated by using MEGA4 (www.megasoftware.net) using the maximum-composite likelihood nucleotide substitution model and complete deletion option. Porcine kobuvirus was used as an outgroup (branch truncated as indicated by slashed lines). Bootstrap values from 1,000 reiterations are depicted next to root points. The final dataset corresponded to nucleotide positions 3,034–3,663 in AiV GenBank accession no. AB040749. Scale bar indicates number of base substitutions per site.

facilitated by the high fecal virus concentrations in some patients. Our data indicate that AiV can be considered an authentic human pathogen that can be transmitted from human to human.

Acknowledgments

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Dr Drexler is a physician and clinical virologist at the University of Bonn. He is currently working on the implementation of methods for affordable viral load monitoring and the characterization of novel human and zoonotic viruses.

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Atypical Pestivirus and Severe Respiratory Disease in Calves, Europe

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In 2010, a HoBi-like pestivirus was isolated from clinically affected calves in Italy. This European virus reproduced a milder form of disease under experimental conditions and was genetically related to previously reported HoBi-like strains. Isolation of this novel virus from a clinical outbreak may have implications for cattle health and prophylactic programs.

Genus *Pestivirus* (family *Flaviviridae*) includes *Bovine viral diarrhea virus type 1* (BVDV-1) and *Bovine viral diarrhea virus type 2* (BVDV-2), *Classical swine fever virus* (CSFV), *Border disease virus* (BDV), and other pestivirus species detected in wild ruminants (*1*,*2*). In 2004, an atypical pestivirus was isolated from a contaminated batch of calf serum originating from Brazil. This virus, named D32/00_HoBi, was proposed as prototype of a new pestivirus species, BVDV-3 (*3*). Although additional HoBi-like strains have been detected in South America (*4*), currently, there is a unique report of natural infection in cattle caused by a HoBi-like strain, Th/04_KhonKaen, which was isolated from a bovine serum sample collected during an epidemiologic survey for BVDV in Thailand (*5*). However, the virus was not associated with any evident clinical signs. Here we report the biologic and genetic characterization of a HoBi-like strain from Europe that was isolated from cattle during an outbreak of respiratory disease in Italy.

The Study

A severe outbreak of respiratory disease occurred in a cattle herd in Calabria region, southern Italy, during December 2009–February 2010. The animals were not screened for pestiviruses before their introduction into the herd but were vaccinated regularly for BVDV. Clinical signs appeared in 26 calves, 6–7 months old, and consisted of fever (39.4°–40.1°C), cough, accelerated pulse and breath, seromucoid nasal discharge, and leukopenia. Most animals recovered progressively within 2 weeks after administration of supportive therapy. Two calves died, and necropsy indicated severe tracheitis and bronchopneumonia involving the apical lung lobes.

The nasal discharge of 6 ill calves and the apical lobes of the dead calves were subjected to traditional or molecular assays to detect the main respiratory pathogens of cattle (*6*). All samples tested positive by a TaqMan assay specific for atypical pestiviruses (*7*) and contained RNA copy numbers ranging from 2.57×10^3 to 5.48×10^5 copies/μL of template. The pestivirus strains (Italy-1/10-1 and Italy-1/10-2) detected in the lung samples of 2 calves were successfully isolated on MDBK cells as shown by the positive results of an immunofluorescence assay by using an anti-NS3 monoclonal antibody pool (3A3, 3H4, IF2). Other viral pathogens of cattle, as well as bacteria and parasites, were not detected in the examined samples, with the exception of the lungs of the dead calves, from which *Streptococcus bovis* and *Vibrio* spp. were isolated.

The near full-length genome (12,104 nt) of strain Italy-1/10–1, representative of the pestiviruses circulating in the herd, was determined through PCR amplifications and subsequent sequencing of overlapping fragments (*8*). The nucleotide sequence (GenBank accession no. HQ231763) obtained was analyzed by using NCBI (www.ncbi.nlm.nih.gov) and EMBL (www.ebi.ac.uk) tools. By sequence comparison with reference sequences, strain Italy-1/10–1 had the same genomic organization of other members of the genus *Pestivirus*, consisting of a unique open reading frame of 11,700 nt flanked by 2 untranslated regions (UTRs). By sequence analysis of the near full-length genome, strain Italy-1/10–1 displayed the closest relatedness to atypical pestivirus Th/04_KhonKaen, whereas the nucleotide identities to BVDV-1 and BVDV-2 reference strains were much lower (Table). Percentage identities were similar between the HoBi-like strain from Italy and BDV and CSFV reference isolates. When the informative genomic regions were analyzed, strain Italy-1/10-1 displayed the closest relatedness to strains D32/00_HoBi and CH-KaHo/cont that had been detected in South America.

Phylogeny was inferred from the full-length genome by using the neighbor-joining method of MEGA4.1 software (*9*). The analyzed pestiviruses clustered into 6 monophyletic clades, with strain Italy-1/10–01 forming a unique cluster with strain Th/04_KhonKaen, which was clearly separated from the other pestivirus species (Figure, panel A). A similar cluster was observed in the trees constructed on the E2, 5′ UTR and N^{pro} sequences, where

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the HoBi-like strain from Europe formed a tight subcluster with South America viruses (Figure, panels B–D).

We evaluated the pathogenic potential of strain Italy-1/10–1 in 2 seronegative 6-month-old calves; an additional calf served as control. The 2 challenged calves showed only mild clinical signs consisting of mucoserous nasal discharge, hyperthermia, and moderate leukopenia. The successful infection was confirmed by viremia and viral shedding through the nasal and fecal routes (data not shown). Seroconversion was demonstrated by using the BVDV-Ab SVANOVIR ELISA (Svanova Biotech AB, Uppsala, Sweden) and virus neutralization (*5*), with mean optical density value and virus neutralizing titer of 0.398 (cutoff 0.200) and 512, respectively.

Conclusions

Traditionally, 4 different species have belonged to the genus *Pestivirus*, but in the past few years, new putative members have been described worldwide. In 2004, an atypical pestivirus, D32/00_HoBi, distantly related to BVDV-1 and BVDV-2 was isolated from a batch of fetal calf serum collected in Brazil (*3*). Strictly related viruses were later identified in the blood of a buffalo in Brazil and in contaminated cell cultures in South America (*4*).

More recently, another atypical pestivirus, Th/04_KhonKaen, was isolated from a bovine serum sample during an epidemiologic survey in Thailand (*5*). Based on the genetic and antigenic divergence from known BVDV-1 and BVDV-2 strains, all these viruses were proposed as members of a new species of the genus *Pestivirus* (*5*,*8*,*10*).

The results of our study show that atypical pestiviruses also are circulating on the European continent. Infection caused by this new virus was associated with overt disease in cattle. Although experimental infection of seronegative calves induced only mild disease, Koch's postulates were

Table. Nucleotide identity of HoBi-like pestivirus strain Italy-1/10-1 with reference pestiviruses in different genomic regions*

Pestivirus species and strain	GenBank accession no.	Nucleotide identity, %			
		Full-length genome	E2	5′ UTR	N^{pro}
HoBi-like pestivirus					
Th/04_KhonKaen	FJ040215	90.0	87.7	92.3	89.4
D32/00_HoBi	AY604725 (E2); AY489116 (5′ UTR); AY735486 (N^{pro})	NA	94.2	98.3	95.2
CH-KaHo/cont	EU385605 (E2); AY895011 (N^{pro})	NA	93.4	NA	95.2
Border disease virus					
H2121 (Chamois-1)	GU270877	66.7	58.1	61.8	64.6
Gifhorn	GQ902940	66.7	58.2	65.5	64.2
X818	NC_003679	66.9	59.3	60.7	66.2
Reindeer	AF144618	66.3	58.6	65.0	63.2
Classical swine fever virus					
Brescia X	AY578687	66.9	58.5	66.6	66.6
HCLV	AF531433	66.5	59.0	65.5	65.4
Brescia	AF091661	66.8	59.0	66.1	65.4
Alfort-A19	U90951	66.8	59.0	66.1	65.8
Shimen/HVRI	AY775178	66.8	59.2	66.6	67.0
Riems	AY259122	66.4	58.7	65.5	65.2
Pestivirus of giraffe, H138	AF144617	65.2	58.0	72.8	64.2
Bovine viral diarrhea virus type 1					
ILLNC	U86600	66.3	60.9	69.8	64.0
ZM-95	AF526381	66.2	62.1	65.7	63.6
Oregon-C24V	AF091605	66.4	61.1	68.8	66.2
CP7-5A	AF220247	66.4	62.1	68.2	66.0
SD1	M96751	66.3	60.1	68.8	66.2
Singer_Arg	DQ088995	66.5	61.4	69.3	65.0
KE9	EF101530	67.0	61.4	70.4	65.8
NADL	M31182	65.3	62.0	69.3	66.2
VEDEVAC	AJ585412	66.4	61.3	70.9	66.2
Bovine viral diarrhea virus type 2					
JZ05-1	GQ888686	67.4	59.5	75.0	63.8
New York'93	AF502399	67.1	58.7	74.4	65.0
XJ-04	FJ527854	67.1	59.3	74.4	64.2
C413	NC_002032	67.1	58.5	74.4	63.8
Hokudai Lab/09	AB567658	67.2	59.3	72.8	65.2

*UTR, untranslated region; NA, sequence not available.

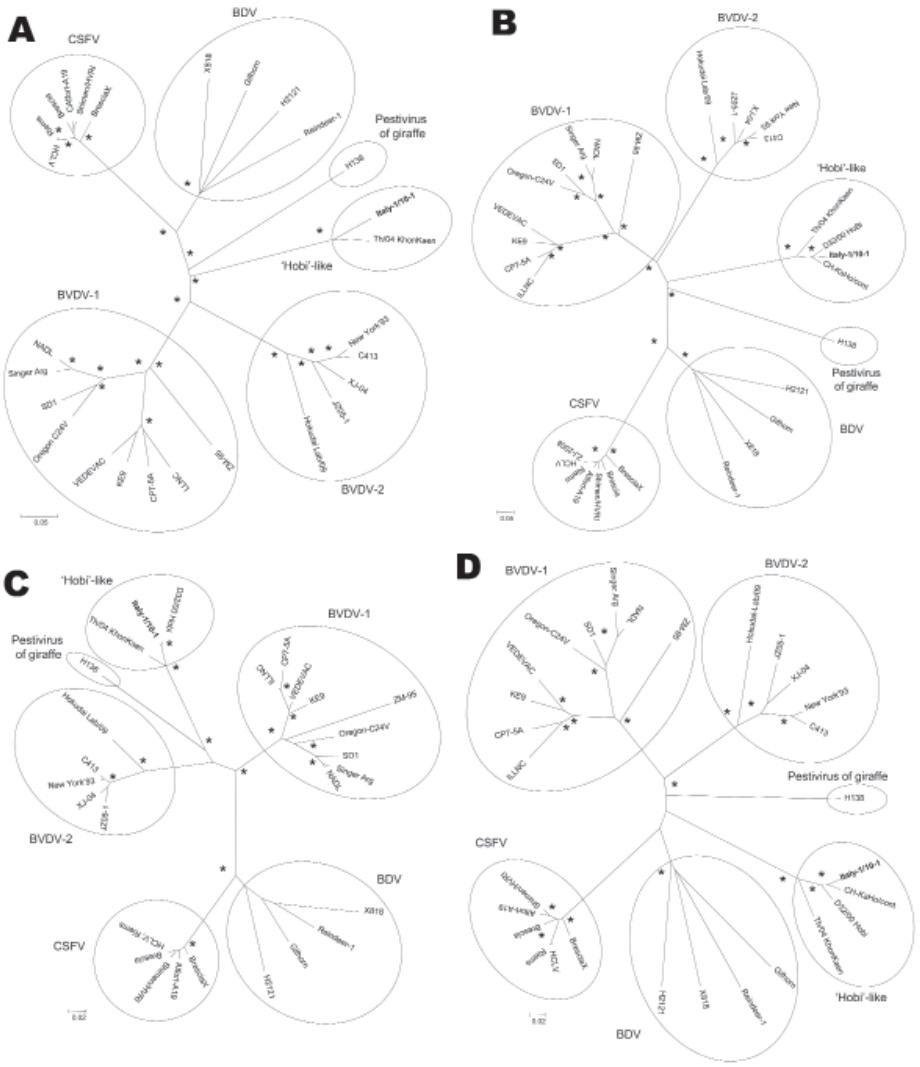

Figure. Neighbor-joining unrooted trees based on the full-length genome (A), E2 (B), 5′ untranslated region (C), and N^{pro} (D) sequences of members of the genus *Pestivirus*. For phylogenetic tree construction, pestivirus sequences listed in the Table were used. Asterisks indicate strong statistical support for a node by a bootstrap value of 75%–100%. Scale bars represent estimated numbers of nucleotide substitutions per site. CSFV, classical swine fever virus; BVDV-1, bovine viral diarrhea virus type 1; BVDV-2, bovine viral diarrhea virus type 2; BDV, border disease virus.

fullfilled, as demonstrated by some clinical signs, viremia, viral shedding, and seroconversion.

At the genetic level, the HoBi-like strain from Italy was more strictly related to the viruses from Brazil than to the isolate from Thailand. Considering that the virus has been repeatedly detected in batches of fetal calf serum (*3*,*11*), a possible introduction of the virus to the European continent through vaccines or other products prepared with contaminated bovine serum should be taken into account. Accordingly, previous reports suggest that BVDV may spread (and cause severe disease) through vaccination with contaminated products (*12*–*14*). By phylogenetic analysis of the full-length genome and E2 and N^{pro} regions, atypical pestiviruses formed a monophyletic cluster that was approximately equidistant from BVDV-1/BVDV-2 and BDV/CSFV, whereas the dendrogram obtained from 5′ UTR showed a closer relatedness to BVDVs. Thus, phylogeny may not support naming HoBi-like strains as BVDV-3 because the proposed nomenclature (*8*,*10*) does not reflect the genetic relationship among different pestivirus species.

Antigenic differences have been found between HoBi-like strains and BVDV-1/BVDV-2 through cross-neutralization assays (*3*,*5*). This difference also was evident in this study in that experimentally infected calves displayed high antibody titers by virus neutralization with the homologous virus, whereas only titers slightly above the cutoff were obtained by a commercial BVDV-1–based ELISA. The genetic and antigenic differences between HoBi-like strains and BVDV-1/BVDV-2 pose intriguing questions about the efficacy of commercially available BVDV vaccines and the need to develop specific vaccines against this new virus. In addition, BVDV surveillance programs may be affected by the poor sensitivity of commonly used PCRs with respect to atypical pestiviruses because of their genetic distance from BVDVs. Continuous

epidemiologic surveillance will help assess the extent to which HoBi-like pestivirus strains are widespread in cattle populations worldwide and their impact on animal health and production, thus requiring specific immunization plans.

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Specimen Collection and Confirmation of Norovirus Outbreaks[1]

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We evaluated data from gastroenteritis outbreaks in Oregon to assess sensitivity of stool testing for norovirus and determine number of specimens needed to confirm norovirus as the cause. Norovirus can be readily confirmed if 3–6 specimens are collected any time ≤7 days after onset of diarrhea and for almost that long after symptoms resolve.

One goal of any outbreak investigation is to identify the causative pathogen (*1*). In a recent analysis of foodborne disease outbreaks in the United States during 2006, only 49% had a confirmed causative pathogen (*2*). A review of foodborne disease outbreaks investigated in the 10-site Foodborne Disease Active Surveillance Network during 1998–1999 found that an etiologic agent was identified for only 29% (*3*). The major limitation in identifying etiologic agents was a lack of specimens; no stool specimens were collected in two thirds of the unconfirmed outbreaks.

In Oregon, outbreaks of illness are reportable to public health authorities, who investigate to determine the causative pathogen and means of transmission and to implement control measures accordingly. Obtaining stool specimens within 3 days of onset has been recommended (*4*), but carrying out this recommendation is frequently not feasible.

Since 1999, specimens from case-patients in outbreaks of acute gastroenteritis have been tested for norovirus at the Oregon State Public Health Laboratory. Noroviruses are a group of related, nonenveloped, single-stranded RNA viruses that cause acute gastroenteritis in humans. We reviewed Oregon data to assess the sensitivity of stool testing for norovirus at different times after illness onset and to determine the number of specimens needed to ensure a high probability of confirming a norovirus outbreak.

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The Study

We reviewed all outbreaks of acute gastroenteritis reported in Oregon from August 23, 1999, through January 31, 2007, for which norovirus was the suspected cause on the basis of incubation period, symptoms, and duration of illness. As part of the outbreak investigations, demographic, clinical and exposure information was gathered, and specimens were solicited with the goal (often unmet) of obtaining at least 3 specimens per cluster. Public health investigators were exhorted to collect specimens "as soon as possible" after a cluster was identified. From 1999 until November 2005, specimens were tested by conventional reverse transcription PCR (RT-PCR) by using consensus primers for norovirus (*5*). From November 2005 onward, specimens were tested by real-time RT-PCR by using primers to a highly conserved region in the open reading frame 1–2 junction (*6*,*7*). Laboratory results were linked to epidemiologic and clinical information ascertained during the outbreak investigation. We defined a confirmed norovirus outbreak as a cluster of compatible illnesses for which norovirus sequences were identified from specimens of at least 2 case-patients (*8*).

From August 23, 1999, through January 31, 2007, a total of 486 (59%) of 824 reported outbreaks of acute gastroenteritis in Oregon were suspected to be caused by norovirus. Norovirus was confirmed as the cause of 355 outbreaks; of these reports, 275 (77%) provided analyzable data. The outbreaks were associated with the following settings: 151 (55%) with nursing homes, long-term-care facilities, or assisted-living centers; 41 (15%) with restaurants or delicatessens; 10 (4%) with schools; 11 (4%) with hospitals; 8 (3%) with private homes; 6 (2%) with camps; 4 (1%) with correction facilities; and 44 (16%) with other settings. From these 275 outbreaks, 1,117 specimens were submitted to the laboratory with a median of 4 (range 2–13) per outbreak; 888 (79%) tested positive for norovirus. Of the 1,117 specimens, dates of diarrhea onset and stool collection were available for 845 (76%). Of those, 698 (83%) tested positive for norovirus. Figure 1 depicts the percentage of specimens that tested positive, by time since diarrhea onset.

Of the 1,117 specimens sent to the laboratory, dates on diarrhea resolution and stool collection were available for 360 (32%) in 153 outbreaks. Of those, 302 (84%) tested positive for norovirus. No association was found between PCR positivity and patient sex (relative risk for male patients 1.06; $p = 0.07$) or age.

To evaluate the number of stool specimens needed to confirm norovirus as the etiologic agent of an outbreak (suspected to have been caused by norovirus), we analyzed

[1]Preliminary report presented at the 2004 International Conference on Emerging Infectious Diseases, February 29–March 3, 2004, Atlanta, Georgia, USA (abstract no. 244).

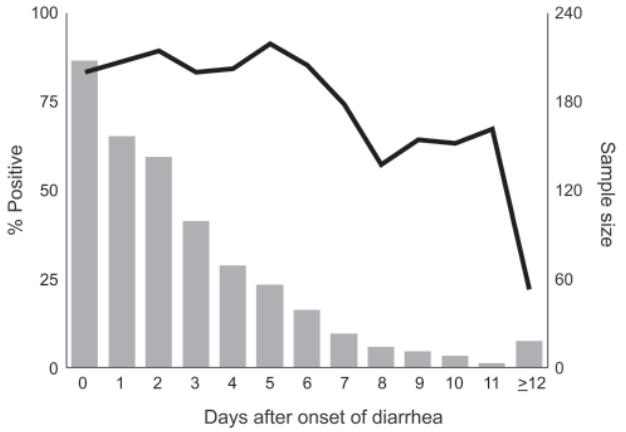

Figure 1. Percentage of specimens positive for norovirus (line), by days after onset of diarrhea, Oregon, USA, August 1999–January 2007.

377 such outbreaks from the same period. A total of 1,532 specimens from these outbreaks were tested, and norovirus was identified in 1,134 (74%). Figure 2 shows likelihood of an outbreak being confirmed as caused by norovirus as a function of the number of specimens tested.

Conclusions

Our data indicate that 3 specimens were sufficient to confirm 91% of norovirus outbreaks, and when 6 specimens were submitted, 97% were confirmed. Testing >7 specimens added nothing to the sensitivity. Therefore, we recommend that to confirm that norovirus is the etiologic agent of an outbreak, at least 3, but not more than 6, specimens should be collected. Norovirus can be readily confirmed as the cause of an outbreak of acute gastroenteritis if specimens are collected at any time during the first 7 days after onset of diarrhea and (though period is somewhat more variable) for almost that long after symptoms resolve. No apparent decline in sensitivity occurs for specimens collected up to 6 days after onset of diarrhea. Sensitivity drops during days 7–14, but remains substantial for up to 2 weeks. The common excuse that "I've already gotten better" can be safely ignored. Several studies have examined the duration of norovirus excretion and found that the average period of shedding is ≈28 days (range 13–56 days), well past the resolution of symptoms (*9*,*10*). Infectivity cannot be inferred from these findings, however.

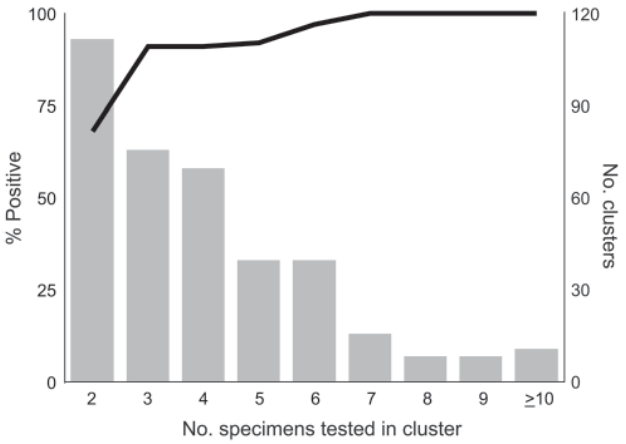

Figure 2. Percentage of outbreaks confirmed as norovirus (line), by number of specimens tested, Oregon, USA, August 1999–January 2007.

These data have several limitations. First, at nursing homes, the responsibility for data collection was often delegated to staff, and no attempt was made to validate the data they submitted. Second, data were incomplete for many cases. Third, few specimens were collected >14 days after diarrhea onset or >7 days after diarrhea cessation, yielding wider confidence intervals around late-sample point estimates. Lastly, a real-time RT-PCR is now being used by the Oregon State Public Health Laboratory (and many others); this assay is up to 10,000× more sensitive than the conventional RT-PCR (*11*), and the number of specimens needed to confirm norovirus outbreaks may decrease as the acceptable time range for collection increases. We noted a trend toward higher positivity rates with the real-time RT-PCR, so fewer specimens might be required; norovirus was confirmed in 100% of outbreaks with ≥5 specimens tested by this method. Despite these limitations, we believe that this information will be helpful to outbreak investigators and to researchers studying sporadic cases of acute gastroenteritis. Taking into account these findings, in turn, will lead to a more accurate picture of the incidence of norovirus infections.

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Mrs Plantenga was a research analyst at the Department of Human Services in Portland, Oregon, when this research was conducted. Her interests include public health surveillance and outbreak investigations.

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African Swine Fever Virus p72 Genotype IX in Domestic Pigs, Congo, 2009

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African swine fever virus p72 genotype IX, associated with outbreaks in eastern Africa, is cocirculating in the Republic of the Congo with West African genotype I. Data suggest that viruses from eastern Africa are moving into western Africa, increasing the threat of outbreaks caused by novel viruses in this region.

African swine fever (ASF) is a serious disease of domestic pigs caused by a DNA arbovirus (African swine fever virus [ASFV]) belonging to the family *Asfaviridae* (*1*). Its highly contagious nature and ability to spread over long distances make it 1 of the most feared diseases of pigs; it causes devastating effects on pig production as manifested in the Caucasus since its introduction from southeastern Africa during 2007 (*2*). Considerable spread of ASF has been reported in western Africa during the past 20 years, and, except for in Côte d'Ivoire, the disease remains endemic (*3*). Because discernible ASFV serotypes are lacking, the field strains are grouped genetically by using sequencing of the C-terminus of the p72 protein, which discriminates 22 genotypes (*4*,*5*). Genotype I is historically associated with outbreaks in western Africa, whereas viruses from southern and eastern Africa have higher heterogeneity, with all 22 known genotypes having been recorded within the region (*5*–*7*).

The Republic of the Congo, located in western-central sub-Saharan Africa, shares borders with the Cabinda enclave of Angola, the Democratic Republic of the Congo, Central African Republic, Cameroon, and Gabon. The last ASF outbreaks in Congo were reported to the World Organization for Animal Health (OIE) during 2003. Since then, the disease has been officially declared endemic but without quantitative data. Sampling and characterization of currently circulating field strains from this region of western-central Africa are needed to fully understand virus spread and maintenance. Such data will have implications for regional control in western Africa.

The Study

During August 2009, a United Nations Food and Agriculture Organization mission was undertaken with local counterparts in Congo to support development of an action plan to control ASF. A key factor in selecting the sites sampled was inclusion of the main pig-producing, marketing, and consuming areas, with a particular focus where suspected ASF outbreaks had been recently reported. From the survey conducted, 86 samples comprising serum (35 samples), whole blood (44 samples), and tissues (7 samples) were collected from 80 domestic pigs in the departments of Brazzaville, Pointe Noire, Kouilou, Bouenza, Niari, and Cuvette (Figure). These departments contain 80%–90% of the country's pig population. Samples also were collected from Pool in southeastern Congo, where a recent hemorrhagic disease outbreak, characterized by case-fatality rates of ≈80%, had been reported. Clinical material was sent to the European Union Reference Laboratory for African Swine Fever (Centro de Investigación en Sanidad Animal, Madrid, Spain) for confirmatory diagnosis and characterization of the ASFV strain(s) responsible for the outbreak(s).

Specific ASF antibodies were detected by using the OIE-prescribed assays (*8*) in 7 of 35 serum samples analyzed. All positive serum samples were from animals that had survived the ASF outbreaks in Bouenza, Niari, and Pointe Noire during 2008. For ASFV genome detection, OIE-prescribed PCRs (*8*) were performed on DNA extracted from 28 serum samples, 44 blood samples, and 7 tissue homogenates. A positive result was obtained in 17 (21%) of samples analyzed, indicating ASF in all the departments where the survey was conducted except for Kouilou.

Subsequently, 5 hemadsorbent Congo ASFVs (Table) were isolated in porcine peripheral blood macrophages (*8*). Viral DNA was extracted, and 3 different sets of primers were used for ASFV genotyping. A region of 478 bp at the C-terminal end of the p72 protein and the full-length sequence of *p54* gene were amplified by using primers p72U/D and 89/722, respectively (*6*). We compared the sequence from each of the p72 and p54 amplicons with homologous sequences representative of each previously

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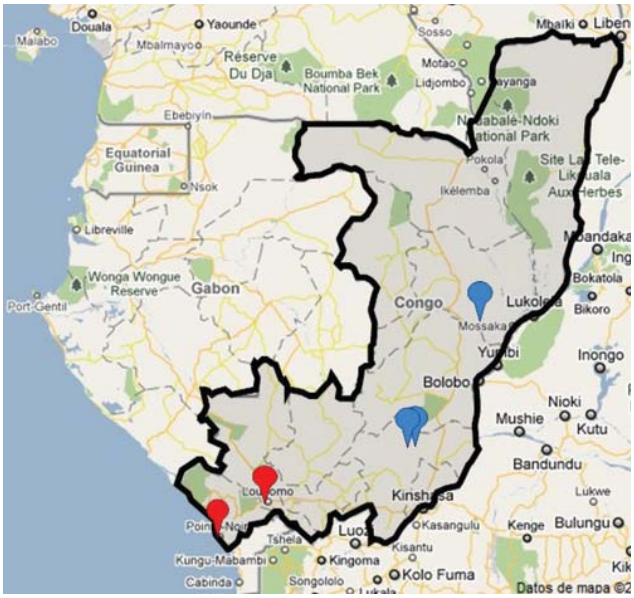

Figure. Republic of the Congo, showing location of the African swine fever virus isolates from eastern (blue) and western (red) Congo selected for genotyping.

described p72 and p54 genotype (*5*,*6*). A rooted minimum-evolution tree was constructed by using MEGA4.0 software and the p-distance nucleotide substitution model (*9*). Two different ASFV phylogenetic profiles were found in western and southeastern Congo. The phylogeny inferred for the ASFV isolates from eastern Congo (Con09/Pk45, Con09/Bzz020, Con09/Abo) placed them within p72 and p54 genotype IX with viruses collected from recent outbreaks in Kenya and Uganda during 2006–2007 (*6*,*7*). By contrast, ASFV isolates from western Congo (Con09/Ni16, Con09/PN003) clustered in p72 genotype I and p54 genotype Ic (*6*) comprising historical isolates from western Africa, such as Ang72 and Kat67, and the first ASFV isolated from Europe, the Portuguese Lisbon57 isolate (online Appendix Figure, www.cdc.gov/EID/content/17/8/101877-appF.htm).

To provide higher resolution, the tetrameric tandem amino acid repeat sequences within central variable region (CVR) of the *B602L* gene were analyzed (*5*–*7*,*10*). Primers CVR1 (5′-ACTTTGAAACAGGAAAC(AT)AATGATG-3′) and CVR2 (5′-ATATTTTGTAATATGTGGGCTGCTG-3′) were designed in this study selected from complete genome sequences of ASFV strains available in GenBank.

Under the same thermal cycling conditions used for full *p54* gene amplification, amplicons of 600–650 bp were generated from the isolates from eastern Congo, whereas the estimated size of the amplicons from western Congo was 500 bp (data not shown). Analysis of the tandem amino acid repeat sequences within the CVR demonstrated 3 different variants cocirculating in the country, 2 within the p72 type I virus genotype. The type of CVR sequence (AABNABTDBNAAAA) identified in the isolate from Pointe Noire (Con09/PN003) was identical to CVR subgroup XIII (*11*) that contains early isolates from Angola (Ang70, Ang72) and Portugal (Lis57). However, the Con09/Ni16, also classified within p72 and p54 genotypes I and Ic, showed a unique CVR sequence (AAAAAAAAAF) not previously described but most similar to viruses from Burundi and Kenya in CVR subgroup XXVI (*7*,*11*). As for p72 and p54 genotyping, isolates from eastern Congo were related to CVR subgroup XXIV, which contains isolates obtained during the 2006 and 2007 outbreaks in Uganda and Kenya (*6*,*7*).

Conclusions

We confirmed ASF in 5 of the 6 departments in which surveillance was conducted during August 2009 in Congo. Genotyping of 5 ASFV isolates from Congo resulted in identification of genetically distinct viruses circulating simultaneously in the country. In eastern districts of Congo, viruses were most genetically similar to those recovered from the outbreaks in Kenya and Uganda during 2006 and 2007. Therefore, genotype IX, associated with ASF outbreaks in

Table. African swine fever virus isolates selected for genotyping from domestic pigs after virus isolation for which nucleotide sequence was determined at 3 loci, Republic of the Congo, 2009*

	Sampling location			p72		p54		Central variable region	
Isolate	Locality	Dept	Province	Geno	GenBank	Geno	GenBank	Subgroup	GenBank
Con09/PN003	Tchimbamba	Ponte Noire	Southwestern	I	HQ645947	Ic	HQ645949	XIII	HQ645957
Con09/Ni16	Kikassa	Niari	Western	I	HQ645943	Ic	HQ645948	Related XXVI	HQ645953
Con09/Pk45	Ferme Champenoise Dimi, Igné	Pool	Southeastern	IX	HQ645944	IX	HQ645952	XXIV	HQ645954
Con09/Bzz020	Ferme Champenoise Dimi, Igné	Pool	Southeastern	IX	HQ645945	IX	HQ645950	XXIV	HQ645955
Con09/Abo	Ferme Ibovi	Abo, Cuvette	Eastern	IX	HQ645946	IX	HQ645951	XXIV	HQ645956

*Dept, department; geno, genotype; GenBank, GenBank accession number.

eastern Africa during the previous 10 years, also occurs in western Africa. By contrast, viruses in western Congo were similar to genotype I viruses that historically circulated in western Africa. Analyses of the CVR identified 2 discrete variants within genotype I. The Pointe Noire ASFV was identical to viruses isolated in neighboring Angola during the 1970s, indicating a prolonged persistence of this virus type in the region. By contrast, the ASFV isolated from Niari was related to variants circulating during the 1980s in Burundi. These results are consistent with recent transfer and dissemination of ASFV genotypes from eastern to western Africa. Broadening of our initial findings requires extending analyses to western-central Africa, particularly to countries such as Cameroon and the Democratic Republic of the Congo, and providing more in-depth analysis in Congo.

The apparent recent movement of a virus genotype previously associated with virulent ASF in eastern Africa to western Africa, where the viruses have hitherto always been classified in p72 genotype I, indicates the possibility of future outbreaks of disease caused by novel viruses in western Africa. The reality of this scenario recently was demonstrated in the Caucasus region. Our study confirms the continuing spread of ASFV.

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Reston Ebolavirus Antibodies in Bats, the Philippines

To the Editor: Filoviruses cause highly lethal hemorrhagic fever in humans and nonhuman primates, except for Reston Ebolavirus (REBOV), which causes severe hemorrhagic fever in macaques (*1*,*2*). REBOV epizootics among cynomolgus macaques occurred in 1989, 1990, 1992, and 1996 (*2*) and among swine in 2008 (*3*). African fruit bats have been suggested to be natural reservoirs for Zaire Ebolavirus and Marburg virus (*4*–*6*). However, the natural reservoir of REBOV in the Philippines is unknown. Thus, we determined the prevalence of REBOV antibody–positive bats in the Philippines.

Permission for this study was obtained from the Department of Environment and Natural Resources, the Philippines, before collecting bat specimens. Serum specimens from 141 wild-caught bats were collected at several locations during 2008–2009. The bat species tested are summarized in the Table. Captured bats were humanely killed and various tissues were obtained. Carcasses were then provided to the Department of Environment and Natural Resources for issuance of a transport permit.

We used immunoglobulin (Ig) G ELISAs with recombinant nucleoprotein (NP) and glycoprotein (GP) of REBOV (*7*) to determine REBOV antibody prevalence. REBOV NP and GP were expressed and purified from Tn5 cells infected with recombinant baculoviruses AcResNP and AcResGPDTM, which express NP and the ectodomain of GP with the histidine tag at its C-terminus. We also used histidine-tagged recombinant Crimean-Congo hemorrhagic fever virus NP as a negative control antigen in the IgG ELISA to confirm specificity of reactivity.

In IgG ELISAs for bat specimens, positive results were detected by using rabbit anti-bat IgG and horseradish peroxidase–conjugated anti-rabbit IgG. Anti-bat (*Rousettus aegyptiacus*) rabbit IgG strongly cross-reacts with IgGs of other bat species, including insectivorous bats (*8*). Bat serum samples were 4-fold serially diluted (1:100–1:6,400) and tested by using IgG ELISAs. Results of IgG ELISAs were the sum of optical densities at serum dilutions of 1:100, 1:400, 1:1,600, and 1:6,400. Cutoff values (0.82 for both IgG ELISAs) were determined by using serum specimens from REBOV antibody–negative bats.

Among 16 serum samples from *R. amplexicaudatus* bats, 5 (31%) captured at either the forest of Diliman (14°38′N, 121°2′E) or the forest of Quezon (14°10′N, 121°50′E) had positive results in the IgG ELISA for REBOV NP, and 5 (31%) captured at the forest of Quezon had positive results in the IgG ELISA for REBOV GP. The REBOV NP antibody–positive bats serum samples were confirmed to be NP antibody positive in the IgG ELISA by using glutathione-S-transferase–tagged partial REBOV NP antigen (*9*). Three samples had positive results in both IgG ELISAs (Table). Serum samples from other bat species had negative results in IgG ELISAs.

All bat serum samples were also tested by indirect immunofluorescence assays (IFAs) that used HeLa cells expressing NP and GP (*10*). In the IFAs, 2 samples from *R. amplexicaudatus* bats captured at the forest of Diliman and the forest of Quezon had high titers (1,280 and 640, respectively) of NP-specific antibodies, and 1 sample from an *R. amplexicaudatus* bat captured at the forest of Quezon had a positive result in the GP-specific IFA (titer 20). All IFA-positive samples were also positive in the IgG ELISA (Table).

The forest of Diliman is ≈30 km from the monkey facility and the Bulacan farm where REBOV infections in monkeys and swine, respectively, were detected. The forest of Quezon is ≈60 km from the monkey facility. Samples from other bat species had negative results in IFAs. We also performed heminested reverse transcription PCR specific for the REBOV NP gene with spleen specimens from all 16 *R. amplexicaudatus* bats but failed to detect any REBOV-specific amplicons.

REBOV-specific antibodies were detected only in *R. amplexicaudatus* bats, a common species of fruit bat, in the Philippines. In Africa, *R. aegyptiacus* bats, which are genetically similar to *R. amplexicaudatus* bats, have been

Table. REBOV-specific IgG in *Rousettus amplexicaudatus* bats and other bats, the Philippines*

Bat ID	Collection site	ELISA optical density		IFA titer	
		REBOV NP	REBOV GP	REBOV NP	REBOV GP
1539	FD	**2.13**	–0.21	**1,280**	<20
1632	FQ1	**0.88**	0.2	<20	<20
1642	FQ1	0.36	**5.22**	<20	**20**
1643	FQ1	**1.26**	**0.92**	<20	<20
1651	FQ1	**1.61**	**1.02**	<20	<20
1657	FQ1	–0.45	**1.69**	<20	<20
1660	FQ1	**3.8**	**2.51**	**640**	<20

*Cutoff optical density of ELISA was 0.82 (sum of optical densities at serum dilutions of 1:100, 1:400, 1:1,600, and 1:6,400). Values in **boldface** are positive results. REBOV, Reston Ebolavirus; Ig, immunoglobulin; IFA, indirect immunofluorescence assay; ID, identification; NP, nucleoprotein; GP, glycoprotein; FD, forest of Diliman at the University of the Philippines Diliman campus; FQ1, forest at the Agricultural College in Province of Quezon, the Philippines. The other 9 *R. amplexicaudatus* bats collected at FQ1 had negative results for all assays. The following bat species also had negative results: 5 *Eonycteris spelaea,* 35 *Cynopterus brachyotis,* 38 *Ptenochirus jagoli,* 6 *Haplonycteris fischeri,* 2 *Macroglossus minimus,* 2 *Rhinolophus rufus,* 1 *Rhinolophus arcuatus*, 9 *Emballonura alecto,* 2 *Pipistrellus javanicus*, 5 *Scotophilus kuhlii,* 8 *Miniopterus australis,* 8 *M. schreibersi,* 1 *M. tristis tritis,* 1 *Hipposideros diadema,* 1 *Myotis macrotarsus*, and 1 bat of unknown species.

shown to be naturally infected with Zaire Ebolavirus and Marburg virus. Thus, *R. amplexicaudatus* bats are a possible natural reservoir of REBOV. However, only 16 specimens of *R. amplexicaudatus* bats were available in this study, and it will be necessary to investigate more specimens of this species to detect the REBOV genome or antigens to conclude the bat is a natural reservoir for REBOV.

We have shown that *R. amplexicaudatus* bats are putatively infected with REBOV or closely related viruses in the Philippines. Antibody-positive bats were captured at the sites near the study areas, where REBOV infections in cynomolgus monkeys and swine have been identified. Thus, bats are a possible natural reservoir of REBOV. Further analysis to demonstrate the REBOV genome in bats is necessary to conclude that the bat is a reservoir of REBOV.

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Acute Hepatitis C Outbreak among HIV-infected Men, Madrid, Spain

To the Editor: In the past decade, hepatitis C virus (HCV) has emerged as a sexually transmitted infection (STI) among HIV-infected men who have sex with men (MSM). The epidemic was originally reported in several northern European countries (England, France, Germany, and the Netherlands) (*1*) and soon after in Australia (*2*) and the United States (*3*). Acute HCV acquisition was associated with group sex, unprotected receptive anal intercourse, and according to some studies, concomitant STI (*4*). Molecular phylogenetic studies suggested evidence of an international transmission network of MSM within northern Europe (*1*). However, expansion of the HCV epidemic among MSM to Spain (*5*) or to other

countries of the Mediterranean area had not previously been reported.

We report 4 cases of acute HCV in HIV-infected MSM in Madrid, Spain, 2010. These patients were monitored at a university-affiliated hospital in downtown Madrid, which provides health care to a large MSM community in the Chueca District. Diagnosis of acute HCV was made by using the following criteria of the European AIDS Treatment Network (*6*): 1) positive HCV RNA; 2) an acute rise in alanine aminotransferase level >5× the normal upper limit, with documented normal alanine aminotransferase level within 12 months; and 3) negative results for anti–hepatitis A virus immunoglobulin M and anti–hepatitis B core immunoglobulin M (when other causes of acute hepatitis were excluded). An HCV RNA load fluctuation of >1 $\log_{10}$ IU/mL, if present, was considered further evidence of acute HCV infection (*7*).

All 4 patients were MSM with well-controlled HIV infection who were receiving antiretroviral treatment. During routine medical screening, they were found to have newly elevated liver transaminase levels, and further assessment confirmed the diagnosis of acute HCV infection (Table). Three patients had received a diagnosis of STI in the previous 6 months, but only 1 patient acknowledged having unprotected anal intercourse. In addition, only 1 patient acknowledged using any recreational drugs (amyl nitrate); all denied using injection drugs (Table). All patients had lived in Madrid for at least 5 years before receiving a diagnosis of acute HCV. No patients reported having sex during international travel, using sex toys, or fisting.

The patients described here lived in the Chueca District of Madrid, the largest MSM community in Spain, which is frequented by MSM traveling from smaller cities in Spain and other countries. Two of the 3 patients were infected with HCV genotype 4, which is unusual in patients from outside the Middle East and Africa (*8*) yet unexpectedly common in northern European HCV outbreaks (*1*), which suggests that the patients reported here may have been part of the social network originating in the north. Further sequencing of these isolates is under way to address this issue. The third patient with an identifiable HCV genotype was infected with HCV genotype 1, the most common genotype among HIV-infected MSM in northern Europe (*1*). These findings suggest that a larger, undetected outbreak of HCV infection is taking place in Madrid.

Although the patients reported here described fewer risks for sexual acquisition of HCV than patients from northern Europe or the United States, 3 had recent STI, which suggests that they underreported their risks for HCV acquisition. This temporal association between STI and acute HCV in these patients suggests that the pattern of emergence of sexually transmitted HCV among MSM in Spain might be similar to that seen in northern Europe, following regional epidemics of syphilis (starting in 2000) (*9*,*10*). We therefore encourage HIV specialists and general practitioners, when investigating an STI, to perform HCV testing on MSM as well as on persons with newly elevated liver aminotransferase levels.

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Table. Description of 4 HIV-infected men with HCV infection, Madrid, Spain, 2010*

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4
Age, y	31	41	39	39
Country of origin	Italy	Ecuador	Spain	Spain
Date of HIV diagnosis	2006 Jun	2000 Jul	1998	2001
Year ART initiated	2008	2000	2006	2006
Prior negative HCV test	2006	None†	None†	2008
Date AHC diagnosed	May 2010	May 2010	May 2010	May 2010
CD4 count, cells/μL	562	327	787	750
HIV viral load	Undetectable	Undetectable	Undetectable	Undetectable
Symptoms at diagnosis	No	Mild asthenia only	No	No
ALT/AST levels at AHC diagnosis, U/L	564/331	304/216	222/114	261/125
HCV genotype	4	4	1a	Indeterminate
HCV RNA load, IU/mL ($\log_{10}$ IU/mL)	950,556 (5.98)	629,875 (5.80)	11,827 (4.07)	2,254,258 (6.35)
HCV RNA load fluctuation within 3 mo, $\log_{10}$ IU/mL	4.32	<1.00	1.33	<1.00
Unprotected anal intercourse	No	No	No	Serosorting‡
STI in previous 6 mo	Proctitis	Syphilis	No	Proctitis
Group sex (>2 persons)	Yes	No	No	No
Drug use	Amyl nitrate only	No	No	No

*HCV, hepatitis C virus; ART: antiretroviral therapy; AHC, acute hepatitis C infection; ALT, alanine aminotransferase; AST, aspartate aminotransferase; STI, sexually transmitted infection.
†Both patients had normal transaminase levels in the 4 y before AHC diagnosis.
‡Unprotected sex between seroconcordant partners (HIV positive).

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Saffold Virus Infection in Children, Malaysia, 2009

To the Editor: Since 2007, a new cardiovirus, named Saffold virus (SAFV), has been isolated from human specimens in the United States, Canada, the Netherlands, and People's Republic of China (*1–4*). Concurrent investigations also showed that SAFV could be detected in feces and respiratory secretions of children in other countries, and genetic analysis showed the circulation of different genetic lineages of SAFVs in various parts of the world. This new virus belongs to the genus *Cardiovirus*, in the family *Picornaviridae* (*5*). Here we report isolation of a new SAFV in Malaysia, designated SAFV-Penang to reflect the locality of isolation in Malaysia.

A 5-year-old girl was brought to a government outpatient clinic on November 18, 2009, with reported fever and sore throat for 3 days. The fever was described as of high grade with occasional episodes of rigor, accompanied by profuse sweating and myalgia, lethargy, and loss of appetite. The child had nasal blockage, mild runny nose, and dry cough. She vomited twice on day 3 of illness and had abdominal pain but no diarrhea. There was no history of similar illness affecting other family members, who lived in a semirural area within the state of Penang, Malaysia. Acute pharyngitis/acute influenza-like illness was provisionally diagnosed, and a throat swab specimen was collected in virus transport medium for virus isolation by using established procedures (*6*).

The throat swab sample was treated with antimicrobial drugs for 1 h before the cells were added to MDCK, Vero, and Hep-2 cells. On the fifth day postinoculation (dpi), a lytic form of cytopathic effect (CPE), similar to the type of CPE from enterovirus infection, was noted in Hep-2 but not in Vero or MDCK cells. The progress of CPE was slow, and full CPE was achieved on 9 dpi. On 8 dpi, a 0.5-mL aliquot containing infected Hep-2 cell suspension was removed and processed for indirect immunofluorescence assay by using a panel of commercial typing monoclonal antibodies for human enteroviruses. The infected Hep-2 cells reacted strongly with broad reactive pan-enterovirus monoclonal antibodies (catalog no. 3360, Chemicon Inc., Temecula, CA, USA) but failed to react with any type-specific monoclonal antibodies (data not shown).

After 3 passages in Hep-2 cells, culture supernatant was subsequently passed into Vero cells. After an additional 3 passages, the virus was fully adapted to grow in Vero cells and was able to induce visible CPE 1 dpi and full CPE by 4 dpi.

Partial genome sequence of the virus was initially obtained by using a random priming and amplification method as described (*7*). Full-length sequence was then determined by using primers designed according to the partial genome sequences of SAFV-Penang and genome sequences of other SAFV strains available in GenBank (primer sequences are available on request). The viral genome of SAFV-Penang is 8,073 nt

(full sequence deposited in GenBank under accession no. HQ162476). As for other SAFVs, a single long open reading frame of 2,295 aa was detected. Phylogenetic analysis based on the complete amino acid sequences of the polyproteins of different SAFVs indicated that SAFV-Penang is a type 3 SAFV (*8*). It is most closely related to the SAFV-3 isolated in the Netherlands in 2007.

To determine the prevalence of SAFV infection among children in Malaysia, we conducted a seroprevalence study by using 400 serum samples collected during 2009 from children 10–12 years of age under the national hepatitis B postvaccination serosurvey. The serum panel comprised 80 samples containing equal numbers of boys and girls from each of the 5 states: Penang (northwestern), Selangor (central-western), and Kelantan (northeastern) of peninsular Malaysia, and Sabah and Sarawak of eastern Malaysia in Borneo Island. Screening for SAFV-specific antibodies was conducted by using an indirect immunofluorescence antibody test on SAFV-infected Vero cells as described (*9*). The results (Table) indicated that >70% of schoolchildren surveyed had been exposed to the virus. The seropositive rate ranged from 67.5% (Penang) to 75.0% (Kelantan and Sabah), with no significant difference among different parts of the country ($\chi^2 = 1.60$, df = 4, p = 0.8091). The seropositive rate did not differ significantly by sex ($\chi^2 = 0.32$, p = 0.5734).

In summary, a new SAFV was discovered in Malaysia by direct virus isolation during an investigation of a febrile patient. Subsequent serologic study indicated that a high percentage of children 10–12 years of age had been exposed to this virus. Further study is required to determine the public health implications of SAFV infection in Southeast Asia, especially in cases in which co-infection with other pathogens might potentially lead to different clinical outcomes.

Table. Prevalence of SAFV-specific IgG in serum from children 10–12 years of age, by sex, Malaysia, 2009*

State of residence	No. children	No. positive serum samples†			
		+	++	+++	Total no. (%)
Penang					
M	40	21	7	0	28 (70.0)
F	40	19	5	2	26 (65.0)
Total	80	40	12	2	54 (67.5)
Kelantan					
M	40	16	8	5	29 (72.5)
F	40	18	11	2	31 (77.5)
Total	80	34	19	7	60 (75.0)
Selangor					
M	40	16	11	5	32 (80.0)
F	40	22	5	0	27 (67.5)
Total	80	38	16	5	59 (73.8)
Sabah					
M	40	16	9	4	29 (72.5)
F	40	24	6	1	31 (77.5)
Total	80	40	15	5	60 (75.0)
Sarawak					
M	40	22	6	3	31 (77.5)
F	40	19	5	4	28 (70.0)
Total	80	41	11	7	59 (73.8)

*SAFV, Saffold virus; Ig, immunoglobulin
†The degree of positive reactivity was classified into 3 groups: weak (titer ≤0–40), medium (titer 80–160), and strong (titer 320–1,280) according to the staining intensity on SAFV-infected Vero cells (data not shown). The classification was further confirmed by correlation with virus neutralizing titer from 20 samples in each group, with + samples having a titer of 0–40, ++ samples 80–160, and +++ samples 320–1,280, respectively.

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Human Bocavirus DNA in Paranasal Sinus Mucosa

To the Editor: Human bocavirus (HBoV) is a newly described parvovirus for which pathogenic potential has not clearly been elucidated (*1*). Recent findings suggest that HBoV may establish persistent infection of mucosal lymphocytes or contribute to tonsillar hyperplasia in children (*2*). In previous reports, we described prolonged HBoV DNA detection in immunocompromised children (*3*,*4*). Partial sequencing of the VP1 gene of HBoV from bronchoalveolar lavage fluid, plasma, and sphenoid sinus samples showed 100% identity, which suggested persistence of the same HBoV strain over a 5-month period (*3*). It remains speculative, however, whether paranasal sinus mucosa represents a site of HBoV persistence. To clarify this, we analyzed samples of paranasal mucosal tissue and nasal polyps from patients with chronic sinusitis for respiratory viruses and atypical bacteria.

A total of 102 tissue samples were obtained from 88 patients (median age 48.5 years, range 13.3–88.1 years) from July 2009 through September 2010 after elective surgery. Indication for surgery was established by otorhinolaryngologists. The most common indication was chronic sinusitis. No patients displayed acute respiratory symptoms at the time of surgery. To detect asymptomatic shedding in the upper respiratory tract and viremia, we collected nasal swabs and EDTA-blood samples concurrently. The study protocol was approved by the Ethics Committee of the University of Freiburg. Informed written consent was obtained from all study participants.

Approximately 25 mg of each tissue specimen was used for nucleic acid extraction by using an RNeasy Mini Kit, as described (*5*) (QIAGEN, Hamburg, Germany). To provide evidence that the QIAGEN RNeasy kit is also suitable for DNA extraction, we spiked HBoV negative samples with different amounts of HBoV DNA before extraction of nucleic acids was done with either the QIAGEN RNeasy Kit or DNA Blood Kit (QIAGEN). Extracted nucleic acids were then subjected to real-time PCR by using primers specific for HBoV. Minimal differences (±1 cycle threshold [C_t] value) in the HBoV PCR were detected; the QIAGEN RNeasy Kit was therefore used throughout the study (data not shown). Nasal swabs and EDTA-blood were purified by using a QIAamp MinElute Virus Spin Kit (QIAGEN). Multiplex PCR for respiratory viruses (Fast-track Diagnostics, Junglinster, Luxembourg) was conducted to detect influenza A (including pandemic [H1N1] 2009) and B viruses; respiratory syncytial virus; human metapneumovirus; HBoV; parainfluenza virus 1–4; human coronaviruses HKU1, NL63, 229E, and OC43; human rhinoviruses; human enteroviruses and parechoviruses; and adenoviruses. *Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophila*, and *Bordetella pertussis* were analyzed as described (*6*–*8*).

A single virus was detected in 22/102 (21.5%) tissue specimens, with HBoV being the most frequent (18/102, 17.6%), followed by rhinovirus (2/102, 1.9%), coronavirus 229E, and influenza A pandemic (H1N1) 2009 virus (1/102, 0.9%). HBoV was detected in specimens collected during July–September (13/18) and during February and March (5/18). All positive results were confirmed by single real-time PCR (*9*). For 14 patients, 2 different mucosal samples were tested and gave identical results. No multiple viral infections and no bacteria were detected. Median patient age was 51.2 years (range 14.4–74.2 years) for HBoV-positive and 47.6 years (range 13.3–88.1 years) for HBoV-negative samples. C_t analysis in single real-time PCR revealed a median C_t of 31 (range 28–38), corresponding to 200 genome equivalents/10^6 cells (range 3–1.8 × 10^4 copies/10^6 cells). No correlation between C_t value and patients' age was observed (r^2 = 0.008; data not shown). No underlying disease was diagnosed for 13/18 HBoV-positive patients, whereas 2/18 and 3/18 patients had chronic obstructive pulmonary and oncologic disease, respectively.

Nasal swabs and EDTA-blood samples were obtained from 17/18 and 7/18 HBoV-positive patients, respectively. No HBoV was detected in any swabs or EDTA-blood samples available, indicating no virus shedding in the respiratory tract and no viremia. However, the 2 patients with rhinovirus-positive samples obtained from biopsy also had rhinovirus RNA detectable in nasal swabs, suggesting rhinovirus infection. Unfortunately, no nasal swab was available from the 2 patients whose sinus biopsy samples

were positive for HCoV 229E and influenza A virus.

In this study, we simultaneously analyzed tissue specimens of paranasal sinuses and nasal polyps, as well as nasal swabs and blood samples, for a broad panel of viruses and atypical bacteria. To avoid seasonal bias, specimens were collected over a 1-year period and exclusively obtained from patients undergoing elective surgery in the absence of acute respiratory symptoms.

The finding that HBoV was present as a single virus in 18/22 virus-positive biopsy samples is intriguing. Moreover, the fact that no HBoV DNA was detected in nasal swabs or EDTA-blood samples indicates no active HBoV infection. In previous studies, HBoV DNA was frequently identified in the adenoids and tonsils of children (*2*,*5*,*10*). However, in contrast with our findings, detection of HBoV was mostly associated with other viruses, suggesting that co-virus–induced cellular damage might contribute to bocavirus reactivation and replication (*5*). Our findings indicate that persistence of viral nucleic acid in sinus mucosa might be a special advantage of HBoV, although the relevance of this observation remains unclear. Whether this presence as a single virus means a dead end for HBoV infection, true latency including the potential of reactivation, or a role in the pathogenesis of clinical conditions requiring surgery warrants future studies.

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Mixed Genotype Infections with Hepatitis C Virus, Pakistan

To the Editor: The prevalence of hepatitis C virus (HCV) infection is high (8% of the population) in Pakistan (*1*). HCV is an RNA virus that has a high mutation rate. This high rate results in extensive genetic heterogeneity, and HCV isolates are found as either quasispecies or genotypes (*2*). Humans can be co-infected with ≥1 genotype (mixed genotype infection) of this virus (*3*). The rate of HCV mixed genotype infections is extremely variable for different regions and for the same group of patients tested by using different assays (*4*). Thus, it is difficult to determine the prevalence of mixed genotype infections by currently available assays, including direct DNA sequencing, because they are designed to identify only the HCV genotype dominant in that particular population. Consequently, genotypes present at lower frequencies could be missed or mistyped (*5*).

To determine the prevalence of HCV mixed genotype infections, we retrospectively analyzed genotyping data for paired serum samples from 22,125 HCV-infected patients during the past 11 years (March 2000–May 2010) for all regions in Pakistan by using molecular-based genotype-specific methods (*6*,*7*). A total of 12,036 (54.4%) were male patients and 10,089 (45.6%) were female patients.

Table. Distribution of mixed genotype infections with hepatitis C virus in 1,007 patients, by age, Pakistan, March 2000–May 2010

Genotype	Mixed genotype infection	Patient age, y								Total no. infections
		0–10	11–20	21–30	31–40	41–50	51–60	≥60	Unknown	
1	1a + 1b	0	0	2	5	6	7	0	1	21
1 + 2	1a + 2a	0	0	2	1	2	0	0	0	5
	1b + 2a	0	0	0	1	2	1	0	1	5
	1b + 2b	0	0	1	0	0	0	0	0	1
1 + 3	1a + 3b	0	3	4	4	3	1	0	0	15
	1a + 3a	0	4	29	51	26	16	2	17	145
	1c + 3a	0	1	2	2	1	0	0	1	7
	1b + 3b	0	2	3	2	1	0	0	0	8
	1b + 3a	0	7	45	90	79	32	3	31	287
1 + 4	1a + 4	0	0	0	0	3	1	0	1	5
2 + 3	2a + 3a	0	2	5	9	4	3	0	1	24
	2a + 3b	0	1	5	8	5	3	0	1	23
	3a + 2b	0	0	1	2	2	0	0	1	6
3	3a + 3b	1	15	94	142	87	46	13	43	441
3 + 4	3b + 4	0	1	0	2	2	1	0	0	6
	3a + 4	0	0	0	2	4	0	0	0	6
3 + 6	3a + 6a	0	0	0	1	0	1	0	0	2
Total		1	36	193	322	227	112	18	98	1,007

The sensitivity and reliability of the assay we used has been assessed and found to be superior to restriction fragment length polymorphism analysis and serotyping methods for detection of mixed genotypes in a viral population. Our method can detect a small amount (8.3%) of HCV RNA in a mixed genotype population (*7*). Restriction fragment polymorphism analysis can detect 2 genotypes only if 1 of them represents ≥41.6% of the genotypes in a mixed genotype population.

Of 22,125 HCV RNA-positive serum samples, type-specific PCR bands were observed in 18,181 (82.2%) samples and 3,944 (17.8%) were not typeable. A total of 1,007 (5.5%) patients had HCV mixed genotype infections.

The distribution of mixed genotype infections in 1,007 patients is shown in the online Appendix Figure (www.cdc.gov/EID/content/17/8/100950-appF.htm). Infection with mixed genotype 3a + 3b was most prevalent (43.79%). Age distribution of patients with mixed genotype infections is shown in the Table. Approximately 33% of patients with mixed genotype infections were 31–40 years of age and 22.5% were 41–50 years of age.

Patterns of HCV mixed genotype infections in Pakistan are similar to those reported from India and Iran (*8*). However, the prevalence of HCV mixed genotype infections was lower (2%) (*8*) for Iran than for Pakistan. This lower rate may have been caused by use of a genotyping kit that can detect only genotypes 1a, 1b, 2, and 3a. Thus, mixed infections with other genotypes would not have been detected. A recent study in Brazil reported that mixed genotype infections were detected in 3.9% of intravenous drug users and 7.1% of former injecting drug users (*9*). These rates were similar to those in our study. In contrast, data from Sweden and Russia showed no mixed genotype infections in serum samples of chronically infected intravenous drug users, hemodialysis patients, and patients with hemophilia (*10*).

Women (288/7,390, 3.89%) in Pakistan had significantly fewer HCV mixed genotype infections than men (719/10,791, 6.66%) ($p<0.01$). This finding might be the result of women having fewer risk factors for contracting mixed genotype infections. Possible risk factors for infection with mixed genotype infections analyzed were blood transfusions and use of blood products (51.3%); multiple use of needles or syringes (18.4%); sharing razors during shaving or circumcision, piercing instruments, nail clippers, and toothbrushes (13.7%); and major or minor dental surgery (9.5%). Mode of transmission was not clear for 7.1% of the patients.

In conclusion, the prevalence of HCV mixed genotype infections in Pakistan is higher than previously reported and higher among men ($p<0.01$). Comprehensive and detailed investigations are warranted to evaluate the clinical role of chronic HCV mixed genotype infections, provide essential information that can be used to determine type and duration of therapy needed, and predict disease outcome.

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West Nile Virus Aseptic Meningitis and Stuttering in Woman

To the Editor: West Nile virus (WNV), a mosquito-borne flavivirus, is closely related to St. Louis encephalitis virus and Japanese encephalitis virus (JEV). Most cases of WNV have been mild, but neuroinvasive disease has been observed, especially among older persons and immunocompromised persons (*1*,*2*). The most common neurologic manifestations of WNV are aseptic meningitis, meningoencephalitis, and encephalitis with or without acute flaccid paralysis (*3*). Other less common neurologic manifestations include Guillain-Barré syndrome, chorioretinitis, stroke-like symptoms, and unilateral brachial plexopathy (*4*,*5*).

We report a case of WNV aseptic meningitis in a 39-year-old immunocompetent woman who had severe headache with new-onset stuttering. Her medical history included lumbar disc herniation and migraines, for which she was taking sumatriptan. Her symptoms started ≈2 weeks before hospitalization and included a severe generalized headache initially thought to be a migraine, but sumatriptan resulted in no improvement. A few days later, she had fever and was intermittently stuttering. She denied recent travel or animal exposure but admitted to having received multiple mosquito bites during the preceding weeks.

At admission, she had a temperature of 101.3°F, pulse rate of 92 beats/min, blood pressure of 130/80 mm Hg, and respiratory rate of 16 breaths/min. She appeared mildly ill but was alert and oriented with no nuchal rigidity, photophobia, rash, or limb weakness. Results of a physical examination were unremarkable, and results of a neurologic examination were notable only for stuttering. Laboratory test results included a leukocyte count of 12,300 cells/mm^3 (63% neutrophils, 29% lymphocytes, 7% monocytes, 1% basophils) and a platelet count of 204,000 cells/mm^3. Other laboratory values were unremarkable, and levels of serum transaminases and creatinine phosphokinase were within reference ranges. Cerebrospinal fluid (CSF) was clear and contained 37 leukocytes/mm^3 (2% neutrophils, 78% lymphocytes, 20% monocytes), 2 erythrocytes/mm^3, a glucose level of 68 mg/dL, a protein level of 36 mg/dL, and a lactic acid level of 2.1 meq/L. No abnormalities were found on a cranial computed tomography scan.

The patient began treatment with acyclovir, 10 mg/kg intravenously, every 8 hours for 3 days. On hospital day 2, she underwent magnetic resonance imaging of the brain; results were within reference limits. On hospital day 3, her headache began to improve and she became afebrile, but she still stuttered occasionally. Results of CSF tests for enterovirus, herpes simplex viruses 1 and 2, and varicella zoster virus and PCR for human herpesvirus 6 were negative, and acyclovir was discontinued. On hospital day 5, she was discharged. Three days later, serum and CSF ELISA results for WNV were positive. A WNV ELISA was performed at ViroMed Laboratories (Minnetonka, MN, USA) by using a Focus Test Kit (Focus Diagnostics, Cypress, CA, USA), and the result was positive. The

patient subsequently reported that her stuttering had ceased.

A high degree of clinical suspicion for WNV infection should be considered in patients with a recent history of mosquito bites and an acute febrile illness associated with neurologic signs and symptoms (*5*). Typical CSF findings of infection with WNV include lymphocytic pleocytosis, elevated protein level, reference glucose and lactic acid levels, and no erythrocytes (*6*).

The clinical presentation of WNV infection varied widely from asymptomatic seroconversion to fatal encephalitis. It is possible, but unlikely, that the stuttering in the patient was an indication of a migraine aura. Initially, the patient reported that the headache might have been a migraine, but later reported that its associated symptoms, e.g., photophobia, were not as severe and did not last as long as her usual migraines. Further argument against migraine aura is the lack of response to her migraine medication and the fact that the stuttering continued after the headache resolved.

Because WNV resembles JEV, it is interesting to note that a case of stuttering in a young adult infected with JEV has been reported (*7*). However, the mechanism of stuttering associated with WNV is unknown. One possible explanation is myoclonic contractions of the tongue, i.e., vocal myoclonus.

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No Evidence of Dengue Virus Circulation in Rural Gabon

To the Editor: Dengue virus (DENV) is a mosquito-borne RNA virus belonging to the family *Flaviviridae*. It is composed of 4 closely related serotypes designated DENV-1–4. There are 2 transmission cycles for this virus. The endemic/epidemic cycle involves humans and the mosquito species *Aedes aegypti* and *Ae*. *albopictus*. The zoonotic or sylvatic cycle involves monkeys and sylvatic *Aedes* spp. mosquitoes (*1*).

Despite occasionally severe clinical forms, human dengue usually consists of a self-limited febrile disease often associated with asthenia, headache, rash, arthralgia, and myalgia. DENV is widely distributed throughout Asia, the Pacific, Central and South America, the Middle East, and Africa (*2*,*3*). In Africa, most DENV outbreaks have been reported in the eastern regions, and episodic cases have occurred in western regions. However, few data are available for central regions.

In Gabon, concurrent of transmission of DENV and chikungunya virus was documented in 2007 during a large outbreak of dengue (*4*). This outbreak affected Libreville and major cities in northwestern Gabon and was caused by DENV-2. DENV isolates were closely related to strains from Asia, suggesting that the outbreak resulted from recent introduction of the virus. Epidemic DENV strains are constantly moving from one region to another, and local DENV transmission from sylvatic to urban areas has been documented in some countries in Africa (*5*,*6*).

To examine possible circulation of DENV in Gabon, we tested the following for antibodies against dengue: villagers living in rural areas, pet monkeys in the same areas, and wild monkeys killed in forests for bushmeat. A total of 4,341 persons and 186 pet monkeys were sampled during July 2005–May 2008 in 220 randomly selected villages, which represented 10.3% of all villages in Gabon. Fifty wild monkeys were also sampled during October 2009–August 2010 in different regions of Gabon (Table).

DENV-specific immunoglobulin (Ig) G and IgM were detected by using capture ELISA kits (Panbio; Brisbane, Queensland, Australia) (*7*) according the manufacturer's instructions. All samples were tested with an IgG assay, which was designed to detect high antibody titers usually associated

Table. Prevalence of IgG and IgM against dengue virus in humans and nonhuman primates, Gabon*

	No. positive/no. tested (% positive)					
	Humans		Pet monkeys†		Wild monkeys‡	
Province	IgG	IgM	IgG	IgM	IgG	IgM
Estuaire	5/286 (1.7)	1/95 (1.1)	0/13 (0)	0/13 (0)	0/1 (0)	0/1 (0)
Haut-Ogooué	1/364 (0.3)	0/80 (0)	0/8 (0)	0/8 (0)	0/1 (0)	0/1 (0)
Moyen-Ogooué	3/558 (0.5)	1/113 (0.9)	0/10 (0)	0/10 (0)	0/7 (0)	0/7 (0)
Ngounié	0/146 (0)	0/60 (0)	0/51(0)	0/51 (0)	0/5 (0)	0/5 (0)
Nyanga	1/774 (0.1)	0/151 (0)	0/33 (0)	0/33 (0)	0/2 (0)	0/2 (0)
Ogooué Ivindo	5/499 (1)	0/105 (0)	0/31(0)	0/31 (0)	0/3 (0)	0/3 (0)
Ogooué-Lolo	0/416 (0)	0/90 (0)	0/11 (0)	0/11 (0)	0/3 (0)	0/3 (0)
Ogooué-Maritime	2/197 (1)	1/72 (1.4)	0	0	0/18 (0)	0/18 (0)
Woleu-Ntem	3/831 (0.4)	2/163 (1.2)	0/29 (0)	0/29 (0)	0/10 (0)	0/10 (0)
Total	20/4,341 (0.5)	5/930 (0.5)	0/186 (0)	0/186 (0)	0/50 (0)	0/50 (0)

*Ig, immunoglobulin.
†*Cercopithecus cephus, C. nictitans, C. neglectus, C. pogonias, C. solatus, Cercocebus torquatus, Gorilla gorilla, Lophocebus albigena, Mandrillus sphinx, Miopithecus ogoouensis*, and *Pan troglodytes* species.
‡*Cercopithecus cephus, C. nictitans, C. neglectus, C. pogonias, Cercocebus torquatus, Mandrillus sphinx*, and *Perodictitus potto* species.

with secondary dengue infection. All IgG-positive human samples, 910 randomly selected IgG-negative human samples, and all monkey samples were also tested by using an IgM capture ELISA.

In humans, overall prevalences of DENV-specific IgG and IgM were 0.5% (20/4341) and 0.5% (5/930), respectively, and only 1 of 20 IgG-positive samples was IgM positive. No IgG or IgM against DENV was detected in pet or wild monkeys. Although the IgG capture ELISA did not enable us to detect low and medium titers, absence of IgM and IgG against DENV in pet and bushmeat monkeys indicates a lack of recent or secondary infections. In humans, the low prevalences of IgM and IgG against DENV suggest that DENV does not circulate actively in rural Gabon, although the 2 assays do not detect late primary DENV infection.

Although these ELISAs have been extensively validated (diagnostic sensitivity of 98.6% and specificity of 99.4%) (*7*), some of our samples may have shown false-positive results because IgG cross-reactivity between flaviviruses can occur (*8*). The rare serum specimen positive for IgG against DENV may also indicate a low level of DENV circulation in rural Gabon, or may be related to travel to and from neighboring countries. After the outbreak in Gabon, DENV surveillance with active detection of febrile human cases is needed to determine whether this virus will become endemic to this area.

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Enteric Coronavirus in Ferrets, the Netherlands

To the Editor: Coronaviruses (CoVs) are enveloped, positive-sense, single-stranded RNA viruses that can cause acute and chronic respiratory, enteric, and central nervous system disease in a variety of animal species (*1*). Recently, a novel ferret enteric CoV (FRECV) was indentified in domesticated ferrets (*Mustela putorius*) in which epizootic catarrhal enteritis had been diagnosed; the illness was characterized by foul-smelling green diarrhea with high mucus content, lethargy, anorexia, and vomiting (*2*). Another ferret CoV emerged in ferrets for which systemic pyogranulomatous inflammation, resembling the clinical and pathologic features of feline infectious peritonitis (FIP), was diagnosed (*3*–*5*).

In 2010, we investigated the prevalence of CoV antibodies in 85 asymptomatic ferrets obtained from 1 ferret farm in the Netherlands. Previous studies have shown that antibodies against different members of the α-CoVs show broad cross-reactivity (*6*). We used FIP virus (FIPV)–infected cells to screen for CoV antibodies in an indirect immunoperoxidase assay. Because 32% of the ferret serum had a titer >20 in this assay, we concluded that these animals most likely had been exposed to a CoV. To test for a CoV in these animals, we analyzed RNA extracted from rectal swabs with a degenerate set of primers to amplify a conserved region within open reading frame (ORF) 1 of CoVs (*7*). Remarkably, 36 (42%) of samples tested were PCR positive, suggesting excretion of a CoV by a substantial proportion of ferrets tested. To corroborate that the CoV detected in the rectal swabs was a ferret CoV (FRCoV), we amplified and sequenced the nucleocapsid protein by using primer pair 5′- TCCC CGCGGGGCTGGCAACGGACAA CGTGT-3′ and 5′-CCCAAGCTTTTA GTTGACTAATAATTTCA-3′. Phylogenetic analyses of 2 of the sequences obtained indicated a variant nucleocapsid that was similar to other FRCoVs described previously but that did not group with 1 of these sequences directly (Figure). Amino acid alignment of 1 of these sequences (FRCoV-511c) with FRECV-MSU2 demonstrated 91.8% identity and 95.7% similarity, whereas this virus shows 89.3% identity and 95.2% similarity to ferret systemic CoV (FRSCV-MSU1).

On the basis of obtained and published nucleocapsid sequences (*2*,*4*), we developed a TaqMan reverse transcription PCR to detect viral RNA using the following primers and degenerate probe: forward, 5′-TTGGAAAGAATG GTGCTAAAACTG-3′; reverse, 5′-CA TTAGGCACGTTACCATCAAATT -3′; and probe, 5′-TAGGAACRCGT GGCACCAACCAA-3′. Using this more specific and sensitive assay, we detected viral RNA in 63% of the rectal swabs tested; other CoVs including FIPV, severe acute respiratory syndrome–CoV, and human CoV NL-63, were not amplified by this assay (data not shown). All samples that had tested positive in the ORF1-CoV PCR were confirmed positive with this TaqMan assay. To analyze FRCoV in ferrets from geographically distinct sites, we tested fecal samples from 90 animals without signs of disease (including epizootic catarrhal enteritis) from 39 different locations in the Netherlands. FRCoV nucleocapsid TaqMan and ORF1-CoV PCR demonstrated that 61% of the fecal samples and 72% of the locations were positive. Multiple testing of fecal swabs at different times and use of FRCoV-specific antibody assays would probably further increase the FRCoV prevalence rate.

Further partial sequence analysis of the spike gene by using primers 5′-AARRTTAATGAGTGTGTGMG DTCA-3′ and 5′- CAACTCTYTTAA GCCARTCAAGG-3′ clearly showed that these viruses are more closely related to systemic FRCoVs than to FRECV (Figure). Amino acid alignment of 1 of these sequences (FRCoV-511c) with FRECV-MSU2 demonstrated 78% identity and 89% similarity, and FRCoV-511c shows 86% identity and 92% similarity to FRSCV-MSU1.

After identification of severe acute respiratory syndrome CoV in humans in 2003 and related viruses in civet cats and bats, an increase in CoV surveillance in different animal species resulted in identification and characterization of a broad range of previously unrecognized CoVs (*8*). Here we report an enteric FRCoV circulating in the Netherlands in a high percentage of asymptomatic ferrets. The ferrets tested did not have a previous record of foul-smelling green diarrhea described previously to be associated with FRECV, a virus detected in the United States and further characterized in 2006 (*2*). On the basis of the phylogenetic analysis of the spike sequences, FRECV-MSU2 might have evolved through recombination with some other unknown CoV. Alternatively, the viruses isolated in the Netherlands grouped more closely with FRCoVs causing systemic disease (e.g., FRSCV-MSU1). Thus far, no evidence indicates that the animals testing positive for the enteric CoV showed clinical disease that pointed to pyogranulomatous inflammation, necrosis with or without perivasculitis, and vasculitis in abdominal and visceral organs associated with the systemic variant. Further genetic characterization of these enteric FRCoVs variants might show genetic differences that could explain the apparent pathotypes of these 2 FRCoVs. FRCoVs might evolve through mutation or deletion into viruses that cause systemic

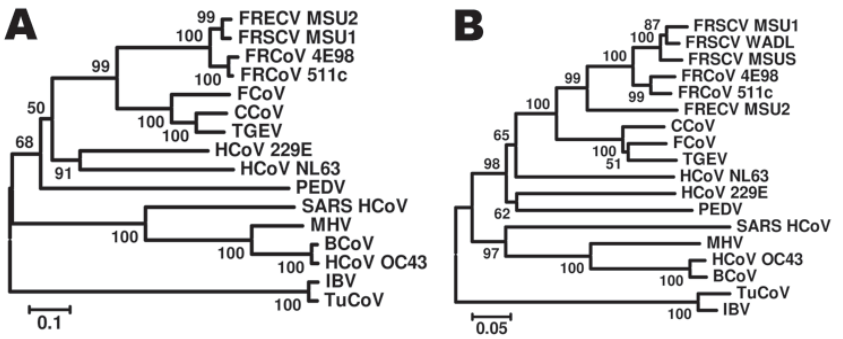

Figure. Phylogenetic tree based on nucleotide sequences of the nucleocapsid (A) and spike gene (B) of ferret coronaviruses (FRCoVs) 4E98 (GenBank accession nos. JF260916 and JF260914, respectively) and 511c (accession nos. JF260915 and JF260913, respectively) and other coronaviruses (CoVs). Partial nucleotide sequences were aligned by using ClustalX (www.clustal.org) and a neighbor-joining Kimura 2-parameter model with 1,000 bootstrap replicates; avian CoVs were used as outgroup sequences (p-distance; allowing gaps or missing data). Other CoVs shown (abbreviation, GenBank accession number): ferret coronavirus (FRECV MSU2, GU338457); ferret systemic coronavirus (FRSCV MSU1, GU338456); feline coronavirus (FCoV, DQ010921); canine coronavirus (CCoV, AY342160); transmissible gastroenteritis virus (TGEV, AF104420); human coronavirus (HCoV NL63, DQ445911); porcine epidemic diarrhea virus (PEDV, AF353511); severe acute respiratory syndrome coronavirus (SARS-HCoV, NC_004718); murine hepatitis virus (MHV, AY700211); bovine coronavirus (BCoV, U00735); infectious bronchitis virus (IBV, AY363968); and turkey coronavirus (TuCov, AF111997). Scale bars indicate nucleotide substitutions per site.

disease, or alternatively, different FRCoVs are circulating, analogous to the hypotheses put forward to explain the occurrence of FIP (*9*,*10*). Given the use of ferrets in testing efficacy of influenza virus vaccines and the propensity of CoVs to cross species barriers, further surveillance and investigation of the biology of these emerging FRCoVs is warranted.

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Seroepidemiology of Saffold Cardiovirus Type 2

To the Editor: Saffold virus (SAFV) is a new human virus belonging to the genus *Cardiovirus* of the family *Picornaviridae* (*1*–*6*). The virus has also been named human Theiler's-like cardiovirus (*4*). To date, 8 SAFV genotypes are known, based on molecular variation in the P1 region, which codes for the viral capsid (*5*). SAFVs are detected in respiratory and fecal samples from infants and children <6 years of age. The virus is generally detected at low frequency (0.5%–3%) in the general population, but in Afghanistan and Pakistan higher incidences have been reported (10%–12%) (*5*). Detection is mainly based on molecular techniques because virus isolation is cumbersome. Other than gastroenteritis, no clear disease manifestations are known (*1*–*8*), although recently, researchers in Japan have suggested that for a select group of children, SAFV infection may cause pharyngitis and tonsillitis (*7*). In rodents, however, cardioviruses are serious pathogens that can cause myocarditis, meningoencephalitis, and pancreatitis.

Previously, we have shown that SAFV-3 infections are ubiquitous and are transmitted early in life (*6*). This conclusion was based on antibody testing by virus neutralization, a highly specific test that can discriminate serotypes within a single species, but formal proof for existence of serotypes was lacking. Extrapolated to other genotypes, the finding would indicate a high infection rate for SAFVs, which is at odds with the low detection frequency reported in most studies. Most SAFV-2 isolates, from our laboratory and others, grow poorly in cell culture, which hampers reading of neutralization. Recently, however, a SAFV-2 strain was isolated in Finland that grows well in HeLa cells and shows clear cytopathic effects (SAFV-2-FIN2008, GenBank accession no. FR682076; S. Blomgvist et al., unpub. data). This strain enabled us to set up a virus neutralization test similar to that described for SAFV-3 (*6*). For comparison, we used strain SAFV-3(NL2007) (GenBank accession no. FM207487), which was isolated in Nijmegen (*6*). The virus neutralization test was performed on HeLa cells with 100 $TCID_{50}$ of virus per serum dilution. Virus–serum mixtures were incubated for 1 hour at 37°C and overnight at 7°C to stabilize virus–antibody complexes (*6*). Human serum samples submitted previously were tested by using 3-fold dilution steps and duplicate testing (*6*). The results are presented in the Table. A low seroprevalence was found for SAFV-2 and SAFV-3 at 9 months of age, which is at the nadir of immunoglobulin G levels in infants. At the age of 24 months, a high seroprevalence of antibodies was found in the Netherlands for SAFV-2 and -3, pointing to early acquisition of infection with both strains. In Finland, the seroprevalence for SAFV-2 and -3 was lower in young children, which suggests somewhat lower infection rate similar to what has been reported for enteroviruses (*9*). A high seroprevalence of 97%–100% was found in persons >4 years of age in Cameroon, Indonesia, and the Netherlands (Table). A high prevalence of SAFV-2 antibodies was recently also reported in blood donors from the United States (*8*). Thus, the seroepidemiology of SAFV-2 is similar to that of SAFV-3. As depicted in the last 3 columns of the Table, on several occasions serum samples independently neutralized either SAFV-2 or SAFV-3, which suggests that the viruses behave as different serotypes.

In conclusion, SAFV-2 and SAFV-3 show an almost identical epidemiologic pattern with infection acquired early in life and with a high seroprevalence in different continents. The outcome is concordant with universal occurrence of infection by both genotypes. Because several times there was a clear discrepancy between antibody titers against one or the other of the 2 genotypes, we conclude that they behave as separate serotypes, although weak cross-reactivity (below the detection limit of 1:15) cannot be excluded. Accepting that SAFV genotypes correspond with existence of different serotypes, the infection rate in the first years of life must be quite high, similar to that for human parechoviruses and enteroviruses, which are found in 15%–18% of stool samples from children <5 years of age (*10*). The

Table. SAFV-neutralizing antibodies in blood samples from humans of different ages and from different geographic regions*

Country	No. samples	Patient age†	Years collected	No. (%) samples				
				SAFV-2 pos‡	SAFV-3 pos‡	SAFV pos§	SAFV-2 pos + SAFV-3 neg	SAFV-2 neg + SAFV-3 pos
Netherlands	29	9 mo	2006–2007	15 (52)	4 (14)	15 (52)	11	0
Netherlands	26	24 mo	2006–2007	21 (81)	20 (77)	25 (96)	5	4
Netherlands	30	18–39 y	2004	30 (100)	29 (97)	30 (100)	1	0
Finland	30	2–2.5 y	1997–1998	10 (33)	21 (70)	24 (80)	3	14
Cameroon	29	5–15 y	1997	28 (97)	28 (97)	29 (100)	1	1
Indonesia	30	4–40 y	1997–1998	30 (100)	30 (100)	30 (100)	0	0

*SAFV, Saffold virus; pos, positive; neg, negative.
†Samples were collected from patients at this age or within this age range.
‡Titer ≥15.
§Cumulative positive for antibodies against SAFV-2 and/or SAFV-3.

low SAFV detection rate remains thereby difficult to explain.

The outcome can be explained by a short duration of virus excretion in stool, which, however, is unlikely for an infection spreading by the fecal–oral route. Alternatively, it may be that the virus is unstable in stool and rapidly degrades, such that fecal samples are inadequate for diagnosis of the infection. Other specimens, however, such as respiratory samples, yielded also low numbers of positive findings (*4*). Remarkably, the study with a high prevalence of positive stool samples made use of primers selected in a conserved region of 2C helicase (*5*), whereas other studies used primers in the 5′ noncoding region (*3*,*4*). Hence, a difference in sensitivity between the different PCRs may be responsible for the discrepancy between seroepidemiology and the low diagnostic yield by PCR. This discrepancy, however, awaits further investigation.

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Alkhurma Hemorrhagic Fever in Travelers Returning from Egypt, 2010

To the Editor: The report of 2 visitors from Italy being infected by Alkhurma hemorrhagic fever virus (AHFV) in southeastern Egypt near the border with Sudan (*1*) provides useful data to help clarify the evolutionary origin of these tick-borne flaviviruses. AHFV was first isolated in Saudi Arabia and is associated with camel ticks (*2*). It is a genetically close relative of Kyasanur Forest disease virus, which was first isolated in India in 1957. Following the original isolation of Kyasanur Forest disease virus, there was no clear explanation for its apparent isolation in the Indian forests. Indeed, its subsequent discovery in southern China (*3*) suggested that migratory birds might carry the infected ticks to or from that region.

The most likely explanation for these outbreaks of hemorrhagic disease now begins to fit a pattern that can be interpreted in terms of the diseases' evolutionary origin in Africa. Thousands of animals are annually transported from Africa and other countries to Mecca, Saudi Arabia, to meet the human demand for food and transport during the Hajj. Many of these animals, including camels, are infested with ticks that may carry AHFV and thus provide the source of this human infectious agent. Phylogenetic evidence had previously suggested that the tick-borne encephalitic flavivirus serocomplex originated in Africa and gradually evolved and dispersed across the Northern Hemisphere of the Old World (*4*,*5*). This concept is totally consistent with the discoveries of AHFV in Saudi Arabia and now in southeastern Egypt. Thus, Africa is a likely source of infected ticks that are regularly moved between Africa

and Saudi Arabia. This concept of an African evolutionary origin for these viruses could readily be tested by serologic investigation of humans and animals and also by analysis of ticks from this region of Africa.

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In Response: The concept suggested by Charrel and Gould (*1*) of an African evolutionary origin for these tick-borne flaviviruses and their dispersal across the Northern Hemisphere raises concerns over possible spread of a new potentially dangerous infection outside its country of origin. This awareness should alert physicians in Western countries to pathogens that cause unspecific, unusual, or totally unknown clinical signs. We agree that more research is needed in human and animal health, as well as in entomologic and environmental studies, especially in light of the recent data suggesting a nonexclusive role of ticks as vectors for human infection with Alkhurma virus and the hypothesis of human-to-human transmission (*2*).

Past experience with emerging diseases in travelers (Crimean-Congo hemorrhagic fever, Lassa fever, Marburg hemorrhagic fever) or with autochthonous spread of imported diseases (chikungunya, West Nile virus disease, malaria) indicates a consistent delay in the diagnosis of first or sporadic cases, leading to inappropriate or untimely treatment of some of the patients. To confront the problem of unusual and emerging pathogens, Western countries must invest in evidence-based and integrated strategies of preparedness and response.

First, the frontline physicians' ability to recognize, diagnose, and treat illnesses caused by unusual pathogens should be improved through training covering rare and tropical diseases. Second, a system of timely information and alerts about threats posed by new infectious diseases should be set up. Third, concentrating clinical samples in virology laboratories with proven experience in detecting emerging pathogens is crucial for comprehensive and rapid differential diagnosis. It must be also remembered that no commercial tests are available for serologic or molecular detection of many rare pathogens or for differential diagnosis. And, finally, laboratory diagnosis is often made difficult by antibody cross-reactivity, as documented in our article (*3*).

For Alkhurma virus, further research is needed in the animal setting because little is known about length and severity of illness, duration of viremia, and modes of animal-to-animal and animal-to-human transmission; we need to better understand the role of vectors to limit the spread of the disease.

Fabrizio Carletti, Concetta Castilletti, Antonio Di Caro, Maria R. Capobianchi, Carla Nisii, Fredy Suter, Marco Rizzi, Alessandra Tebaldi, Antonion Goglio, Cristiana Passerini Tosi, and Giuseppe Ippolito

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Max eld Parrish (1870–1966). ***Masquerade*** **(1922) Oil on board (43.2 cm × 35.6 cm).** High Museum of Art, Atlanta, Georgia, USA. Gift of Ruth Jernigan McGinty

We are such stuff / as dreams are made on

—William Shakespeare, The Tempest

Polyxeni Potter

"We are all doubles… but at night… we meet our sleeper," wrote Tom Stoppard in his play "Hapgood" (1988). He was approaching a common theme, identity, and how we use disguises to change it. In literature, as in life, we turn the world into a carnival to address personal, social, and cultural objectives. In microbiology carnival de-structuring abounds within the living cells of organisms. And in art, identity and its mysteries and disguises have occupied many, who examine it from all angles, exposing its multifaceted and diverse nature. Maxfield Parrish takes a playful look at it in his own *Masquerade*, on this month's cover.

"I don't know what people find or like in me," Parrish once said. "I'm hopelessly commonplace."And although he might not have intended it this way, a modest and unassuming man, he had become ubiquitous in his lifetime. In 1925, one in four American households owned one of his prints. His wildly successful calendar of nature scenes alone sold more than a million copies. His box covers for Crane's Chocolates flew off the shelves. His massive murals, magazine covers, and book illustrations captured the imagination of the public. "As far as the sale of expensive reproductions is concerned, the three most popular artists in the world are Vincent van Gogh, Paul Cézanne, and Maxfield Parrish," asserted Time magazine in 1936.

A native of Philadelphia, Pennsylvania, Parrish knew none of the financial hardships of the proverbial starving artist. His privileged family recognized and nurtured his talent from early childhood. They traveled to Europe for extended periods to broaden his art horizons and appreciation. In his native state he attended Swarthmore

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College's preparatory school; Haverford College, where he studied architecture; the Pennsylvania Academy of Fine Arts; and the Drexel Institute of Art.

His painted dreams, imaginary landscapes, mythical animals, romantic creatures, and classical figures in vivid distinctive hues became fixtures of storybooks and part of the vernacular. All the while he shaped the Golden Age of Illustration and went on to influence Norman Rockwell, who once said about Parrish, "He was one of my Gods."

Though his long life was not entirely immune to trials, each crisis seemed to propel him to a higher level of greatness. Typhoid fever in his early youth and prolonged convalescence confined him enough to allow etching and drawing lessons from his father, also an artist. And when tuberculosis sent him to Arizona for the hot and dry climate, the Desert Southwest transformed him from illustrator to landscape painter. "I'm done with girls on rocks," he declared. "I'm quitting my rut now while I'm still able."

His commercial art ventures gained early national attention. He was interested in the business of art. He created works for the color print market and romantic scenes for a public that craved them. "I've always considered myself a popular artist." His early success enabled him to build a home and studio in Cornish, New Hampshire, setting of his *Land of Make Believe*, where he lived and worked for the rest of his life in the natural environment he loved and recreated in his works. One of his four children, Jean Parrish, became an accomplished artist in her own right.

Parrish embraced use of technology in his work. He had a machine shop in the studio to make his own tools and used photography extensively, often several photographs in one painting. He produced costumes for his fantastic landscape sets. The dramatic palette came from an elaborate technique, secret of the old masters. Over a smooth white ground, he applied several thin layers of transparent paint, each followed by a layer of glaze to achieve luminescence. A certain blue color was named after him, "Parrish blue."

He devised innovative techniques that anticipated op art, a method of abstraction. Movement, flashing, or other similar optical effects could be achieved by combining photography with geometric shapes to distort figures. Parrish belonged to no formal movement or school. He developed his own unique style. He influenced the likes of Andy Warhol and has been compared to Salvador Dali.

In *The Masquerade*, many Parrish elements come into play. Like many of his paintings, this one has a mixture of fantasy and humor, realism, a dream-like quality, and a generous sprinkling of pageantry and optical illusion. The large red mantle is draped against a repetitive geometric pattern, alternating yellow and black squares that vibrate under direct scrutiny. Otherworldly hues form a natural backdrop of trees, flowers, and leaves.

The mosaic effect of the predominant fabric in this work, along with the carnival disguise, invites a Tom Stoppard interpretation of identity, its changes, and the masquerade. This issue of Emerging Infectious Diseases, with its emphasis on viral infections, provides a tempting overview of de-structuring opportunities in virology. Its own mosaic of viral diseases, the Table of Contents provides an overview of the tiny agents that invade living cells of organisms, spread in many different ways and cause serious illnesses, from gastroenteritis to dengue. Viruses inside living organisms are carnival experts. Their particles enter the host cell and, masquerading as the regular intracellular messengers, subvert the cellular machinery to make new viral components. When the new virus particles are assembled, sometimes with costume elements modified to escape recognition, they leave the cell and "party on" in new host cells. Red cape goes in, checkered robe comes out, until "… our little life / is rounded with a sleep."

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Risk-based Estimate of Effect of Foodborne Diseases on Public Health, Greece

Endemic Scrub Typhus–like Illness, Chile

Seroepidemiologic Study of Pandemic (H1N1) 2009 during Outbreak in Boarding School, England

Leptospirosis as Frequent Cause of Acute Febrile Illness in Southern Sri Lanka

Role of *Chlamydia trachomatis* in Miscarriage

Classical and Atypical Bovine Spongiform Encephalopathy in a Homologous Bovine Prion Protein Context

Pandemic (H1N1) 2009 Outbreaks and Preparedness at Children's Hospitals

Multiple Reassortment of Pandemic (H1N1) 2009 and Endemic Influenza Viruses in Swine, United States

Mycobacterium chelonae-abscessus Complex and Sinopulmonary Disease, Northeastern United States

Central Line–associated *Nocardia* Bacteremia in Cancer Patients

Intrahousehold Transmission of Pandemic (H1N1) 2009, Victoria, Australia

Geographic Distribution of Endemic Fungal Infections among Older Persons, United States

Effects of Pandemic (H1N1) 2009 on Remote and Indigenous Groups, Northern Territory, Australia, 2009

Invasive *Haemophilus influenzae* Disease in Adults, Utah, USA

Epidemiologic Modeling with FluSurge for Pandemic (H1N1) 2009, Queensland, Australia

Estimating Effect of Antiviral Drug Use during Pandemic (H1N1) 2009 Outbreak, United States

Hospitalized Patients with Pandemic (H1N1) 2009, Kenya

Wild Rodents as Natural Hosts of *Candidatus* Neoehrlichia mikurensis, Southern Sweden

Complete list of articles in the September issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

August 8–19, 2011
12th International Dengue Course
Havana, Cuba
http://www.ipk.sld.cu/cursos/dengue2011/index.htm

August 27–31, 2011
2011 Infectious Disease Board Review Course—16th Annual Comprehensive Review for Board Preparation
Ritz-Carlton, Tysons Corner
McLean, VA, USA
http://www.IDBoardReview.com

September 17–20, 2011
51st Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)
McCormick Place Chicago
Chicago, IL, USA
http://www.icaac.org

October 12–15, 2011
The Denver TB Course
Denver, CO, USA
http://www.njhealth.org/TBCourse

October 20–23, 2011
49th Annual Meeting of the Infectious Diseases Society of America
Boston, MA, USA
http://www.idsociety.org/idsa2011.htm

November 6–8, 2011
2011 European Scientific Conference on Applied Infectious Diseases Epidemiology (ESCAIDE)
Stockholm, Sweden
http://www.escaide.eu, or email escaide.conference@ecdc.europa.eu

November 16–19, 2011
7th World Congress of the World Society for Pediatric Infectious Diseases (WSPID 2011)
Melbourne, Victoria, Australia
http://www.kenes.com/wspid2011/mailshot/ms3.htm

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Article Title

Deaths Associated with Human Adenovirus-14p1 Infections, Europe, 2009–2010

CME Questions

Activity Evaluation

1. Based on the above case series by Dr. Carr and colleagues, which of the following statements about demographic, genetic, and transmission-related factors associated with human adenovirus-14p1 (HAdV-14p1) infections recently detected in Ireland is most likely correct?

A. These HAdV-14p1 variants are phylogenetically different from those previously detected in the United States
B. Most of the patients were young women
C. Geographic clustering was observed
D. Efficient transmission of HAdV-14p1 appears to require close physical contact

2. You are a public health of cial asked to consult with local healthcare facilities in a European region where HAdV-14p1 infections have been detected. Based on the above study, which of the following statements about anticipated clinical characteristics and complications associated with HAdV-14p1 infections is most likely to appear in your report?

A. Initial presentation is usually with gastrointestinal symptoms
B. A very small proportion of patients are likely to have significant comorbidity
C. A little less than half of patients are likely to have an unremarkable full recovery
D. No death has been reported in an immunocompetent individual

3. As the public health of cial described in question 2, which of the following recommendations would you be most likely to make in your report, based on the clinical and public health implications of the case series described above?

A. Surveillance for HAdV-14p1 should remain the same
B. Healthcare workers should be advised that HAdV-14p1 infection is significantly more severe than that of other HAdV serotypes
C. HAdV-positive specimens should be retrospectively investigated for HAdV-14p1, particularly from untyped viruses from patients who had severe or fatal disease without established etiology
D. HAdV-14p1 should not be included in the differential diagnosis of community-acquired pneumonia

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Risk Factors for Pandemic (H1N1) 2009 Seroconversion among Adults, Singapore, 2009

CME Questions

Activity Evaluation

1. You are a member of a public health task force with the assignment to reduce the impact of in uenza on your community. The task force director asks you to investigate risk factors for infection with pandemic (H1N1) 2009.

Which of the following demographic variables was associated with a higher risk for pandemic (H1N1) 2009 seroconversion?

A. Age between 20 and 29 years
B. Age older than 60 years
C. Male sex
D. 3-room or smaller dwelling

2. Which of the following factors most likely reduced the risk for pandemic (H1N1) 2009 seroconversion?

A. Malay ethnicity
B. More international travel
C. Working outside of the home
D. Regular tea consumption

3. Which of the following public health interventions may be most effective in preventing the spread of pandemic (H1N1) 2009?

A. Limiting theater seating to every other seat
B. Closing restaurants
C. Improving air circulation on public transportation
D. Limiting seating at places of worship

4. Which of the following laboratory values was associated with a reduced rate of pandemic (H1N1) 2009 seroconversion?

A. High baseline antibody titers to pandemic (H1N1) 2009
B. Low baseline antibody titers to pandemic (H1N1) 2009
C. Normal serum glucose levels
D. High serum white blood cell counts

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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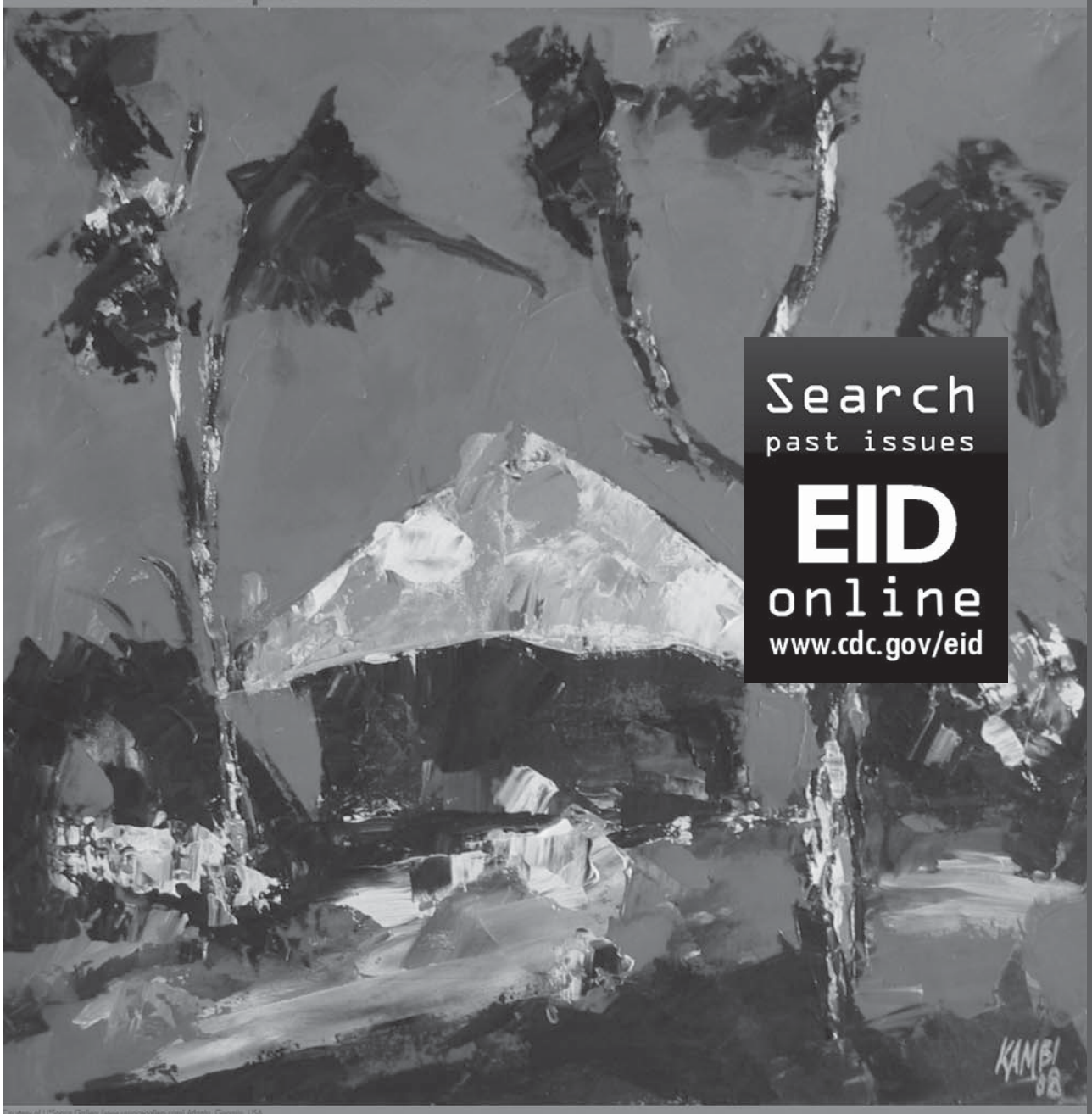
July 2011

Parasitic and Tropical Infections



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Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

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 figures as separate files, in the native format when possible (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpg or .tif files. Other file formats may be acceptable; contact fue7@cdc.gov for guidance. Figures should not be embedded in the manuscript file. Use color only as needed. Use Arial font for figure lettering. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced to print size. Large figures may be made available online only. Place figure keys within the figure; figure legends should be provided at the end of the manuscript file.

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Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

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

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only and should contain 500–1,000 words. They should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.