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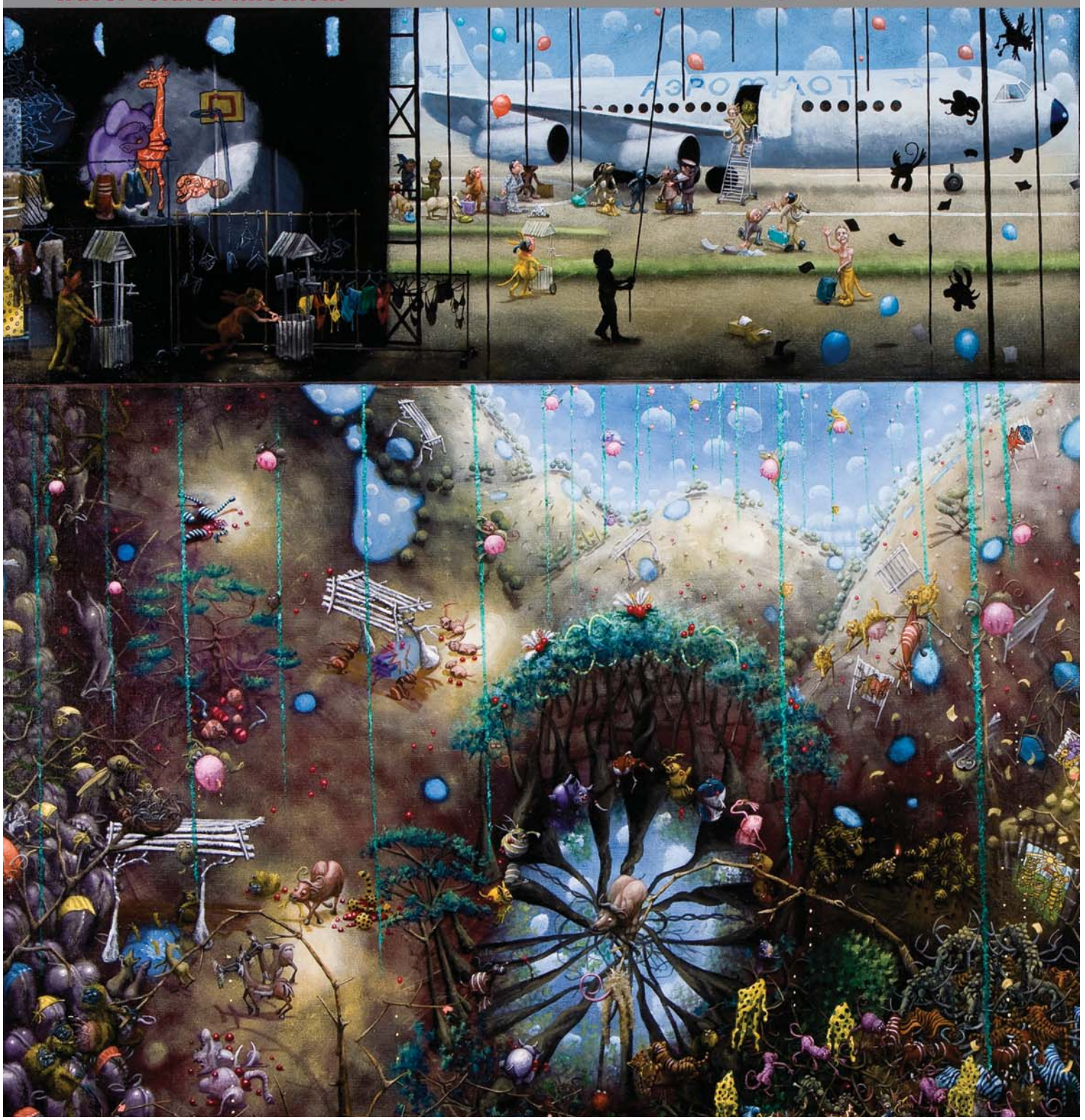

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On the Cover

Cameron Hayes (b. 1969)
The Russians knew perfectly well that the happiness of the African animals was that they had such low expectations—before the pets were introduced (detail) (2008)
Oil on linen (203.2 cm × 254 cm)
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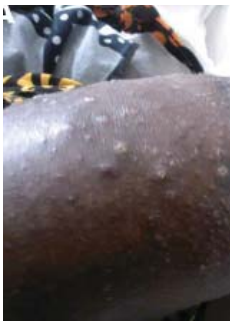

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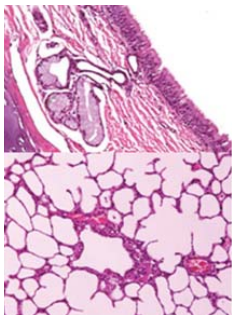
January 2009



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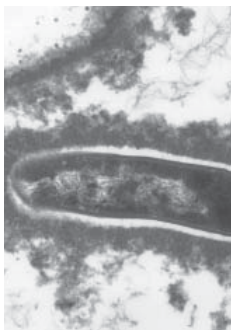

Another Dimension

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News and Notes

Past, Present, and Future of Japanese Encephalitis

Tobias E. Erlanger, Svenja Weiss, Jennifer Keiser, Jürg Utzinger, and Karin Wiedenmayer

Japanese encephalitis (JE), a vector-borne viral disease, is endemic to large parts of Asia and the Pacific. An estimated 3 billion people are at risk, and JE has recently spread to new territories. Vaccination programs, increased living standards, and mechanization of agriculture are key factors in the decline in the incidence of this disease in Japan and South Korea. However, transmission of JE is likely to increase in Bangladesh, Cambodia, Indonesia, Laos, Myanmar, North Korea, and Pakistan because of population growth, intensified rice farming, pig rearing, and the lack of vaccination programs and surveillance. On a global scale, however, the incidence of JE may decline as a result of large-scale vaccination programs implemented in China and India.

Japanese encephalitis (JE) is a vector-borne viral disease that occurs in South Asia, Southeast Asia, East Asia, and the Pacific (*1*). An estimated 3 billion persons live in countries where the JE virus is endemic (*2*), and the annual incidence of the disease is 30,000–50,000 cases (*1*). The disease can cause irreversible neurologic damage. The JE virus (JEV) is mainly transmitted by the mosquito *Culex tritaeniorrhynchus*, which prefers to breed in irrigated rice paddies. This mosquito species and members of the *Cx. gelidus* complex are zoophilic. Wading ardeid water birds (e.g., herons and egrets) serve as virus reservoirs, but the virus regularly spills over into pigs, members of the family of equidae (e.g., horses and donkeys), and humans. The annual number of human deaths is 10,000–15,000, and the estimated global impact from JE in 2002 was 709,000 disability-adjusted life years (DALYs) (*1*,*3*). However, these statistics should be interpreted with care because the transmission of JE is highly dynamic; hence, the disease usually occurs in epidemics, and there is considerable fluctuation in estimates of its global impact. In 1999, JE caused 1,046,000 DALYs; in the 2 subsequent years, it caused 426,000, and 767,000 DALYs, respectively (*3*). Underlying factors that might explain these fluctuations are contextual determinants (mainly environmental factors) and spillover effects into the human population, which trigger epidemics.

Reporting of JE cases depends on the quality of health information systems and the ability to clinically and serologically diagnose the disease. JE is often confused with other forms of encephalitis. Differential diagnosis should therefore include other encephalitides (e.g., conditions caused by other arboviruses and herpesviruses) and infections that involve the central nervous system (e.g., bacterial meningitis, tuberculosis, and cerebral malaria) (*4*).

Figure 1 shows the transmission of JE and highlights contextual determinants. Because infected pigs act as amplifying hosts, domestic pig rearing is an important risk factor in the transmission to humans (*1*). Two distinct epidemiologic patterns of JE have been described. In temperate zones, such as the northern part of the Korean peninsula, Japan, China, Nepal, and northern India, large epidemics occur in the summer months; in tropical areas of southern Vietnam, southern Thailand, Indonesia, Malaysia, the Philippines, and Sri Lanka, cases occur more sporadically and peaks are usually observed during the rainy season (*5*). Thus far, the reasons for the spread of JE are not fully understood. Bird migration might play a role in dispersing JEV (*6*). Accidental transportation of vectors, human migration, and international travel seem to be of little importance because viremia in humans is usually low and of short duration and because humans are dead-end hosts (*1*). JE was likely introduced into northern Australia by windblown mosquitoes from Papua New Guinea (*7*) (Figure 1).

The main pillar of JE control is the use of a live attenuated vaccine for humans, which was developed some 40 years ago (*8*). Currently available JE vaccines are relatively safe and effective, but a drawback is that multiple doses are required (*1*,*9*). Effective delivery of the vaccines

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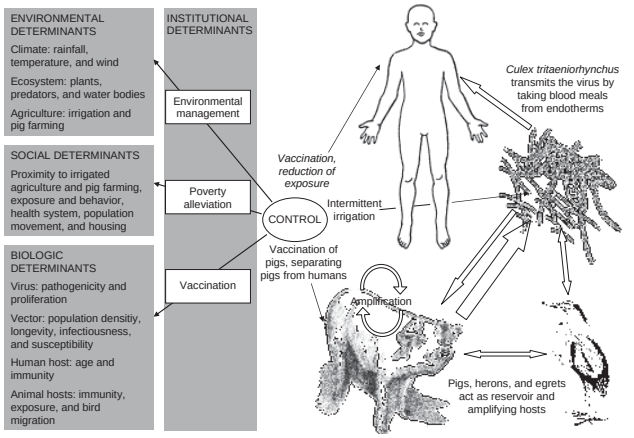

Figure 1. Contextual determinants and transmission of Japanese encephalitis.

to poor, rural communities therefore remains a formidable challenge, and compliance and delivery costs have to be considered (*10*). Two vaccine candidates are in late-stage clinical development. The first one is a second-generation, live inactivated, single-dose vaccine grown in Vero cells. It is the yellow fever virus–based chimeric vaccine and will soon enter the market (*1*). The second candidate is an attenuated SA 14–14–2 virus strain, adjuvanted with aluminum hydroxide and also grown in Vero cells (*9*,*11*).

The vaccination of pigs represents another potential strategy to control JE, but it is not widely used for 2 main reasons. First, the high turnover in pig populations would require annual vaccination of newborn pigs, which would be costly. Second, the effectiveness of live attenuated vaccines is decreased in young pigs because of maternal antibodies (*12*).

Environmental management for vector control, such as alternative wetting and drying of rice fields (also known as intermittent irrigation), can substantially reduce vector breeding while saving water, increasing rice yields, and reducing methane emission (*13*). However, an effective irrigation requires well-organized educational programs, sufficient water at specific times during the rice-growing cycle, and an adequate infrastructure. In addition, because vectors are largely dispersed, intermittent irrigation should be applied to all rice fields over large areas and during the entire cropping season, which is often not feasible (*14*). Environmental management measures are most viable if they are readily integrated into a broader approach of pest management and vector management (*15*).

Chemical control of vector populations with insecticides such as pyrethroids, organophosphates, and carbamates plays a marginal role in JE control. In some circumstances (for example, when an outbreak of JE occurs in a densely populated area), space spraying can break the transmission cycle in the short term. However, rising levels of insecticide resistance have compromised the effectiveness of this emergency measure. Indeed, JE vectors that prefer manmade habitats, such as irrigated rice fields, are often heavily exposed to pesticide selection pressure. Although JE vectors are prone to develop insecticide resistance, usually this issue arises with insecticides that are not directly targeted to JE control, but rather are targeted to control of other pests (*16*).

We provide a historic account of the origin of JE and disease epidemics, describe the current situation, and discuss several factors that might explain the rise of JE incidence in some countries and its decline in others. Finally, we speculate about possible future trends.

Historic Account

Genetic studies suggest that JEV originated from an ancestral virus in the area of the Malay Archipelago. The virus evolved, probably several thousand years ago, into different genotypes (I–IV) and spread across Asia (*17*).

The history of the clinical recognition and recording of JE dates to the 19th century. JE appeared as recurring encephalitis outbreaks in the summer season. The first clinical case of JE was recorded in 1871 in Japan. Half a century later, also in Japan, a large JE outbreak involving >6,000 cases was documented. Subsequent outbreaks occurred in 1927, 1934, and 1935. In 1924 an agent from human brain tissue was isolated; 10 years later, it was proven to be JEV by transfection into monkey brains. The role of *Cx. tritaeniorhynchus* as a vector and the involvement of wading ardeids and pigs as reservoir hosts were demonstrated in 1938 (*18*).

Table 1 shows when the first JE cases were described in countries currently considered JE-endemic. On the Korean Peninsula, the first JE cases were recorded in 1933. On the Chinese Mainland, the first JE cases were documented in 1940. In the Philippines, first reports of JE cases occurred in the early 1950s (*19*). Eventually, the JE epidemic reached Pakistan (1983) as the furthest extension in the West, and Papua New Guinea (1995) and northern Australia (Torres Straight) as the furthest south. In parts of southeastern Russia (Primorje Promorsij), a few JE cases have been reported occasionally (e.g., 2 cases from 1986 to 1990) (*18*). JE is potentially endemic to Afghanistan, Bhutan, Brunei Darussalam, and the Maldives, but to our knowledge, no cases have been reported in these countries in the past 30 years. According to the World Health Organization (WHO), JE is endemic to the Western Pacific Islands, but cases are rare (*20*). The enzootic cycle on those Pacific Islands might not sustain viral transmission; hence, epidemics occur only after introduction of virus from JE-endemic areas. Subtle changes in the spatiotemporal distribution of JEV are difficult to track; thus, the year when a first case of JE in a country is reported does not necessarily correspond with the actual first occurrence of JE in that country (Table 1) (*21*–*35*).

Table 1. First reported case and current situation of Japanese encephalitis in the main disease-endemic countries

Country	First reported case	Total population in rural JE-endemic areas (% of total)*	Annual incidence†	DALYs in 2002‡	Trend of JE incidence§	Vaccination program†	National diagnostic center†	References
Australia	1995	NA§	<1	<1¶	Stable	Yes	Yes	(*21*)
Bangladesh	1977	106,385,000 (75)	NA	24,000	Increasing	No	No	(*22*)
Cambodia	1965#	11,293,000 (80)	NA	4,000	Increasing	No	NA	(23)
China	1940	422,532,000 (32)	8,000–10,000	281,000	Decreasing	Yes	Yes	(*18*)
India	1955	597,542,000 (54)	1,500–4,000	226,000	Increasing	No	Yes	(*24*)
Indonesia	1960	116,114,000 (52)	NA	23,000	Increasing	No	NA	(*25*)
Japan	1924	43,969,000 (34)	<10**	<1	Stable	Yes	Yes	(*26*)
North Korea	1933	8,606,000 (38)	NA	6,000	NA	NA	NA	(*27*)
South Korea	1933	9,194,000 (19)	<20	6,000	Stable	Yes	Yes	(*27*)
Laos	1989	4,643,000 (78)	NA	5,000	Increasing	No	Yes	(*28*)
Malaysia	1952††	8,854,000 (35)	50–100	2,000	Decreasing	Yes	Yes	(*29*)
Myanmar	1965	35,077,000 (69)	NA	13,000	Increasing	No	NA	(*18*)
Nepal	1978	4,567,000 (20)	1,000–3,000	5,000	Stable	Yes	Yes	(*30*)
Papua New Guinea	1995	5,109,000 (87)	NA	2,000	NA	NA	NA	(*21*)
Pakistan	1983	18,536,000 (12)	NA	82,000	Increasing	NA	NA	(*27*)
The Philippines	1950	31,081,000 (37)	10–50	8,000	Stable	No	Yes	(*19*)
Singapore	1952	0	<1	260	Stable	No	Yes	(*31*)
Sri Lanka	1968	16,381,000 (79)	100–200	1,000	Decreasing	Yes	Yes	(*32*)
Thailand	1964	43,364,000 (68)	1,500–2,500	5,000	Decreasing	Yes	Yes	(*18*,*27*)
Vietnam	1960	61,729,000 (73)	1,000–3,000	11,000	Stable	Yes	Yes	(*27*,*33*)

*Japanese encephalitis (JE)–-endemic areas drawn from map provided in (*34*); rural population estimates derived from United Nations Urbanization Revisions (*2*).
†Information retrieved from questionnaires answered by employees from Ministry of Health and World Health Organization country offices.
‡Estimates for 2002 from World Health Organization (*20*). DALYs, disability-adjusted life years.
§<1% of the country is JE-endemic (Torres Straight islands, Cape York Peninsula). NA, not available.
¶Two deaths since 1995 (*35*).
#Virus isolated from mosquito (*29*).
**JE cases are very rare; highest risk is on Okinawa, Miyako, and Ishigaki islands. Between 1995 and 2005, 3 cases were reported (all in 2002) (*26*).
††First isolated from humans. During World War II, an outbreak that was probably due to JE occurred (*31*).

Present Situation

Nearly half of the human population currently lives in countries where JEV occurs. As shown in Figure 2, JE is concentrated in China, India, and the Southeast Asian peninsula.

Current epidemiologic data on JE are summarized in Table 1. These data were gathered from a diversity of sources, including peer-reviewed literature, specialized text books, and reports from national health departments and international organizations, such as WHO and the Food and Agriculture Organization. We also contacted national health ministries and WHO country offices for up-to-date information regarding country-specific JE statistics. This was accomplished by administering a standardized questionnaire.

There are 2 distinct trends in JE incidence. In countries such as Bangladesh, Cambodia, India, and Laos, where no specific diagnostic centers, vaccination programs, and surveillance systems are in place, the incidence of JE appears to have increased in recent years. On the contrary, in China, Japan, Nepal, South Korea, Sri Lanka, and Thailand, where vaccination programs are being implemented and regular surveillance is pursued, the incidence of JE is stable or declining. Despite the availability of WHO estimates, the situation in North Korea, Myanmar, Pakistan, and Papua New Guinea remains largely unknown.

However, underreporting is substantial in most JE-endemic countries; hence, it is conceivable that annual JE incidence is considerably higher than heretofore reported. For example, an estimate that used a representative incidence of 25/10,000 (not immunized), and a 1994 population estimate of 700 million children <15 years of age who live in JE-endemic areas suggested 175,000 cases annually with 43,750 deaths, and 78,750 cases with lasting sequelae. Adjusted for vaccine coverage, the estimate is 125,000 cases per year (*36*).

Emerging JE

The emergence of JE can probably be explained by 2 factors. First, JE-endemic countries experienced an unprecedented population growth in recent decades. For example, in Eastern Asia, South-Central Asia, and Southeast Asia, the population more than doubled, from 1.7 billion in the mid-1950s to 3.5 billion 50 years later (*2*). Second, pig rearing has grown exponentially and rice-production systems, particularly irrigated rice farming, have increased both in cropping area and cropping intensity. In China, for example, pork production doubled from 1990 to 2005.

Figure 2. Disability-adjusted life years (DALYs) per 100,000 persons in Japanese encephalitis–endemic countries. Numbers in parentheses indicate estimated number of deaths in 2002 according to the World Health Organization (*20*). NA, not available.

Today, the total rice-harvested area of all JE-endemic countries (excluding the Russian Federation and Australia) is 1,345,000 km^2, an increase of 22% in the past 40 years. Over the same time span, the total rice production in these countries has risen from 226 million tons to 529 million tons (+134%) (*37*).

Table 2 presents information on 3 key environmental contextual determinants of JE transmission, stratified by country. The following issues are offered for consideration. First, the number of people living in close proximity to irrigated areas reflects the fraction of the population that potentially is at an elevated risk of acquiring JE. Methods for calculating those numbers have been described elsewhere (*13*). Second, the absolute and relative change of irrigated rice area and, third, the relative change in pork production can be used as proxies for alterations in the risk of acquiring JE. In absolute numbers (116.6 million) and in relative terms, most people living in close proximity to irrigated areas are from Bangladesh (82%); the second largest population lives in India (107.8 million), followed by China (22.0 million). The largest irrigated rice area in 2005 was found in India (41.9 million ha), followed by China (29.0 million ha), Bangladesh (10.5 million ha), and Thailand (10.0 million ha). Highest increases in irrigated rice areas in the past 15 years were estimated for Myanmar (+47%) and Cambodia (+30%). Highest increases in pork production occurred in Myanmar (+381%), Vietnam (+147%), and China (+87%). On the other hand, pork production declined in Malaysia (–47%), North Korea (–35%), and Japan (–23%).

Despite the fact that irrigated rice production and pig rearing are key factors in the transmission of JE, crude numbers fail to completely explain the complex interplay of various contextual determinants of the disease. Clearly, where rice production and pig rearing overlap, the impact on JE transmission is stronger than in areas where both activities are physically separated. This is the case, for example, in Malaysia, where the Malays mainly grow rice in 1 area and the Chinese rear pigs in another area. Here, the social determinant of religion (most Malays are Muslim) plays a decisive factor (Table 2).

Conclusion and Outlook

Discovered 125 years ago, JE has spread widely in the 20th century. Almost half of the human population now lives in countries where the disease is endemic. JE is a vector-borne epidemic with several features that are typical of an emerging infectious disease. The failure to halt the spread of JE in Asia and the Pacific region, despite the availability of an effective and inexpensive vaccine for 40 years, is of considerable public health concern. A similar conclusion has been drawn for yellow fever, a disease that can also be prevented by vaccination yet is rampant (*38*). Similar to schistosomiasis, malaria, food-borne trematodiasis, lymphatic filariasis, and dengue, one of the main reasons for the proliferation of JE is the ecologic transformation caused by water resources development and management that create suitable breeding sites for vectors and intermediate hosts, which in turn influence the frequency and transmission dynamics of these diseases (*39*).

High-quality data on transmission and incidence of JE are lacking in various countries. Although clinical and serologic methods to diagnose and monitor JE are available, health systems in many developing countries are unable to differentiate encephalitis diagnoses. Information regarding the distribution and public health importance of JE in Bangladesh, Cambodia, Indonesia, North Korea, Laos, Myanmar, Papua New Guinea, and Pakistan is inadequate. Such epidemiologic information, however, is mandatory for advocacy and allocation of resources for the control of JE. Examples of countries with successful JE control programs are Japan and South Korea. Before the 1950s, these countries experienced JE outbreaks, but incidence rates have remained stable for >2 decades. The following key control strategies and developments might explain the successful decline of JE in these countries: 1) large-scale immunization programs for humans, 2) pig immunization and the separation of pig rearing from human settlements, 3) changes in agricultural practices (e.g., enhanced mechanization and decrease of irrigated land), and 4) improved living standards (e.g., better housing and urbanization).

Table 2. Rice irrigation and pork production by Japanese encephalitis–endemic country, 1990 and 2005*

Country	Persons in close proximity to rice irrigation†	Rice paddy area, 1990†	Rice paddy area, 2005†	% Change in paddy area	% Change in pork production†
Australia	NA	NA	NA	NA	NA
Bangladesh	116,600,000	10,435	10,524	+1	NA
Cambodia	1,426,000	1,855	2,415	+30	+46
China	22,019,000	33,519	29,087	–13	+87
India	107,785,000	42,687	41,907	–2	–8
Indonesia	7,169,000	10,502	11,802	+12	–13
Japan	1,947,000	2,074	1,706	–18	–23
North Korea	414,000	600	590	–2	–35
South Korea	921,000	1,244	980	–21	+69
Laos	164,000	NA	NA	NA	+21
Malaysia	181,000	681	676	–1	–47
Myanmar	3,120,000	4,760	7,008	+47	+381
Nepal	554,000	1,455	1,542	+6	+12
Papua New Guinea	110	NA	NA	NA	NA
Pakistan	513,000	2,113	2,621	+24	NA
The Philippines	12,200,000	3,319	4,200	+27	+18
Singapore	0	0	0	0	NA
Sri Lanka	2,232,000	828	915	+10	+21
Thailand	8,330,000	8,792	10,042	+14	+80
Vietnam	18,648,000	6,043	7,329	+21	+147

*NA, not available.
†Sources of data and method of calculation are described by Keiser et al. (*13*).
‡Source: Food and Agriculture Organization (*37*).

We speculate that JE incidence is increasing mainly in low-income countries. However, because reliable figures about JE emergence are lacking due to the absence of rigorous monitoring systems, more research is needed to support or refute this claim. In any event, lack of political will and financial resources are 2 important reasons why JE is often given low priority. These factors might explain the paucity of JE immunization programs for children in low-income countries where the disease is endemic. Nevertheless, Sri Lanka and Nepal, 2 countries with limited health budgets, and Thailand and Vietnam have managed to successfully control JE.

The national situations with respect to JE in the near future could develop as follows. We hypothesize that in Cambodia, Laos, and Myanmar, severe JE outbreaks could occur in the near future, partially explained by increases in irrigated rice farming and enhanced pig rearing. The JE situation in North Korea is not well understood, but on the basis of the population's general health status, we predict that JE will likely remain a substantial public health issue in the years to come. Bangladesh and Pakistan are among the worst affected and most populous countries in which JE is endemic, and yet effective surveillance is missing. Outbreaks are likely to occur but will remain largely undetected. Muslim countries such as Bangladesh and Pakistan have traditionally been JE free. JEV transmission ends in Pakistan, even though the JE vector is abundant further to the West. The recent rise in JE in those countries has yet to be fully investigated and shows the complexity of transmission of this disease. In Indonesia, Malaysia, the Philippines, and Singapore, JE incidence has usually been low, and transmission will remain stable at a relatively low level. Given the paucity of data in Indonesia, a monitoring system should be established to document changes over time. Occasional small JE outbreaks might also occur in Papua New Guinea with spillover to Australia. Awareness of the disease and vaccination coverage rates are high in Australia, particularly in the region of the Torres Strait; hence, it seems unlikely that larger epidemics will occur anytime soon.

The overall trend of JE has been declining over the past 3 decades, and we anticipate that this trend will continue in the long term. Indeed, China and India influence JE figures on a global scale because most people living in JE-endemic areas are concentrated in these 2 countries. The incidence of JE in China has declined since 1971, coincident with economic growth and development. Meanwhile, the national JE vaccination program has been integrated into the Expanded Program on Immunization, and, at present, >110 million doses of a live, attenuated vaccine (SA14–14–2 strain) are produced annually. However, social, economic, and health policy changes in the face of privatization and a more market-based economy have led to reduced funding for immunization programs and somewhat reduced salaries for public health workers, particularly in the poorest provinces. As a consequence, these changes have contributed to increasing disparities in immunization coverage rates between the wealthy coastal and the less developed rural provinces and thus to the recently observed differences in levels of JE incidence between those regions (*40*).

The incidence of JE in India is still increasing, and the case-fatality rate of reported cases is high, i.e., 10%–30% (online Technical Appendix, supplementary reference *41*; available from www.cdc.gov/EID/content/15/1/1-Techapp.pdf). India currently has no national vaccination program, but the Ministry of Health has recently drawn up a plan in which children 1–12 years of age will be immunized. In Tamil Nadu and Uttar Pradesh, immunization programs are already running; thus, JE incidence might stabilize in those regions. However, overall trends for India are difficult to predict because JE endemicity is heterogeneous and because socioeconomic conditions for control differ substantially from 1 state to another (online Technical Appendix, supplementary reference *42*).

Coverage of immunization programs and changes in agricultural practices will further influence JE transmission. In Taiwan, for example, the average age for the onset of confirmed JE cases shifted from children <10 years toward adulthood, explained by a high coverage of vaccinated children (online Technical Appendix, supplementary reference *43*). Interestingly, the peak JE transmission, which occurred in August in the 1960s, shifted to June beginning in the 1980s. Improvements in pig-feeding technologies, which resulted in shorter periods from birth to pregnancy of female pigs, has been proposed as an important reason explaining the shift in transmission (online Technical Appendix, supplementary reference *44*).

Climate change has been implicated in the increase of transmission of several vector-borne diseases (online Technical Appendix, supplementary reference *45*). For example, a potential effect of climate change has been shown empirically for dengue virus, which is closely related to that of JE (online Technical Appendix, supplementary reference *46*). Although JE vector proliferation might be influenced in a similar way than that predicted for dengue vectors, the potential impact of climate change on JE remains to be investigated. Indeed, climate change could not only directly increase JE vector proliferation and longevity but could also indirectly increase disease because of changing patterns of agricultural practices such as irrigation (online Technical Appendix, supplementary references *47*,*48*). Areas with irrigated rice-production systems may become more arid in the future, and the impact of flooding will be more dramatic, which in turn might result in JE outbreaks. Generally, extreme rainfall after a period of drought can trigger outbreaks in situations in which vector populations rapidly proliferate and blood feeding is spilling over to humans. Climate change may also influence migration patterns of birds, which may result in JEVs being introduced into new areas. However, little is known about reservoir bird migration patterns; hence, this issue remains to be investigated (*6*).

The culicines that transmit JE are usually highly zoophilic, and human outbreaks are therefore the result of a spillover of the virus from the animal reservoir into the human population. Studies in Sri Lanka showed that spillovers happen when there is rapid and dramatic buildup of *Culex* spp. populations to the extent that the number of human blood meals passes a threshold after which virus transmission begins (online Technical Appendix, supplementary reference *49*). Such rapid buildups are a result of extreme weather conditions or of rice fields in semi-arid areas being flooded before rice is transplanted. Information on vector population dynamics would be very useful in early warning systems and could also help improve targeting of control programs.

In conclusion, JE can be controlled, with effective surveillance systems and vaccines playing key roles. Although currently available vaccines are effective, the need for 3–4 injections compromises compliance and increases delivery costs (*10*). The advent of second-generation, cell-culture–derived vaccines will continuously replace mouse-brain and hamster kidney cell–derived vaccines. Such developments will hopefully boost current vaccination programs and deliver safer, more efficacious, and cheaper vaccines that comply with regulatory norms. Political will and commitment, financial resources, intersectoral collaboration (between the Ministries of Health and Agriculture and other stakeholders to set up vaccination programs for young children), as well as changing agricultural practices, pig vaccination, rigorous monitoring, and surveillance will go a long way in controlling JE.

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Dr Erlanger is an affiliate of the Swiss Tropical Institute in its Department of Public Health and Epidemiology. His main research interests pertain to environmental epidemiology of vector-borne diseases and health impact assessment.

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Threat of Dengue to Blood Safety in Dengue-Endemic Countries

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Dengue, the most common arbovirus infection globally, is transmitted by mosquito vectors. Healthcare-related transmission, including transmission by blood products, has been documented, although the frequency of these occurrences is unknown. Dengue is endemic to Singapore, a city-state in Asia. Using mathematical modeling, we estimated the risk for dengue-infected blood transfusions in Singapore in 2005 to be 1.625–6/10,000 blood transfusions, assuming a ratio of asymptomatic to symptomatic infections of 2:1 to 10:1. However, the level of viremia required to cause clinical dengue cases is person-dependent and unknown. Further studies are needed to establish the magnitude of the threat that dengue poses to blood safety in countries where it is endemic. It will then be possible to assess whether screening is feasible and to identify approaches that are most cost-effective on the basis of characteristics of local populations and seasonality of dengue.

Emerging infectious diseases pose threats to the general human population, including recipients of blood transfusions. Dengue is an expanding problem in tropical and subtropical regions and is now the most frequent arboviral disease in the world, with an estimated annual 100 million cases of dengue fever, 250,000 cases of dengue hemorrhagic fever, and 25,000 deaths per year (*1*). Dengue virus infections have been reported in >100 countries; 2.5 billion people live in areas where dengue is endemic (*1*). It is also increasingly reported to occur in international travelers (*2*). Dengue is of major international public health concern because of the expanding geographic distribution of the virus and competent mosquito vectors, increased frequency of epidemics, cocirculation of multiple virus serotypes, and emergence of dengue hemorrhagic fever in new areas.

Dengue virus belongs to the family *Flaviviridae* and is transmitted by mosquitoes of the genus *Aedes* (*1*). Flaviviruses are small, lipid-enveloped, positive-stranded RNA viruses (*1*). Dengue infection has a viremic phase that lasts 4–8 days (*3*), and most infections remain subclinical (*4*). Viremia can precede the onset of symptoms in persons with clinical disease. Plasma viral RNA levels range from $10^{5.5}$ to $10^{9.3}$ copies/mL, and blood collected during this phase may be infective when transfused into susceptible hosts (*5*).

A literature search conducted in March 2008 on blood transfusion and dengue did not identify any published reports of blood transfusion–associated dengue in dengue-endemic countries. Healthcare-associated transmission is difficult to ascertain in dengue-endemic countries, although there is 1 report of possible transmission of dengue in a healthcare setting in Hong Kong, Special Administrative Region, People's Republic of China, attributed to a viremic resident of Hong Kong (*6*). In contrast, healthcare-acquired dengue infections in countries in which dengue is not endemic can be determined. Several reports have described travelers who returned from dengue-endemic countries to those not endemic for dengue and transmitted dengue infection to healthcare workers by needlestick injury or mucocutaneous exposure to blood (*7,8*).

Healthcare-associated transmission of viruses among humans has been recently reviewed for dengue and other flaviviruses such as West Nile virus (WNV) and yellow fever virus (*9*). Routes of transmission include percutaneous, mucous membrane, bone marrow transplant, organ transplant, hemodialysis, and transfusion of blood products (*6–8,10–25*) (Table) However, despite good evidence for its transmission in healthcare settings, dengue is currently not considered a risk to blood safety.

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We draw parallels from recent experiences with West Nile fever and encephalitis in the United States. WNV first appeared in the United States in 1999 and has since spread throughout the country, resulting in thousands of cases of disease (*26*). Approximately 80% of WNV infections are asymptomatic (*27*). By 2002, 23 patients in the United States were confirmed to have acquired WNV through transfused blood and blood products (erythrocytes, platelets, and fresh-frozen plasma) (*23*). The estimated risk for virus transmission through transfusion during the 1999 WNV epidemic in New York was 1.8/10,000–2.7/10,000 donations, and ≈2.0 viremic donations/10,000 donors in the borough of Queens (*28*). Of the 2.5 million blood donations screened for WNV from June through December 2003, 0.05% were positive at the first screening and 0.02% were confirmed (*29*). In response to these findings, by 2003, essentially all blood donations in the United States were being tested for WNV. In contrast, no screening of blood products is conducted for dengue, although dengue virus is estimated to affect >100 million persons annually in tropical and subtropical regions. A recent study in Puerto Rico reported nucleic acid testing for dengue virus in the blood supply and found the viral RNA prevalence to be 7.3/10,000 U of blood donations, which approximates the prevalence of WNV in the United States during the transmission season (*30*). In addition, screening of donors in Honduras and Brazil has identified dengue virus RNA (0.37% and 0.06% of blood donations or 37/10,000 and 6/10,000 blood donations, respectively) by using a transcription-mediated amplification assay (*31*).

We postulate that dengue virus poses a greater threat worldwide to blood safety than WNV but that this hypothesis has been neglected because dengue occurs predominantly in developing countries. We used mathematical modeling to estimate the risk for dengue in Singapore. Singapore is an industrialized Asian city-state in which dengue is endemic. This city-state has the capacity to implement blood screening for dengue. The dengue seroprevalence rate in the adult population in Singapore, which has a population of ≈4 million persons, is 45% (*32*). In 2005, 14,209 cases of symptomatic dengue infections were reported in Singapore, a large proportion (≈80%) of which were in adolescents or adults (*33*). We calculated the force of infection in this population. The force of infection is defined as follows: per capita new cases in a finite period = rate of contacts by potentially infective persons × (no. viremic persons in a population in a finite period/total population in a finite period).

If one assumes that there were 11,367 reported adult cases in Singapore in 2005 (80% of 14,209) and a ratio of asymptomatic to symptomatic cases of 2:1, a total of 34,101 viremic adults in 2005 would have viremia that lasts ≤1 week. This finding indicates there would be 656 infective persons per week, which translates to 0.00016 viremic persons in a finite period per total population in a finite period. If one considers that there are ≈80,000 blood transfusions in Singapore per year, there would be 1,538 transfusions per week. Therefore, the force of infection for 2005 in Singapore was ≈13 infective blood donations.

However, if we assume a ratio of asymptomatic to symptomatic cases of 10:1, we then have 125,037 viremic persons, or ≈2,405 infective persons per week. This finding indicates that the number of viremic persons in the popu-

Table. Reported healthcare-associated transmission of flaviviruses

Virus	Route of transmission	Comment	References
Dengue	Percutaneous	Several healthcare workers were infected after needlestick injuries during care of returned travelers who had diagnoses of dengue.	(*8,10–13*)
	Mucocutaneous	A healthcare worker became infected with dengue 3 virus after being splashed in the face by blood from a febrile traveler who had a diagnosis of dengue.	(*7,14*)
	Blood transfusion	A 17-year-old man from Hong Kong, Special Administrative Region, People's Republic of China, donated blood in July 2002, from which erythrocytes were transfused to a 72-year-old woman, in whom febrile illness consistent with dengue fever developed 3 d later.	(*15*)
	Bone marrow transplant	A 6-year-old child from Puerto Rico became infected with dengue 4 virus from a bone marrow transplant and died.	(*16*)
	Renal transplant	Dengue hemorrhagic fever developed after a living donor renal transplant.	(*17*)
Yellow fever	Laboratory	A laboratory technician acquired yellow fever after obtaining blood and performing a blood count on a yellow fever patient; he died subsequently. Yellow fever was transmitted to at least 30 other scientists and laboratory workers after contact with mouse or monkey blood or tissues or handling infected animals.	(*18–20*)
West Nile	Percutaneous	Virus was transmitted to 2 microbiologists by laceration or needlestick injuries in laboratory.	(*21*)
	Transfusion	Virus was transmitted to numerous recipients of blood products.	(*22,23*)
	Organ transplant	Virus was transmitted to transplant recipients from kidneys, liver, and heart of an infected donor.	(*24*)
	Hemodialysis	Virus infection in a cluster of 3 hemodialysis patients suggested transmission through a common dialysis machine.	(*25*)

lation in a finite period/total population in a finite period is 0.00060115. We would then end up with a force of infection for 2005 of 48 infective donations. Depending on the ratio of asymptomatic to symptomatic infections, there were ≈13–48 dengue infected blood donations in Singapore in 2005. With ≈80,000 blood transfusions annually in Singapore, the proportion of infected blood transfusions would be ≈1.625–6/10,000 transfusions, which is consistent with reported dengue RNA prevalence in blood donations in Puerto Rico, Brazil, and Honduras (*30*,*31*). This proportion is similar or even higher than the estimated risk for WNV transmission by transfusion during the 1999 epidemic in New York, which was reported to be 1.8/10,000–2.7/10,000 donations (*28*). A total of 1.625–6/10,000 blood transfusions would translate to 3.25–12 potentially infective blood transfusions/1 million persons in Singapore, if one assumes a ratio of asymptomatic to symptomatic case between 2:1 and 10:1. Further, a recent report provides well-documented evidence of a cluster of blood transfusion–associated dengue infections in Singapore (*34*).

Why has transfusion-associated dengue not yet been widely recognized as a problem in dengue-endemic countries? Lack of recognition is likely due to lack of awareness that dengue is transmitted not only by vectors but also by blood products. Because of the effects of infection and recurring epidemics in dengue-endemic countries, isolated cases of healthcare-acquired infections will go unnoticed. In many healthcare facilities, patients are not protected from mosquitoes, and it is therefore difficult to ascertain whether infections were related to blood transfusions or exposure to vectors. Further, risk for transmission by transfusion may depend on the level of viremia, which has been shown to correlate with severity of disease (*35*). It is likely, although not proven, that viremia is lower and shorter in duration in asymptomatic persons than in symptomatic persons. The risk for transfusion-associated dengue will vary greatly from 1 country to another, depending on the epidemiologic pattern of dengue and the immunity level in the population. In countries where dengue is mainly a childhood disease, risk for blood transfusion–transmitted dengue will be lower because of lack of overlap of infected and blood-donating populations. However, risk for nosocomial transmission from needlesticks and other blood exposures would exist in all areas with dengue.

Blood transfusion–related dengue will likely represent only a small proportion of all dengue cases in dengue-endemic countries. Screening blood for dengue antigens in dengue-endemic countries would be costly and should therefore be recommended only after careful assessment of risk for infection and cost per blood product–associated dengue infection averted. Therefore, the first step is to quantify this risk in a systematic study. Risk will vary by geographic region and season and may change over time. We suggest targeted nucleic acid amplification testing of individual donations in high-prevalence regions, a strategy that was implemented successfully for screening of WNV in the United States in 2004 (*36*); nucleic acid amplification tests of minipools of several samples of donated blood have also been proposed (*36*). A prototype nucleic acid test, which is suitable for high-throughput screening, has been developed for detection of dengue virus RNA in blood donations (*31*). The initial study should be conducted during the dengue transmission season to identify maximum incidence of viremic donations. This testing would provide a baseline estimate of risk for transmission of infective blood. If the risk is found to be substantial, healthcare providers would need to decide at what threshold screening should be instituted. Policies will also be influenced by economic resources available and healthcare priorities of a country or region.

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Sphingomonas paucimobilis Bloodstream Infections Associated with Contaminated Intravenous Fentanyl[1]

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CME ACTIVITY

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Learning Objectives

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

- Describe the types of bacterial contamination associated with different compounding pharmacy medications.
- Describe the features of *Sphingomonas paucimobilis* bacteria.
- Identify the types of exposure associated with transmission of *S. paucimobilis* infection with contaminated fentanyl.
- Describe a strategy that would limit the occurrence of compounding pharmacy product contamination.

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Nationally distributed medications from compounding pharmacies, which typically adhere to less stringent quality-control standards than pharmaceutical manufacturers, can lead to multistate outbreaks. We investigated a cluster of 6 patients in a Maryland hospital who had *Sphingomonas paucimobilis* bloodstream infections in November 2007. Of the 6 case-patients, 5 (83%) had received intravenous fentanyl within 48 hours before bacteremia developed. Cultures of unopened samples of fentanyl grew *S. paucimobilis;* the pulsed-field gel electrophoresis pattern was indistinguishable from that of the isolates of 5 case-patients. The contaminated fentanyl lot had been prepared at a compounding pharmacy and distributed to 4 states. Subsequently, in California, *S. paucimobilis* bacteremia was diagnosed for 2 patients who had received intravenous fentanyl from the same compounding pharmacy. These pharmacies should adopt more stringent quality-control measures, including prerelease product testing, when compounding and distributing large quantities of sterile preparations.

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[2]These authors contributed equally to this article.

Compounding pharmacies are licensed or registered by state pharmacy boards to combine "reasonable quantities" of ingredients to fill a valid prescription from a licensed practitioner for an individual patient (*1*). Some pharmacies, however, have moved beyond this role and, in anticipation of receiving routine orders, prepare larger quantities of compounded preparations for national distribution to healthcare facilities (*2*,*3*). Nationally distributed medications from compounding pharmacies, which typically adhere to less stringent quality-control standards than pharmaceutical manufacturers, can lead to multistate outbreaks that may be difficult to detect. Sunenshine et al. recently reported a 5-state outbreak of *Serratia marcescens* bloodstream infections associated with contaminated intravenous magnesium sulfate from a compounding pharmacy (*3*). Other reported outbreaks caused by contaminated medications from compounding pharmacies include the following: *S. marcescens* infections associated with betamethasone injection, *Pseudomonas putida* septicemia caused by use of contaminated flush solutions in a special-care nursery, *Burkholderia cepacia* infections caused by contaminated intravenous flush solutions, *Pseudomonas fluorescens* bloodstream infections associated with a heparin/saline flush, and *Exophiala dermatitidis* infections caused by injection of contaminated steroids (*4*–*10*; Table 1).

Sphingomonas paucimobilis is an aerobic bacterium found in soil and water; it is a rare cause of healthcare-associated infections (*11*,*12*). *S. paucimobilis* has been reported to cause outbreaks of bacteremia among immunocompromised patients in hematology and oncology units; these outbreaks are possibly related to bacterial colonization of hospital water systems (*13*,*14*). An *S. paucimobilis* outbreak in mechanically ventilated neonates was linked to contaminated temperature probes (*15*). In November 2007, The Johns Hopkins Hospital Department of Hospital Epidemiology and Infection Control initiated an outbreak investigation after being notified by the hospital's microbiology laboratory of the growth of *S. paucimobilis* in several patients' blood cultures over a 2-week period.

Methods

The Johns Hopkins Hospital is a 926-bed, tertiary-care, academic hospital in Baltimore, Maryland, USA. For this investigation, we defined a case-patient as any patient in our hospital whose cultures of blood or of other normally sterile body sites grew *S. paucimobilis* in November 2007. *S. paucimobilis* isolates were characterized as gram-negative rods that are yellow-pigmented, glucose nonfermenting, and weakly oxidase positive; they were preliminarily identified by the BD Phoenix Automated Microbiology System (BD Diagnostics, Inc. Sparks, MD, USA). All *S. paucimobilis* isolates were confirmed by cell wall fatty acid analysis using gas liquid chromatography (Sherlock Microbial Identification System version 4.5, library 5.0; MIDI, Inc. Newark, DE, USA). Microbiology records from January 2006 through November 2007 were examined to identify case-patients and to establish the baseline rate of *S. paucimobilis* bacteremia. We identified common exposures for case-patients and focused on intravenous infusions, medications, and contrast agents and on case-patients' clinical signs, treatments, and outcomes. Because most of the blood cultures growing *S. paucimobilis* were collected in BacT/Alert FA bottles containing activated charcoal (bioMérieux, Durham, NC, USA), we cultured noninoculated bottles from clinical units by placing them directly into the blood culture instrument to assess for intrinsic contamination. On the basis of information from the medical record review, samples for bacterial culture were taken from 4 implicated lots of intravenous fentanyl mixed in 0.9% sodium chloride solution. All 4 lots came from an out-of-state compounding pharmacy,

Table 1. Recently published reports of infectious outbreaks associated with contaminated medications prepared at compounding pharmacies, United States, 2002–2007

Reference	Organism	Infection (no. patients)	Mode of transmission	Location of outbreak
(*3*)	*Serratia marcescens*	Bloodstream infections (18)	Intravenous magnesium sulfate	California, New Jersey, North Carolina, New York, Massachusetts
(*4*)	*S. marcescens*	Meningitis, epidural abscess, or joint infection (11)*	Epidural or intra-articular injection of betamethasone	California
(*6*)	*Burkholderia cepacia*	Bloodstream infections and sepsis (2 pediatric patients)	Intravenous antibiotic-lock flush solution	Connecticut
(*7*)	Hepatitis C virus	Acute hepatitis C (16)	Injected radiopharmaceutical for myocardial perfusion study	3 clinics in Maryland
(*8*,*10*)	*Pseudomonas fluorescens*	Bloodstream infections (64)	Heparin/saline intravenous flush	Missouri, New York, Texas, Michigan, South Dakota
(*9*)	*Exophiala dermatitidis*	Meningitis (5)†	Epidural injection of methylprednisolone‡	2 pain management clinics in North Carolina

*3 case-patients died.
†1 case-patient died.
‡Prepared by a compounding pharmacy in South Carolina and supplied to hospitals and clinics in 5 states.

hereafter called pharmacy A. All *S. paucimobilis* isolates were strain typed by pulsed-field gel electrophoresis (PFGE) after digestion with *XbaI* using standard methods and interpreted according to criteria established by Tenover et al. (*16*).

We investigated the possibility that fentanyl had been tampered with or diverted by tracing the narcotic chain of custody, reviewing controlled substance procedures, visually inspecting fentanyl bags for signs of tampering, testing fentanyl concentrations, and analyzing personnel access records from the automated medication management system (Pyxis, Cardinal Health; www.cardinal.com/us/en/providers/products/pyxis/index.asp). The investigation was coordinated with public health authorities, including the Centers for Disease Control and Prevention (CDC) and the Baltimore City Health Department. CDC performed a multistate case-finding investigation by working with the compounding pharmacy to trace the distribution of implicated lots of intravenous fentanyl and by asking recipient healthcare institutions whether they had identified cases of *S. paucimobilis* bacteremia. We notified the US Food and Drug Administration (FDA) of our findings, and the FDA investigated compounding practices at pharmacy A. The Johns Hopkins University Institutional Review Board approved this study and waived informed consent.

Results

S. paucimobilis was isolated from the blood cultures of 6 patients; the samples were collected from November 11 through November 23, 2007. The organism was not isolated from cultures of any other body site. Case-patients had various underlying medical conditions and had been admitted to different hospital units: neurologic intensive care unit (3 patients), medical intensive care unit (1 patient), oncology center (1 patient), and medicine unit (1 patient) (Table 2). *S. paucimobilis* grew in multiple sets of blood cultures (5 patients) and from blood cultures collected on >1 date (3 patients). In the preceding 22 months, *S. paucimobilis* had been isolated from blood cultures of 4 patients (Figure 1). As a result of *S. paucimobilis* bloodstream infections, 5 of the case-patients reported here received antimicrobial drug treatment and had central intravenous catheters or implanted medication ports removed and replaced. One of these patients had an adverse reaction (rash and renal insufficiency) to antimicrobial drug treatment. All 5 patients treated with antimicrobial agents became free of *S. paucimobilis* bloodstream infection and survived. One patient (patient 5, Table 2) died of group A streptococcal sepsis before blood culture results for *S. paucimobilis* were available.

No breaches of infection control procedures or inappropriate blood culture practices were identified. Blood for culture was collected by staff in each unit rather than by

Table 2. Demographic and clinical characteristics of patients with *Sphingomonas paucimobilis* bloodstream infection, United States, 2007*

Patient no./ age, y/gender	Hospital unit (US state)	Clinical diagnosis	Date(s) of fentanyl administration†	Date(s) of infection	Treatment	Outcome	PFGE strain‡
1/65/M	Medicine (MD)	Osteomyelitis; MRSA wound infection	NA	Nov 14	ADT, central line removed and replaced	Survived	Unique
2/64/M	NCCU (MD)	Subarachnoid hemorrhage	Oct 29–31, Nov 2–3, Nov 11–22	Nov 14, Nov 18	ADT, central line removed and replaced	Survived	A
3/46/F	NCCU (MD)	Subarachnoid hemorrhage	Nov 10–11 Nov 16–18	Nov 11 Nov 17 Nov 19	ADT, central line removed and replaced	Survived	A
4/69/F	NCCU (MD)	Subarachnoid hemorrhage	Nov 18	Nov 18	ADT, central line removed and replaced	Survived	A
5/38/F	MICU (MD)	Group A streptococcal sepsis	Nov 16	Nov 16	ADT§	Died	A
6/38/M	Oncology (MD)	Head and neck tumor	Nov 20, Nov 26	Nov 20, Nov 23	ADT, implanted medication port removed and replaced	Survived	A
7/38/M	SICU (CA)	Temporal lobe hemorrhage	Oct 29	Oct 29	ADT	Survived	NA¶
8/59/M	SICU (CA)	Entero-cutaneous fistula; aorto-bifemoral bypass surgery	Nov 8	Nov 8	ADT	Survived	NA¶

*All infections (except in patient 1) developed after administration of intravenous fentanyl compounded at an out-of-state pharmacy; MD, Maryland; CA, California; NCCU, neurologic critical care unit; MICU, medical intensive care unit; SICU, surgical intensive care unit; NA, not applicable; ADT, antimicrobial drug therapy; MRSA, methicillin-resistant *Staphylococcus aureus;* PFGE, pulsed-field gel electrophoresis.
†Intravenous fentanyl in 0.9% sodium chloride solution from a 250-mL (10 μg/mL) bag prepared at compounding pharmacy A.
‡Strain A is the outbreak strain that was indistinguishable by PFGE from the fentanyl isolates.
§The patient died of Group A streptococcal sepsis before the blood culture results for *S. paucimobilis* became available.
¶Isolates from patients 7 and 8 in the California hospital were not available for strain typing.

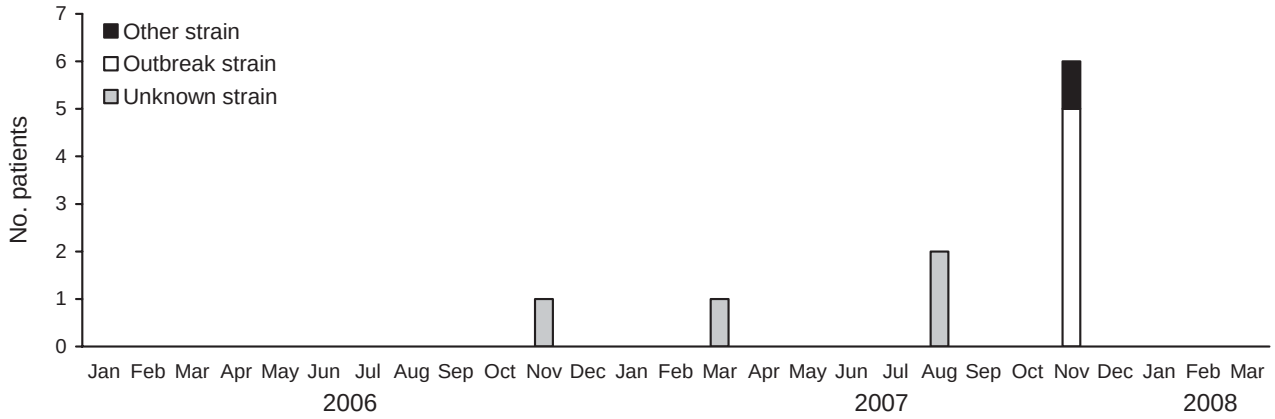

Figure 1. Epidemiologic curve showing the number of patients with *Sphingomonas paucimobilis* bacteremia at The Johns Hopkins Hospital, Baltimore, Maryland, USA, January 2006 through March 2008.

a central vascular access team. No growth occurred from cultures of 50 noninoculated activated charcoal blood culture bottles; this finding was therefore not consistent with a pseudo-outbreak or intrinsic contamination of the bottles. Pharmacy records showed that 5 (83%) of 6 case-patients had received at least 1 intravenous dose of fentanyl (10 μg/mL in 250 mL 0.9% sodium chloride) within the 48 hours before *S. paucimobilis* bacteremia developed. No other common exposure was identified. We found that our hospital had outsourced preparation of 250-mL fentanyl bags to pharmacy A, which shipped several lots to the hospital every 2 weeks.

Cultures of unopened samples from 1 implicated fentanyl lot grew *S. paucimobilis* that had a PFGE pattern indistinguishable from that of the isolates of the 5 patients who had received intravenous fentanyl (Figure 2). A sixth case-patient (patient 1; Table 2), who did not receive intravenous fentanyl, had *S. paucimobilis* with a unique PFGE pattern. Of 26 unopened bags from the implicated lot of fentanyl that were cultured, 16 (62%) grew *S. paucimobilis*. Cultures of 9 samples from 3 other fentanyl lots in use during the outbreak period produced no growth.

CDC's multistate case-finding investigation determined that pharmacy A had distributed the contaminated fentanyl lot to 4 hospitals in different states. A California hospital that had received the implicated lot subsequently identified 2 additional case-patients who had *S. paucimobilis* bacteremia after administration of intravenous fentanyl from pharmacy A (Table 2). After diagnosis of bacteremia, these 2 case-patients received treatment with appropriate antimicrobial drugs without removal of existing central lines and were subsequently discharged from the hospital without complications from the bacteremia. At the California hospital, no unopened bags of the implicated fentanyl lot were available for culture, and the bloodstream isolates were not available for PFGE analysis. Specific lot numbers of fentanyl administered to patients were not available at either hospital. The 2 other hospitals that had received the implicated fentanyl lot did not detect any case-patients. The outbreak ended after the implicated fentanyl lot was removed from clinical areas. All bags of the implicated lot that could be located were tested for sterility at The Johns Hopkins Hospital and CDC before being discarded. The rest of the lot had either been used or was expired and destroyed. No product was recalled by pharmacy A.

Investigation found no evidence of tampering with or diversion of fentanyl within the Maryland hospital. We found stringent procedures in place to document and secure the narcotic chain of custody. No signs of tampering were visible on implicated bags of fentanyl that later grew *S. paucimobilis,* and the bags contained the expected concentration of fentanyl. We found that it was possible, although difficult, to remove and replace safety seals on the bags, which potentially could allow diversion of fentanyl without visible signs of tampering. Analysis of personnel access records from the automated medication and supply management system (Pyxis, Cardinal Health) did not identify any 1 person who had accessed >1 of the machines where the fentanyl was stored in the 4 hospital units with case-patients.

These 250-mL bags of intravenous fentanyl in 0.9% sodium chloride solution (10 μg/mL) are currently available only from pharmacy A and 1 other out-of-state compounding pharmacy. This preparation is the most frequently used at our institution and is not available from any pharmaceutical manufacturer. Because commercially available opioid alternatives at the desired concentration are not available, and to avoid disruption of patient care, our hospital continued to purchase the compounded preparation from pharmacy A. For 3 months after the outbreak, our microbiology laboratory performed sterility testing by culturing samples from each fentanyl lot received. None of

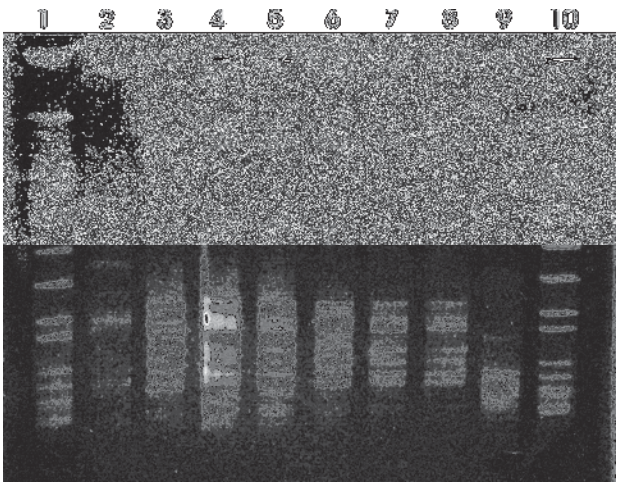

Figure 2. Results of pulsed-field gel electrophoresis (PFGE) of *Sphingomonas paucimobilis* isolates obtained in November 2007. Lanes 1 and 10, molecular weight marker; lanes 2–7, bloodstream isolates from patients 1–6, respectively; lane 8, isolate from contaminated fentanyl; lane 9, unrelated control isolate. Patients 2 through 6 received intravenous fentanyl within 48 hours before *S. paucimobilis* bacteremia developed and had isolates with a PFGE pattern indistinguishable from that of fentanyl isolates. Patient 1 did not receive intravenous fentanyl and had *S. paucimobilis* bacteremia with a distinct PFGE pattern.

these samples grew any bacterial or fungal pathogens. We do not have access to the results of FDA's investigation into compounding practices at pharmacy A.

Discussion

We describe a multistate outbreak of *S. paucimobilis* bacteremia that was associated with contaminated intravenous fentanyl prepared at an out-of-state compounding pharmacy. Astute observation by microbiology laboratory staff, good communication, and swift implementation of an epidemiologic investigation led to expeditious characterization of the source of the infections and termination of the outbreak. Pharmacy A prepares large quantities of compounded pharmaceutical preparations and distributes them to many states in anticipation of orders. In contrast to pharmaceutical manufacturers, traditional compounding pharmacies are not routinely inspected by FDA to ensure that they have the capacity to consistently produce high-quality drugs. In response to other infectious outbreak incidents, the FDA has warned compounding pharmacies that the high-volume production of pharmaceutical preparations in response to bulk orders constitutes manufacturing and is inconsistent with traditional pharmacy compounding (*17*,*18*). Current good manufacturing practice regulations, as defined by FDA, require end-product sterility testing, among other stringent controls, when sterile pharmaceutical products are manufactured (*19*). Although traditional pharmacy compounding fills a commercial void when FDA-approved, commercially available drugs cannot meet patients' medical needs, the lack of end-product sterility testing and other standards of good manufacturing practice is a serious potential threat to patient safety when compounding pharmacies produce and distribute large quantities of sterile pharmaceutical preparations. Multistate distribution of compounded preparations makes outbreaks and clusters of infections even more difficult to detect and manage.

In 2001, as a result of a dispute over advertising restrictions, the Supreme Court ruled that the compounding provisions of the FDA Modernization Act of 1997 were unconstitutional (*20*–*22*). In 2002, the FDA issued guidance to clarify the FDA's position on pharmaceutical compounding; the guidance identified factors that are considered in deciding whether to initiate enforcement action with respect to compounding (*1*). FDA historically has not taken enforcement actions against pharmacies engaged in traditional pharmacy compounding, but it has directed its enforcement against establishments whose activities are normally associated with a drug manufacturer. However, much of the investigation, regulation, and enforcement of compounding falls to state licensing boards (*2*,*18*). The American Society of Health-System Pharmacists (ASHP), the United States Pharmacopeia (USP), and the National Association of Boards of Pharmacy have issued practice and quality assurance guidelines for sterile compounding of pharmaceutical preparations (*23*,*24*). In 2004, USP chapter 797 put forth a set of enforceable standards for the compounding of sterile preparations (*25*); recently revised standards took effect on June 1, 2008 (*24*). These standards do not require end-product sterility testing for most compounded preparations (*24*). Some states require compounding pharmacies to comply with USP 797 and ASHP standards; however, a national survey found that many pharmacies are not fully compliant with ASHP quality assurance guidelines (*26*), and a 2006 survey documented incomplete awareness and implementation of USP 797 standards (*27*). From 1990 through 2002, the FDA received reports of >55 quality problems associated with compounded preparations (*28*); Table 1 shows reports of 6 such incidents published since 2002. An FDA survey in 2001 found 34% of tested preparations from compounding pharmacies failed to meet analytic testing standards, although none failed sterility tests (*28*).

Our investigation had limitations. Neither hospital could trace the lot numbers administered to each patient, so we could neither confirm which patients received contaminated lots of fentanyl nor assess an attack rate for receipt of the contaminated lots. We did not conduct a case–control study to calculate an odds ratio for administration of fentanyl being the primary risk factor for infection. Isolates

from California case-patients were not available for strain typing. Finally, we do not have access to FDA's investigational findings regarding pharmacy A, which might indicate the source of the contamination. Despite these limitations, the available evidence strongly suggests that contaminated fentanyl from pharmacy A was the source of this multistate outbreak of *S. paucimobilis* bloodstream infections.

FDA continues to face many serious and complex challenges (*29*,*30*). Our investigation, along with other similar published reports, indicates that the issue of large-scale pharmaceutical production and distribution by compounding pharmacies is also an urgent concern that requires attention. Pharmacies that compound and distribute large quantities of sterile pharmaceutical preparations without prescriptions for individual patients should be considered manufacturers and should be required to follow good manufacturing practices, including end-product sterility testing.

Until stricter regulations are imposed and enforced, hospital pharmacists and administrators must be cautious when outsourcing compounded pharmaceutical preparations and should consider the possibility of contaminated sterile pharmaceutical products when unusual organisms or patterns of disease are detected. Hospital personnel may be unaware that preparation of pharmaceutical products has been outsourced to a compounding pharmacy (*3*) and may not recognize the different regulatory requirements and potential implications of this decision. Healthcare facilities should strongly consider recording lot numbers of compounded medications administered to individual patients because this would aid investigations. Increased outsourcing of compounded pharmaceutical preparations makes surveillance for unexpected untoward events increasingly important. Compounding pharmacies should adhere to the standards set forth in USP 797 and should adopt more stringent quality control measures, including prerelease product testing, when preparing and distributing large quantities of sterile preparations.

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Human Infection with Highly Pathogenic Avian Influenza Virus (H5N1) in Northern Vietnam, 2004–2005

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We performed a retrospective case-series study of patients with influenza A (H5N1) admitted to the National Institute of Infectious and Tropical Diseases in Hanoi, Vietnam, from January 2004 through July 2005 with symptoms of acute respiratory tract infection, a history of high-risk exposure or chest radiographic findings such as pneumonia, and positive findings for A/H5 viral RNA by reverse transcription–PCR. We investigated data from 29 patients (mean age 35.1 years) of whom 7 (24.1%) had died. Mortality rates were 20% (5/25) and 50% (2/4) among patients treated with or without oseltamivir (p = 0.24), respectively, and were 33.3% (5/15) and 14.2% (2/14) among patients treated with and without methylprednisolone (p = 0.39), respectively. After exact logistic regression analysis was adjusted for variation in severity, no significant effectiveness for survival was observed among patients treated with oseltamivir or methylprednisolone.

Human infection with the highly pathogenic avian influenza A virus (H5N1) was discovered in Hong Kong Special Administrative Region, People's Republic of China, in 1997 (*1–3*). It has since been identified in other countries, primarily in Southeast Asia. Among 100 confirmed infected patients, 46 have died in Vietnam since 2003 (*4,5*).

Severe viral pneumonia accompanied by diffuse alveolar damage develops in patients infected with influenza virus (H5N1) (*6*). High viral load causes intense cytokine reactions and inflammation (*7*). Clinical factors that might be associated with severity include age, delayed consultation, lower respiratory tract lesions, and leukopenia (*4,8–10*). However, few cases have reported which factors, including patient management, affect outcomes. Our study reviews the clinical courses of patients treated in Hanoi, Vietnam, and investigates the association between clinical findings and survival.

The effects of oseltamivir and other neuraminidase inhibitors have been demonstrated in experimental models (*11–13*), but their outcomes in humans have not been verified. Randomized controlled trials would be optimal for investigating the effectiveness of oseltamivir compared with placebo, but they are not an option because of ethical issues. Therefore, this issue can only be addressed through observational studies. Despite limited empirical evidence, the World Health Organization (WHO) reported that oseltamivir improved survival (*14*) and recommended treatment with oseltamivir because of high mortality rates associated with influenza A virus (*14,15*). Patients from northern Vietnam are described in detail.

Methods

We investigated patients infected with influenza A virus (H5N1) who were referred to the National Institute of Infectious and Tropical Diseases in Hanoi, Vietnam, from other local hospitals from January 2004 through July 2005. Pediatric patients were admitted to another institution in Hanoi and were excluded from the present study. A WHO inspection team at the National Institute for Hygiene and Epidemiology in Hanoi virologically confirmed H5N1 subtype infection in the patients by using a reverse transcrip-

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tion–PCR (RT-PCR) for influenza A/H5. We investigated only patients with H5N1 subtype infection determined from symptoms of acute respiratory tract infection, a history of high-risk exposure, or chest radiographic findings such as pneumonia. All patients were reported to WHO as having confirmed infection with avian influenza virus (H5N1). We excluded other patients with positive RT-PCR results because of reasons described below. The study was reviewed and approved by the ethics committees at the International Medical Center of Japan and the National Institute of Infectious and Tropical Diseases in Vietnam.

Data were obtained for general characteristics, history of high-risk exposure, medical history, symptoms, signs, microbiologic and biochemical test results, chest radiographic findings, treatment strategies, and outcomes from medical records from April through October 2006.

We investigated associations between clinical findings and survival by using univariate analysis. Initial laboratory and chest radiographic findings after hospitalization were recorded in the medical charts and used. The relationship between survival and treatment with oseltamivir or methylprednisolone was investigated by adjusting for factors related to severity in an exact logistic regression analysis, which is appropriate for small amounts or unbalanced binary data (*16*). Because the study cohort was small, deaths were few and overfitting was possible (*17*,*18*); only 1 covariate could be added for adjustment into the logistic regression model. Therefore, we used leukocyte counts, platelet counts, aspartate aminotransferase (AST) levels, and urea nitrogen levels as an adjustment for severity because these values are associated with reported outcomes (*14*). Also, many missing observations prevented adjustment using albumin levels. Data were analyzed by using the Wilcoxon test, χ^2 test, and Fisher exact test when appropriate and the statistical package SAS version 8.2 (SAS Institute, Cary, NC, USA).

Results

Among 41 patients who were hospitalized from January 2004 through July 2005 and had positive RT-PCR results, 12 were excluded from the study (3 patients whose medical records were unavailable; 2 patients related to persons with confirmed H5N1 subtype pneumonia who were asymptomatic, positive for viral RNA, and treated with prophylactic oseltamivir; and 7 patients who had some illnesses, particularly respiratory diseases, which complicated interpretation of the clinical course or chest radiographic findings). We therefore studied 29 patients with clinically and virologically confirmed influenza A (H5N1) infection.

Table 1 shows the general characteristics of the patients, and the Figure shows the clinical course from onset of disease to hospitalization and discharge. Patients ranged in age from 14 to 67 years with a mean age of 35.1 years. A total of 25 patients were given 150 mg/day of oseltamivir, and 15 were treated with methylprednisolone (initial dose 40–160 mg/day, median dose 80 mg/day). Seven (24.1%) of the 29 patients died. No significant associations were found between mortality rates and age (p = 0.57), sex (p = 0.68), history of high-risk exposure

Table 1. Characteristics of 29 patients infected with highly pathogenic avian influenza virus (H5N1), northern Vietnam, 2004–2005*

Characteristic	Value
Age, y, mean ± SD	35.1 ± 14.4
M:F sex (%)	15:14 (52:48)
High-risk exposure, no. (%)†	
Poultry	19 (65.5)
Sick poultry	12 (41.4)
Family infected with H5N1 virus subtype	6 (20.7)
Sick poultry or person	15 (51.7)
Hospitalization after disease onset, median, d (IQR)	6 (4–8)
Hospital stay, median, d (IQR)	14 (9–17)
Treated with oseltamivir, no. (%)	25 (86.2)
Began treatment with oseltamivir after disease onset, median, d (IQR)	7 (5–10)
Treated with methylprednisolone, no. (%)	15 (51.7)
Died, no. (%)	7(24.1)

*IQR, interquartile range.
†Poultry, a history of exposure to sick or healthy poultry; sick poultry or person, a history of exposure to sick poultry or a family infected with avian influenza (H5N1).

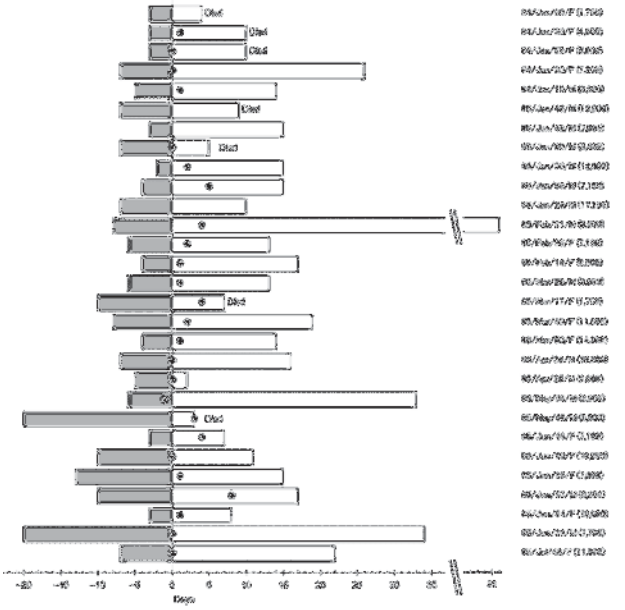

Figure. Clinical course of 29 patients infected with highly pathogenic avian influenza virus (H5N1), northern Vietnam, 2004–2005. Zero days on horizontal axis represent days of hospitalization at the National Institute of Infectious and Tropical Diseases. Shaded bars, days between disease onset and hospitalization; open bars, days between hospitalization and discharge; dots, start of oseltamivir treatment. Information on the right shows date of hospitalization, age in years, sex, and leukocyte count per microliter.

(contact with poultry [p = 1.00], contact with sick poultry [p = 1.00], and contact with sick poultry or persons [p = 1.00]). Three of 6 patients from a family infected with H5N1 subtype died, and 4 of 23 patients without such an association died (p = 0.13). Duration between onset of disease and hospitalization was not associated with higher mortality rates (p = 0.98).

Table 2 shows initial laboratory findings at hospitalization. Leukopenia (neutropenia), thrombocytopenia, hypoalbuminemia, and increased AST and urea nitrogen levels were associated with increased deaths.

Five (20.0%) of the 25 patients treated with oseltamivir died, as did 2 (50.0%) of 4 who were not treated (odds ratio 0.25, 95% confidence interval [CI] 0.03–2.24, p = 0.24). To adjust for variation in disease severity among patients, exact logistic regression was performed by using leukocyte counts, platelet counts, AST levels, and urea nitrogen levels. Adjusted odds ratios for deaths among patients treated with oseltamivir were 0.15 (95% CI 0.00–2.57, p = 0.19), 0.16 (95% CI 0.00–2.23, p = 0.17), 0.54 (95% CI 0.02–11.85, p = 1.00), and 0.28 (95% CI 0.01–5.16, p = 0.55), respectively, for the 4 adjustments for disease severity.

The time between the onset of symptoms and initiation of treatment with oseltamivir varied (Table 1, Figure). The mortality rates were 20% (3/15) and 20% (2/10) when treatment with oseltamivir was started within and after 7 days of disease onset.

Methylprednisolone was given to 15 of 29 patients. Five (33.3%) of these 15 patients died, and 2 (14.3%) of 14 patients who were not given this drug died (odds ratio 3.0, 95% CI 0.48–18.93, p = 0.39). Exact logistic regression after adjustment for severity by using leukocyte counts, platelet counts, AST levels, or urea nitrogen levels showed odds ratios for deaths among patients treated with methylprednisolone of 0.74 (95% CI 0.00–9.57, p = 0.82), 1.82 (95% CI 0.18–25.48, p = 0.89), 1.14 (95% CI 0.07–18.92, p = 1.00), and 2.43 (95% CI 0.28–31.69, p = 0.61), respectively.

Thirteen patients were treated with oseltamivir and methylprednisolone. The regression model that included these 2 drugs and interactions did not show effectiveness of either drug.

Discussion

The overall mortality rate of 24.1% in this study was lower than rates in previous studies and WHO reports. Table 3 summarizes the characteristics of patients from previous studies. Patients in the present study were older because pediatric patients were excluded because of treatment elsewhere. WHO has reported that the mortality rate of 73% for infection with H5NI subtype is highest in persons 10–19 years of age, and that patients 20–39 years of age account for >60% of the deaths (*22*). The expected mortality rate would be 51.8% if our case-patients had the same age-specific mortality rate as in a WHO report (*14*). The lower mortality rate in our study could not be explained by an age difference. The relatively high leukocyte count and factors related to outcomes suggest that a reasonably large number of mildly infected patients might have been included, although chest radiographs showed variable progression in lesions from mild to severe.

Persons who died were concentrated in the early period of the study, especially in 2004. Virus genotype and load data could provide useful information on pathogenesis and outcome. However, these data were not available.

Factors affecting outcome were leukocyte and platelet counts, and albumin, ALT, and urea nitrogen levels. Results were consistent with previous findings (*14*) and

Table 2. Initial laboratory and chest radiographic results for 29 patients infected with highly pathogenic avian influenza virus (H5N1), northern Vietnam, 2004–2005*

Characteristic	Survived, median (interquartile range), n = 22	Died, median (interquartile range), n = 7	p value
Leukocytes, × $10^3/\mu L$	7.8 (7.1–12.0)	3.4 (1.7–5.6)†	0.0093
Neutrophils, ×$10^3/\mu L$	6.8 (4.8–9.9)	2.3 (1.1–3.8)†	0.0101
Hemoglobin, g/L	130 (107–137)	121 (103–138)	0.6102
Platelets, × $10^3/\mu L$	214 (181–284)	86 (38–139)†	0.0101
Albumin, g/L	34.5 (31.2–35.1)	21.7 (10.4–29.4)†	0.0265
AST, U/L	45 (28–69)	327 (77–352)†	0.0077
Total bilirubin, μmol/L	10.3 (7.6–16.8)	11.4 (7.0–27.1)	0.7921
Urea nitrogen, mmol/L	4.5 (3.4–5.5)	9 (3.4–14.3)†	0.0462
Initial chest radiographic findings‡			
No or slight lesion	7	1	0.6510
Moderate lesion	10	3	
Severe lesion	5	3	

*Not all laboratory findings and chest radiographic results were available. Results were derived from the following numbers of patients: complete blood count, 29; albumin 12; AST, 25; total bilirubin, 17; urea nitrogen, 27. AST, aspartate aminotransferase.
†p<0.05, by Wilcoxon test or Fisher exact test.
‡No or slight lesion, no lesion or localized (occupying less than one third of unilateral lung fields) in 1 lung; moderate, diffuse in 1 lung or localized but evident in both lungs; severe, diffuse in both lungs.

Table 3. Comparison of studies of influenza A virus (H5N1)–infected patients*

Author (reference)	Year	Patient age, y, mean ± SD	Leukocyte count, median, × 10^3/μL (IQR)	Hospitalization after disease onset, d, median (IQR)	Outcome	
					No. alive	No. died
Present study	2009	35.1 ± 14.4	7.2 (3.9–11.3)	6 (3.5–8)	22	7
Yuen at al. (*8*)†	1998	17.6 ± 20.4	5.6 (2.47–10.7)	3 (2–4)	5	5
Tran et al. (*4*)	2004	13.7 ± 6.4	2.1 (1.9–2.8)	6 (5–7)	2	8
Chotpitayasunondh et al. (*9*)	2005	22.0 ± 21.4	4.43 (2.75–5.64)	NR	4	8
Oner et al. (*19*)	2006	10.1 ± 4.0	3.8 (1.8–5.75)	6 (4–7)	4	4
Kandun et al. (*20*)	2007	15.4 ± 14.9	3.59 (2.605–6.3)	7 (5–7)	4	4
Buchy et al. (*21*)	2007	16.0 ± 9.9	4.2 (3.6–4.7)	6 (5–7)	0	6

*IQR, interquartile range; NR, not reported.
†Outcomes of 2 patients were not reported.

suggested that outcome is related to lesions in several organs.

Although the mortality rate was lower among patients treated with oseltamivir, differences were not significant. Exact logistic regression after adjustments for laboratory results yielded an odds ratio of 0.15–0.54 for death. The small number of patients prevented valid adjustment, and confounding factors might not have been sufficiently eliminated. A larger patient cohort should be able to adjust for severity of disease.

If one considers the possibility of confounding factors, the reason oseltamivir was not prescribed should be investigated. If oseltamivir was withheld from patients with severe infections and administered only to those with milder symptoms, the drug would apparently be more effective. Among 4 patients who were not prescribed oseltamivir, initial RT-PCR results were negative for 1 patient, who subsequently died. Oseltamivir was unavailable for treatment of another patient who died. The other 2 surviving patients were not prescribed oseltamivir because their chest radiographs showed only minimal lesions. Therefore, withholding oseltamivir was not associated with disease severity.

Higher doses of oseltamivir or longer drug administration have improved outcomes in animal models (*23*,*24*). Because all patients in our study were given oseltamivir at a dose of 150 mg/day, we could not investigate the effect of a higher dose.

Mortality rates were higher in patients treated with methylprednisolone than in those not treated with this drug. This finding can be explained by disease severity because severely ill patients were more likely to be given methylprednisolone. However, even after we adjusted for this confounding effect, no beneficial effect of methylprednisolone was observed. Further, an experimental model has recently raised doubt about the effect of cytokine suppression (*25*).

Our study described patients infected with influenza A virus (H5N1) in Hanoi, Vietnam. These patients had lower mortality rates than those reported in other studies. The reason for the low mortality rate could not be investigated thoroughly without virologic information. Oseltamivir was prescribed in 25 of 29 patients, and their mortality rate was apparently decreased, although the patient cohort was too small to generate sufficient statistical power. In addition, since our study was an observational study, these findings might have been influenced by confounding factors. Further detailed observations from a larger number of patients are required.

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Enhanced Hygiene Measures and Norovirus Transmission during an Outbreak

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Control of norovirus outbreaks relies on enhanced hygiene measures, such as handwashing, surface cleaning, using disposable paper towels, and using separate toilets for sick and well persons. However, little is known about their effectiveness in limiting further spread of norovirus infections. We analyzed norovirus outbreaks in 7 camps at an international scouting jamboree in the Netherlands during 2004. Implementation of hygiene measures coincided with an 84.8% (95% predictive interval 81.2%–86.6%) reduction in reproduction number. This reduction was unexpectedly large but still below the reduction needed to contain a norovirus outbreak. Even more stringent control measures are required to break the chain of transmission of norovirus.

Gastroenteritis is one of the most common causes of illness (*1*). Recent findings indicate norovirus is the most common cause of gastroenteritis (*2*,*3*). Of all gastroenteritis outbreaks reported in the Netherlands during 2002, 54% were caused by norovirus (*4*). Norovirus is predominantly transmitted through the fecal–oral route, either indirectly through contaminated food or surfaces or directly from person to person (*5*). It can be transmitted through small infectious droplets (aerosols) after a vomiting episode (*6*,*7*) and can survive for a very long time in the environment (*5*,*8*). Most norovirus outbreaks are seen in settings where clusters of vulnerable, susceptible persons live closely together, such as nursing homes, hospitals, and daycare centers (*4*), and in settings in which turnover of susceptible persons is high, such as hotels and cruise ships (*9*,*10*).

Norovirus infection can cause serious medical complications, such as dehydration, in persons with underlying illness (*11*). No antiviral treatment exists for norovirus infection, and although norovirus vaccines are in development (*12*), none are available yet. Early studies suggested that norovirus outbreaks could be contained by rapid implementation of enhanced hygiene measures, such as washing hands, thoroughly cleaning contaminated surfaces, avoiding contact between sick and healthy persons, and requesting caretakers and cleaning staff to wear gloves and aprons (*13*–*16*). However, in nursing homes or on cruise ships, norovirus can cause consecutive outbreaks, even after implementation of strict hygiene protocols (*9*,*17*,*18*). No quantitative estimates exist of the results of such enhanced hygiene measures on reducing further transmission of norovirus. To our knowledge, the effect of enhanced hygiene measures has not been investigated in randomized controlled trials or in statistical analyses of outbreaks.

We investigated the effect of enhanced hygiene measures on reducing norovirus transmission during an outbreak. We measured the effectiveness of enhanced hygiene measures as the relative reduction in the reproduction number—defined as the average number of secondary cases caused by 1 typical case—in the absence of and after enhanced hygiene measures. The value of this reproduction number provides crucial information about transmission potential: if the reproduction number exceeds the threshold value of 1, the number of new cases will increase over time; if it is <1, the number of new cases will decline over time, and eventually the chain of transmission will break.

The time course of the reproduction number during an outbreak can be inferred from the epidemic curve (*19*,*20*).

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We obtained a detailed epidemic curve of a norovirus outbreak at an international scout jamboree in the Netherlands from July 26 through August 10, 2004. This outbreak was ideally suited to estimating the effects of enhanced hygiene measures because the date enhanced hygiene measures began was recorded. Moreover, because the scouts were divided into 7 camps, the jamboree provided a natural experiment in which the camps could be regarded as "experimental units," with varying durations between introduction of the virus and implementation of enhanced hygiene measures.

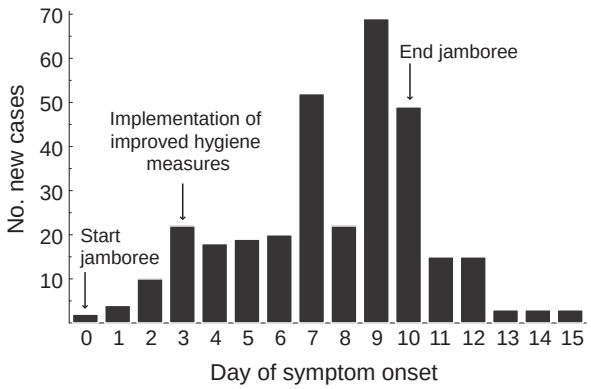

Figure 1. Epidemic curve of an outbreak of norovirus at an international scout jamboree in the Netherlands, starting July 26, 2004 (day 0).

Methods

Data

An outbreak of norovirus infection occurred at an international scout jamboree in the Netherlands during the summer of 2004 (*21*). Approximately 4,500 persons from 32 countries attended this event. At the start of the scout jamboree on July 26, 2004, two participants became ill with symptoms of gastroenteritis. The outbreak affected at least 326 persons with typical, generally mild symptoms of gastroenteritis (case-patients). Most ill persons experienced vomiting (258) and/or diarrhea (195). Ninety-two ill persons visited a local first aid tent; another 54 were admitted to a local hospital for rehydration.

The jamboree was held on a large site, ≈600 m × 1,000 m. Jamboree participants were divided into 7 camps according to age: 3 camps each for participants 11–14 and 15–17 years of age and 1 camp for staff ≥18 years of age. The 7 camps were situated around a central field for joint activities; most activities were organized within the camps. The camps were labeled A–G, according to the day the first participants became sick. For 296 (91%) of 326 case-patients, the camp label was known (Figure 1, Table).

On July 29 (day 3 of the jamboree), the Municipal Health Service "Hart voor Brabant" in 's-Hertogenbosch provided advice on enhanced hygiene measures (*22*), instructed participants about proper hand hygiene and use of soap pumps and disposable paper towels, and assigned separate toilets for sick participants. In addition, the Municipal Health Service provided guidelines for cleaning toilets and contaminated surfaces with a 1,000-ppm chlorine solution. Sick participants were instructed to go to a first aid tent. Sick participants were not allowed to prepare food until 3 days after their last symptoms. Persons working in the jamboree's field hospital were instructed to wear gloves, aprons, and surgical masks and to minimize the number of patients per nurse. The scout jamboree ended on August 5.

Norovirus was epidemiologically implicated as the causative agent (*21*) of the outbreak and was confirmed in stool samples through a standard reverse transcription-PCR protocol (*23*). Typing of 7 samples from case-patients in whom symptoms first developed 7–9 days after the outbreak began resulted in 3 norovirus genotypes: 2 samples typed as norovirus genotype GI.4, 1 sample typed as genotype GI.5, and 4 samples typed as genotype GII.4–2004. We did not detect any multiple infections.

During the outbreak, the Municipal Health Service assessed the number of new cases from typical gastroenteritis symptoms self-reported by participants and staff. After the jamboree, participants and staff were given a questionnaire asking them to report to the Municipal Health Service whether gastroenteritis had developed within a week after departure. The questionnaire asked the date of symptom onset, symptoms, camp label, and hospital admission.

Reproduction Number

Estimation of Reproduction Numbers

We estimated the reproduction number for every case during the norovirus outbreak at the jamboree. Using the date of symptom onset for each case, we applied statistical methods to reconstruct likely patterns of who infected whom (online Technical Appendix 1, available from www.cdc.gov/EID/content/15/1/24-Techapp1.pdf). We first calculated the difference in day of symptom onset for all combinations of case pairs. To calculate the probability of transmission between any pair of cases, we needed information from the distribution of generation times (defined as the time between day of symptom onset in a secondary case and day of onset in its primary case) (*19*,*24*). To estimate the generation time distribution for norovirus infections, we used observations of generation times from several large norovirus outbreaks in child daycare centers in Sweden during 1999 (*25*). These generation times were well described by a gamma distribution (Figure 2), for which the parameters were estimated by the method of maximum likelihood (online Technical Appendix 1). The frequency

Table. New norovirus cases during outbreak at international scout jamboree, the Netherlands, starting on Jul 26, 2004 (day 0), by day of symptom onset and camp label

Day of onset	Camp, no. new cases/d								Total (n = 4,500)
	A (n = 485)	B (n = 721)	C (n = 729)	D (n = 499)	E (n = 735)	F (n = 825*)	G (n = 506)	Unknown	
0	1	1	0	0	0	0	0	0	2
1	1	0	1	0	0	0	0	2	4
2	0	2	2	1	1	3	0	1	10
3	2	7	9	0	2	1	0	1	22
4	3	4	2	1	2	2	4	0	18
5	0	10	1	2	1	1	2	2	19
6	0	12	3	2	2	0	0	1	20
7	2	19	14	2	3	3	6	3	52
8	3	5	8	2	2	1	1	0	22
9	7	10	14	0	2	10	24	2	69
10	5	4	3	2	0	16	8	11	49
11	3	2	4	1	0	1	1	3	15
12	4	1	4	2	1	1	0	2	15
13	0	0	1	0	0	0	1	1	3
14	0	0	1	0	0	1	0	1	3
15	0	0	1	0	1	1	0	0	3
Total†	31	77	68	15	17	41	47	30	326

*This number is estimated.
†Overall attack rate: 7.2. Attack rate by camp: A, 6.4; B, 10.7; C, 9.3; D, 3.0; E, 2.3; F, 5.0; G, 9.3.

distribution of generation times was used to assign a likelihood of transmission for any pair of cases, allowing estimation of the transmission probabilities. We then used a powerful statistical sampling algorithm to generate a large sample of plausible transmission patterns (for technical details, see online Technical Appendix 1). The expected value of the reproduction number for a specific case was the sum of all transmission probabilities of outgoing infectious contacts to all other cases in the outbreak. For cases in which symptoms began the same day, we calculated the mean minimum and maximum values of the reproduction number. For the entire sample of transmission probabilities, we obtained the 0.025 and 0.975 quantiles for these 3 metrics as predictive intervals.

Host Population Structure and Pathogen Genotype

We incorporated additional information about the camp label of almost all case-patients and the pathogen genotype for 7 case-patients into the estimation procedure by adding a ”weight” to the transmission probabilities between pairs of cases. Here we considered 2 extreme cases for mixing between camps. The first extreme case was homogeneous mixing between all participants of the jamboree, as we assumed in the analysis described above; to achieve this, we assigned a weight of 1 to any pair of cases. The second extreme case was mixing within camps only and no mixing between camps. In this instance, the transmission probabilities for pairs of case-patients that stayed in different camps were assigned a weight of 0, and the transmission probabilities for pairs of case-patients that stayed in the same camp were given a weight of 1. The transmission probabilities for pairs of cases with known different genotypes were assigned a weight of 0, and the transmission probabilities for pairs of cases with known identical genotypes were assigned a weight of 1.

Expected Time Course of Reproduction Number

If the enhanced hygiene measures resulted in a sudden decline in transmission, the expected decline of the reproduction number would be gradual. Four factors determined the expected time course: the day enhanced hygiene measures began, the cumulative frequency distribution of generation times, the reproduction number without enhanced hygiene measures, and the relative reduction of the reproduction number attributed to hygiene measures. We express the reproduction number as a function of these 4 factors (online Technical Appendix 1) and fitted this function to every sampled time course of the mean reproduction number for days 0–5, with least squares regression to obtain point estimates and 95% predictive intervals for the parameters describing the reproduction number in the absence of hygiene measures and relative reduction of the reproduction number resulting from the hygiene measures.

Testing of the Estimation Procedures

We tested the estimation procedure by simulating epidemic curves with known reproduction numbers. The interval estimates for reproduction numbers covered the actual values for days 0–7. We detected a slight downward bias for the estimated value of reproduction numbers and a slight downward bias for the estimated realtive reduction of reproduction numbers after implementation of

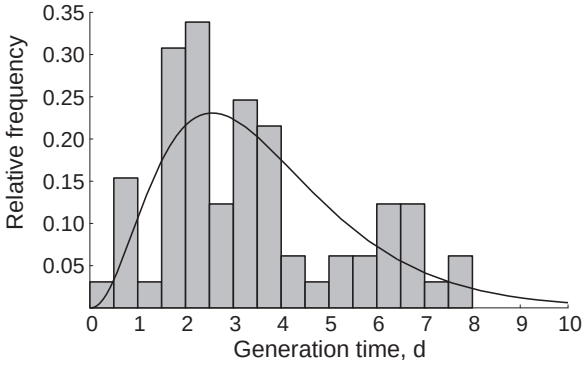

Figure 2. Generation time distribution for norovirus infections. Generation time is the time between onset of symptoms in successive case-patients. The histogram gives the relative frequency in norovirus outbreaks in Sweden in 1999 (*25*); the black line indicates the maximum-likelihood fit of the gamma distribution.

enhanced hygiene measures, indicating that the values obtained by the estimation procedure are conservative (online Technical Appendix 2, available from www.cdc.gov/EID/content/15/1/24-Techapp2.pdf).

Results

The estimated reproduction numbers decreased over time as the norovirus outbreak spread through the international scout jamboree (Figure 3). We estimated an initial reproduction number of 7.26 secondary cases per primary case (95% predictive interval 5.26–9.25); 5 days after the enhanced hygiene protocol began, the estimated reproduction number dropped below 1 (Figure 3, black diamonds). Under the hypothesis that transmission potential decreased abruptly when enhanced hygiene measures began, we estimated a reproduction number of 14.05 secondary cases per primary case (95% predictive interval 9.96–17.98) without enhanced hygiene measures and a reproduction number of 2.13 secondary cases per primary case (95% predictive interval 1.88–2.40) with enhanced hygiene measures (Figure 3, black solid line). This decrease corresponded to a relative reduction in reproduction number of 84.8% (95% predictive interval 81.2%–86.6%).

The disease attack rate varied among different camps, from 2.3% to 10.7%; overall attack rate was 7.2% (Table). For camps A and B, the estimated time course of reproduction number was initially high for the 2 index case-patients (Figure 4, black diamonds). Repeating the analysis with additional information about the host population structure and pathogen genotypes resulted in similar point estimates of the reproduction numbers (Figure 4, gray boxes) but with narrower predictive intervals. The value of the initial reproduction number in each camp followed a time course consistent with 85% reduced transmission when enhanced hygiene measures were implemented (Figure 4, black solid lines), indicating the time course of the reproduction numbers did not depend on the time of introduction of norovirus in the camp—because this differed between camps—but on the time the enhanced hygiene protocol began, which was identical for all camps.

Discussion

We have shown that during an outbreak of norovirus, implementation of enhanced hygiene measures coincided with an 85% reduction of norovirus transmission, from 14.05 secondary cases per primary case before enhanced hygiene measures to 2.13 secondary cases per primary case after enhanced hygiene measures. This estimate is consistent with the time course of reproduction numbers in different camps in which infection was introduced at different times. Our estimates confirm the alleged high epidemic potential of norovirus and suggest that the enhanced hygiene measures were not sufficient to reduce the reproduction number below the threshold value of 1. This estimate explains why the number of new cases per day continued to increase and why norovirus infection spread to new camps, even after implementation of enhanced hygiene measures. It is tempting to speculate that our findings could be extrapolated to other hygiene measures to explain the typical pattern in several subsequent norovirus outbreaks on cruise ships and in hotels (*9*,*26*,*27*).

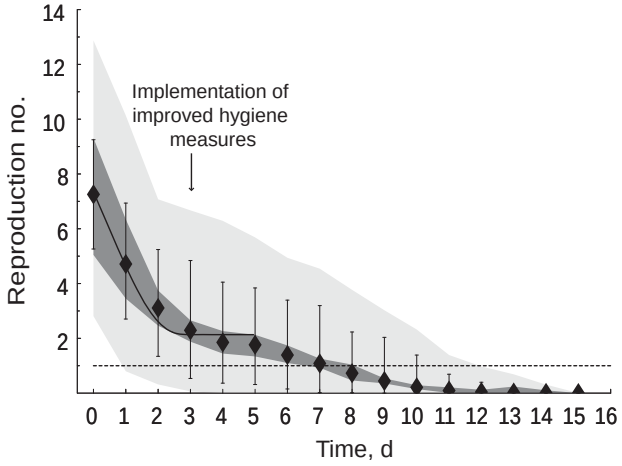

Figure 3. Time course of the reproduction number for norovirus at an international scout jamboree, starting July 26, 2004 (day 0), in the Netherlands. Black diamonds show the mean value for the reproduction number over all sampled transmission matrices; vertical lines, mean minimum and maximum values for the reproduction number over all sampled transmission matrices. The dark gray area shows the uncertainty range (0.025 and 0.975 quantiles) in the mean reproduction number; light gray area, the uncertainty range (0.025 and 0.975 quantiles) of the maximum and minimum estimates of the reproduction number. The solid black line represents the fitted time course of reproduction numbers if decrease in the mean reproduction number results from an instantaneous decline in transmission when enhanced hygiene measures began; dashed line, the threshold value of 1, below which the outbreak was controlled.

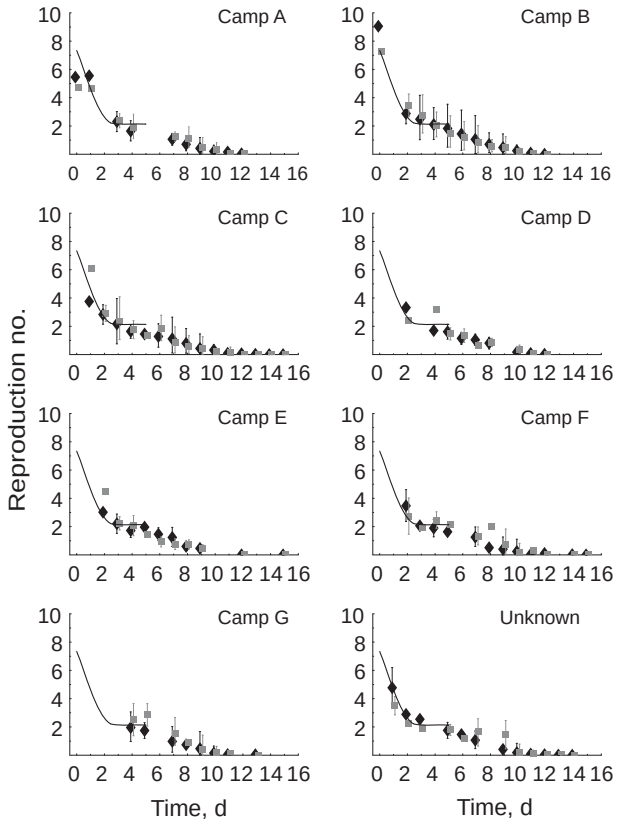

Figure 4. Time course of the reproduction number for norovirus for 7 camps of an international scout jamboree. Black diamonds show the mean value of the reproduction number without additional information about population structure and genotypes. Gray boxes show the mean value of the reproduction number when additional information about population structure and genotypes is used. The vertical lines show the mean minimum and maximum reproduction number over all sampled transmission matrices. The solid black line represents the time course of reproduction numbers if decrease in the mean reproduction number results from an instantaneous decline in transmission when enhanced hygiene measures begin. The camps are in order of the day of symptom onset of the first case-patient. Top panels indicate first introduction, bottom panels the last introduction.

The estimation procedure for the time course of the reproduction number has several limitations. It requires a frequency distribution for the generation time, which may be unknown for many diseases that are less well studied than norovirus. The procedure also requires reporting of symptom onset of case-patients over intervals on the order of the mean generation time or smaller. Here, because the mean generation time was 3.6 days, we cannot use weekly reports of time of symptom onset. The procedure also requires a large outbreak so the effects of chance events on the course of the epidemic are minimized. Small outbreaks would lead to estimates of reproduction numbers that are highly uncertain and have questionable value for making generalizations about transmission.

Our main result is that the observed decline in the reproduction number coincided with implementation of enhanced hygiene measures. This extrapolation is highly suggestive of a causal relationship, which implies that hygiene measures can effectively reduce transmission of norovirus. However, several alternatives can explain the declining reproduction number, as discussed below.

First, the decrease in reproduction number may be due to chance events. Here we explicitly estimated the reproduction numbers from times of symptom onset and the generation time distribution for norovirus infections, whereas earlier work relied on transforming epidemic curves to reproduction numbers (*19*,*20*). The tests of our explicit estimation procedure indicate that the interval estimates cover the actual values of reproduction numbers and the reduced reproduction numbers after the implementation of hygiene measures. The predictive interval for the relative reduction of 81.2%–86.6% clearly shows the change is statistically significant because it excludes the null hypothesis of a change of 0%. The tests also indicate a slight bias in the estimated values toward lower values, which suggests that the estimated 85% reduction after enhanced hygiene measures began should be treated as a conservative estimate. Therefore, the reduction in transmission is highly unlikely to be due to chance.

Second, it might be that jamboree participants differed in susceptibility, and the pool of highly susceptible persons was depleted during the first days of the outbreak. However, preexisting immunity for the genotypes involved seems highly unlikely: GI.5 and GI.4 rarely are detected in Europe, and the GII.4–2004 genotype caused a large epidemic during the winter after the jamboree (*28*). The number of persons infected before implementation of enhanced hygiene measures was smaller than the total number of case-patients, and the total number of case-patients was smaller than the number of jamboree participants. Depletion of susceptible persons or different susceptibility is highly unlikely to explain the sudden decrease in transmission around day 3 of the outbreak.

Third, the decline in reproduction number could be because many infections were asymptomatic and many symptomatic cases were not reported. The request to report any symptoms might not have reached all participants because of the event's large size and because participants came from many different countries. During norovirus outbreaks, asymptomatic cases occur; in almost half of the outbreaks in the Netherlands during 2002, stool samples from ≥1 healthy persons tested positive for norovirus (*4*). Volunteer and outbreak studies demonstrate that 30% of collected stool specimens of exposed, asymptomatic persons were positive for norovirus (*29*–*31*). However, both the proportion of asymptomatic infections and the reporting rate, as long as they remain constant, do not influence the value of the reproduction number because the reproduc-

tion number is estimated as the ratio of the number of secondary cases to the number of primary cases: both the proportion of asymptomatic infections and the reporting rate affect both the numerator and the denominator of this ratio, thereby canceling out in this calculation. Therefore, how the proportion of asymptomatic infections or the reporting rate would have resulted in a similar decline in reproduction number in the different camps is difficult to imagine.

Fourth, different genotypes of norovirus could have spread at different times during the outbreak. From genotyping data of 7 cases of the norovirus outbreak during the jamboree, we know that 3 different norovirus genotypes circulated during this outbreak from genogroup I and II. Recent work (*32*) showed first signs of a different viral load, which could indicate different transmissibility and different generation times between genogroup I and genogroup II. However, all genotyped strains were found during days 7–9 of the outbreak; although we cannot rule out the possibility that genotype replacement occurred, the most transmissible type is highly unlikely to have dominated during the first 3 days before giving way to less transmissible types.

Finally, a change of the generation time distribution during the outbreak could explain the decline in reproduction number. The method we used to estimate the time course of reproduction number depends crucially on a correct specification of the generation time distribution. Here we obtained this distribution from a study of a norovirus outbreak in child daycare centers in Sweden (*25*) and estimated that the generation time distribution peaked at 2.6 days (Figure 2). This estimation agrees with results from volunteer studies in which adults showed a peak in virus shedding at 1–3 days postchallenge (*31*). Further, this peak agrees with the time between exposure and symptom onset of 2 days in primary-school children during a norovirus outbreak after a vomiting event (*6*), whereas 80% of case-patients reported vomiting during the scout jamboree. Overall, the most plausible explanation for the decrease in reproduction number is implementation of enhanced hygiene measures.

We have quantitatively estimated the effectiveness of enhanced hygiene measures in containing an outbreak of norovirus. Because the reproduction number did not fall below the threshold value of 1, implementation of the hygiene measures was not sufficient to effectively break the chain of person-to-person transmission of norovirus during this outbreak. To contain an outbreak of norovirus, more rigorous interventions are required. These might range from better compliance with hygiene protocols to strict isolation of case-patients and quarantine of their contacts. We recommend quantifying the effectiveness of interventions against norovirus to provide the necessary evidence to justify use of existing hygiene protocols during outbreaks and to direct the development of better intervention measures. Although such quantifying would require analysis of more norovirus outbreaks with different sets of intervention measures, it would enable identification of the best possible intervention strategies to control the spread of one of the most common pathogens of humans.

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Selection Tool for Foodborne Norovirus Outbreaks

Linda P.B. Verhoef, Annelies Kroneman, Yvonne van Duynhoven, Hendriek Boshuizen, Wilfrid van Pelt, and Marion Koopmans, on behalf of the Foodborne Viruses in Europe Network[1]

Detection of pathogens in the food chain is limited mainly to bacteria, and the globalization of the food industry enables international viral foodborne outbreaks to occur. Outbreaks from 2002 through 2006 recorded in a European norovirus surveillance database were investigated for virologic and epidemiologic indicators of food relatedness. The resulting validated multivariate logistic regression model comparing foodborne (n = 224) and person-to-person (n = 654) outbreaks was used to create a practical web-based tool that can be limited to epidemiologic parameters for nongenotyping countries. Non–genogroup-II.4 outbreaks, higher numbers of cases, and outbreaks in restaurants or households characterized (sensitivity = 0.80, specificity = 0.86) foodborne outbreaks and reduced the percentage of outbreaks requiring source-tracing to 31%. The selection tool enabled prospectively focused follow-up. Use of this tool is likely to improve data quality and strain typing in current surveillance systems, which is necessary for identification of potential international foodborne outbreaks.

Globalization of the food industry, centralized production, and the wide geographic distribution of products support the need for increased international surveillance of foodborne viral outbreaks, which may occur in clusters in different countries. Because control of pathogens in the food chain requires hazard analysis critical control points and verification of measures taken, detection of the pathogen is an important step (*1*). However, viral contamination of food is less likely to be recognized than bacterial contamination due to the infrequency of testing for viruses (*2*). Moreover, foods acceptable by bacterial standards are not necessarily safe from viral contamination. For example, norovirus may be present in shellfish and still meet the European Union *Escherichia coli* standard for human consumption (*3*). Consequently, foodborne viral infections are common, despite successful measures to reduce bacterial contamination. Recognition of foodborne viral outbreaks with international consequences would benefit from a linked and consistent reporting network among countries.

The challenge for surveillance systems is to obtain a complete dataset for the reported outbreaks (*4*). The European Food Safety Authority (EFSA) recognized this challenge and began developing guidelines for an international reporting system for foodborne outbreaks caused by bacteria, viruses, or parasites (working group of foodborne outbreak surveillance, www.efsa.europa.eu). The need for a better surveillance system has also been recognized by the Foodborne Viruses in Europe (FBVE) network, which has conducted virus-specific surveillance of gastroenteritis outbreaks since 1999 (*5*). Although the name FBVE suggests a foodborne focus, the network actually investigates outbreaks from all modes of transmission to obtain a comprehensive overview of viral activity in the community. A total of 13 countries are participating in the FBVE surveillance network, 11 of which are capable of collecting integrated epidemiologic and virologic surveillance data (*6*).

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Because of the etiologic dominance of viruses, the network's primary focus is on norovirus infections (*7*) that have been more frequently reported in recent years after emergence of novel variant strains in the population (*8*,*9*). Kroneman et al. described strengths and limitations of the FBVE data collection (*6*) but stated that outbreak reports need to be interpreted with caution; the number and content of these reports may vary considerably among countries because surveillance databases may be different. Most of these reports link outbreaks to person-to-person transmission; international interventions and follow-up are rare. In ≈40% of the outbreaks, no suspected mode of transmission was reported. Therefore, epidemiologic or virologic criteria should be used during the early stages of an outbreak investigation to determine whether foodborne sources should be considered. Given that surveillance systems are overwhelmed during norovirus peak seasons, use of these criteria would assist in focusing follow-up activities.

Our objective was to retrospectively derive, from surveillance data, a predictive model that could serve prospectively in the selection of norovirus outbreaks potentially related to food. Such a tool could be used to warn food safety authorities (FSAs) earlier, to improve the quality of outbreak report data, and to provide better estimates of the effects of viral foodborne disease. Our study demonstrates the added value of a reporting system amalgamated across countries; the FBVE dataset can form the basis of this tool, which may be a first step towards detection of diffuse outbreaks.

Methods

Categorizing Surveillance Systems

Because surveillance systems are known to vary in terms of design, effectiveness, and priorities (*6*,*9*–*11*), a structured telephone survey/questionnaire (available from the authors) was conducted among FBVE participants to categorize national surveillance systems of the involved countries. If a participating country reported that their national system labeled outbreaks "person-to-person" as a diagnosis of exclusion, the data were excluded to avoid potential misclassification of foodborne outbreaks and consequent dilution of differentiating parameters.

Dataset

Combined epidemiologic and virologic outbreak reports from countries capable of detecting foodborne outbreaks derived from the FBVE network were collected in a protected web-based database on the basis of a structured questionnaire (www.fbve.nl/attachments/questionnaire.pdf). Collection of sequence results focused on region A of the genome (www.rivm.nl/bnwww), but allowed other entries (regions B, C, and D) because of the lack of standardization between cooperating laboratories (*12*). Norovirus outbreaks were selected from this database if they fulfilled the following minimum dataset: date of onset from January 1, 2002, through December 31, 2006; norovirus detected as the only causative agent; and presence of a known norovirus sequence or genotype. The surveillance database from April 2007 was used and accounted for the median reporting lag (*6*) and enabled completion of data entry for outbreaks in 2006.

Parameters for Evidence

Data items used in the construction of the predictive model were derived from the EFSA draft guidelines (www.efsa.europa.eu), which are being developed to achieve consensus on the minimal variables to be reported for all foodborne outbreaks and on additional variables to be reported for thoroughly investigated foodborne outbreaks. The list has been amended with data items for foodborne outbreaks as described in comprehensive overviews (*13*,*14*) and with data items required to enable interventions by an FSA (Table 1).

Definitions

Outbreaks were reported when they satisfied the agreed-upon case definition (a cluster of ≥2 patients within 2 days showing signs of acute gastroenteritis indicative of norovirus) (*5*,*15*). A gastroenteritis outbreak was ascribed to norovirus based on compatible descriptive epidemiology and laboratory confirmation according to agreed upon criteria (*16*). Because norovirus outbreaks typically occur in winter, an off-seasonal period was defined as May through September; a seasonal period was defined as October through April of the following year. An outbreak was considered foodborne when infection was related to consumption of food contaminated during production or preparation. Where there was laboratory evidence of norovirus in food or analytical epidemiologic evidence for a food source through a case-control or cohort study, the outbreak was defined as confirmed foodborne. When descriptive epidemiologic data indicated a link to food, the outbreak was defined as probably foodborne. A random 50% of the total dataset was used as the training sample to build a model that distinguishes modes of transmission. The remaining 50% was used as the validation sample to validate the model. Sensitivity, or true positives, of the model for foodborne outbreaks was the proportion of the number of foodborne outbreaks correctly labeled as foodborne. Specificity, or true negatives, of the model for foodborne outbreaks was the proportion of the number of outbreaks reportedly due to person-to-person transmission that are indeed classified as person-to-person transmission. The receiver operating characteristics (ROC) curve was the graphic representation of the tradeoff between false negatives and false positives

Table 1. Consensus list of parameters for optimal reporting of foodborne (viral) outbreaks as defined by expert opinion, and completeness for data collected in the FBVE surveillance database*

Parameters for outbreak data	Variable	Foodborne outbreak data (% missing), n = 224	Other mode outbreak data (% missing), n = 654
EFSA (confirmed/probable)†			
Type of outbreak: general or household	Yes	224 (0)	654 (0)
No. human cases‡	Yes	217 (3)	651 (0)
No. hospitalizations‡	Yes	78 (65)	295 (55)
No. deaths‡	Yes	66 (70)	195 (70)
Foodstuff implicated	*Yes*	*93 (58)*	NA‡
Causative agent§	Yes	224 (0)	654 (0)
Setting	Yes	224 (0)	654 (0)
Contributory factors	Yes	202 (10)	482 (26)
Origin of foodstuff	No	NA	NA
Strength of evidence food	Yes	*224 (0)*	NA
EFSA (thoroughly investigated)†			
Reason reporting	No	NA	NA
Laboratory results food	Yes	*202 (10)*	NA
Place food produced	No	NA	NA
Place food consumed/purchased	Descriptive	*106 (52)*	NA
Age-affected persons	Categorical	11 (95)	73 (89)
Gender-affected persons	Yes	27 (88)	106 (84)
Additional information on agent	Yes	224 (0)	653 (0)
Additional parameters in literature			
Attack rate†	Yes	121 (46)	226 (59)
Seasonality	Yes	149 (33)	484 (26)
Duration of the outbreak†	Yes	90 (60)	265 (59)
Epidemic curve/point source	No	202 (10)	496 (24)
Sequence or variant	Yes	224 (0)	654 (0)
Link with other outbreaks	Yes	22 (90)	15 (98)
Additional parameters VWA experts			
Incubation period	Yes	51 (77)	65 (90)
Illness in food handlers and their family	Partially	*202 (10)*	NA
Presence of ill persons in setting	No	NA	NA

*FBVE, Foodborne Viruses in Europe network; EFSA, European Food Safety Authority; NA, not applicable; VWA, Food and Consumer Products Safety Authority. Parameters listed in italics could not be included in univariate analyses.
†Not restricted to viral.
‡A systematic retrospective check of Dutch data showed that variables for no. cases involved were reported to the national institute by regional health services when the outbreak was ongoing, and that these numbers were not updated when the outbreak had finished. The same situation was reported for other countries during the telephone survey.
§Inclusion criterion.

for every possible cut-off. The area under curve (AUC) was used to determine how well the predictor (based on several variables) was able to discriminate between groups (1 = perfect, 0.5 = no discrimination). Positive predictive value (PPV) was the proportion of outbreaks that met the model's foodborne criteria that are correctly labeled as such, indicating efficiency in reducing the workload of FSAs.

Data Analysis

Selected norovirus outbreaks were divided among 3 groups: confirmed or probable foodborne outbreaks; outbreaks resulting from person-to-person transmission; and outbreaks with an unknown mode of transmission. Data analysis was performed stepwise. First, completeness of data in the FBVE database with respect to the data-items in the consensus list (Table 1) was determined for outbreaks from foodborne and person-to-person transmission. Descriptive data were given for items relevant to foodborne outbreaks but not applicable to, or available for, outbreaks by person-to-person or unknown transmission. This included information concerning the food vehicle, product-handling hygiene, and place of preparation or consumption.

Second, the consensus list of predefined data items was used to compare foodborne and person-to-person outbreaks in the training sample by using logistic regression models. Variables were included in a multivariate model if they were statistically significant with $p<0.10$ during univariate analyses and if completeness of the variable was sufficient (80%) to result in a valid model. Because a logistic regression model can only be considered valid if the number of parameters is $\leq$10% of the number of outbreaks in the smallest group, analyzed variables were included as continuous where possible. The variables remained in the multivariate model if p values were <0.10, while the back-

ward selection procedure was used or if they were found to be confounding factors for other variables in the model (β values changing at least 10%). The optimal cut-off value was determined in the training sample and validated in the validation sample. When the validated model performed well, the β values for the final model were based on the total dataset, i.e., the validation and the training set together.

Third, the final model was used to create a web-based tool to assist public health workers in selecting outbreaks for further investigation when they receive outbreak reports, and to calculate the predicted individual probability of each outbreak with unknown mode of transmission caused by food. Individual probabilities of food relatedness were summarized to estimate the number of foodborne outbreaks in the unknown group and in the total dataset.

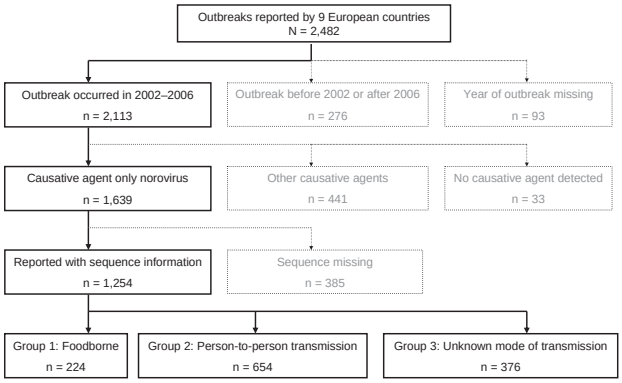

Figure 1. Outbreaks reported to the Foodborne Viruses in Europe network from January 2002 through December 2006, by suspected or confirmed cause and completeness of year and month of the outbreak, sequence information, and mode of transmission. Other causative agents include rotavirus, hepatitis A, and various bacteria.

Results

Categorizing Surveillance Systems

Of the surveillance systems in the 13 participating countries, 11 met the FBVE network's reporting criterion of linked laboratory and epidemiologic norovirus outbreak data. Of these 11 countries, 9 were included for analysis of parameters differentiating foodborne from person-to-person outbreaks: Denmark, Finland, France, Hungary, Italy, the Netherlands, Slovenia, Spain, and Sweden. As a result of legislation, surveillance systems in 4 of these 9 countries focused on foodborne outbreaks. Six of 9 countries reported at least 1 typed outbreak per million inhabitants per year (intensive surveillance). Surveillance systems were categorized as follows: 1) intensive surveillance with focus on food; 2) intensive surveillance without focus on food; and 3) no intensive surveillance.

Data Analysis

A total of 1,639 norovirus outbreaks occurring from January 1, 2002, through December 31, 2006, were reported by the countries included. Figure 1 shows the selection of the final dataset comprising 77% (1,254/1,639) of outbreaks; the remaining 23% were excluded due to missing laboratory confirmation of norovirus. Table 1 shows the completeness of analysis-set with respect to the parameters in the consensus list. The level of evidence for food-relatedness was confirmed for 24 (11%) of 224 outbreaks and was probable for 200 (89%) foodborne outbreaks. Thirty food categories were associated with outbreaks, including shellfish, fruit, fancy cakes, buffets, sandwiches, and salads. In 1 foodborne outbreak, poor personal hygiene was mentioned as a contributory factor; an infected food-handler was reported in 16 outbreaks, with 1 cook being involved in 2 outbreaks; and hygiene rather than preparation or consumption of food was mentioned in 2 outbreaks. Completeness of FBVE data with respect to the consensus list of data-items varied between items and between foodborne and person-to-person outbreaks (Table 1). Completeness of data items varied between 2% (link to other outbreaks) and 100% (type of outbreak, setting, causative agent, implicated strain). Data concerning hospitalization, attack rate, epidemic curve, incubation period, and links to other outbreaks were more likely to be reported for foodborne outbreaks. On the other hand, seasonality and contributory factors were more frequently reported for outbreaks with other modes of transmission.

The items in italics in Table 1 could not be included during univariate analyses because they played a role only when foodborne transmission occured or because data were not requested in the FBVE surveillance system. Risk factors resulting from univariate analyses are presented in Table 2 and show that foodborne outbreaks were found more often in households or restaurants and less often in healthcare settings, involved nongenogroup (GG) II.4 strains relatively more frequently, were more likely to occur during off-seasonal months, and involved more cases when notified compared to outbreaks from person-to-person transmission. Table 2 shows all parameters included in the multivariate analysis that remained in the model during the backward selection procedure. The AUC in the training sample and in the validation sample was 0.92 and 0.90, respectively, indicating the model performs very well in distinguishing foodborne outbreaks from person-to-person transmission (Figure 2). In the validation sample, the optimal cut-off value (the value of the ROC curve closest to the upper left corner) resulted in a sensitivity of 0.72, a specificity of 0.92, and a PPV of 0.64; a follow-up of outbreaks would have focused on 24% of the total number of reported outbreaks. The probability that an outbreak was attributed to food was calculated by using the following final model, based on the complete dataset of

Table 2. Factors (8 of 17) of borderline significance during univariate logistic regression in a random selection of 50% of the dataset for comparison of foodborne outbreaks (group 1) and outbreaks from other modes of transmission (group 2)*

Indicator	Category/measure	Group 1 (n = 112)	Group 2 (n = 327)	Univariate, OR (95% CI)	Univariate adjusted for country, OR (95% CI)	Multivariate adjusted for country, OR (95% CI)
1	General	105	325	Reference	Reference	Reference
	Household	7	2	10.8 (2.2–52.9)	10.1 (1.6–64.3)	0.1 (0.0–1.0)
2	No. cases†	–	–	1.1 (1.0–1.1)	1.0 (0.9–1.1)	1.1 (1.0–1.2)
7	Residence	7	2	Reference	Reference	Reference
	Restaurant‡	36	1	10.3 (0.8–129.4)	13.2 (0.7–234.0)	>999†
	Healthcare institute	27	267	0.0 (0.0–0.1)	0.0 (0.0–0.1)	0.0 (0.0–0.0)
	Daycare	2	15	0.0 (0.0–0.3)	0.1 (0.0–0.9)	0.0 (0.0–0.1)
	Hotel/guest house	9	12	0.2 (0.0–1.3)	0.1 (0.0–1.3)	0.0 (0.0–0.1)
	School	11	9	0.3 (0.1–2.1)	0.3 (0.0–2.7)	0.0 (0.0–0.2)
	Other	20	21	0.3 (0.1–1.5)	0.3 (0.0–2.1)	0.0 (0.0–0.2)
17	Non-GGII.4	55	48	Reference	Reference	Reference
	Genogroup II.4	57	278	0.2 (0.1–0.3)	0.2 (0.1–0.4)	0.4 (0.2–1.0)
18	Attack rate*	–	–	*14.0 (3.6–54.0)*	*6.7 (1.5–34.3)*	–
19	May–Sep	*20*	*35*	*Reference*	*Reference*	–
	Oct–Apr	*47*	*208*	*0.4 (0.2–0.7)*	*0.5 (0.3–1.2)*	
20	Duration in hours*	–	–	*0.9 (0.8–0.9)*	*0.9 (0.8–1.0)*	–
21	No point source	*60*	*242*	*Reference*	*Reference*	
	Point source	*43*	*3*	*58.8 (17.3–192.7)*	*44.7 (11.8–167.7)*	–

*Significant factors were included in multivariate analyses to construct the final model. –, entered as a continuous variable. Parameters in italics could not be included in multivariate analysis because of missing values. OR, odds ratio; CI, confidence interval; GG, genogroup.
†A systematic retrospective check of Netherlands data showed that variables for no. of cases involved were reported to the national institute by regional health services when the outbreak was ongoing, and that these numbers were not updated when the outbreak had finished. The same situation was reported for other countries during the telephone survey.
‡The parameter restaurant was set to 0 because the variable was a linear combination of other variables as follows: Restaurant = intercept – household – health care – day care – hotel – school – other >999.

878 records and corrected for characteristics of national surveillance systems:

$$\text{Odds (foodborne = 1)} = \frac{P(\textit{foodborne} = 1)}{P(\textit{foodborne} = 0)}$$

= exp (1.5477 + 0.8065 when in household)
+ (0.0322 for each involved case when notified)

+ (3.0999 when in restaurant)
or – (1.2963 when in hotel)
or – (2.6616 when in hospital)
or – (1.9912 when in daycare)
or – (0.5289 when in school)

+ (0.3190 when GGnonII.4)

– (1.0270 if intensive surveillance and focus food)
or – (2.0540 if intensive surveillance and no focus food)

This final model (sensitivity = 0.80, specificity = 0.86, PPV = 0.65) can be prospectively applied to calculate potential food-relatedness of reported norovirus outbreaks and reduce the number of outbreaks to 31% of all reported outbreaks. The practical web-based tool created with the model can be found in the online Technical Appendix, available from www.cdc.gov/EID/content/15/1/31-Techapp.xls. If this tool is used by a nongenotyping country, intensive surveillance can be considered to be at least 2 reported outbreaks (instead of 1 typed outbreak) per 1 million inhabitants per year. For the nongenotyping countries, the unknown genotype in the model resulted in an additional 5 unrecognized foodborne outbreaks coexisting with a slight reduction of sensitivity (0.78), equal specificity (0.86), PPV (0.65), and 30% of the outbreaks requiring follow-up.

Of 376 outbreaks with an unknown mode of transmission, data for 352 (94%) were sufficient to calculate the probability of a foodborne outbreak. Summarizing individual probabilities resulted in 100 (29%) of 352 potential foodborne outbreaks in the unknown group; summarizing probabilities in the total dataset resulted in an estimated 280 (22%) of 1,254 reported outbreaks being possibly foodborne.

Discussion

We built and validated a model to estimate the likelihood that a norovirus outbreak was related to food. This study was the basis for a practical tool that can prospectively be applied in near real-time in the European setting to identify potential foodborne viral outbreaks in both genotyping and nongenotyping countries. The model can also retrospectively estimate the true contribution of food to norovirus outbreaks in Europe, and may contribute to studies estimating the effects of foodborne gastroenteritis. Moreover, the user-friendly tool may support more consistent reporting and typing of viral outbreaks. Our approach is innovative for norovirus surveillance and provides a new estimate for the proportion of foodborne outbreaks that is

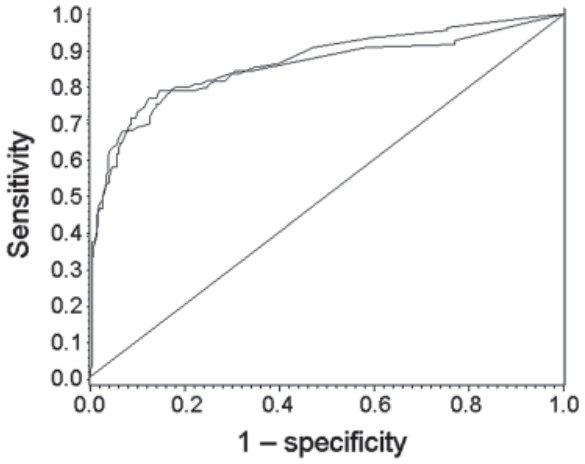

Figure 2. Receiving operator characteristics curves for distinction of foodborne outbreaks from person-to-person outbreaks in the training sample (upper graph, 435/439 records used) and in the validation sample (lower graph, 432/439 records used). The area under curve in the validation sample was 0.90, indicating good performance of the model.

higher (22%) than the recognized proportion of foodborne outbreaks in countries that can separate transmission modes (18%). However, this higher estimate is based on reported outbreaks and therefore does not account for underreporting or overreporting.

Using this selection mechanism prospectively for identification of outbreaks requiring detailed follow-up, FSAs can focus on 31% of all reported outbreaks and accept that 1 of 5 foodborne outbreaks will be missed. This finding may appear to lack sensitivity, but at present only a few foodborne outbreaks are investigated sufficiently to provide information on the basis of which FSAs can act, which we will illustrate. In 2007, a year when an unusually high number of norovirus outbreaks were reported, 1 of the 37 Municipal Health Service agencies in the Netherlands reported a total of 28 norovirus outbreaks. Applying this model to reduce the number of outbreaks needing investigation from 28 to 3 is likely to greatly improve the potential for intervention and the quality of surveillance data for foodborne outbreaks. The tool, based on this model, will be implemented and evaluated in the Netherlands in 2009.

As previously identified, restaurants were the most common setting for foodborne outbreaks (*17*,*18*). Our model output, however, also provides a strong first indication that the epidemiology of norovirus outbreaks differs between genotypes because the proportion of non-GGII.4 outbreaks was higher in foodborne outbreaks. Non-GGII.4 outbreaks indicated source contamination, altering the probability of outbreaks being related to food (online Technical Appendix). Unfortunately, many countries cannot take advantage of this result because genotyping is not among their routine procedures. For this reason, the practical tool was adjusted so that it can be restricted to epidemiologic parameters only. However, a rapid assay should be developed that discriminates GGII.4 from non-GGII.4, which would enable earlier and more targeted measures by FSAs on a large scale.

The difference identified between GGII.4 and non-GGII.4 is a first step towards identification of international foodborne outbreaks, of which some examples are known (*19*–*21*). Detailed strain type and sequence information may provide the linking conditions for such outbreaks. Unfortunately, analysis of strain types did not give statistically significant results but did suggest the existence of differences, which should be separately investigated. More data are needed about the diversity of noroviruses belonging to rare genotypes to reliably use the data when identifying a probable source of infection. This diversity is illustrated through a recent example. In the spring of 2006, an unusually high number of norovirus outbreaks was reported that involved passengers on cruise ships in European waters (*22*). The finding that several of the outbreaks were caused by a distinct strain of GGII.4 norovirus triggered an outbreak investigation which tested the hypothesis that these outbreaks might have resulted from a common source (*23*). More detailed molecular characterization, outbreak investigations, and use of molecular strain data from surveillance of land-based outbreaks showed that the new variant strain viruses could be found across Europe, thus reflecting a widespread epidemic rather than a common source event. Much less is known about noroviruses belonging to the rare genotypes. For instance, if these viruses behave in an opportunistic fashion, they will circulate in the community without causing outbreaks, going undetected because routine surveillance for sporadic cases is rare (*24*). Until further investigation can show epidemiologic characteristics of rare genotypes, the selection tool using information on setting, genogroup, and number of cases enables quick screening of outbreaks of interest.

Several countries have conducted studies using methods to estimate the proportion of foodborne gastroenteritis (*25*–*27*). These studies have identified foodborne norovirus infections varying from 1/33 inhabitants in the United States to 1/780 in the United Kingdom. Prospective cohort studies are usually the most accurate method of ascribing illness to food, but are costly. Deriving estimates from existing data collections has its weaknesses (*28*), but the data collections available provide a good tradeoff between costs and providing useful information for public health. Numbers based on surveillance data need to be interpreted and extrapolated with caution, as international differences in surveillance systems can introduce bias.

Although our approach is innovative in categorizing norovirus outbreaks in a surveillance system, it is commonly applied in medical research to predict critical diagnostic outcomes (*29*–*31*). A limitation of our data is that

selection of outbreaks may have occurred in the database, e.g., if outbreaks were not reported until they had reached a certain size because of (secondary) person-to-person transmission (*32*). Conversely, outbreaks likely to be foodborne may have an origin other than food (*33*), and confirmation of this is rare. In addition, the definition applied for a foodborne outbreak may differ substantially among countries. We reduced the chance of misclassification by using retrospectively applied uniform definitions, and by selecting those countries clearly discriminating transmission modes. During the survey for categorization of surveillance systems, we confirmed that proof of a foodborne outbreak is often difficult to obtain; the transmission mode consequently may remain unknown relatively more frequently than that of person-to-person outbreaks. However, the slight difference between the foodborne proportion of outbreaks among the outbreak with unknown (28%) and known (26%) transmission does not suggest a difference in the estimated and reported proportion.

The European norovirus surveillance system, like most surveillance systems, has to cope with reporting delays and missing values (*4,6,10*). Because virologic and epidemiologic distinctive parameters were of interest, strict selection criteria were used in this study. Incompleteness in our selection criteria left us with 1,254 (66%) of 1,639 norovirus outbreaks in our analysis dataset. Greater completeness of our analysis dataset may have resulted in an extended model that included a larger variety of indicators as proposed by EFSA, which requested an extensive minimal dataset for foodborne outbreaks from differing causes. However, our model was able to distinguish norovirus foodborne outbreaks with far fewer indicators than those prescribed by EFSA. Because an optimal surveillance system for detection of diffuse foodborne outbreaks is dependent on completeness of a minimal dataset, use of the tool is likely to be a first step towards such a system.

We developed a practical tool that can distinguish food-relatedness of norovirus outbreaks and is likely to improve surveillance data quality. A model that predicts foodborne outbreaks regardless of causative agents, and that links conditions for viral outbreak, should be the focus of future studies. The requested minimum dataset for surveillance of foodborne outbreaks with potential for international consequences needs to be clearly defined. The more information needed, the less the compliance; priority should therefore be given to information essential for initiating interventions.

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Venetian Rule and Control of Plague Epidemics on the Ionian Islands during 17th and 18th Centuries

Katerina Konstantinidou, Elpis Mantadakis, Matthew E. Falagas, Thalia Sardi, and George Samonis

During the 17th and 18th centuries, measures were taken by the Venetian administration to combat plague on the Ionian Islands. At that time, although the scientific basis of plague was unknown, the Venetians recognized its infectious nature and successfully decreased its spread by implementing an information network. Additionally, by activating a system of inspection that involved establishing garrisons along the coasts, the Venetians were able to control all local movements in plague-infested areas, which were immediately isolated. In contrast, the neighboring coast of mainland Greece, which was under Ottoman rule, was a plague-endemic area during the same period. We conclude that even in the absence of scientific knowledge, close observation and social and political measures can effectively restrain infectious outbreaks to the point of disappearance.

Plague is a zoonotic infection circulating among small animals, usually black rats and their fleas; it is caused by the bacillus *Yersinia pestis*. This disease is transmitted from animals to humans by the bite of infected fleas, direct contact, inhalation, and, rarely, ingestion of infective material. Untreated plague has a high case-fatality rate (*1*,*2*).

Y. pestis is a global pathogen that has active foci in all continents except Australia and Antarctica (*3*). Plague represents an exotic disease in North America; it usually affects prairie dogs (*Cynomus luduvicianus*) and has eliminated large colonies of these animals in the northwestern United States. Although these animals are susceptible, it is believed that other rodents and their fleas are the reservoirs and spread the disease during epizoonotics and maintain the pathogen (*4*).

Three forms of plague are known: bubonic, septicemic, and pneumonic. The bubonic form is most common and results from the bite of an infective flea. The bacillus enters through the bite, travels through the lymphatic system to the lymph nodes, and results in painful inflammation. The septicemic form occurs when the infection spreads through the bloodstream. The pneumonic form results from inhalation of aerosolized infective droplets and can also be transmitted between humans (*1*,*2*).

Bubonic plague, historically also known as Black Death, swept across Europe during the late medieval period in an epidemic that started in 1347 (*5*–*9*). The disease got its name from the deep purple, almost black discoloration of infected persons caused by subcutaneous hemorrhages. Wars, poverty, hunger, and malnutrition made Europe of the 14th century an ideal ground for plague epidemics. The first major outbreak occurred in Sicily in 1347, spread through Europe, and killed nearly half of the population (≈25 million persons) in 3 years. The disease became endemic and haunted the continent throughout the 14th–18th centuries. Major outbreaks occurred in Italy in 1629, London in 1665, and Vienna in 1679 (*5*–*9*).

It is not known where the pandemic started. It most likely originated in central Asia and was carried west by Mongols and traders along the Silk Road. It was imported to Europe through Crimea, from which it spread to Sicily. The total number of deaths worldwide from the pandemic is estimated to be 75 million (*5*–*9*).

Urban rat-borne plague has been controlled since the beginning of the 20th century by modern sanitation practices. Epidemics caused by rats transferred on ships to port

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cities are no longer a threat. However, the disease still occurs in rural areas because *Y. pestis* infects various wild rodents (*1*,*2*).

The continuing potential for reemergence of plague is evident by reports of outbreaks of the infection in Africa and India. The World Health Organization reports 1,000–3,000 new cases every year in impoverished rat-infested rural areas of Africa, Southeast Asia, and South America (*1*,*2*). However, an investigation has created some doubts about the nature of Black Death by implicating other possible causes such as Ebola-like viruses or other infectious agents (*10*).

Since 1347, when plague appeared in Europe, and especially after 1493, when syphilis was observed in Europe, theories on infectious and communicable diseases were formulated. Many scientists during these times developed ideas of contagion, which had an effect on public health regulations and the structure of cities. Moreover, decisions on plague control during that period reinforced the idea of public health measures for prevention of infectious diseases, an idea that was previously vague.

Since the 11th century, Venice, a naval and commercial power, had a special interest in the Eastern Mediterranean and later took advantage of the redistribution of the land of the Byzantine Empire after the Fourth Crusade (1204). Gradually and through conflicts after this crusade, this city-state on the Adriatic Sea gained control of the Ionian Islands, Crete, and some coastal cities of mainland Greece and established a network of trading posts. The islands of Corfu (Greek name Kerkyra), Zante (Zakynthos), Cephalonia, and Leukada were incorporated into the Venetian State in 1386, 1485, 1502, and 1684, respectively, and remained part of it until its demise in 1797 (*11*).

Our study investigates plague on the 4 islands during the 17th and 18th centuries. This period was selected because after the second half of the 17th century plague was observed only sporadically with limited epidemics. Plague was last observed in Venice in 1630, whereas in southeastern Europe, plague was observed until the 19th century (*12*). During the early 18th century, changes took place in the Venetian health policies, and the strategic and economic role of the islands increased after the gradual loss of the great trade routes of the Mediterranean Sea.

The 4 large islands in the Ionian Sea (Figures 1, 2) are a useful area for research because they had been under Venetian rule and were located just off the western coast of mainland Greece. This location made them a gateway to and from the Ottoman Empire and a frontier of Venice to the East. However, despite their proximity to mainland Greece, political and institutional differences were substantial between these islands and the neighboring Greek coast that was under Ottoman rule. Research regarding plague in this area has been limited. Moreover, comparisons of Venetian health policies regarding plague and their effectiveness on the Ionian Islands with those of neighboring Greece have not been made.

Figure 1. Map of the wider geographic area of reference.

Our study had 4 goals. The first goal was to identify epidemics of plague that struck the Ionian Islands during the 17th and 18th centuries. The second goal was to reconstruct the course of the epidemics. The third goal was to highlight differences in the prevalence of infection on the Ionian Islands during the 17th and 18th centuries and discuss the epidemiologic status of the islands compared with that of the neighboring coast of the Greek peninsula. The fourth goal was to investigate and describe measures taken by the Venetian authorities on these islands against plague during the study period.

Historical Sources

On-site research was conducted in the Venetian state archive (Archivio di Stato di Venezia). Unpublished archival material dealing with the Ionian Islands during the 17th and 18th centuries was investigated with emphasis on periods of epidemiologic crises. We studied the following: 1) total number of registers of legislative bodies of the Venetian Republic (Senato mar and Senato rettori) for 1600–1797 to locate laws dealing with curtailment of plague and organization of health services on the Ionian Islands, 2) daily correspondence of Venetian authorities of the islands with Venice through the *proveditori da terra et da mar* and *senato* (secreta) *dispacci rettori*, and 3) archives of the Venetian health inspectors (*provveditori alla sanità*) regarding the Venetian health policy for the Ionian Islands. Additionally, several historical sources providing information about plague epidemics in the Mediterranean area during the 17th and 18th centuries were reviewed. This investigation was related to the doctoral dissertation of this article's first author (K.K.), a historian at the University of Athens. This dissertation (*13*) reviews the subject

Figure 2. The 4 large Ionian Islands, which were under Venetian rule, and the Greek peninsula, which was under Ottoman rule, during the period studied (17th and 18th centuries).

historically. This article examines the subject from medical and epidemiologic points of view.

Findings and Discussion

Archival sources show that most cases of plague on the Ionian Islands during the 17th and 18th centuries were imported from the neighboring coast of mainland Greece and ports in the southwestern Ottoman Empire. Only 2 epidemics were imported from the trade routes of the Mediterranean Sea. Of 11 epidemics, 8 occurred during the 17th century and 3 occurred during the 18th century. Plague struck Corfu in 1611, 1630, 1648, and 1673; Zante in 1617, 1646, 1692, and 1728; and Cephalonia in 1646 and 1760. Leukada had a disastrous epidemic in 1743, a few decades after the island became part of the Venetian Republic.

During the 18th century, plague had waned despite an outbreak along the southwestern coast of the Ottoman Empire, a short distance from the coasts of the Ionian Islands. In the 18th century, the southern Balkans had repeated waves of plague in 1718–1720, 1728–1731, 1733–1740, 1756–1765, 1782–1784, 1787–1789, and 1790–1793 (*13*). Only 14 plague-free years are described for the Greek peninsula during the 18th century (*14*). These epidemics affected cities and villages in western Peloponnese and western mainland Greece, which, because of trade, were in constant contact with the Ionian Islands. Because of commercial interests, contact between inhabitants of the islands and mainland Greece could not be halted. However, vigorous attempts by Venetian authorities stopped all communication between these areas during plague outbreaks.

Under these conditions, waning of plague epidemics during the 18th century may be attributed to sanitary measures taken by the Venetian government. These measures, among other regulations, dictated strict control of population movements, particularly during periods of epidemiologic crises. The efficacy of these measures is better appreciated because plague during the 17th century spread from *lazzarettos* (institutions where those with plague or other similar diseases were isolated) mainly because of negligence. In contrast, 2 of the 3 epidemics during the 18th century were caused by incorrect diagnoses or delayed notification of the authorities. Success of these measures became apparent in Corfu where plague was eradicated after 1673, only to reappear during British rule in the early 19th century (*13*). The importance of Corfu to Venice, particularly after the loss of Crete in 1669, resulted in creation of an effective invisible wall against plague until the end of the Venetian domination in 1797.

The city-states of northern Italy, including Venice, organized their defense against plague from the time of the Black Death (*9*). Experience and observation provided the first tools against epidemics because scientific information about the cause of plague was not obtained until several centuries later at the end of the 19th century, through laboratory research conducted by Alexandre Yersin (*15*). The Venetian State, on the basis of the belief in the miasmatic and contagious nature of plague and being a pioneer in organization of public health services conceived in the late Middle Ages, established regulations and practices in the city of the Doges and its conquests. These regulations and practices included quarantine (period of isolation ranging from 14 to >40 days and occasionally even longer, depending on the health of the port of origin), *lazarettos*, public health offices, and *cordoni di sanità*, which on the Ionian Islands were coastal garrisons that controlled access to Venetian territories (*16*).

Archival sources showed that health board officers in Corfu and Zante were initially elected around 1545. However, it is likely that health boards had previously existed. The first *lazzarettos* were established in Corfu and Zante in 1588 and in Cephalonia and Leukada at the beginning of the 18th century.

The Venetian health policy was reformed in the 18th century. These reforms likely emphasized disease prevention and dealt with health emergencies (*17*). The new policy was based on daily reports of health conditions on

the islands and suspicious areas in the eastern Mediterranean, and included creation of a common public health framework (identical laws for plague control, decrees, and institutions and infrastructures) for all islands. This policy would facilitate interventions and change the way in which local health officers were selected.

The main goal of these reforms was improvement of the structure and function of *lazzarettos*. During the 18th century, new *lazzarettos* were established, and existing ones underwent extension and changes. In 1726, a new statute defining the obligations and responsibilities of the heads of the institutions was introduced. According to this statute, during periods with no or low disease activity, *lazzarettos* referred directly to Venetian authorities and bypassed authority of local health boards (*13*).

Despite these new regulations, Venetian archives reported occasional problems in health services of the islands because of poorly trained health officers, who were elected from the local upper class and were eager to acquire greater political autonomy. This situation resulted in conflicts between Venetian representatives and local health officials, as well as between local factions known as *cittadini* (*18*).

Archives show that until the end of the Venetian rule on the Ionian Islands, *lazzarettos* functioned as protective shields for Venice and transferred responsibility of plague control from Venice to peripheral areas (*19*). When there was evidence or even suspicion that plague was present on an island, all links to Venice were immediately discontinued for the duration of the threat. Islands were isolated by order of the *senato*, and trade commenced only after the state of emergency had ended. During these periods, the Venetian government did not intervene in the responsibilities and actions of local health inspectors because such intervention could provoke social unrest (*13*). Thus, the cost for Venice was minor. However, this action did not indicate negligence by the state mechanism each time plague affected an island and threatened Venice.

Prevention was based on widespread use of an information network of daily reports of Venetian consuls in Mediterranean areas to Venetian authorities, detailed interrogation of sailors who arrived in Venetian ports, effective control of all local movements in plague-infested areas, and activation of the *cordoni di sanità*. Additionally, when plague occurred, residents were separated by health authorities into groups of healthy and sick persons regardless of social hierarchies. Persons affected by plague were kept in *lazarettos*, and large numbers of infected persons were kept in hospitals, houses, or neighborhoods on the assumption that plague in these persons remained isolated. Isolation was ensured by military force. Thus, plague-stricken areas resembled a large institution under constant inspection and surveillance and disconnected from the rest of society (*13*).

In contrast to measures taken on the Ionian Islands during the 17th and 18th centuries, mainland Greece, which was under Ottoman rule, had a different mentality in dealing with plague. Isolation of patients and quarantine were not common practices. Thus, plague continued to cause epidemics in mainland Greece during the first half of the 19th century. These epidemics had devastating demographic and financial consequences. After 1830, when the Ottoman administration implemented sanitary measures such as quarantine, spread of plague in mainland Greece decreased substantially (*20*).

Although modern medical regimens can successfully treat patients with plague and stop its spread, prevention and isolation policies can contribute to control of this disease. This finding has been demonstrated by successful management of a plague epidemic in Surat, India, in 1994, in which prevention techniques similar to those used during Venetian rule were used (*21*)

In conclusion, although the scientific basis of plague was not known, the Venetian administration recognized the infectious nature of this disease and took successful measures that dramatically decreased the spread of the plague epidemic on the Ionian Islands during the 18th century. The results of these measures are more impressive if compared with those in the neighboring coastal region of the Greek peninsula, which under Ottoman rule had endemic plague during the same period. Results of the present historical investigation lead to the conclusion that even in the absence of scientific knowledge, observation and well-organized public health services can effectively restrain infectious outbreaks to the point of disappearance, as occurred with plague in Corfu during the 18th century.

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Parapneumonic Empyema Deaths during Past Century, Utah

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Bacterial pneumonia with empyema is a serious complication of influenza and commonly resulted in death during the 1918 influenza pandemic. We hypothesize that deaths caused by parapneumonic empyema are increasing in Utah once again despite advances in critical care and the availability of antimicrobial drugs and new vaccines. In this study, we analyzed the historical relationship between deaths caused by empyema and influenza pandemics by using 100 years of data from Utah. Deaths caused by empyema have indeed increased from 2000–2004 when compared with the historic low death rates of 1950–1975. Vaccine strategies and antimicrobial drug stockpiling to control empyema will be important as we prepare for the next influenza pandemic.

An influenza pandemic is thought to be the most likely and most severe biological emergency facing the United States (*1–3*). Bacterial pneumonia is a serious complication of influenza infection and was likely a major cause of the excess deaths seen during the 1918 influenza pandemic (*4–8*). Even today, though the overall disease mortality rate due to infectious diseases is declining in the United States, death from pneumonia and influenza remains one of the top 10 causes of death overall (*9*,*10*).

Parapneumonic empyema, a serious complication of bacterial pneumonia frequently caused by *Streptococcus pneumonia* and *Staphylococcus aureus*, is increasing in North and South America, Europe, and Asia (*11–21*). As we prepare for an influenza pandemic, changes in the microbiology of pneumonia and the increasing rates of empyema must be considered. In this study, we analyzed the historical relationship between deaths due to empyema and influenza pandemics using 100 years of data from Utah.

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Methods

Utah Population Database

The Utah Population Database (UPDB) is a computerized genealogical database linking multiple data sources. The resource includes genealogical records of the original Utah pioneers (members of the Church of Jesus Christ of Latter Day Saints) who settled in Utah in 1847 and their descendants. These records have been linked to disease data for Utah, including death certificate records dating back to 1904. We used a version of the database without individual identifiers that spans 100 years (1904–2004).

Identification of Patients

The institutional review boards for both the University of Utah and the UPDB reviewed and approved this study. Each death record in the UPDB contains a primary cause of death that was coded with the International Classification of Diseases (ICD) nomenclature. All death certificates between 1956 and 2004 were encoded by using ICD revisions 6 through 10. For death certificates from 1904 through 1955, a nosologist with a University of Utah research project used the literal information and coded cause of death to ICD revision 10 using the 2000 Medical Data System and supplemented this system with hand coding. During 1985–1995, the Bureau of Vital Statistics added selected secondary causes of death, including pneumonia, empyema, and influenza. Beginning in 1996, death certificate records contained multiple secondary causes of death and classified persons with each. We searched the UPDB for deaths associated with empyema on the basis of ICD

codes: ICD-10 J869, ICD-9 510.9, ICD-8 510, ICD-7 518, and ICD-6 518. For influenza deaths, we used the following ICD codes: ICD-10 J10, J11, ICD-9 487, ICD-8 470–474, ICD-7 480–483, and ICD-6 480–483.

Statistical Analysis

We analyzed empyema-related deaths by decade and population to examine trends in death related to empyema. We analyzed empyema deaths in 2 age groups: children (0–18 years of age) and adults (>18 years of age). Utah population was determined based on national census data. Utah's population data have been available every 10 years from 1900 through 2000 and for 2005. Using available data, we fitted a cubic curve of log-transformed population for 1 year to estimate Utah's population for other years. We defined 3 notable periods in Utah history. Period 1, 1917–1920, represents the Spanish influenza pandemic. Period 2, 1950–1975, represents the post–antimicrobial drug era and encompasses smaller influenza pandemic periods of 1957–58 and 1968–69. Period 3, 2000–2004, includes the period of increasing incidence of empyema in children (*15*,*22*) and recent increases in empyema deaths. A Poisson model was used to estimate risk for death in different years and compare estimates among the 3 identified periods.

Results

Empyema and influenza death rates in Utah over a 100-year period are shown in the Figure. A high rate of deaths caused by empyema occurred during 1900–1909. Deaths caused by empyema peaked at 18/10,000 person-years during the decade 1910–1919, which included the 1918–19 Spanish influenza pandemic (period 1). Empyema deaths steadily decreased in the decades after the Spanish influenza pandemic. This decline is most apparent in the 1930s, coincident with the widespread introduction of sulfonamide antibiotics. With the introduction of penicillin during World War II, deaths caused by empyema leveled off substantially in the 1940s–1950s. During the period from 1950 through 1975 (period 2), empyema deaths remained at a nadir of 0.4–0.8/10,000 person-years. The rate of deaths increased significantly during the final 5-year period from 2000–2004 (period 3). Compared with period 2, the death rate for persons with empyema in Utah during period 3 was 3.2/10,000 person years, >6-fold higher (rate ratio 6.6; 95% confidence interval 3.2–13.4: $p<0.005$).

During the pre–antimicrobial drug era and during the 1918–19 influenza pandemic, children 0–18 years of age accounted for 31%–37% of all empyema deaths. Between 1940 and 1999, deaths among children decreased more dramatically than among adults and accounted for ≤4% of all empyema deaths. Almost all (97%) of the empyema deaths seen recently in period 3 are in adults >18 years of age, most among persons >65 years of age.

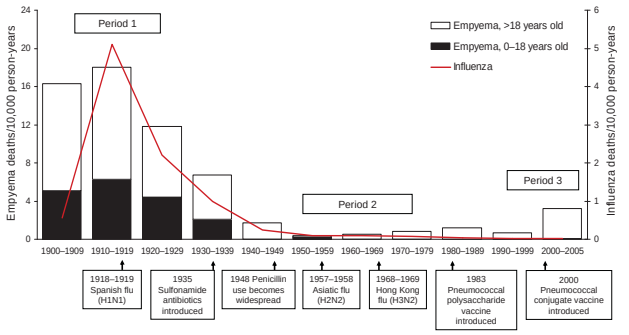

Figure. Average rates of deaths in Utah caused by parapneumonic empyema and influenza, by decade, 1900–2005.

Death rates attributed to influenza peaked dramatically during the decade 1910–1919, associated with the peak in empyema deaths. After 1970, the number of influenza deaths declined steadily and has remained very low.

Discussion

The rates of empyema leading to death in Utah have significantly increased between 2000–2004. This increase occurred in the absence of a major influenza pandemic and in spite of advances in medical care. The recent increase in empyema deaths is unexplained and may have broad implications for future influenza pandemics.

Empyema caused substantial illness and death in the era before antibiotics. In the 8th edition of his book, published just before the Spanish influenza pandemic, Osler described empyema as "a most common complication [of pneumonia] occurring in 2.2 percent of clinical cases" seen over an 8-year period at Johns Hopkins Hospital (*22*). He described pneumococcus as the most common pathogen leading to empyema. Pneumonia and empyema were, at the time, "the most fatal of all acute diseases" (*22*). Osler himself died of pneumonia and empyema in 1919.

Influenza has historically been linked to pneumonia deaths. According to our study, deaths caused by empyema peaked during the Spanish influenza pandemic of 1918–19. The estimated worldwide death toll attributed to Spanish influenza has been estimated at 21–50 million (*23*). Pneumonia, especially due to *S. pneumoniae*, is thought to be a major contributor to the excess deaths seen during the 1918 influenza pandemic (*5*,*6*). As seen in the Figure, data from the UPDB demonstrate an increase in deaths caused by empyema during this period, although it is not as dramatic as the increase in deaths attributed to influenza. This finding suggests that empyema contributed to the deaths from pneumonia associated with the influenza pandemic.

The number of deaths due to empyema fell moderately during the decades of the 1920s and 1930s but fell dramatically after 1940 and World War II. This decrease in

empyema deaths corresponds with the beginning of the antimicrobial drug era. During the 1950s and 1960s, when the pandemics of 1957 and 1968 occurred, the rate of deaths caused by empyema remained low. We did not observe a significant increase in the deaths caused by influenza over the same period. This lack of an increase may have been due to the relatively mild nature of these pandemics compared with the 1918 pandemic, the availability of antimicrobial drugs, improvements in the management of pneumonia and its complications, and perhaps the insensitivity inherent in examining death rates by decade.

We noted a statistically significant increase in the number of empyema deaths in Utah at the turn of the 21st century (2000–2004) when compared with the mid-20th century (1950–1975). This increase in empyema deaths has taken place without the advent of an influenza pandemic and in the setting of continued advances in medical care. The increase in death rates/person years caused by empyema is primarily seen in the adult population and is most apparent in patients >65 years of age. What might account for this increase in empyema deaths? We speculate that increased mortality rates from empyema are caused by changes in the organisms that cause pneumonia and empyema worldwide.

S. pneumoniae is thought to have been the major cause of death from secondary bacteria in prior pandemic influenza outbreaks. Comparing all 3 of the influenza pandemics of the twentieth century, 1 study estimates that 50% of bacterial pneumonia cases associated with influenza were caused by *S. pneumoniae* (*24*). Experimental as well as epidemiologic data support the association between serious *S. pneumoniae* infections and influenza. Studies by McCullers and others have shown that influenza virus infection preferentially predisposes mice to fatal infections with *S. pneumoniae* (*25–27*). Recent studies have demonstrated a clear temporal association between seasonal influenza and invasive pneumococcal disease in children (*28*).

Changes in circulating serotypes of *S. pneumoniae* have been reported from many regions worldwide (*11*,*14*,*16*,*17*,*29–32*). These changes may play a role in the increase in deaths caused by empyema. The annual incidence of invasive disease caused by *S. pneumoniae* has decreased significantly in all age groups with the introduction of the 7-valent pneumococcal conjugate vaccine (PCV-7) in 2000 (*33*). Further, disease caused by resistant *S. pneumoniae* has also decreased significantly with the introduction of the PCV-7 vaccine, which targeted the resistant serotypes (*33*,*34*). Thus, antimicrobial drug resistance to *S. pneumoniae* seems unlikely to be responsible for the increase in empyema deaths since the introduction of PCV-7. In spite of the recent decreases in invasive pneumococcal disease, hospitalizations for empyema are increasing in US children (*20*). Recent reports further show that the incidence of empyema due to non–PCV-7 serotypes, especially types 1, 3, and 19A, has increased significantly worldwide in the post PCV-7 era (*11–17*,*21*,*32*). These serotypes are historically associated with severe invasive disease, particularly empyema, and might contribute to the increased rates of deaths caused by empyema among adults (*17*,*35*,*36*).

S. aureus is increasingly recognized as a significant cause of complicated pneumonia. A recent study from France demonstrated a mortality rate of >50% in patients infected with *S. aureus* that contained Panton-Valentine leukocidin (PVL), which caused necrotizing pneumonia (*37*). A study among children from Houston demonstrated a marked increase in complicated pneumonia in patients with PVL-containing *S. aureus* infection (*29*). The PVL gene is now considered one of the identifying features of community-associated methicillin-resistant *S. aureus* (CA-MRSA) and is at least a marker for invasiveness and virulence, although the exact contribution of PVL remains unclear (*38*).

CA-MRSA has become more widespread over the past decade and has been responsible for increasing amounts of severe complicated pneumonias in the community (*19*). The Centers for Disease Control and Prevention reported a significant increase in CA-MRSA pneumonia associated with influenza infection in the United States during 2006–2007, including deaths among previously healthy children (*18*).

History reminds us that we must be prepared to deal with severe bacterial pneumonia when planning for future influenza threats. Good evidence exists that influenza will interact with bacterial pathogens to cause severe pneumonia and increased mortality rates. Thus, the recent increase in deaths caused by empyema has potential implications for pandemic influenza preparedness. The rise of pneumococcal serotypes with a propensity to cause complicated pneumonia and increasing rates of community acquired pneumonias due to CA-MRSA should be considered when developing strategies to prevent and treat influenza complications. These strategies might include broadening recommendations for existing or enhanced pneumococcal vaccines that cover serotypes associated with empyema, such as 1, 3, and 19A. Determining who should be vaccinated with these pneumococcal vaccines during an influenza pandemic would have to be done on the basis of risk and the availability of vaccines. Further, the stockpiling of antimicrobial drugs active against CA-MRSA and other resistant pathogens may also be needed. Currently, the US Department of Health and Human Services Pandemic Influenza Plan from 2005 does not specifically account for secondary bacterial infections or the need for bacterial vaccines (*1*,*39*).

However, the Infectious Diseases Society of America called for improved antibacterial agents and vaccines as a key need in pandemic influenza preparedness (*40*). Our data

provide support for this concept. Extending the range of conjugate pneumococcal vaccines to include the serotypes now commonly associated with empyema and encouraging broader use of the polysaccharide vaccine may help lessen the effects of *S. pneumoniae* infection on a pandemic. If high vaccination rates cannot be routinely achieved, stockpiles of pneumococcal vaccine might be needed. Antimicrobial drug stockpiling, particularly agents with activity against MRSA, should be included in the discussion of antiviral drug stockpiling as well. Further, because the treatment for empyema frequently requires drainage either through chest tubes or surgical procedures, planning for these healthcare resources should also be considered.

This study has some limitations. Cause of death in death certificate data is not always accurate, and the accuracy may vary by disease. The clinical manifestations of empyema were well described by 1900 (*22*). Empyema was common during the pre–antimicrobial drug era and relatively easy to document with clinical examination and chest radiographs. Thus, it seems unlikely that recognition or reporting of empyema changed with time during the period covered in this historical study. There is no reason to suspect that empyema was more readily diagnosed in the final 5 years of the study, that definitions changed, or that it was more readily listed as a cause of death. Influenza, in contrast, is more difficult to diagnose clinically and may have been more readily listed as a cause of death during known pandemics and after virologic testing became available. Our data do not include microbiology results, so the association between empyema deaths and specific organisms is speculative.

We observed a significant increase in deaths caused by bacterial empyema during the period of the influenza pandemic of 1918–19 and an unexplained increase from 2000 through 2005. As secondary bacterial pneumonia historically has been a significant cause of illness and death in influenza pandemics, understanding the recent increase in empyema deaths is critical as we prepare for the next influenza pandemic. Changes in prevalent bacteria, including *S. pneumoniae* serotypes and the virulence of *S. aureus,* should be further explored. Pneumococcal vaccines targeting the serotypes most associated with empyema and antimicrobial agents against resistant bacteria such as CA-MRSA should be key components in national and international influenza pandemic planning.

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Microsporidiosis and Malnutrition in Children with Persistent Diarrhea, Uganda

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We show that the microsporidian fungus *Enterocytozoon bieneusi* is associated with lower rates of weight gain in children in Uganda with persistent diarrhea. This relationship remained after controlling for HIV and concurrent cryptosporidiosis. Children with microsporidiosis were predicted to weigh 1.3 kg less than children without microsporidiosis at 5 years of age.

Enterocytozoon bieneusi is an important cause of persistent diarrhea, intestinal malabsorption, and wasting in HIV-positive adults. Mucosal damage associated with microsporidiosis is more extensive than that related to other opportunistic intestinal infections (*1*,*2*) and leads to substantial malabsorption of carbohydrates, fat, and essential nutrients (*2*–*5*). Although microsporidiosis is common in children <5 years of age, particularly those who live in developing countries (*6*,*7*) or who are HIV positive (*6*,*8*,*9*), the effects of infection on nutritional health of these vulnerable populations are not well documented. We reexamined anthropometric data of children in Uganda with persistent diarrhea (*6*) and used regression analysis to determine whether there is an association between microsporidiosis and reduced growth rates.

The Study

A total of 243 children ≤60 months of age with persistent diarrhea (>14 days) were enrolled at Mulago Hospital in Kampala, Uganda, from November 2002 through May 2003. After informed consent was obtained, demographic, anthropometric, and clinical information was collected from each child. *E. bieneusi* spores were detected in stool specimens by using a nested PCR with *E. bieneusi*–specific primers (*7*). *Cryptosporidium* oocysts were detected by using immunofluorescence microscopy, with confirmation and genotyping subsequently determined by PCR–restriction fragment length polymorphism analysis (*10*). HIV status was determined by using established methods, and children positive for HIV were referred to the Mulago Hospital Pediatric Infectious Disease Clinic for further care. The study population and results of primary analysis are described in more detail elsewhere (*6*).

A complete set of anthropometric measures (age, weight, height, weight-for-age z-score, height-for-age z-score, and weight-for-height z-score) was available for 224 children. Wasting was twice as likely in children with microsporidiosis than in children without the infection (Table 1). Microsporidiosis was strongly associated with HIV and concurrent cryptosporidiosis. These infections likely compound the poor nutritional status of children with microsporidiosis, although this assessment is limited by sample size (Figure 1).

Linear regression models were used to describe weight and height gain in study children. Weight and height were treated as continuous dependent variables. When regressed on age, the slopes of the curves represent the rate of weight and height gain, respectively. All variables were transformed to the natural logarithmic scale.

For initial data exploration, several simple models were applied with children stratified according to HIV and *Cryptosporidium* spp. status. Within each strata, slope parameters were compared by using *t* tests to identify differences in growth rates between *E. bieneusi*–positive and –negative children. Growth rates were reduced in children with microsporidiosis across all HIV and *Cryptosporidium* strata (Table 2). In HIV-positive children, the rate of weight gain was lower in children with microsporidiosis than in those without microsporidiosis (model 1a vs. model 1b), and some evidence showed that this was also true for HIV-negative children (model 1c vs. model 1d). In children concurrently infected with *Cryptosporidium* spp., rates of weight (model 1e vs. model 1f) and height gain (model 2e vs. model 2f) were lower in children with microsporidiosis.

Adjusted growth rate estimates were obtained by fitting a multiple linear regression model that controlled for the effect of sex, HIV status, and concurrent cryptosporidiosis. The independent variable of interest was an interaction term between *E. bieneusi* and age, which reflected the difference in the growth rates of children with and without microsporidiosis. Interaction terms between *E. bieneusi*, *Cryptosporidium* spp., HIV, and age were also explored but were excluded from the final model because they did not improve model fit. When we simultaneously adjusted for sex, HIV status, and concurrent cryptosporidiosis, rate of weight gain remained significantly lower in children with microsporidiosis ($p = 0.014$). However, rate of height gain

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Table 1. Clinical features of 224 children with persistent diarrhea with and without cryptosporidisis, Uganda*

Feature	Total	Microsporidiosis		Crude OR (95% CI)	p value†
		Yes	No		
No. patients	224	68	156		
Age category, mo					
≤6, no. (%)	32 (14.3)	5 (7.4)	27 (17.3)	1.0	
7–12, no. (%)	108 (48.2)	31 (45.6)	77 (49.4)	2.2 (0.8–6.2)	0.137
13–24, no. (%)	69 (30.8)	24 (35.3)	45 (28.8)	2.9 (1.0–8.4)	0.048
≥25, no. (%)	14 (6.3)	8 (11.8)	6 (3.8)	7.2 (1.7–29.9)	0.004
Female sex, no. (%)	89 (39.7)	29 (42.6)	60 (38.5)	0.8 (0.5–1.5)	0.556
Nutritional status‡					
Mean WHZ (SD)	–1.44 (1.79)	–1.76 (1.83)	–1.30 (1.76)		0.077
Mean WAZ (SD)	–2.61 (1.41)	–2.76 (1.60)	–2.55 (1.32)		0.356
Mean HAZ (SD)	–2.16 (1.77)	–2.10 (1.90)	–2.19 (1.73)		0.750
Wasted, no. (%)	94 (42.0)	37 (54.4)	57 (36.5)	2.1 (1.2–3.7)	0.013
Underweight, no. (%)	148 (66.1)	47 (69.1)	101 (64.7)	1.2 (0.7–2.2)	0.525
Stunted, no. (%)	121 (54.0)	39 (57.4)	82 (52.6)	1.2 (0.7–2.2)	0.508
Concurrent cryptosporidiosis, no. (%)	63 (28.1)	55 (80.9)	8 (5.1)	78.3 (30.8–199.1)	<0.001
HIV+, no. (%)	77 (34.4)	58 (85.3)	19 (12.2)	41.8 (18.3–95.4)	<0.001
HIV+ and cryptosporidiosis, no. (%)	54 (24.1)	51 (75.0)	3 (1.9)	153.0 (43.1–543.5)	<0.001

*OR, odds ratio; CI, confidence interval.
†Continuous variables compared by using 2-sided *t* test. Categorical variables were compared by using Pearson χ^2 test.
‡WHZ, weight-for-height z-score; WAZ, weight-for-age z-score; HAZ, height-for-age z-score. Children were considered wasted, underweight, or stunted if WHZ, WAZ, or HAZ were ≤–2.0, respectively.

was not significantly different between children with and without microsporidiosis (p = 0.151). Predicted weight-for-age growth curves are shown in Figure 2, which also displays reference curves for healthy Ugandan children (*11*). The growth trajectory of children with microsporidiosis was such that by age 5, these children were predicted to weigh ≈1.3 kg less than children without microsporidiosis. This finding exceeded the predicted difference in weight in children with and without HIV (0.74 kg) at the same age.

Conclusions

Given consistent reports of severe wasting and malnutrition in HIV-positive adults with microsporidiosis, it is conceivable that *E. bieneusi* infection early in life may result in malnutrition. Two cross-sectional studies attempted to correlate microsporidiosis with poor anthropometric status but did not find a significant association (*7,12*). However, the dichotomous method used for these assessments is sensitive to the choice of cut-off values. Although children who fall ≥2 SDs below the reference growth curves are conventionally categorized as malnourished, this cut-off does not denote a biologically meaningful distinction between healthy and malnourished children.

Using regression analysis, we present evidence that microsporidiosis is associated with growth faltering in children in Uganda. In this approach, anthropometric data were treated as continuous variables, thus avoiding arbitrary categorization of children into malnourished and normally nourished groups. Lack of evidence for an independent effect of microsporidiosis on linear growth might be explained by the fact that these children were currently infected with *E. bieneusi*, and longer intervals are needed to document decelerating linear growth. In contrast, weight-for-age reflects chronic and acute nutritional states. Because all study participants had a history of persistent diarrhea and stool was generally collected on the day of hospitalization, nosocomial acquisition of microsporidiosis was unlikely.

There are several limitations to this analysis. The causal role of *E. bieneusi* in childhood malnutrition cannot be inferred because of the retrospective and cross-sectional study design. Although intestinal infection in children may impair absorption of nutrients (as documented in adults),

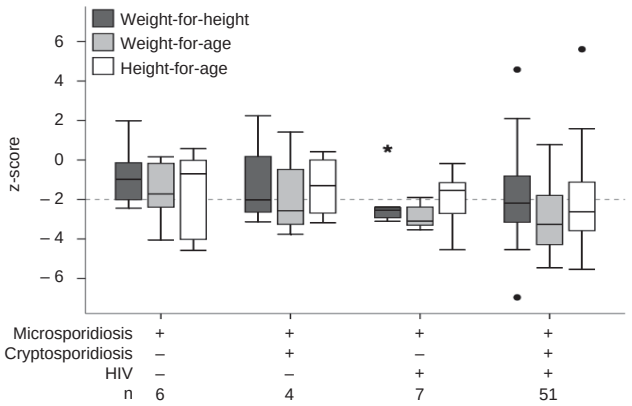

Figure 1. Nutritional status of children in Uganda with microsporidiosis with or without HIV or concurrent cryptosporidiosis. The infection status and number of children in each infection category are shown. Outliers are indicated with dots (1.5–3 interquartile ranges [IQRs]) or the asterisk (>3 IQR). The dashed horizontal line indicates conventional cut-off for malnutrition (z-score <2), horizontal lines in each column indicate the median score, and error bars indicate the highest and lowest z-scores excluding outliers.

Table 2. Rate of weight (model 1) and height gain (model 2) in children with and without microsporidiosis when stratified by HIV and *Cryptosporidium* infection, Uganda*

Model	No.	Infection status			Measurement at age:			R^2	Rate (95% CI)	Difference†	p value‡
		Micro	Crypto	HIV	12 mo	36 mo	60 mo				
1a	58	+		+	6.63	8.46	9.48	0.27	0.222 (0.13–0.32)	–0.147	0.016
1b	19	–		+	6.69	10.03	12.11	0.73	0.369 (0.25–0.49)		
1c	10	+		–	7.60	8.77	9.37	0.28	0.130 (–0.04–0.30)	–0.177	0.056
1d	137	–		–	6.92	9.70	11.35	0.33	0.307 (0.23–0.38)		
1e	55	+	+		6.65	7.98	8.69	0.17	0.166 (0.07–0.27)	–0.338	<0.001
1f	8	–	+		6.50	11.30	14.62	0.82	0.504 (0.27–0.74)		
1g	13	+	–		7.40	9.50	10.67	0.51	0.227 (0.08–0.37)	–0.081	0.336
1h	148	–	–		6.91	9.69	11.34	0.39	0.308 (0.25–0.37)		
2a	58	+		+	69.55	82.83	89.84	0.63	0.159 (0.13–0.19)	–0.035	0.123
2b	19	–		+	70.46	87.20	96.28	0.82	0.194 (0.15–0.24)		
2c	10	+		–	71.25	81.47	86.70	0.71	0.122 (0.06–0.19)	–0.040	0.206
2d	137	–		–	69.17	82.64	89.77	0.55	0.162 (0.14–0.19)		
2e	55	+	+		69.49	82.03	88.61	0.59	0.151 (0.12–0.19)	–0.085	0.009
2f	8	–	+		68.75	89.10	100.51	0.81	0.236 (0.12–0.35)		
2g	13	+	–		71.49	82.73	88.55	0.75	0.133 (0.08–0.18)	–0.034	0.240
2h	148	–	–		69.41	83.38	90.81	0.61	0.167 (0.15–0.19)		

*For ease of interpretation, predicted measurements at various ages are shown in addition to the rate of growth in ln(kg or cm)/ln(months). Micro, microsporidiosis; Crypto, cryptosporidiosis; CI, confidence interval.
†Rate of growth of children with microsporidiosis minus rate of growth of children without microsporidiosis in ln(kg or cm)/ln(months).
‡By *t* test comparing rate of growth of children with and without microsporidiosis.

malnourished children may also have immune defects that predispose them to *E. bieneusi* infection. Because the PCR was specific for *E. bieneusi*, we cannot rule out that some children were infected with other microsporidian species. However, *E. bieneusi* is the more common of 2 species known to cause intestinal microsporidiosis (*13*,*14*). In previous studies at Mulago Hospital, 16.8% of children with acute diarrhea and 16.8% of children without diarrhea had microsporidiosis (*7*). Because all children in the current study had persistent diarrhea, direct comparison between groups was possible without the need to control for diarrhea status. However, we cannot comment on the effect of acute or subclinical infection on nutritional health. Residual confounding may exist through sociodemographic factors not accounted for in the analysis. Sociodemographic data were limited to the accessibility of safe drinking water and type of sanitary facility in the household, neither of which were associated with microsporidiosis (data not shown). Finally, use of cross-sectional data is a major limitation because measurements obtained at a single point in time do not capture individual growth trends. Such data make it difficult to assess the effect of a particular episode of illness on growth attainment (*15*). To this extent, longitudinal anthropometric assessment is the only means of detecting growth faltering that results from *E. bieneusi* infection in childhood.

Although our results suggest an association between reduced weight gain and microsporidiosis, further studies are required to determine the role of *E. bieneusi* in childhood malnutrition. Longitudinal studies enabling comparison of preinfection and postinfection weights in individual children are needed to establish the direction of causation. It will be particularly useful to identify whether the period of reduced weight gain is followed by catch-up growth. Because microsporidiosis is highly prevalent in children in developing countries, the finding that the infection has

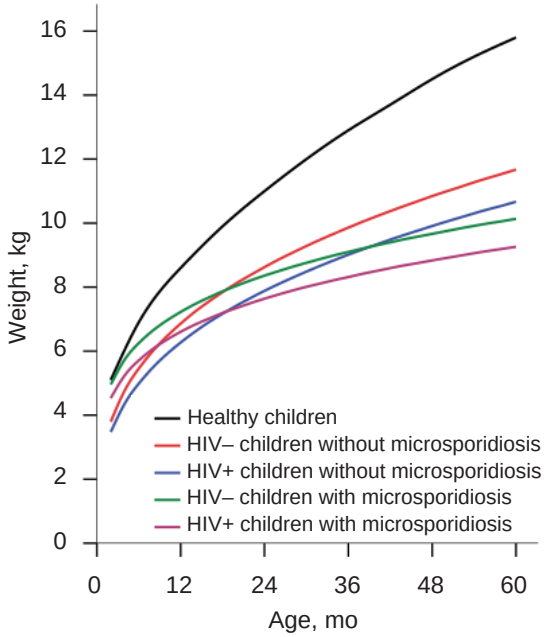

Figure 2. Weight-for-age growth curves of study children (as modeled by multiple linear regression) and reference populations in Uganda (*11*). Curves represent the median weight-for-age, averaged between boys and girls and controlling for concurrent *Cryptosporidium* spp. infection. The difference, 95% confidence interval, and significance of the interaction term between *Enterocytozoon bieneusi* and age reflect the difference in growth rates of children with and without microsporidiosis in ln(kg)/ln(age). R^2 = 0.42, difference = –0.133, 95% confidence interval –0.23 to –0.03, p = 0.009.

a lasting effect on growth would highlight the importance of nutritional rehabilitation and provide impetus to develop therapeutics suitable for use in young children.

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Invasive Disease Caused by Nontuberculous Mycobacteria, Tanzania

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Data on nontuberculous mycobacterial (NTM) disease in sub-Saharan Africa are limited. During 2006–2008, we identified 3 HIV-infected patients in northern Tanzania who had invasive NTM; 2 were infected with "*Mycobacterium sherrisii*" and 1 with *M. avium* complex sequevar MAC-D. Invasive NTM disease is present in HIV-infected patients in sub-Saharan Africa.

In sub-Saharan Africa, mycobacterial infections are predominantly caused by *Mycobacterium tuberculosis* (*1*). In more developed countries, *M. avium* and *M. simiae* are responsible for disseminated disease in HIV-infected persons (*2*). To better understand invasive nontuberculous mycobacterial (NTM) infections in HIV-infected persons in sub-Saharan Africa, we studied patients at 2 hospitals in northern Tanzania.

The Study

From July 2006 through August 2008, we collected blood from 723 patients ≥13 years of age who had axillary temperatures ≥38ºC and who had been admitted to Kilimanjaro Christian Medical Centre and Mawenzi Regional Hospital in Moshi, Tanzania. Standardized clinical information was collected from all patients. For mycobacterial culture, 5 mL from each patient was inoculated into a BacT/ALERT MB bottle and monitored in a BacT/ALERT 3D (bioMérieux, Durham, NC, USA) automated liquid culture instrument. Other tissue samples (not blood) were obtained from patients with suspected invasive mycobacterial disease and incubated on Middlebrook 7H10 and Lowenstein-Jensen media at 36°C. We used AccuProbe MTB and MAC kits (GenProbe, San Diego, CA, USA) to identify members of *M. tuberculosis* complex and *M. avium* complex. NTM were further identified by INNO-LiPA Mycobacteria v2 reverse line blot (Innogenetics, Gent, Belgium). All assays were used according to the manufacturer's instructions. All reverse line blot identifications were confirmed by performing additional sequencing of the complete 16S rDNA gene, the 16S–23S internal transcribed spacer (ITS), and the heat shock protein 65 (*hsp65*) gene (*3*,*4*).

Of the 723 patients, 30 (4.1%) had mycobacterial bloodstream infections, of which 2 (9%) were NTM. In 1 additional patient, NTM was identified in a tissue specimen. We describe the 3 patients with NTM infections.

The first patient was a 49-year-old man with cough and weight loss. His sputum contained acid-fast bacilli, and he simultaneously received a diagnosis of HIV infection with a CD4-positive T-lymphocyte count (CD4 count) of 9 cells/mm^3. Tuberculosis therapy was begun and comprised isoniazid, rifampin, pyrazinamide, and ethambutol; he was also started on a fixed-dose combination of zidovudine, lamivudine, and abacavir. DNA was extracted from the initial sputum smear taken at the time of presumptive tuberculosis diagnosis according to previously published methods (*5*). The GenoType CM/AS reverse line blot assay (Hain Lifesciences, Nehren, Germany) was weakly positive for *M. tuberculosis* complex. The patient's cough resolved, and he completed a 9-month course of tuberculosis therapy. When fever subsequently developed, he was admitted to the hospital; CD4 count was 13 cells/mm^3. Mycobacterial blood culture grew acid-fast bacilli after 12 days of incubation; results of AccuProbe MTB and MAC tests were negative. Heat-killed cells from the positive blood culture were identified as *M. simiae* by the INNO-LiPA reverse-line blot. Sequencing of the full 16S rDNA gene, ITS, and *hsp65* gene identified the isolate as "*M. sherrisii*." The 16S rDNA and *hsp65* sequences were identical to the *M. sherrisii* American Type Culture Collection (ATCC; Manassas, VA, USA) BAA-832 strain sequences deposited in the GenBank sequence database under accession nos. AY353699 (16S rDNA) and AY365190 (*hsp65*). The ITS sequence was identical to that of *M. sherrisii* strain FI-95229 (accession no. DQ185132), isolated from sputum of a patient in Italy (*6*). The Tanzania patient was treated with azithromycin, 500 mg/day, and ethambutol, 800 mg/day. His fever abated

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and he remained well, with 109 CD4 cells/mm^3 as of last follow-up in 2008.

The second patient was a 36-year-old HIV-infected man with a 3-month history of fever and weight loss and 31 CD4 cells/mm^3. He had been taking fixed-dose combination stavudine, lamivudine, and nevirapine for 5 months, but his adherence to therapy was poor. A mycobacterial blood culture grew acid-fast bacilli after 15 days of incubation; AccuProbe MTB and MAC test results were negative. Heat-killed cells from the positive blood culture were identified as *M. simiae* by the INNO-LiPA reverse-line blot and again as *M. sherrisii* by sequencing of the full 16S rDNA gene, ITS, and the *hsp65* gene. The 16S rDNA gene had a single base-pair difference when compared with the *M. sherrisii* ATCC BAA-832 strain sequence in GenBank. We deposited the new 16S rDNA sequence in GenBank under accession no. EU883389. The *hsp65* sequence was identical to the *M. sherrisii* ATCC BAA-832 strain sequence (accession no. AY365190); the ITS sequence was identical to the *M. sherrisii* strain FI-95229 (accession no. DQ185132) sequence (*6*). The patient was treated with azithromycin, 500 mg/day, and ethambutol, 800 mg/day; fever abated. At follow-up in 2008, the patient was continuing treatment with azithromycin and ethambutol but had abdominal pain and hepatosplenomegaly. Abdominal ultrasonography showed retroperitoneal lymphadenopathy. Follow-up mycobacterial blood cultures have been negative.

The third patient was a 36-year-old HIV-infected woman with a 4-month history of bilateral skin lesions affecting the lower extremities (Figure) and 206 CD4 cells/mm^3. HIV infection had been diagnosed 18 months earlier; baseline CD4 count was 6 cells/mm^3. She began fixed-dose combination stavudine, lamivudine, and nevirapine soon after her HIV diagnosis. An incisional biopsy from the active margin of a leg lesion showed several foci of dermal necrosis with dense lymphocytic infiltrate and Langhans-type giant cells consistent with granulomatous inflammation of tuberculosis (Figure). Culture of biopsy material was positive for *M. avium* complex. The isolate reacted only with the *M. avium-intracellulare-scrofulaceum* complex probe of the INNO-LiPA reverse-line blot. The 16S rDNA gene and ITS sequences were identical to the *M. avium* complex ATCC 35770 (Melnick) strain sequences published by Böddinghaus et al. (*7*) and available in the Ribosomal Differentiation of Microorganisms database (http://rdna.ridom.de). The ITS sequence was also identical to the MAC ATCC 35770 strain sequence available in GenBank (ITS sequevar MAC-D, accession no. L07851). The *hsp65* sequence was identical to the ATCC 35770 sequence (accession no. U85637). Because the full 16S rDNA gene sequence of this strain was not available in GenBank and only a small fragment of *hsp65* was available, we deposited our sequences under accession nos. EU815938 (16S rDNA) and EU935586 (*hsp65*). This patient was treated with azithromycin, 500 mg/day, ethambutol, 800 mg/day, and rifampin, 600 mg/day. Her lesions abated over the subsequent weeks, and she remained well as of follow-up in 2008.

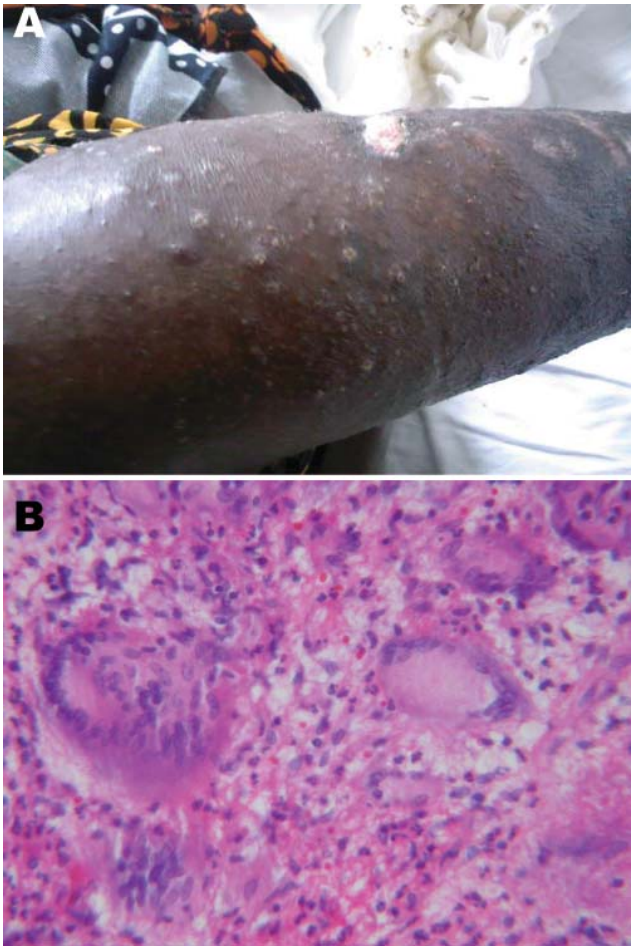

Figure. A 36-year-old HIV-infected woman with *Mycobacterium avium* disease. A) Photograph of skin lesions on right leg, taken before treatment. B) Histopathologic appearance of skin biopsy specimen from right leg lesion (stain, hematoxylin and eosin; magnification ×40).

Conclusions

Improved laboratory techniques enabled us to demonstrate that invasive NTM infections occur in northern Tanzania and include *M. sherrisii* and *M. avium* complex. *M. sherrisii* still awaits official recognition (*8*). Of *M. sherrisii* infections reported to date (*6*,*9*–*12*), most have been in HIV-infected patients from Africa (*9*–*11*). Although recommendations for the antimicrobial drug management of these infections have not yet been established, our 2 patients with *M. sherrisii* disseminated disease responded clinically to the optimization of their antiretrovi-

ral therapy regimen and to the combination of ethambutol and azithromycin.

The *M. avium* complex isolated from our third patient is remarkable for its ITS sequevar type. MAC-D has not previously been associated with invasive disease in HIV-infected patients, in which *M. avium* sequevars, mainly Mav-A and -B, are most common (*13*). The *M. avium* complex ATCC 35770 reference strain was the first reported strain with a MAC-D ITS. The ATCC 35770 strain, however, was isolated from a sputum sample in a symptomatic patient in the United States (*14*). The isolate from our third patient and the ATCC 35770 strain are genetically divergent from other *M. avium* complex members and may represent a separate species within the *M. avium* complex.

Invasive NTM disease in HIV-infected populations in sub-Saharan Africa demands more attention in terms of identification of etiologic agents, clinical relevance, and management. Further insights would be gained if current and future studies on tuberculosis in the region included liquid culture and molecular identification to confirm *M. tuberculosis* infection and establish the epidemiology and clinical relevance of NTM.

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Experimental Infection of Dogs with Avian-Origin Canine Influenza A Virus (H3N2)

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Susceptible dogs were brought into contact with dogs experimentally infected with an avian-origin influenza A virus (H3N2) that had been isolated from a pet dog with severe respiratory syndrome. All the experimentally infected and contact-exposed dogs showed elevated rectal temperatures, virus shedding, seroconversion, and severe necrotizing tracheobronchitis and bronchioalveolitis.

Transmission of highly pathogenic avian-origin canine influenza A viruses (H3N2) that spread across South Korea during May through December 2007 was observed repeatedly in the country's animal clinics (*1*). These viruses share ≥97% nucleotide sequence homology, suggesting that whole viruses were transmitted directly from birds to dogs. To determine whether these viruses can be transmitted directly from dog to dog, we experimentally infected beagles by direct contact. Dog-to-dog transmission of the virus raises questions about the interspecies transmission of avian influenza viruses and adaptation of these viruses to canine physiology.

The Study

Dogs in the study comprised 3 groups of beagles housed in different rooms of the isolation facility at Green Cross Veterinary Products (Yong-in, South Korea). The virus used was avian-origin canine influenza virus A/canine/01/2007, subtype H3N2, which had been isolated from a pet dog with severe respiratory syndrome. In the first group (challenge group), 4 beagles were inoculated intranasally with a $10^{6.5}$ 50% egg infectious dose (EID_{50}). Two hours later, the second group of 4 uninfected dogs (exposure group) was housed in the same contaminant room. These uninoculated dogs had frequent direct nose-to-nose contact with the inoculated dogs. The third group of 4 dogs (control group) was housed separately as uninoculated controls. Rectal temperatures were checked and nasal swab samples were collected daily. We monitored clinical signs of infection 7 days postinoculation (dpi) and examined nasal swabs obtained 10 dpi for virus shedding; serum samples were collected at 0, 3, 7, 9, and 13 dpi. Serum antibodies against nucleoprotein were detected by using a commercial competitive ELISA (Animal Genetics, Inc., Suwon, South Korea). On 7 and 13 dpi, 2 dogs from each group were euthanized for gross and histopathologic examination. All organs from the dogs were rapidly immersed in 10% neutral formalin buffer to prevent autolysis and stored overnight. All animal experiments complied with the current laws of South Korea. Animal care and treatment were conducted in accordance with guidelines established by the Seoul National University Institutional Animal Care and Use Committee. A p value <0.01 was considered statistically significant.

Clinical signs, including sneezing, nasal discharge, and coughing, were observed 2–8 dpi in the challenge group and 5–8 dpi in the exposure group. Twenty-four hours after inoculation, fever developed in dogs in the challenge group (mean rectal temperature 39.85°C–39.75°C) that lasted until 3 dpi. Fever (39.5°C) developed in dogs in the exposure group 72 hours after exposure; mean rectal temperature was 38.65°C a day later. Fever and clinical signs were not observed in the control group. Necropsy examination found gross lesions in the lung, including multifocal to coalescing reddish consolidations, consistent with influenza-induced lung lesions in other species. These gross lesions were observed in the challenge group on dpi 7 and 13 but in the exposure group only on dpi 13. Histopathologic examination showed severe necrosis and inflammation of the upper and lower respiratory tracts. Infected dogs shared the following histopathologic features (Figure 1): 1) severe multilobular or diffuse necrotizing tracheobronchitis with suppurative inflammation in the lumina and squamous metaplasia of the tracheobronchial epithelium and 2) severe multilobular bronchiolitis and alveolitis. We observed these histopathologic lesions in the trachea and lungs in the challenge group on dpi 7 and 13 but in the exposure group only on dpi 13. For the challenge group, mean virus titers in respiratory tract tissues were $10^{3.9}$ EID_{50}/mL on dpi 7, but no viruses were detected in the tissues on dpi 13. For the contact group, mean virus titers in respiratory tract tissues were $10^{4.7}$ EID_{50}/mL on dpi 7, but no viruses were detected in the tissues on dpi 13. We detected viruses in nasal discharge of dogs in the challenge group on dpi 2–6 and in the exposure group on dpi 5–8 ($p<0.01$). Shedding peaked on

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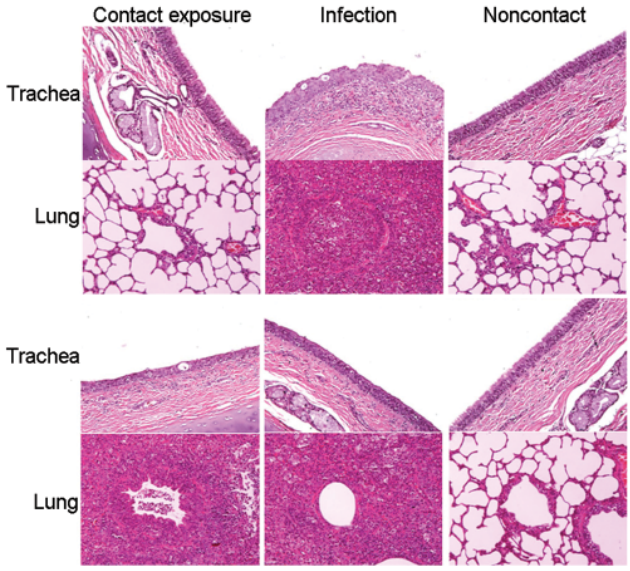

Figure 1. Histopathologic appearance of tissue of dogs experimentally exposed to canine influenza virus by contact with infected dogs. Severe necrotizing, suppurative tracheitis and bronchioalveolitis were observed in the contact-exposure group on day postinoculation (dpi) 13. However, influenza-associated lesions were not yet present in these dogs on dpi 7. Original magnification was ×200 for all images. Hematoxylin and eosin stain.

dpi 2 in the challenge group and dpi 6 in the exposure group (Figure 2). Nucleoprotein-specific ELISAs showed that all dogs lacked nucleoprotein-specific antibodies before inoculation and that dogs in the control group remained negative throughout the experiment. However, the percentage inhibition values were positive on dpi 7, 9, and 13 for dogs in the challenge group ($p<0.01$) and positive on dpi 9 and 13 for dogs in the exposure group ($p<0.01$, cut-off ratio of percentage inhibition >50) (Figure 2).

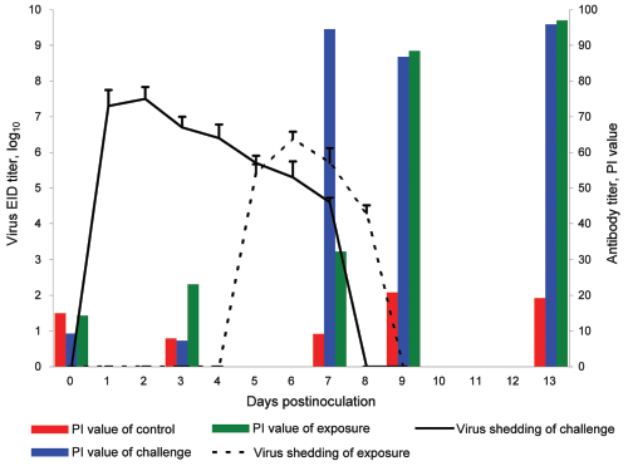

Figure 2. Virus shedding and serologic response of beagles after experimental contact transmission of canine influenza virus. EID, egg infectous dose; PI, percentage inhibition

Transmission of virus from 1 host to another species—in general, essentially unaltered by direct transfer—is an important feature of the ecology of influenza virus (*2*). Examples of this interspecies transmission mechanism include the recent human infections with the H5N1 subtype of avian influenza virus (*3*,*4*). Dogs infected with avian subtype H3N2 recently were identified in South Korea, suggesting that an avian influenza virus with high pathogenicity that can rapidly spread from dogs to dogs has made the interspecies leap**.** Most whole influenza viruses that are transmitted directly from the natural host species to a different species do not achieve sustained transmission in the new host species (*5*), suggesting that multiple virus–host interactions are needed before the virus can replicate and be transmitted horizontally in a new host species (*2*). Here we showed that close contact between canine influenza virus-infected and –noninfected dogs results in spread of the virus to the uninfected dogs, which then develop clinical signs of the disease.

Conclusions

We showed that an avian-origin canine influenza virus isolated from a pet dog can spread from dog to dog by contact infection. We observed a transient rise in rectal temperature in the challenge and exposure dogs and seroconversion in the exposure dogs. These dogs also had viral RNA in their nasal swabs and histopathologic changes in their upper and lower respiratory tracts. Our results demonstrate that an avian-origin canine influenza virus readily infects dogs and is easily transmissible among dogs.

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Personal Protective Equipment and Risk for Avian Influenza (H7N3)

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An outbreak of avian influenza (H7N3) among poultry resulted in laboratory-confirmed disease in 1 of 103 exposed persons. Incomplete use of personal protective equipment (PPE) was associated with conjunctivitis and influenza-like symptoms. Rigorous use of PPE by persons managing avian influenza outbreaks may reduce exposure to potentially hazardous infected poultry materials.

In April 2006, an outbreak of avian influenza occurred on 3 poultry farms in Norfolk, England (*1*). Reverse transcription–PCR (RT-PCR) of poultry blood samples and cloacal swabs detected low-pathogenic avian influenza (H7N3) on 1 farm, and veterinary investigation confirmed influenza subtype H7N3 on the 2 adjacent farms. Surveillance and protection zones were established around all infected premises, and all birds were culled. Persons who had been exposed were offered oseltamivir prophylaxis; those with influenza symptoms were offered oseltamivir treatment and influenza vaccination. All persons at risk were orally instructed to wear personal protective equipment (PPE).

The Study

We conducted a retrospective cohort study of all persons who had been potentially exposed to infectious material by handling live and dead poultry, poultry products, or litter derived from infected premises. Our objective was to measure associations between potential exposure to infectious material, completeness of use of PPE, and taking and timing of oseltamivir prophylaxis with having symptoms consistent with or confirmed as resulting from influenza virus A (H7N3) infection. We pretested and then administered a questionnaire by telephone after poultry culling ended (median 66 days, range 60–143 days). For persons who did not respond to the questionnaire (n = 39), we extracted data recorded in the outbreak records to describe their activities in relation to the outbreak, their use of oseltamivir prophylaxis, and their seasonal influenza vaccine status. Only persons who were interviewed and completed the questionnaire (n = 103) were included in the statistical analysis. Persons were invited to provide an acute-phase blood sample during the outbreak and a convalescent-phase sample 28 days after their last potential exposure. Exceptions were those at low risk, e.g., incinerator workers and lorry drivers.

Possible case-patients were those who reported conjunctivitis or influenza-like symptoms (≥1 of the following: fever, sore throat, cough, shortness of breath, body/muscle pain, runny nose) in the 7 days after last potential exposure. Confirmed case-patients were those for whom virus was detected by culture and RT-PCR of material from the conjunctiva or respiratory tract and/or confirmed by serologic testing. Influenza virus (H7N3) from the conjunctiva of the index case-patient was prepared by growth in embryonated eggs. Serum samples were screened by using microneutralization (MN) and hemagglutination inhibition (HI) tests (*2*,*3*). We defined MN >20 as evidence of seroreactivity. When either test gave a positive result, we performed confirmatory Western blot analysis, using purified influenza (H7N3) virus (*4*).

We calculated odds ratios (ORs), 95% confidence intervals (CIs), and p values for being a possible or confirmed case-patient. Independent variables are shown in the online Technical Appendix, Table A, available from www.cdc.gov/EID/content/15/1/59-Techapp.pdf. All risk factors with $p \leq 0.2$ in the single-variable analysis were initially included in a logistic regression model and then removed, least significant first, until all had $p \leq 0.1$. Confounding variables (those that caused ≥10% change in the ORs of covariates) were retained regardless of p value.

In total, 142 persons were potentially exposed. Questionnaires were completed for 103 (73%) persons (21 could not be contacted, 10 declined, 7 had no contact information, and 1 questionnaire was lost). Characteristics, potential exposures, and preventive measures differed little between persons who did or did not complete the questionnaire (Table 1). Of 46 persons who reported symptoms, 19 reported conjunctivitis with influenza-like symptoms and 27 reported influenza-like symptoms only. PPE reported as "always used" were protective coveralls (81%), protective footwear (82%), disposable gloves (67%), face-fitted mask (51%), other mask (24%), and protective goggles (19%) (online Technical Appendix, Table B).

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Fifty-six (54%) persons reported complete use of PPE. Single-variable analysis indicated that working on an infected premise (OR 2.76, 95% CI 1.17–6.50) was significantly associated with being a possible or confirmed case-patient (online Technical Appendix, Table A). Higher levels of exposure to potentially infected poultry (OR 2. 20, 95% CI 0.96–5.04) and only partial use compared with full use of PPE (OR 2.16, 95% CI 0.97–4.83) were also associated

Table 1. Characteristics and preventive measures taken by all persons potentially exposed to influenza A virus A (H7N3)–infected materials

Characteristic	Completed questionnaire (n = 103), no. (%)*	Did not complete questionnaire (n = 39), no. (%)†
Male gender	81 (79)	34 (87)
Oseltamivir prophylaxis		
Yes	98 (95)	30 (77)
No	5 (5)	6 (15)
Unknown		3 (8)
Seasonal influenza vaccine		
Received before outbreak	5 (5)	1 (3)
Received during outbreak	12 (12)	1 (3)
Received before and during outbreak	66 (64)	29 (74)
Not received	8 (8)	2 (5)
Unknown	12 (12)	6 (15)
Activities on infected premises‡	65 (63)	22 (56)
Any activity with potentially high exposure§	62	6
Catching poultry	39	4
Culling poultry	24	7
Inspecting or collecting biological/environmental samples	21	2
Loading dead poultry for transport	32	0
Disinfecting and cleaning	17	2
Activities off infected premises	38 (37)	12 (31)
Running incinerator	16	8
Transporting dead poultry	10	0
Testing biological/environmental samples	4	1
Other	8	3
Activities unknown	0	5 (13)
Use of personal protective equipment¶		
Complete	56 (54)	–
Incomplete	47 (46)	–
Exposure to poultry during 6 mo before outbreak		
Never	20 (19)	–
Occasional	34 (33)	–
Frequent	46 (45)	–
Unknown	3 (3)	–
Symptoms reported 7 d postexposure		
Conjunctivitis only	0	–
Influenza-like symptoms only	27 (26)	–
Conjunctivitis and influenza-like symptoms	19 (18)	–
Influenza-like symptoms#	46 (45)	–
Body/muscle pain	23	–
Sore throat	22	–
Runny nose	16	–
Cough	15	–
Shortness of breath	8	–
Fever (subjective, not measured)	5	–

*Median age (range) 40 (15–64) y.
†Median age (range) 41 (19–74) y.
‡For the 39 persons who did not respond to the study questionnaire, we used activities recorded in the outbreak records. Some persons had >1 exposure on site.
§High exposure includes ≥1 of the following activities: entering poultry sheds, coming within 1 m of live poultry, handling live or dead poultry, contact with chicken litter or feathers, and handling eggs or egg products.
¶Complete use of personal protective equipment defined as always using gloves, coveralls, footwear, face-fitted N95 respirator, or other mask (unspecified), and goggles.
#Reported by the 27 patients with influenza-like symptoms only and the 19 with conjunctivitis and influenza-like symptoms.

with being a possible or confirmed case-patient, but 95% CIs were <1.0. Characteristics not associated with being a possible or confirmed case-patient were age >30 years; male sex; being a Department for Environment, Food and Rural Affairs employee; smoking; having had a prior influenza vaccination; timing of starting oseltamivir prophylaxis; and exposure to potentially infected poultry in the preceding months. Multivariable analysis showed the association with being a possible or confirmed case-patient to be statistically significant for incomplete use of PPE and weakly significant for working on an infected premise (Table 2).

Serum samples were available from 91 persons: 33 acute- and convalescent-phase pairs, 49 acute-phase samples, and 9 convalescent-phase samples. Only the serum from the index case-patient showed reactivity in both the MN (titer 40) and HI (titer 32) tests and also showed reactivity in Western blot. No acute-phase sample from this person was available. All other acute- and convalescent-phase samples were negative in both tests. During the outbreak, eye, nose, and throat swabs were taken from 14 persons (1–8 days after symptom onset); 10 reported influenza-like symptoms (2 without eye involvement), 2 reported no symptoms, and 2 had no clinical information available. Comprehensive molecular diagnostic tests for common human viral respiratory pathogens (enteroviruses, rhinoviruses, adenoviruses, respiratory syncytial viruses, parainfluenza viruses) were also performed and did not provide evidence of alternative causes of infection. A vaccine strain of avian paramyxovirus (Newcastle disease virus) was recovered from 1 person with conjunctivitis, which suggests that at least 1 case of conjunctivitis was caused by avian paramyxovirus. Serologic testing for seasonal influenza infection (HI tests on all paired serum samples) did not indicate any recent human infections.

Table 2. Multivariable analysis of factors associated with possible or confirmed cases*

Factor	Odds ratio	95% CI	p value
Defra employee			
No	1.00		
Yes	2.07	0.72–5.94	0.17
Working on an infected premise			
No	1.00		
Yes	7.53	0.68–83.41	0.064
Potential exposure level			
Low	1.00		
High	0.26	0.02–2.75	0.22
Use of personal protective equipment			
Complete	1.00		
Incomplete	3.26	1.22–8.73	0.015
Use of oseltamivir relative to first potential exposure			
Before	1.00		
On the same day	2.19	0.84–5.71	0.27
After	1.33	0.29–6.09	

*Based on 96 questionnaires with complete information. CI, confidence interval; Defra, Department for Environment, Food and Rural Affairs.

Our study had a number of limitations. Because workers were interviewed a minimum of 2 months after the outbreak, they may not have accurately recalled their exposures. In addition, we relied on self-reported data. Difficulties recalling symptoms were less likely as we actively followed up persons for 7 days after last exposure. In the absence of a control group, such as farmers from noninfected premises, whether the incidence of influenza-like illness and conjunctivitis in this cohort was different is unclear, although during the outbreak, influenza activity in the general population was low and no isolates of seasonal influenza were reported. We did not measure dust exposure as an alternative explanation for conjunctivitis in some or all persons, apart from the index case-patient who reported this symptom. The results from laboratory testing were limited because convalescent-phase serum was not available from all persons who reported influenza-like illness. However, a wide range of molecular diagnostic tests for human viral pathogens were performed on samples from persons who were not well at the time of the outbreak. Because the kinetics of appearance and disappearance of human antibodies to avian influenza are poorly understood, timing of the collection of samples may not have been optimal in this outbreak and we may have missed the opportunity to diagnose some infections. Moreover, because serologic tests for influenza virus A (H7N3) may not correlate well with infection (*5*), we could not rule out influenza A virus (H7N3) infection among symptomatic persons, even in the presence of convalescent-phase serum that was negative for H7.

Conclusions

Strict compliance with PPE use should be reinforced when outbreaks of avian influenza among poultry are being managed, as recommended in current guidance from the United Kingdom (*6*) and the European Centre for Disease Prevention and Control (*7*). Compliance tends to be suboptimal (*8*), possibly because of low risk perception among poultry workers (*9*). Understanding what obstacles prevent workers from wearing complete PPE is needed. Our study suggests that rigorous use of PPE by persons managing avian influenza outbreaks reduces influenza-like symptoms and conjunctivitis and potentially hazardous exposure to infected poultry materials.

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Imported Case of Poliomyelitis, Melbourne, Australia, 2007

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Wild poliovirus–associated paralytic poliomyelitis has not been reported in Australia since 1977. We report type 1 wild poliovirus infection in a man who had traveled from Pakistan to Australia in 2007. Poliomyelitis should be considered for patients with acute flaccid paralysis or unexplained fever who have been to poliomyelitis-endemic countries.

The World Health Organization declared the Western Pacific region, including Australia, polio free in 2000 (*1*). The last known case of wild poliomyelitis in Australia occurred in 1977 (*2*). The National Polio Reference Laboratory (NPRL), in collaboration with the Australian Paediatric Surveillance Unit, coordinates surveillance for acute flaccid paralysis in children <15 years of age and investigates all suspected cases of polio (*3*). However, with increasing time since widespread poliomyelitis occurred in Australia, concern has been raised about whether poliomyelitis would be recognized (*4*). Indeed, we report a Pakistani patient in whom poliomyelitis was first considered only after magnetic resonance imaging (MRI).

The Study

The patient was a 22-year-old Pakistani student in Melbourne, Australia; he had received at least 3 doses of oral polio vaccine as a child. On March 13, 2007, the student returned home to Kharian, Pakistan, to recuperate from herpes zoster, which had developed in February 2007. From June 4 through June 7, he toured the North West Frontier Province of Pakistan, then returned to Kharian. On June 22, he felt unusually hot. Over the next 2 days he had fever, diaphoresis, nausea, vomiting, and pain in his low back and legs. On June 24, he noted lower limb weakness, especially in his left leg. He was not hospitalized, and after several days, all symptoms except the pain resolved. On July 2, he traveled back to Melbourne.

Starting on July 3, the patient's pain increased and lower limb weakness subsequently returned, accompanied by upper limb tremors. He had neither systemic symptoms nor bladder or bowel dysfunction. He was referred to Boxhill Hospital, Eastern Health, emergency department on July 6. Physical examination found that the patient's legs were tender to palpation and that strength was mildly reduced in the entire left leg and proximal right leg. Lower limb reflexes and sensation, as well as cranial nerve and upper limb function, were within normal limits. Laboratory results of blood tests were within reference ranges.

The next day, MRI was highly suggestive of poliomyelitis. It showed a globular pattern of increased signal on T2-weighted sequences, limited to the anterior horn region throughout the spinal cord, without enhancement with gadolinium (Figure 1). The Department of Human Services, Victoria, was notified of the clinical diagnosis of poliomyelitis; the patient was admitted to a single room, and contact precautions were instituted. Panenterovirus reverse transcription–PCR (RT-PCR) of feces, serum, and throat swab produced negative results, but the samples were forwarded to the NPRL for cell culture 2 days later (Table). Cerebrospinal fluid contained 1×10^6 polymorphonuclear cells, 8×10^6 lymphocytes, 24×10^6 erythrocytes, a protein concentration of 1.9 g/L, and glucose levels within normal limits.

Given the patient's recent history of herpes zoster, intravenous acyclovir (10 mg/kg every 8 hours) was begun to treat possible varicella zoster virus–related myelitis. The patient had recovered completely by 48 hours, and this treatment was stopped. Laboratory identification of wild poliovirus type 1 was reported on day 7 after hospital admission, 5 days after fecal samples were submitted. The patient was quarantined in the hospital until 2 fecal specimens taken 7 days apart were negative for poliovirus by cell culture and RT-PCR, a total of 34 days (Table). The public health response, directed by the Department of Human Services, included vaccination of potentially susceptible persons who had been exposed to the index case-patient and home quarantine of his household contacts until poliovirus shedding was excluded. No secondary cases were identified.

At the NPRL, the initial fecal specimen was extracted according to recommended procedures (*5*) and incubated with 4 continuous mammalian cell lines: L20B, RD-A, Hep2 Cincinnati, and human embryonic lung. After 4 days, enterovirus cytopathic effect was observed in all cell lines except RD-A. This finding was after passage onto fresh cell lines to reduce toxicity.

Confirmatory testing for enterovirus isolation and intratypic differentiation for poliovirus was performed (*5*). The virus was identified by RT-PCR and ELISA as

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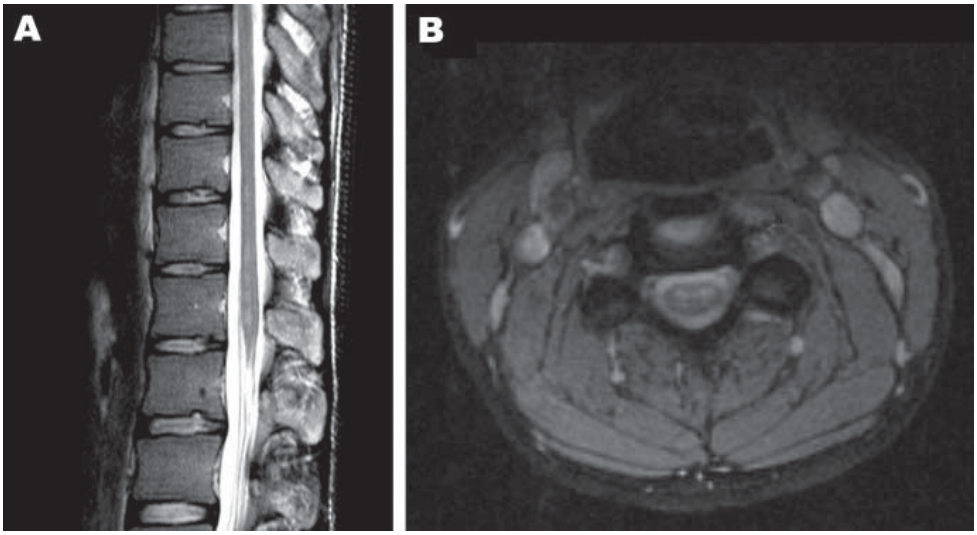

Figure 1. A) Sagittal image of the conus and B) coronal image of the cervical cord, demonstrating increased signal on T2 weighted sequences in the region of the anterior horns. There was no enhancement with contrast.

non-Sabin–like poliovirus type 1. Sequences for comparative analysis of the virus capsid protein 1 genomic region were acquired by using the BLAST algorithm (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Phylogenetic analysis showed a common ancestor with a wild poliovirus isolated from Pakistan in 2000 and clustering within a group of isolates originating in Pakistan, Afghanistan, and Iran (Figure 2). The National Polio Laboratory of Pakistan reported that the isolate from Australia had 98.8% nucleotide identity with isolates from Pakistan in 2006, which are not available in the public domain (S. Zaidi and S. Sharif, pers. comm.).

Acute- and convalescent-phase serum specimens were collected on days 15 and 47 after symptom onset, respectively, and tested for total immunoglobulin poliovirus neutralizing antibodies according to recommended methods (*6*). Antibodies to all 3 poliovirus serotypes were detected in both specimens. The potency of antibodies to authenticated Sabin poliovirus type 1 was 212 and 424 IU for the acute- and convalescent-phase samples, respectively. To have determined the titer of neutralizing antibodies at the onset of symptoms, earlier collection of acute-phase serum would have been needed.

Doubt was cast on the diagnosis of poliomyelitis because of the patient's age, vaccination history, and the initial panenterovirus PCR–negative fecal specimen. However, previous vaccination does not exclude poliomyelitis; reduced seroconversion to polio vaccine is well described in polio-endemic regions (*7*). Furthermore, although this patient had received oral polio vaccine during childhood, he received no booster dose before his recent travel.

Several factors support an argument against the negative predictive value of the initial fecal panenterovirus RT-PCR. Fecal shedding of poliovirus by infected persons can be intermittent; hence, 2 fecal specimens from suspected case-patients are recommended (*5*). Panenterovirus RT-PCR was subsequently positive for the second fecal specimen, provided 2 days after the first. In retrospect, the virus titer in the first specimen may have been below the limits of detection for RT-PCR; this limitation was overcome by virus multiplication in cell culture. The panenterovirus RT-PCR is a heminested assay adapted from Zoll et al. (*8*) by addition of an internal second round primer. This assay's performance in an external proficiency testing program conducted by the Royal College of Pathologists of Australasia (*9*) has met a high standard. Repeat testing of the first fecal specimen by this assay showed 1 of 15 replicates to be positive, consistent with a low poliovirus copy number.

Table. Virus diagnostic tests for poliovirus conducted for 22-year-old male Pakistani student in Melbourne, Australia, 2007*

Specimen	Time after symptom onset, d	EV RT-PCR	Cell culture	PV1 ELISA	ITD PCR
Feces	15	Negative	Positive	PV1NSL	PV1 wild
Swab (throat)	15	Negative	Negative		
CSF	15	Negative	Negative		
Feces	17	Positive	Positive	Not tested	PV1 wild
Feces	25	Negative	Negative		
Feces	28	Negative	Negative		
Feces	30	Negative	Negative		
Feces	37	Negative	Negative		

*EV RT-PCR, panenterovirus reverse transcription–PCR for direct detection from original clinical specimen; PV1, poliovirus type 1; ITD PCR, intratypic differentiation PCR; PV1NSL, poliovirus type 1 non–Sabin-like; CSF, cerebrospinal fluid.

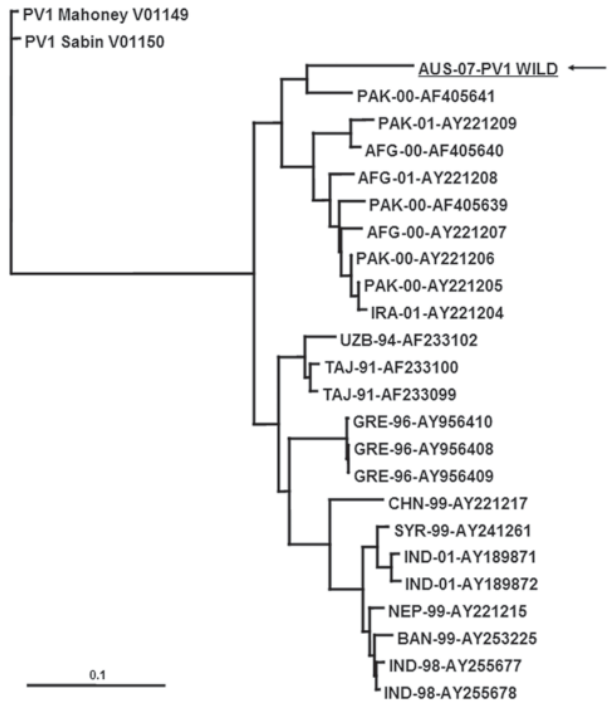

Figure 2. VP1 phylogenetic tree constructed by using cognate sequence available in the public domain was created by using the PHYLIP DNA maximum-likelihood algorithm with 100 bootstrap replicates. Marker represents relative phylogenetic distance. AFG, Afghanistan; AUS, Australia; BAN, Bangladesh; CHN, China; GRE, Greece; IND, India; IRA, Iran; NEP, Nepal; PAK, Pakistan; SYR, Syria; TAJ, Tajikistan; UZB, Uzbekistan. Scale bar represents 0.1 nucleotide substitions per site.

Furthermore, childhood vaccination may have attenuated the duration of virus shedding. Because the travel history, clinical illness, and radiographic appearance were highly suggestive of poliomyelitis, the presumptive diagnosis remained poliomyelitis until confirmation by fecal culture.

Conclusions

Until polio is completely eradicated, polio-free regions remain at risk for importation and subsequent transmission of poliovirus. Poliomyelitis should be considered in patients who have acute flaccid paralysis or a febrile illness without an alternate diagnosis and who have travelled from countries with endemic poliovirus transmission; neither previous vaccination nor a single negative fecal enteroviral PCR excludes poliomyelitis. The high level of vaccination in Australia reduces the risk for local transmission after poliovirus importation. However, Australia has a large migrant population who might not be immune, and Aboriginal and Torres Strait Islanders exhibit reduced seroconversion rates after polio vaccination (*10*). Risk for local outbreaks can be minimized by widespread vaccination, early recognition of an index case, and prompt and appropriate public health measures.

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Isolation of Candidatus *Bartonella melophagi* from Human Blood[1]

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Candidatus *Bartonella melophagi* was isolated by blood culture from 2 women, 1 of whom was co-infected with *B. henselae*. Partial 16S rRNA, RNA polymerase B, and citrate synthase genes and 16S–23S internal transcribed spacer sequences indicated that human isolates were similar to Candidatus *B. melophagi*.

During the past decade, the number of *Bartonella* species that are documented human pathogens has rapidly increased (*1*). Currently, *B. bacilliformis*, *B. quintana*, *B. henselae*, *B. elizabethae*, *B. vinsonii* subsp. *berkhoffii*, *B. vinsonii* subsp. *arupensis*, *B. koehlerae*, *B. alsatica*, *B. washoensis*, *B. rochalimae*, and *B. tamiae* have been isolated or sequenced from patient samples (*1*–*7*). Sheep are the most likely reservoir hosts for Candidatus *B. melophagi* and sheep keds may be a vector for their transmission among sheep. We report isolation of Candidatus *B. melophagi* from blood cultures from 2 women. This study was reviewed and approved by the North Carolina State University Institutional Review Board.

The Study

Patient 1 was a previously healthy, 51-year-old woman. During July 2004, she visited family residing in rural Ohio and participated in a variety of outdoor activities. Although she saw many wild animals, including deer, she did not report tick attachment or insect bites. Within 24 hours of her return home to North Carolina, a nonpuritic, slightly raised, circular red lesion, approximately the size of a quarter, was noted on the medial aspect of her thigh. Within 3 days, the lesion expanded to the size of a hand. Two weeks later, she exhibited a dry cough, fatigue, muscle pain in the upper body, severe chills, and extreme pain in both feet.

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During the next 2 years, these symptoms persisted, along with exertional chest pains, a previously undiagnosed ausculted II to III/VI holosystolic murmur, headaches, difficulty speaking, difficulty sleeping, weakness involving the arms, joint pain, and facial tremors. No abnormalities were shown on an electrocardiogram. An echocardiogram identified mildly thickened aortic and mitral valve leaflets, mild aortic insufficiency, and mild mitral regurgitation.

After the acute illness, the woman reported cycles of illness every 3 to 4 weeks. Results of numerous complete blood counts were normal, with the exception of persistently low neutrophil counts of 2,000–2,500 neutrophils/μL. All serum biochemical parameters remained within normal reference ranges during the 2-year illness. *Borrelia burgdorferi* C6 peptide and immunoglobulin (Ig) M and IgG antibodies to *Babesia microti* were not detected. Results of PCRs specific for *Anaplasma phagocytophilum*, *B. microti*, and *B. burgdorferi* were negative. Oral antimicrobial drugs resulted in transient improvement; however, symptoms returned within days after the use of these drugs was stopped. Blood culture resulted in the detection of Candidatus *B. melophagi* and isolation of *B. henselae*. Her serum was not reactive with *B. henselae* or *B. vinsonii* subsp. *berkhoffii* antigens.

Treatment with rifampin and azithromycin, started in January 2006, resulted in some overall improvement in symptoms. Cefuroxime was added in February, and the combination resulted in substantial improvement, after which the drugs were selectively withdrawn. For 15 years before the onset of illness, this person had worked as an animal shelter manager in West Virginia and as a veterinary office manager in Virginia. Animal contact was minimal, but she had been bitten by fleas and mosquitoes. Travel history was limited to the eastern and central United States.

Patient 2 was a 65-year-old woman whose condition had been diagnosed as pericarditis of undetermined etiology in September 2004. Six months later, because of residual fatigue and muscle weakness in the arms and legs, mostly on her right side, a blood sample was cultured in *Bartonella* alpha proteobacteria growth medium (BAPGM).

The woman lived on a farm in southern California with her husband and managed a large animal sanctuary that also housed ≈100 cats and ≈100 dogs. She had resided in southern California for 50 years but occasionally traveled to the southeastern United States and other countries. She was directly involved in daily care of animals and had exposure to pet cattle and sheep, wolf hybrids, lamas, emus, pigs, horses, and numerous pet bird species. Bites and scratches were a daily occurrence, and exposure to cattle and sheep occurred at least weekly. In addition, the woman

[1]Results of this study were presented in part at the 21st Meeting of the American Society for Rickettsiology, Colorado Springs, Colorado, USA, September 8–11, 2007.

reported daily exposure to biting flies, occasional exposure to ticks and mosquitoes, and infrequent exposure to fleas or lice. Sheep keds had never been observed on sheep by the attending veterinarian. Blood culture resulted in isolation of Candidatus *B. melophagi*. Serum was reactive at a titer of 64 to *B. henselae*, *B. vinsonii* subsp. *berkhoffi*, and *B. quintana* antigens.

We used BAPGM and other published blood culture methods to test blood samples from both women (*2*,*8*,*9*). Candidatus *B. melophagi* DNA was amplified directly from blood of patient 2, and from the respective BAPGM enrichment cultures and 14-day subculture colonies from both patients. Sequence analysis of respective colony isolates showed *B. henselae* (internal transcribed spacer [ITS] sequence identical to Houston 1 strain, data not shown) and Candidatus *B. melophagi* from patient 1 and Candidatus *B. melophagi* (isolate 05-HO-1) from patient 2. Both isolates were composed of extremely small gram-negative bacilli consistent with *Bartonella* spp. Sequence analyses for both isolates are summarized in the Table. Unfortunately, attempts to separate *B. henselae* and Candidatus *B. melophagi* colonies from the sample of patient 1 by serial passage were unsuccessful. *Bartonella* sp. DNA was not amplified from an uninoculated BAPGM control culture or from sheep blood used as a supplement. Flagella, as visualized in the Candidatus *B. melophagi* strain K-2C isolated from sheep blood (Figure), were not visualized in the human 05-HO-2 strain by transmission electron microscopy.

Conclusions

Based on 16S rRNA, citrate synthase and RNA polymerase B genes, and the 16S–23S ITS region, the bacteria detected in these woman was most likely Candidatus *B. melophagi*, which was recently isolated from sheep blood and sheep keds (*10*; M. Kosoy, unpub. data). ITS sequences were nearly identical to those of *Wolbachia melophagi* detected in a tick removed from sheep in Peru (*11*). Similar to electron micrographs of the *Bartonella* sp. isolated from sheep blood (*1*), no flagella were observed by transmission electron microscopy of the 05-HO-1 human isolate, whereas the sheep ked isolate contains flagella. Because both women had had frequent contact with numerous domestic and wild animals and potential insect vectors, the route of transmission is unknown.

The clinical relevance of Candidatus *B. melophagi* infection in these women remains to be established. Efforts to passage Candidatus *B. melophagi* in our laboratory and others (D.A. Bemis, *10*) have not been successful. Therefore, development of a serologic assay was not pursued. Nonspecific abnormalities, including difficulty sleeping, muscle weakness, joint pain, and facial tremors, have been reported in association with isolation of *B. henselae* and *B. vinsonii* subsp. *berkhoffi* (*2*,*12*). Pericardial or pleural effusions are infrequent complications of *B. henselae* infection in association with classical cat-scratch disease (*13*,*14*).

Before the report of Candidatus *B. melophagi* in commercial sheep blood sources in 2007 (*10*), sheep blood was used as a BAPGM supplement in our laboratory. With the exception of these 2 patients, Candidatus *B. melophagi* was never detected by PCR in >2,250 BAPGM enrichment blood cultures or subculture isolates obtained from animals or humans. In addition, Candidatus *B. melophagi* DNA was never amplified from >250 BAPGM uninoculated BAPGM enrichment control cultures, and bacterial colonies were never observed after subculture. Beginning in 2007, we also found that some batches of commercial sheep blood contained Candidatus *B. melophagi* DNA. Therefore, we no longer use blood as a BAPGM supplement. Recently, BAPGM was used to facilitate isolation of *B. tamiae* from human patients in Thailand (*7*), and another laboratory has published data supporting the utility of insect cell culture media for growing *Bartonella* spp. (*15*).

Table. Sequence similarities for 16S–23S ITS and 3 genes from 2 patient isolates and available GenBank sequences*

Sequence or gene	Basepair homology (%)	Basepair homology (%)
ITS	*Bartonella* sp. tick†	*Bartonella melophagi*‡
Patient 1	405/408 (99.3)	385/388 (99.2)
Patient 2	405/408 (99.3)	385/388 (99.2)
gltA	*Bartonella* sp. sheep blood§	*B. melophagi*¶
Patient 1	131/134 (97.8)	183/187 (97.9)
rpoB		*B. melophagi*#
Patient 1	NA	651/656 (99.2)
16S rRNA	*Wolbachia melophagi***	*B. melophagi*††
Patient 1	670/671 (99.8)	631/633 (99.7)

*ITS, internal transcribed spacer; gltA, citrate synthase; *rpoB*, RNA polymerase B; NA, not available.
†Uncultured *Bartonella* sp. clone BT7498 sequenced from a tick from Peru (GenBank accession no. AF415209).
‡Candidatus *B. melophagi* strain K-2C isolated from a sheep ked (M. Kosoy, unpub. data).
§*Bartonella* sp. isolated from commercial sheep blood agar plates (GenBank accession no. EU020109).
¶Candidatus *B. melophagi* strains K-9B and K-2C isolated from sheep ked (GenBank accession nos. AY724769 and AY724769).
#Candidatus *B. melophagi* strain K-2C isolated from sheep ked (GenBank accession no. EF605288).
***W. melophagi* sequenced from *Melophagus ovinus* sheep keds (GenBank accession no. X89110).
††Candidatus *B. melophagi* strain K-2C isolated from a sheep ked (GenBank accession no. AY724770).

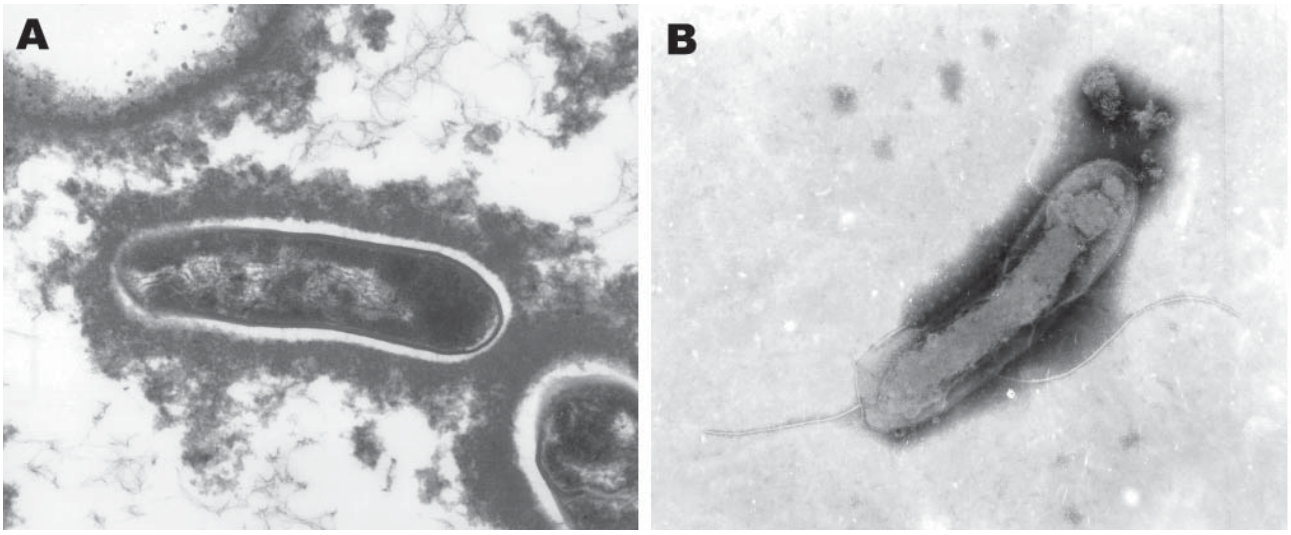

Figure. Transmission electron micrographs of Candidatus *Bartonella melophagi*–like isolate 05-HO-1 from a human (A) (image provided by the North Carolina State University–College of Veterinary Medicine Electron Microscopy Facility, Raleigh, NC, USA) and Candidatus *B. melophagi* isolate from a sheep ked (B) (image provided by V. Popov, University of Texas Medical Branch, Galveston, TX, USA). Magnification ×41,000 in A and ×62,700 in B.

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Botulism from Drinking Pruno

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Foodborne botulism occurred among inmates at 2 prisons in California in 2004 and 2005. In the first outbreak, 4 inmates were hospitalized, 2 of whom required intubation. In the second event, 1 inmate required intubation. Pruno, an alcoholic drink made illicitly in prisons, was the novel vehicle for these cases.

Foodborne botulism is a rare paralytic disease caused by ingestion of preformed botulinum toxin in food contaminated with *Clostridium botulinum*, an anaerobic, spore-forming bacterium that is ubiquitous in the environment. The other 2 main categories of botulism are infant botulism caused by intestinal colonization with *C. botulinum* and wound botulism caused by wound contamination with *C. botulinum*. In each of these latter categories, illness results from in situ production of botulinum toxin. In California, wound botulism caused by injection drug use has increased since 1994 (*1*).

Of the 7 botulinum toxin types (A–G), types A, B, and E are associated with most human cases. Symptom onset generally occurs 12–36 hours after ingestion of contaminated food. Symptoms start as cranial nerve palsies and are followed by a symmetric descending flaccid paralysis that can lead to respiratory failure and death if respiratory support is not provided (*2*). Botulism antitoxin can stop progression of paralysis if given early in the course of illness.

In July 2004, the Riverside County Department of Public Health and the Division of Communicable Disease Control (DCDC), California Department of Health Services (CDHS), investigated 4 suspected cases of botulism, all in male inmates from a California state prison in Riverside County. In May 2005, DCDC and the Monterey County Health Agency investigated suspected botulism in another male inmate from another California state prison. In both instances, pruno (also known as prison wine, jailhouse hooch, juice, or brew) was found to be the cause of foodborne botulism in these patients.

The Investigations

On July 1, 2004, the 4 inmates from the California state prison in Riverside County were hospitalized with signs and symptoms consistent with clinical botulism, including blurry vision, dysarthria, dysphagia, shortness of breath, and generalized muscle weakness. All 4 men reported symptom onset on June 30. They were 19–35 years of age and lived in the same building. None had a history of injection drug use and had no needle track marks or skin abscesses. All had reportedly drunk from the same batch of pruno on June 27. All 4 men received botulism antitoxin; 2 required mechanical ventilation and all survived.

Prison and hospital records were reviewed for other potential cases of clinical botulism. Serum, stool, and gastric specimens from suspected case-patients were requested and forwarded to the CDHS Microbial Disease Laboratory (MDL) for testing. No sample of the reported pruno batch was available for testing, but a cup with traces of pruno, belonging to 1 of the hospitalized patients, was submitted to MDL. Testing for botulinum toxin was conducted by using a mouse bioassay (*2*), and bacterial culture was conducted on stool and gastric aspirate specimens and on washings from the cup. Laboratory results are shown in the Table. The 4 case-patients had laboratory-confirmed botulism; botulinum toxin type A was detected in their pretreatment serum (3/4), directly from their stool (1/4), or from their stool culture (3/4). Cup washings were negative for botulinum toxin but culture positive for *C. botulinum* type A. No other botulism cases were confirmed from this prison.

From information gathered, one of the hospitalized inmates began making the pruno on June 21 using "unpeeled potatoes smuggled from the kitchen, apples from lunches, one old peach, jelly, and ketchup." On June 25, this inmate "heated water with an immersion heater and added it to the mixture." Correctional officers estimated that ≈2 gallons of pruno were made. On June 27, each of the 4 inmates drank ≈16 ounces or more of the pruno, which they described later to a prison nurse as being "magenta in color" and "smelling like baby-poop."

In May 2005, DCDC was notified of clinical botulism in another inmate of another California state prison in Monterey County. A 30-year-old male inmate was admitted to a local hospital with ptosis, ophthalmoplegia, dysarthria, dysphagia, and upper extremity weakness and was

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Table. Laboratory results for botulism outbreak in California, 2004*

Patient no.	Botulinum toxin detected in pretreatment serum	Botulinum toxin detected directly from stool	Botulinum toxin detected in stool culture	*Clostridium botulinum* isolated from stool culture
1	+	–	+	–
2	QNS	–	+	+
3	+	+	+	–
4	+	QNS	QNS	QNS

*QNS, quantity not sufficient.

intubated. At first, the patient was thought to have Miller-Fisher variant of Guillain-Barré syndrome, but subsequent testing of his serum showed a positive result for botulinum toxin type A. Upon further questioning, the patient admitted to making and drinking pruno in the prison; he had used potatoes in making the pruno. Pruno mash was found in his cell, and culture at MDL yielded *C. botulinum* that produced toxin type A. The patient required prolonged ventilatory support but eventually recovered.

Conclusions

The homemade prison alcohol called pruno caused botulism in 5 California prison inmates in 2 separate instances. In the 2004 outbreak, all 4 inmates drank from the same batch of pruno and 3 days later had laboratory-confirmed botulism type A. The same *C. botulinum* type was recovered from a cup that had held that pruno. In the 2005 event, botulism was confirmed for another California inmate at another state prison; this inmate had drunk pruno, and the same *C. botulinum* type A was cultured from leftover pruno mash.

Pruno has been described on the Internet as "an alcoholic beverage made from apples and/or oranges, fruit cocktail, ketchup, sugar, and possibly other ingredients including bread … originated in (and remains largely confined to) prisons" (http://en.wikipedia.org/wiki/Pruno). Although alcoholic beverages on prison grounds are considered contraband in California (Title 15, California Code of Regulations, Section 3016 [a]), pruno appears to be popular in prisons, and recipes are available on the Internet; most recipes call for some form of fruit, hot water, ketchup, and sugar (*3*,*4*). The ingredients are reportedly mixed in a plastic bag at different intervals and fermented with intermittent warm heating over several days.

In our investigations, the potatoes used in the pruno could have been the source of botulinum toxin. *C. botulinum* is commonly found in the soil, and its spores have been found on raw potatoes (*5*). Several outbreaks of botulism caused by eating potatoes have occurred in the United States (*6*–*8*), and laboratory studies have shown that *C. botulinum* spores on the surface of raw potatoes can survive baking and lead to production of botulinum toxin (*5*). The warm anaerobic fermentation process of making pruno probably predisposes toward production of botulinum toxin, particularly if any ingredient happens to be contaminated with *C. botulinum* or its spores, such as the potatoes used in these 2 instances.

Pruno is popular in prisons across the country, and it is somewhat surprising that botulism caused by pruno consumption has not been previously reported. This lack of reporting may be due to the fact that potatoes are not generally used in the making of pruno; recipes for making pruno and references to pruno found on the Internet do not mention potatoes as an ingredient (*3*,*4*). Occasional crackdowns on making pruno in some prisons could have driven some inmates to look for alternative ingredients, including potatoes. Nonetheless, with >2 million inmates in prisons and jails in the United States, this illicit homemade alcoholic drink may put more inmates at risk for botulism. Anecdotally, making pruno has been attempted outside prisons, possibly extending the potential risk for foodborne botulism carried by this novel vehicle beyond the prison walls. Risk for botulism from consuming pruno should be conveyed to inmates, prison staff, the medical community, and the general public. Any inmate with clinical botulism should be examined for an infected wound caused by drug injection and queried about recent drug use and drinking pruno.

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Isolation of *Bordetella avium* and Novel *Bordetella* Strain from Patients with Respiratory Disease

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Bordetella avium is thought to be strictly an avian pathogen. However, 16S rRNA gene sequencing identified 2 isolates from 2 humans with respiratory disease as *B. avium* and a novel *B. avium*–like strain. Thus, *B. avium* and *B. avium*–like organisms are rare opportunistic human pathogens.

Several *Bordetella* species have been associated with respiratory disease in humans. Although *B. avium* is thought to be strictly an animal pathogen that causes tracheobronchitis in wild and domesticated birds (*1*,*2*), infections in birds share many of the clinical and histopathologic features of disease in mammals caused by *B. pertussis* and *B. bronchiseptica* (*3*). Human cases of respiratory disease associated with *B. avium* have only recently been reported in patients with cystic fibrosis (*4*). We describe 2 isolates, *B. avium* and a novel strain resembling *B. avium*, isolated from 2 patients with pneumonia, thereby demonstrating that *B. avium* and *B. avium*–like organisms are opportunistic human pathogens.

The Cases

Patient 1, a 68-year-old man with left lower lobe pneumonia and hemoptysis, sought care at a community hospital. The patient had a history of end-stage renal disease requiring hemodialysis, chronic obstructive pulmonary disease (COPD), long-term tobacco use, and ischemic cardiomyopathy being treated with anticoagulation. He had a 10-day history of increasing shortness of breath and cough associated with mild hemoptysis. The initial chest radiograph demonstrated an extensive left-sided infiltrate, which progressively worsened; the patient ultimately required endotracheal intubation and mechanical ventilation. Bronchoscopy showed purulent secretions in the left mainstem bronchus, complete obstruction of the bronchus, and frothy secretions in the right airways. Gram-stained bronchoalveolar lavage (BAL) fluid showed many polymorphonuclear leukocytes but no organisms. Routine bacterial culture of the fluid isolated a pure culture that was phenotypically identified by API NFT rapid test strip (bioMérieux, Hazelwood, MO, USA) as *B. avium.* Antimicrobial drug–susceptibility testing performed using Etest (AB Biodisk, Solna, Sweden) resulted in the following MICs: ceftriaxone 2 μg/mL, azithromycin 4 μg/mL, piperacillin/tazobactam 0.125/4 μg/mL. The patient was initially treated with azithromycin and ceftriaxone and completed empiric therapy with piperacillin/tazobactam and vancomycin. By day 7, the patient's respiratory status improved enough that he could be extubated. Follow-up chest radiographs showed substantial resolution of the left-sided infiltrates, and on day 11, the patient was discharged to a rehabilitation care facility.

Patient 2, a 61-year-old homeless man, was admitted to the Houston DeBakey Veteran Affairs Medical Center with a 4-month history of productive cough and a 1-month history of sporadic hemoptysis. He denied fever, chills, shortness of breath, and chest pain. His medical history included COPD, pulmonary tuberculosis, and long-term use of tobacco and alcohol. HIV ELISA result was negative. Chest radiographs and computed tomography scan showed marked emphysematous changes with bullae in the posterior left lobe, numerous calcified granulomas compatible with old granulomatous disease in the left lung, and pleural thickening and reaction in the left apex. A lobulated 2-cm soft tissue density in 1 of these cavities was also identified. No acute infiltrates were seen. The patient began treatment for COPD and for suspected pulmonary tuberculosis (ethambutol 1,600 mg/day, rifampicin 600 mg/day, pirazinamide 1,500 mg/day, and isoniazid 300 mg/day). Gram-stained BAL fluid showed many polymorphonuclear leukocytes and small gram-negative rods. Routine bacterial culture of BAL fluid isolated a pure culture that was identified as *Bordetella* spp. but that could not be identified to the species level by API NFT rapid test strip. Susceptibility testing was performed by using the Kirby-Bauer disk-diffusion method. Although no susceptibility guidelines for *Bordetella* spp. have been established, the isolate appeared to be sensitive to amikacin, trimethoprim-sulfamethoxazole, gentamicin, ceftazidime, and imipenem and resistant to ampicillin, ampicillin/sulbactam, and aztreonam. An oral cephalosporin drug was added to the patient's regimen. Three sputum samples were negative for acid-fast bacilli,

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and treatment for tuberculosis was discontinued. The patient was discharged 9 days after hospital admission.

Isolates of nonlactose fermenting, small, gram-negative rods were recovered from BAL fluid by using trypticase soy agar supplemented with 5% sheep blood, chocolate agar, and MacConkey agar incubated at 35°C in 5% CO_2. The isolate from patient 1 was phenotypically consistent with *B. avium* (catalase positive, oxidase positive, nitrate reduction negative, urease negative). The isolate from patient 2 was phenotypically inconsistent with all other well-characterized *Bordetella* spp. (catalase positive, oxidase positive, nitrate reduction positive, urease negative) and could not be phenotypically identified to the species level.

Sequence analysis was performed by PCR targeting the 16S rRNA gene as previously described (*5*). Samples were prepared by using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and sequencing reactions were performed by using the ABI PRISM 3100 Analyzer according to manufacturer's instructions. Sequences were aligned by using Sequencher software and were submitted to GenBank BLAST database. The sequence from patient 1 was genetically identical to *B. avium* type strain ATCC 35086 (477 bp evaluated, 100% sequence similarity), and the sequence from patient 2 was related to *B. avium* type strain ATCC 35086 (509 bp evaluated, 98% sequence similarity [submitted as GenBank accession no. EU352642]). Several 16S rRNA gene sequences of type strains from the genus *Bordetella* were retrieved from GenBank, and a phylogenetic tree was constructed by using a neighbor-joining algorithm with *Achromobacter xylosoxidans xylosoxidans* as the outgroup (Figure). Concise alignment of the sequence from patient 2 showed 11-bp and 15-bp differences compared with *B. avium* (type strain ATCC 35086) and *B. trematum* (type strain DSM 11334), respectively. Thus, it represents a novel uncharacterized strain most closely related to *B. avium* (i.e., *B. avium*–like; Figure). Both the genotype of *B. avium* and the novel strain are distinguishable from other *Bordetella* spp. according to 16S rRNA gene sequencing of the first 500 bp (≈2% bp difference). This finding is in contrast to the indistinguishable sequences of *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, and the closely related *B. homlseii,* which can be a limitation of 16S rRNA gene sequencing for the genus *Bordetella*, particularly when only the first 500 bp are evaluated (Figure).

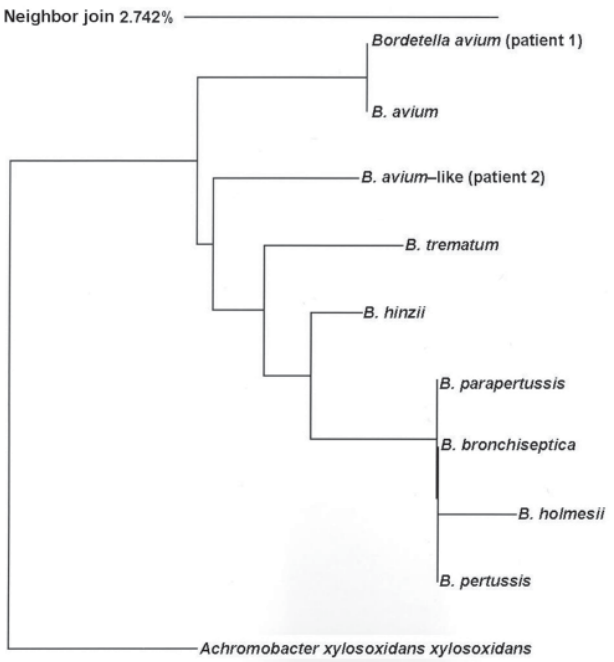

Figure. 16S rRNA dendrogram. Phylogenetic tree of relationships among *Bordetella* spp. inferred on the basis of aligned 16S rRNA gene sequences from type strains (first 500 bp); a neighbor-joining algorithm with *Achromobacter xylosoxidans xylosoxidans* is used as an outgroup. *B. avium,* ATCC35086; *B. bronchiseptica,* ATCC19395; *B. hinzii,* ATCC51783; *B. holmseii,* ATCC51541; *B. parapertussis,* ATCC15311; *B. pertussis,* ATCC9340; *B. trematum,* DSM11334. Scale bar, indicates percentage genetic distance.

Conclusions

Although the first report of human disease associated with *B. avium* was recently described in patients with cystic fibrosis (*4*), the role of *B. avium* in respiratory infection as opposed to colonization in patients with cystic fibrosis is unclear. The cases in this report provide a more direct clinical association of human respiratory infection and *B. avium* and *B. avium*–like organisms. Each of the 2 isolates was obtained in pure culture from BAL fluid. Although neither patient was conventionally immunocompromised (e.g., no HIV, hematologic disorders, or immunosuppressive therapy), each was an older person who had underlying pulmonary problems along with other medical conditions, and each belonged to a patient population typically susceptible to opportunistic infections. Each patient had signs and symptoms clinically consistent with respiratory syndromes caused by *Bordetella,* and each responded to treatment. Clinicians should be aware that human infections with *B. avium* may pose some antimicrobial drug treatment challenges. A previous report demonstrated that cultures of *B. avium* were consistently resistant to penicillin and cefuroxime but susceptible to mezlocillin, piperacillin, gentamicin, amikacin, and cefoperazone (*6*). Although *B. avium* has been shown to share several virulence factors with *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, it does not carry genes that encode pertussis toxin or adenylate cyclase toxin (*7*). *B. avium* and *B. avium*–like organisms have yet-unidentified virulence factors, which may contribute to their ability to cross over from an animal host to an opportunistic human pathogen.

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Clonal Multidrug-Resistant *Corynebacterium striatum* Strains, Italy

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We assessed the clinical relevance and performed molecular characterization of 36 multidrug-resistant strains of *Corynebacterium striatum.* Pulsed-field gel electrophoresis confirmed a single clone, possessing *erm*(X), *tet*A/B, *cmx*A/B, and *aph*A1 genes, but few related subclones. This strain is emerging as a pathogen in Italy.

Isolation of *Corynebacterium* spp. as the only organism from clinical specimens from patients, mostly with varying degrees of immunocompromisation and severe infections, is increasing in Italy. Therefore, we evaluated the microbiologic characteristics, resistance profiles, and similarities among genomes of multidrug-resistant (MDR) *C. striatum* strains.

The Study

We evaluated 36 strains of MDR *C. striatum,* isolated from 3 hospitals in Italy during 2005–2007. Fourteen strains were from bronchoalveolar lavage (BAL) fluid, 3 from blood, 7 from central venous catheter tips, 5 from tracheal aspirates, 4 from wound specimens, 1 from BAL and pleural fluid, 1 from urine, and 1 from a lung biopsy specimen. To assess the clinical relevance of these strains, we used the Centers for Disease Control and Prevention 2004 definition for nosocomial infections (www.cdc.gov/ncidod/dhqp/nnis_pubs.html) (*1*) and tracked antimicrobial drug–resistance determinants.

We identified all strains as putative *C. striatum* by using the commercial system API 20 Coryne (bioMérieux, Marcy l'Etoile, France). *C. striatum* was differentiated from *C. amycolatum* by supplementary tests, i.e., tyrosine hydrolysis, N-acetylglucosamine assimilation, and phenylacetic acid assimilation (*2*); it was reconfirmed by sequencing the internal fragment of the 16S rRNA gene (*3*). The American Type Culture Collection (ATCC) 6940 *C. striatum* strain was included as phenotypic and molecular control. All strains were stored at –80°C until use.

MICs were determined by using microdilution in cation-adjusted Mueller-Hinton broth in accordance with guidelines of the Clinical and Laboratory Standards Institute (CLSI) (*4*). The following antimicrobial drugs were tested: tigecycline and piperacillin/tazobactam, oxacillin, gentamicin, kanamycin, levofloxacin, erythromycin, clindamycin, piperacillin, vancomycin, teicoplanin, tetracycline, moxifloxacin, imipenem, meropenem, quinupristin/dalfopristin, linezolid, and daptomycin. Etest strips (AB-BIODISK, Solna, Sweden) were used for vancomycin, teicoplanin, linezolid, and daptomycin. Daptomycin Etests were performed by using Muller-Hinton agar (Oxoid, Milan, Italy), supplemented to a final concentration of 50 mg/L calcium.

In the absence of approved breakpoints for *Corynebacterium* spp., we used those for α-hemolytic streptococci of the *viridans* group. Results were read after incubation at 37°C for 18–24 h. Susceptibility to daptomycin was defined as MIC ≤1 mg/L (*5*); CLSI guideline MIC breakpoints were used for all other drugs tested (*4*).

To further characterize the *C. striatum* isolates, we used 2 DNA fingerprinting techniques: automated ribotyping (RiboPrinter Microbial Characterization System; DuPont Qualicon, Wilmington, DE, USA) with *Eco*RI as restriction enzyme and pulsed-field gel electrophoresis (PFGE) macrorestriction analysis with 2 enzymes (*Xba*I and *Swa*I; New England Biolabs, Beverly, MA, USA). We had used 4 enzymes (*Xba*I, *Swa*I, *Sfi*I, and *Pac*I) to test 10 random strains, but because *Xba*I and *Swa*I enzyme-restriction patterns gave a better resolution for low and high molecular weight fragments, respectively, we used only these 2 restriction enzymes to type all 36 strains.

Whole genomic DNA chromosomal extraction, macrorestriction digestion, and PFGE (CHEF-DR II apparatus; Bio-Rad, Hercules, CA, USA) were performed as previously reported (*6*). Macrorestriction fragments were separated on 1% (wt/vol) ultrapure agarose gels (Sigma Aldrich, St. Louis, MO, USA) at 6 V/cm, for 21 h at 14°C with pulse times of 0.1–5 s, to separate *Xba*I fragments, and for 23 h with pulse times of 1–70 s, to separate *Swa*I fragments. Lambda DNA concatemers (New England BioLabs) were used as molecular size markers. Similarities among macrorestriction patterns were identified according to established criteria (*7*).

The sequence of pTP10 (GenBank accession no. AF024666) (*8*) was used to design the primers for *erm*(X), *tet*A and *tet*B, *cmx*, *aph*A1, and *rep*B genes. The VectorNTI program (Invitrogen, www.invitrogen.com) was used

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for this purpose. The presence of pTP10 was confirmed first by amplification and sequencing of the resistance determinants and the replication gene (*rep*B) and then by *Xba*I and *Swa*I PFGE hybridizations, performed with the specific probes (*erm*(X), *tet*AB, *cmx*, and *aph*A1), following a protocol previously described (*9*). The PCR amplifications were performed in a Techne TC412 thermal cycler (Barloworld Scientific, Staffordshire, UK). All primers and the related probe regions used in hybridization experiments are shown in Table 1.

All *C. striatum* isolates were recovered from hospitalized patients who had undergone surgery or been admitted to intensive care units (Table 2). We documented 19 cases of infections and discarded 17 as contaminants. The isolates that were considered causes of infections were responsible for 8 cases of ventilator-associated pneumonia (including 1 with associated pleural empyema), 2 cases of pneumonia, 1 case of catheter-related sepsis, 2 cases of ventilator-associated tracheobronchitis, and 6 cases of wound infections.

The 36 strains showed an MDR phenotype, including resistance to ≥3 classes of drugs; MICs required to inhibit growth of 90% (MIC_{90}) were penicillins ≥256 mg/L, carbapenems ≥256 mg/L, gentamicin 32 mg/L, levofloxacin 256 mg/L, tetracycline ≥256 mg/L, lincosamides ≥256 mg/L, and erythromycin 32 mg/L. *C. striatum* strains were susceptible to only the most recent drugs used for treatment of infections with gram-positive organisms, such as glycopeptides and tigecycline (MIC_{90} 1 mg/L), quinupristin/dalfopristin and daptomycin (MIC_{90} 0.25 mg/L), and linezolid (MIC_{90} 2 mg/L). A discrepancy was found when susceptibility testing using a disk-diffusion method was performed on different strains; the inhibition zone of erythromycin was always in the intermediate range, even if MICs for this drug were in the low-resistance range.

Ribotyping gave a unique profile for all strains in this study. PFGE enabled us to discriminate the right number of macrorestriction fragments (*5*,*10*,*11*) for pattern comparison.

Analyses of *Swa*I digestion patterns showed that of the 36 strains, only 1 clone had 3 different subtypes (30 strains subtype a1, 4 strains a2, and 2 strains a3). Macrorestriction analysis with *Xba*I showed almost comparable results (27 strains A1, 7 strains A2, and 2 strains A3) (Figure). This genotyping method and the enzymes used were defined as appropriate, comparing PFGE patterns of our clinical isolates with *C. striatum* ATCC 6940 type strain, which was different with respect to the epidemic strains. This result demonstrates that single MDR *C. striatum* clones had been selected and were circulating in the 3 hospitals.

Further, the molecular characterization of some of the resistance genes in the 36 *C. striatum* isolates demonstrated the presence of *erm*(X), codifying for the resistance to erythromycin and clindamycin; *tet*A, and *tet*B, codifying for the resistance to tetracycline, oxytetracycline, and oxacillin; and *cmx* and *aph*A1, responsible for resistance to aminoglycosides and chloramphenicol, respectively. The presence of pTP10 carrying all these determinants was confirmed by amplification and sequencing of these genes and the replication gene of the plasmid, together with hybridization experiments demonstrating that all resistance determinants were localized in the same hybridization band generated by each probe onto $PFGE_{XbaI}$ (≈15 kb) and $PFGE_{SwaI}$ (≈280 kb) membranes (Figure).

Conclusions

We report isolation of MDR *C. striatum* from clinical specimens responsible for cases of pneumonia, catheter-related bacteremia, and wound infections. Infections sustained from this species are strongly associated with devices, not only tubes or catheters (91%) but also sternal surgical wound wires.

The MDR phenotype of these strains was immediately observed and was responsible for the alarm that led to the subsequent in-depth examination of these strains. Their clonal nature, as demonstrated in our study, is of particular concern. Further, the MDR phenotype correlated to the

Table 1. Primer conditions, PCR products, and related sequences confirmed by BLAST analysis of 36 strains of multidrug-resistant *Corynebacterium striatum,* Italy, 2005–2007*

Primer	Related resistance	Sequence (5′ → 3′)	Temperature, °C	Size, bp	BLAST from–to, bp
*erm*X up	Erythromycin and clindamycin	AACCATGATTGTGTTTCTGAACG	57	566	2,285–2,850
*erm*X down		ACCAGGAAGCGGTGCCCT			
*tet*A up	Tetracycline, oxytetracycline, and oxacillin	TTAGCGTTCGGCGACCTGG	58	1,829	5,496–7,324
*tet*B down		AACTGGGTGCCTTCAGGGTC			
*cmx*B up	Cloramphenicol (2 identical subunits)	AGTCGGTATGGTCGTCGGC	57	879	16,031–16,909
*cmx*A down		GCTCCGATATTCAATGCTGCG			36,078–36,956
*aph*A1 up	Aminoglycoside	GGCAAGATCCTGGTATCGGTCT	57	480	41,859–42,338
*aph*A1 down		AGACTAAACTGGCTGACGGCAT			
*rep*B up	Replicase	CGATCTGGAATTTGTCTGCCGT	57	875	32,523–33,397
*rep*B down		CTGGTTGATAGACCCCGTGT			

*BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis of each gene with pTP10 sequence (GenBank accession no. AF024666) showed nucleotide identities >99%.

Table 2. Clinical diagnoses for 36 patients with *Corynebacterium striatum* infection, Italy, 2005–2007*

Specimens	No. isolates			Diagnosis
	Total	From ICU	From non-ICU wards	
BAL fluid, pleural fluid, blood, tracheal aspirate	8	7	1	Ventilator-associated pneumonia
BAL fluid	2	2	0	Ventilator-associated tracheobronchitis
BAL fluid, lung biopsy	2	0	2	Pneumonia
Blood, CVC tip	1	1	0	CVC-related bacteremia
CVC tip	1	1	0	CVC exit-site cellulites
Blood, surgical wound	5	1	4	Sternal wound cellulites and infections
Tracheal aspirate	10	10	0	Ventilator-associated respiratory tract colonization
CVC tip	6	4	2	CVC-exit site colonization
Urine	1	0	1	Urinary tract catheter colonization
Total	36	26	10	

*ICU, intensive care unit; BAL, bronchoalveolar lavage; CVC, central venous catheter.

presence of the pTP10 plasmid, which demonstrates that these MDR microorganisms acquired not only the capability to cause infections but also increased resistance and the ability to spread by virtue of their clonal nature. The only drugs still active against these MDR strains are glycopeptides, linezolid, quinopristin/dalfopristin, daptomycin, and tigecycline. To avoid using drugs that appear active in vitro but that could be ineffective in vivo, clinicians should be aware of the circulation of these MDR strains.

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Dr Campanile is a researcher at the Department of Microbiology, University of Catania. She is involved in the fields of antimicrobial drug resistance, molecular typing, evolutionary relationships among strains of diverse sources, and horizontal exchange of antimicrobial drug resistance determinants by mobile genetic elements.

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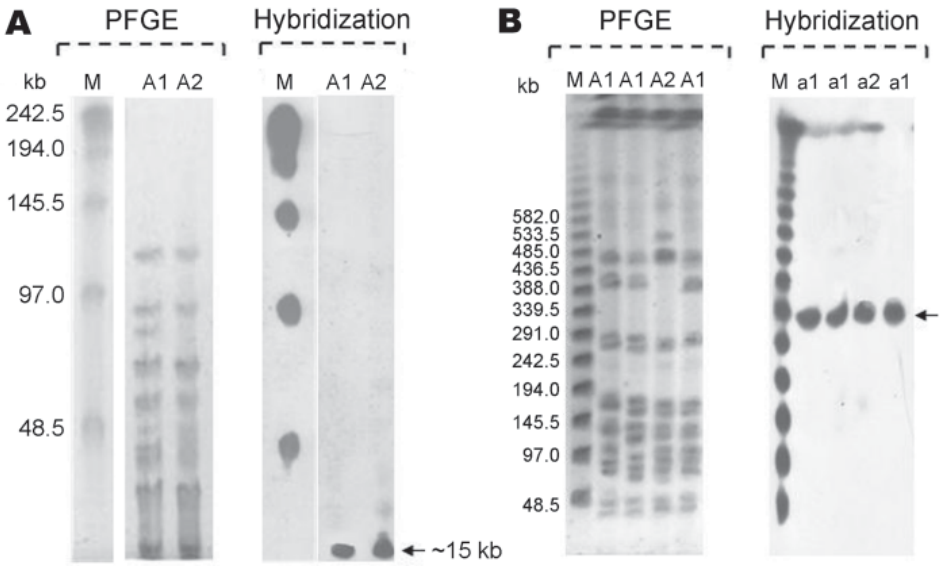

Figure. Pulsed-field gel electrophoresis (PFGE) patterns of *Corynebacterium striatum* and their representative hybridizations obtained with probes corresponding to the resistance genes *erm*(X), *tet*A-*tet*B, *cmx*, and *aph*A1 (m, lambda ladder PFG marker). A) *Xba*I (A1 and A2 profiles); B) *Swa*I (a1 and a2 profiles).

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Enterovirus 71 Outbreak, Brunei

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Enterovirus 71 (EV71) outbreaks occur periodically in the Asia-Pacific region. In 2006, Brunei reported its first major outbreak of EV71 infections, associated with fatalities from neurologic complications. Isolated EV71 strains formed a distinct lineage with low diversity within subgenogroup B5, suggesting recent introduction and rapid spread within Brunei.

Enterovirus 71 (EV71), a member of the family *Picornaviridae* and the genus *Enterovirus*, is a common cause of hand, foot, and mouth disease in children. Infection with this virus is rarely complicated by severe neurologic disease, such as meningitis, brain stem encephalitis, neurogenic pulmonary edema, and acute flaccid paralysis. EV71 was first isolated in 1969 (*1*), and during the subsequent 30 years, outbreaks were reported in the United States, Europe, and Asia (*2*). Since 1997, several major outbreaks with deaths have occurred in the Asia-Pacific region, notably in Sarawak (East Malaysia), Peninsular Malaysia, Taiwan, Australia, Singapore, Japan, and Vietnam (*3*–*10*).

Brunei is situated on the island of Borneo (4°30′N, 114°E) and has a population of ≈370,000. From February through August 2006, Brunei experienced its first reported major outbreak of EV71. More than 1,681 children reportedly were affected, with 3 deaths resulting from severe neurologic disease. We report the virologic findings from this outbreak.

The Study

During March through October 2006, samples from at least 100 patients from Brunei diagnosed with hand, foot, and mouth disease or herpangina were received at the University Malaya Medical Center, Kuala Lumpur, Malaysia. Samples were inoculated into Vero and A549 cell cultures for virus isolation. EV71 was isolated from 34 patients (including 2 who died of severe neurologic complications), and an additional 7 isolates were obtained from Malaysian patients seen at the University Malaya Medical Center during the outbreak period in Brunei (Table 1). Adenovirus also was isolated from stool or rectal swabs of 4 patients, of whom 2 were coinfected with EV71; none had neurologic disease.

Enteroviral RNA was extracted from cell cultures using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), and reverse transcription–PCR was performed to amplify the viral capsid protein (VP1) gene at nt positions 31–861. The primers used were VP1F 5′-CAGGCTAGCA TGGGAGATAGGGTGGCAGATGTGATCGAGAGC-3′ and VP1R 5′-GGTGGATCCCAAAGGGTAGTAATGGC AGTACGACTAGTGCCGGT-3′. The 831-nt partial VP1 gene fragments were sequenced, and phylogenetic relations of the sequences were examined using selected enterovirus reference strains obtained from GenBank (Table 2). Sequences were aligned and phylogenetic trees were drawn using the neighbor-joining method (Figure), as described (*12*). Maximum-likelihood tree showed similar clustering and is not shown. The prototype coxsackievirus A16 (CoxA16-G10) was used as the outgroup virus for construction of the phylogenetic tree.

The phylogenetic tree, drawn on the basis of the alignment of the VP1 gene sequences, showed 3 independent genogroups (A, B, and C) with the prototype BrCr strain as the only member of genogroup A (*11*). Within each of genogroups B and C, 5 additional subgenogroups were identified, designated B1–B5 and C1–C5 (*8*,*10*). Although no definitions have been established, generally there is nucleotide variation of ≈16%–20% between genogroups and differences of ≈6%–12% between subgenogroups within each genogroup (*5*,*11*).

All Brunei and Malaysia isolates from 2006 clustered into subgenogroup B5, except for 1 Brunei isolate, which grouped to subgenogroup B4. Nucleotide sequences of the VP1 gene were highly similar (96%–100%) among all strains in subgenogroup B5. All Brunei B5 isolates were clustered in an independent lineage within subgenogroup B5 (99.9% bootstrap support), separate from the established Sarawak and Yamagata isolates from 2003 (*8*). Amino acid sequences were highly conserved among the Brunei B5 isolates, with 99%–100% similarity. No amino acid sequence changes were observed in the 2 isolates from patients who died.

Conclusions

The different genogroups of EV71 are widely distributed around the world (*2*). The continuing appearance of new EV71 subgenogroups in recent years in the Asia-Pacific region suggests that the virus is continuously evolving (*5*,*8*,*9*). The annual rate of evolution is estimated at 1.35 $\times 10^{-2}$ substitutions per nucleotide, similar to poliovirus (*11*). In some countries, outbreaks occur in a cyclical pattern every 3 years, predominantly caused by strains that are distinct from previous outbreaks (*3*,*9*). These strains often

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have been detected in other countries in the region in years preceding the outbreak. In some EV71 outbreaks, other enteroviruses cocirculate, particularly coxsackievirus A16 or EV71 from a different subgenogroup (*3,8,10*). On the basis of the samples received in the study, the Brunei 2006 EV71 outbreak was caused by subgenogroup B5 virus. Apart from the single isolate from subgenogroup B4, no other enteroviruses were isolated, although 2 patients also had adenovirus. Occasional EV71 and adenovirus co-infection has been reported (*13*), also without association with severe disease. The low sequence diversity and predominance of the Brunei B5 isolates in this outbreak suggest recent introduction and subsequent rapid spread, without the concurrent spread of other genogroups, subgenogroups, or enteroviruses.

Other than its northern coastline, Brunei is surrounded entirely by the East Malaysian state of Sarawak. In 2006, an outbreak of EV71 affected approximately 14,400 children in Sarawak (*14*). Thus, temporally and geographically, the Brunei and Sarawak outbreaks were related, raising the possibility that the same strains were involved. Sarawak had experienced EV71 outbreaks every 3 years (1997, 2000, and 2003), caused by subgenogroups B3, B4, and B5, respectively (*3*). However, no sequence results from the Sarawak 2006 outbreak are available for comparison. All subgenogroup B5 isolates reported seem to have diverged

Table 1. Enterovirus 71 from Brunei and Malaysia isolated in 2006

Isolate	GenBank accession no.	Subgenogroup	Specimen type	Origin
EV71/BRU/2006/33930	FM201328	B5	Rectal swab	Brunei
EV71/BRU/2006/34095	FM201329	B5	Rectal swab	Brunei
EV71/BRU/2006/34099	FM201330	B5	Rectal swab	Brunei
EV71/BRU/2006/34111	FM201331	B4	Skin swab	Brunei
EV71/BRU/2006/34235	FM201332	B5	Throat swab	Brunei
EV71/BRU/2006/34355	FM201333	B5	Throat swab	Brunei
EV71/BRU/2006/34456	FM201334	B5	Swab*	Brunei
EV71/BRU/2006/34597	FM201335	B5	Stool	Brunei
EV71/BRU/2006/34700	FM201336	B5	Stool	Brunei
EV71/BRU/2006/34701	FM201337	B5	Stool	Brunei
EV71/BRU/2006/35053	FM201338	B5	Rectal swab	Brunei
EV71/BRU/2006/35207	FM201339	B5	Stool	Brunei
EV71/BRU/2006/35245	FM201340	B5	Rectal swab	Brunei
EV71/BRU/2006/35247	FM201341	B5	Rectal swab	Brunei
EV71/BRU/2006/35334	FM201342	B5	Swab*	Brunei
EV71/BRU/2006/35335	FM201343	B5	Blister swab	Brunei
EV71/BRU/2006/35338	FM201344	B5	Swab*	Brunei
EV71/BRU/2006/35341	FM201345	B5	Swab*	Brunei
EV71/BRU/2006/35379	FM201346	B5	Rectal swab	Brunei
EV71/BRU/2006/35479	FM201347	B5	Rectal swab	Brunei
EV71/BRU/2006/35640	FM201348	B5	Rectal swab	Brunei
EV71/BRU/2006/35641	FM201349	B5	Rectal swab	Brunei
EV71/BRU/2006/35643	FM201350	B5	Rectal swab	Brunei
EV71/BRU/2006/35645	FM201351	B5	Rectal swab	Brunei
EV71/BRU/2006/35646	FM201352	B5	Rectal swab	Brunei
EV71/BRU/2006/35649	FM201353	B5	Rectal swab	Brunei
EV71/BRU/2006/35652	FM201354	B5	Rectal swab	Brunei
EV71/BRU/2006/35653	FM201355	B5	Rectal swab	Brunei
EV71/BRU/2006/35728	FM201356	B5	Swab*	Brunei
EV71/BRU/2006/35730	FM201357	B5	Swab*	Brunei
EV71/BRU/2006/35731	FM201358	B5	Swab*	Brunei
EV71/BRU/2006/35732	FM201359	B5	Swab*	Brunei
EV71/BRU/2006/35754	FM201360	B5	Rectal swab	Brunei
EV71/BRU/2006/35755	FM201361	B5	Rectal swab	Brunei
EV71/MY/2006/1764281	FM201321	B5	Stool	Malaysia
EV71/MY/2006/1764283	FM201322	B5	Rectal swab	Malaysia
EV71/MY/2006/1764454	FM201323	B5	Nasopharyngeal swab	Malaysia
EV71/MY/2006/1764589	FM201324	B5	Stool	Malaysia
EV71/MY/2006/1764739	FM201325	B5	Stool	Malaysia
EV71/MY/2006/1765017	FM201326	B5	Stool	Malaysia
EV71/MY/2006/1765058	FM201327	B5	Stool	Malaysia

*Site of swab not known.

Table 2. Reference Enterovirus 71 sequences used for phylogenetic analysis*

Isolate	GenBank accession no.	Subgenogroup	Origin	Year	Clinical details	Reference
BrCr-CA-70	U22521	A	USA	1970	Encephalitis	(*11*)
S11051-SAR-98	AF376081	C1	Sarawak	1998	HFMD	(*6*)
1M-AUS-12-00	AF376098	C1	Australia	2000	HFMD	(*6*)
2M-AUS-3-99	AF376103	C2	Australia	1999	Myelitis	(*6*)
2644-AUS-95	AF135949	C2	Australia	1995	NA	(*11*)
KOR-EV71-09	AY125973	C3	South Korea	2000	NA	UD
KOR-EV71-10	AY125974	C3	South Korea	2000	NA	UD
F2-CHN-00	AB115491	C4	China	2000	NA	UD
H26-CHN-00	AB115493	C4	China	2000	NA	UD
1091S/VNM/05	AM490143	C5	Vietnam	2005	NA	(*10*)
999T/VNM/05	AM490163	C5	Vietnam	2005	NA	(*10*)
2609-AUS-74	AF135886	B1	Australia	1974	Meningitis	(*11*)
2258-CA-79	AF135880	B1	USA	1979	Tremors	(*11*)
7673-CT-87	AF009535	B2	USA	1987	NA	(*11*)
2222-IA-88	AF009540	B2	USA	1988	Fever	(*11*)
MY104-9-SAR-97	AF376072	B3	Sarawak	1997	Cardiogenic shock	(*6*)
26M-AUS-2-99	AF376101	B3	Australia	1999	HFMD	(*6*)
1067-Yamagata-00	AB213625	B4	Japan	2000	HFMD	(*8*)
2027-SIN-01	AF376111	B4	Singapore	1997	Acute flaccid paralysis	(*6*)
CN04104-SAR-00	AF376067	B4	Sarawak	2000	HFMD	(*6*)
5511-SIN-00	AF376121	B5	Singapore	2000	HFMD	(*6*)
2716-Yamagata-03	AB177816	B5	Japan	2003	HFMD	(*8*)
2419-Yamagata-03	AB213647	B5	Japan	2003	HFMD	(*8*)
S19841-SAR-03	AY258310	B5	Sarawak	2003	NA	UD
SB12869-SAR-03	AY905545	B5	Sarawak	2003	NA	(*3*)

*HFMD, hand, foot, and mouth disease; NA, not available; UD, unpub. data.

from an ancestral strain related to strain 5511/SIN/00 (GenBank accession no. AF376121), isolated in Singapore as early as 2000 (*3*). Subsequently, subgenogroup B5 emerged in Japan (*8*) and Sarawak (*3*) in 2003, before appearing in Peninsular Malaysia and Brunei in 2006. The source of the Brunei outbreak remains unclear, and it may not be one of these countries where subgenogroup B5 has already been reported. However, EV71 subgenogroup B5 clearly continues to diverge, and further subgenogroups are likely to arise.

In summary, the first reported major outbreak of EV71 in Brunei was caused by strains from subgenogroup B5 that were distinct from other reported B5 isolates, suggesting a recent introduction from an as-yet-unidentified source. Hence, continued molecular surveillance of EV71 in Asia is required to further our understanding of factors influencing the evolution of the virus and its association with emergence of outbreaks in the region.

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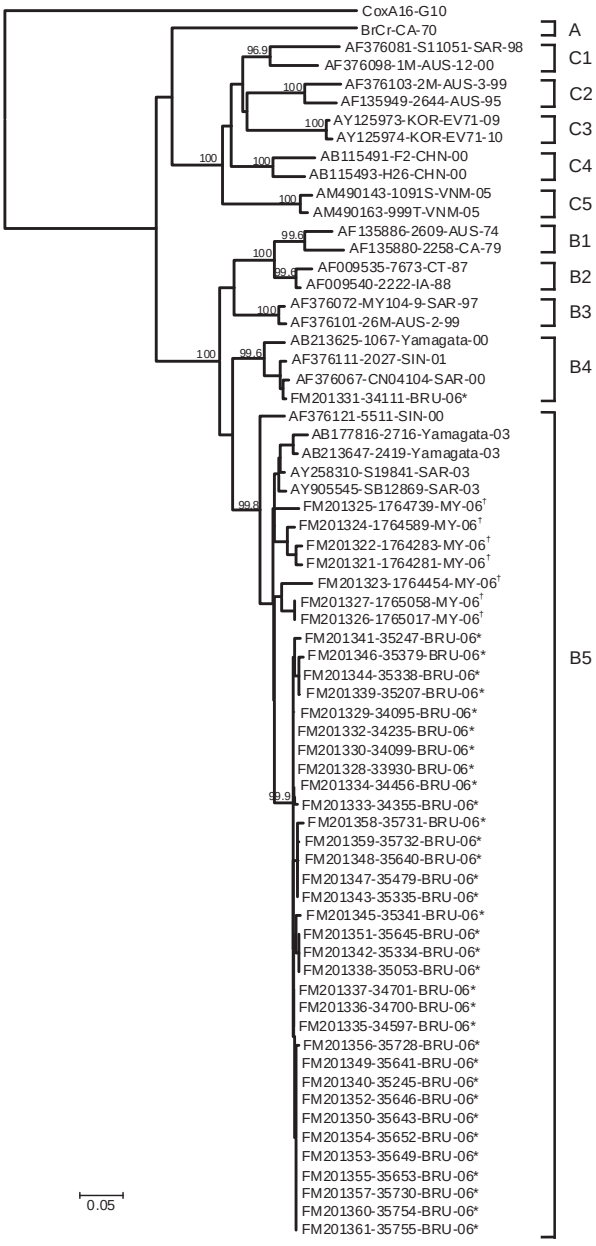

Figure. Phylogenetic relationships of enterovirus 71 partial viral protein (VP1) gene sequences. The prototype coxsackievirus A16 (CoxA16-G10) was used as the outgroup virus. The phylogenetic tree shown was constructed by using the neighbor-joining method. Bootstrap values (>95%) are shown as percentages derived from 1,000 samplings at the nodes of the tree. Scale bar denotes number of nucleotide substitutions per site along the branches. Isolates from this study are indicated by * (Brunei) and † (Peninsular Malaysia).

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Novel Human Rotavirus Genotype G5P[7] from Child with Diarrhea, Cameroon

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We report characterization of a genotype G5P[7] human rotavirus (HRV) from a child in Cameroon who had diarrhea. Sequencing of all 11 gene segments showed similarities to ≥5 genes each from porcine and human rotaviruses. This G5P[7] strain exemplifies the importance of heterologous animal rotaviruses in generating HRV genetic diversity through reassortment.

Group A rotaviruses are a major cause of severe diarrheal disease in infants, young children, and a variety of animals. In humans, rotavirus gastroenteritis results in deaths and hospitalizations; most deaths have occurred in developing countries (*1*).

Rotavirus surveillance and strain characterization, in support of rotavirus vaccine development programs, have detected many new human rotavirus (HRV) genotype specificities and highlighted the importance of mechanisms such as reassortment and zoonotic transmission in the evolution of rotaviruses (*2*). However, more comprehensive analyses of gene fragments (*3*) or entire genes (*4*) are needed to clarify the origin of rotavirus gene segments for common and uncommon strains. To elucidate the possible origin of the novel G5P[7] HRV strain from the African Rotavirus Surveillance Network (ARN), we determined its genomic composition and compared its gene sequences with rotavirus sequences in GenBank.

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The Study

During ARN surveillance conducted from 1998 through 2004, a total of 215 rotavirus-positive stool samples could not be typed by standard reverse transcription–PCR genotyping methods. Among untypeable samples, we identified a G5P[7] strain (designated 6784/2000/ARN), which represented a rare G genotype and a new P genotype specificity in humans. This strain was isolated from a stool specimen from a child with gastroenteritis in Kumba, Cameroon. Because G5 and P[7] genotype specificities are common in pigs, we studied the entire genomic composition of this strain to determine if it was an example of a strain that arose through direct interspecies transmission from a particular animal host, or by reassortment with heterologous rotavirus strains.

Gene fragments of the 11 gene segments of strain 6784/2000/ARN were amplified by using consensus primers for structural protein 4 (VP4), VP6, and VP7 (*5*–*8*) and newly designed consensus primers for VP1, VP2, VP3, nonstructural protein 1 (NSP1), NSP2, NSP3, NSP4, and NSP5 (Table 1). The fragments were sequenced by using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Dye-labeled products were sequenced in an ABI 3130 sequencer (Applied Biosystems). Similarity and phylogenetic relationships were inferred by using aligned nucleotide and deduced amino acid sequences by the neighbor-joining method and p-distance algorithm of MEGA4 software (*9*).

Similarity matrices and phylogenetic trees based on nucleotide and amino acid sequences were constructed and compared with cognate gene sequences of human and animal rotaviruses. Except for the 2 gene segments, which encode neutralizing antigens VP7 and VP4, respectively, and are commonly encountered in porcine rotaviruses, the remaining 9 gene segments of 6784/2000/ARN were grouped in a common phylogenetic clade in which reference human strains of the Wa genogroup and related porcine rotaviruses also clustered (online Appendix Figure, available from www.cdc.gov/EID/content/15/1/83-appF.htm). However, VP1, NSP3 (likely), and NSP5 genes were more closely related to cognate gene sequences of porcine strains (Gottfried, PRICE, CMP034, and OSU) than to HRVs and shared an nt identity of 92%–99%. VP2, VP3, VP6, NSP1, NSP2, and NSP4 genes showed a stronger genetic relationship with human strains of the Wa genogroup (90%–99% nt identities) than with known porcine rotaviruses (Table 2).

Sequence analysis of the VP7 gene demonstrated that 6784/2000/ARN had 85%–91% nt and 92%–100% aa identities with representative G5 rotaviruses from humans and animals, respectively. Although the VP7 gene was highly divergent from other human G5 isolates detected in South

[1]Member of the African Rotavirus Network.

Table 1. Primers used for amplification and sequencing of rotavirus genes

Primer*	Sequence (5′ → 3′)†	Gene‡	Nucleotide position, strand	Amplicon size, bp	Strain	Reference
MDEVP1F	AAT CAC AAT CTG CAG TTC AAA	VP1	68–89, +	337	Ku	This article
MDEVP1R	AAT GAA TCA GTG TAT TCT TCG	VP1	405–384, –		Ku	This article
MDEVP2F	CTG ACA AAG TGC TAT CAC A	VP2	156–175, +	300	Ku	This article
MDEVP2R	AGG TAA TTG TCT TGG TTC	VP2	456–438, –		Ku	This article
MDEVP3F	TTG CTA GAT TGT CAA ATC GTG	VP3	597–618, +	327	Ku	This article
MDEVP3R	AAT AAG ATG GAG CTG AAC C	VP3	924–905, –		Ku	This article
MDENSP1F	GAG ACC RTC AAC TCC TAC YAA	NSP1	120–141, +	344	Wa	This article
MDENSP1R	ATT GTA AYG TTA TTG GCA T	NSP1	464–445, –		Wa	This article
MDENSP2F	GCT TGC TTT TGT TAT CCT	NSP2	58–76, +	327	Ku	This article
MDENSP2R	ATT TTC CAA ATG TCT AAC AG	NSP2	385–365, –		Ku	This article
MDENSP3F	GCC ACT TCA ACA TTA GAA	NSP3	101–119, +	303	Ku	This article
MDENSP3R	TAC ACT AAA ACA AGC ATT AAG	NSP3	404–383, –		Ku	This article
MDENSP5F	AGC GCT ACA GTG ATG TCT CT	NSP5	10–29, +	337	Ku	This article
MDENSP5R	CCA TTT GAT CGC ACC CA	NSP5	347–330, –		Ku	This article
JRG30	GGC TTT TAA AAG TTC TGT T	NSP4	1–19, +	737	Wa	This article
JRG31	ACC ATT CCT TCC ATT AAC	NSP4	738–721, –		Wa	This article
Con3	TGG CTT CGC TCA TTT ATA GAC A	VP4	11–32, +	876	Ku	(*5*)
Con2	ATT TCG GAC CAT TTA TAA CC	VP4	887–868, –		Ku	(*5*)
9con1-L	TA GCT CCT TTT AAT GTA TGG TAT	VP7	37–59, +	896	Wa	Modified from (*6*)
VP7-Rdeg	GAC GGV GCR ACT ACA TGG T	VP7	933–914, –		Wa	Modified from (*7*)
VP6-F	GAC GGV GCR ACT ACA TGG T	VP6	747–766, +	379		(*8*)
VP6-R	GTC CAA TTC ATN CCT GGT G	VP6	1126–1106, –			(*8*)

*F, forward; R, reverse.
†R, A or G; Y, C or T; V, A, C, or G; N, A, C, G, or T.
‡VP, structural protein; NSP, nonstructural protein.

America and Asia, it was identical to a human serotype G5 rotavirus isolated in Cameroon (*10*) and clustered with 2 porcine strains from Argentina (online Appendix Figure). Genetic analysis of the VP8* portion of the VP4 gene of strain 6784/2000/ARN had higher similarity (90% nt and 89% aa) with porcine genotype P[7] strains, e.g., OSU and JL94, than with strains of other genotypes (39%–85% nt and 55%–72% aa). This finding suggests that 6784/2000/ARN also belongs to genotype P[7].

Although we did not sequence the minimum 500 bp/gene, we propose a tentative genotype classification based on ≈300–350 nucleotides sequenced by using the scheme of Matthijnssens et al. (*11*). VP1-, VP2-, VP3-, VP6-, NSP1-, NSP2-, NSP3-, NSP4-, and NSP5-encoding gene segments of strain 6784/2000/ARN form a close phylogenetic cluster with human and animal rotavirus strains of the Wa-like genogroup, respectively, in R1, C1, M1, I1, A1, N1, T1, E1, and H1 genotypes (*11*). Nucleotide sequences deposited in GenBank are FM179285 (VP1), FM179286 (VP2), FM179287 (VP3), FM179288 (VP4), FM179289 (VP6), FM179290 (NSP1), FM179291 (NSP2), FM179292 (NSP3), FM179293 (NSP4), FM179294 (NSP5), and EF218667 (VP7).

Conclusions

Serotype G5 rotaviruses, which are common in pigs but also detected in horses and cattle, were identified in the 1990s in children from Brazil who had diarrhea (*12*). This serotype has also been reported in children with severe diarrhea in Paraguay, Cameroon, Argentina, Vietnam, and the People's Republic of China (*2*,*13*,*14*), which suggests that G5, although uncommon overall in humans, is found worldwide. Partial molecular analyses showed that human G5 strains are reassortants with various genetic compositions. Some human G5 strains from Brazil, China (LL36755), and Vietnam (KH210) contain a genotype P[6] VP4 gene, but their other genes have not been characterized (*12–14*). The novel 6784/2000/ARN strain characterized here shares a VP6 subgroup II specificity and a long RNA electrophoretic pattern with prototype human G5 strain IAL-28 but differs in subgroup and electropherotype from the Cameroon isolate MRC3105 (*10*). Strain 6784/2000/ARN has a P[7] VP4 genotype and represents a human strain with this VP4 specificity.

Detection of G5 rotaviruses with different genetic compositions from children in Cameroon raises questions about the origin of these strains. MRC3105 not only represents a reassortant strain between porcine rotaviruses and HRVs but also may have obtained gene segments from isolates of human Wa and DS-1 genogroups, as suggested by unusual combinations in its RNA profile, subgroup specificity, and P type (*10*). In contrast, 6784/2000/ARN seems to have obtained its outer capsid combination from a porcine rotavirus, and its overall genomic composition showed genetic exchange between a porcine parental strain and a human strain of the Wa genogroup. We hypothesize that these

Table 2. Nucleotide/amino acid identities of rotavirus 6784/2000/ARN gene segments with cognate gene sequences of 36 known human and animal rotavirus sequences from GenBank*

Strains†	Nucleotide/amino acid identity, %										
	VP1	VP2	VP3	VP4	VP6	VP7	NSP1	NSP2	NSP3	NSP4	NSP5
Ku/G1P[8]/Hu	88/94	**96**/96	90/94	61/55	90/98	74/79	85/88	**91**/94	94/94	89/93	94/96
DRC88/G8P[8]/Hu	79/85	76/77	69/73	–	76/93	74/81	74/75	85/93	84/90	79/83	90/93
OSU/G5P[7]/Po	–	–	86/90	**90**/89	80/90	86/94	86/89	–	89/95	88/96	**98**/100
RMC321/G9P[19]/Hu	82/93	84/94	–	–	80/91	–	81/87	88/91	89/93	88/95	97/98
Tb-chen/G2P[4]/Hu	79/83	77/79	69/72	62/56	77/91	–	77/76	88/92	85/90	81/83	89/92
AU-1/G3P[9]/Hu	78/84	77/84	74/76	59/56	76/91	78/85	69/72	81/89	82/93	78/82	93/97
ST-3/G4P[6]/Hu	–	–	91/94	–	–	–	94/94	–	97/97	88/91	–
69M/G8P[10]/Hu	–	–	71/75	–	–	–	77/76	–	82/88	–	94/95
T152/G12P[9]/Hu	–	–	–	60/57	76/91	75/82	70/71	–	–	–	–
R14a/G9P[8]/Hu	–	–	–	–	–	–	**97**/97	–	–	–	–
Wa/G1P[8]/Hu	85/94	93/93	**95**/96	62/55	88/98	74/78	85/88	88/91	97/97	**90**/95	95/95
DS-1/G2P[4]/Hu	79/84	–	70/75	62/56	79/92	72/74	76/76	88/94	83/89	81/84	–
30/96/G3P[14]/Lp	79/85	79/85	73/82	61/56	–	–	–	86/95	83/90	79/83	91/96
PRICE/Po	–	–	–	–	–	–	–	–	**98**/98	–	–
RRV/G3P[3]/Si	–	–	–	69/72	–	–	–	–	82/92	79/82	–
PO-13/G18P[17]/Av	67/61	63/56	62/57	39/20	70/77	65/58	–	56/46	60/58	–	63/52
KJ75/G5P[5]/Bo	–	–	–	–	–	86/93	–	89/94	–	–	–
US1205/G9P[6]/Hu	–	–	–	61/57	76/91	77/84	–	–	–	80/83	–
EW/G16P[16]/Mu	–	–	–	62/60	–	–	–	–	–	62/62	–
KUN/G2P[4]/Hu	–	–	–	–	–	–	–	–	–	81/83	90/93
CU-1/G3P[3]/Ca	–	–	–	–	–	–	–	–	–	78/84	–
FRV-1/G3P[9]/Fe	–	–	–	–	–	–	–	–	–	79/82	–
EHP/G16P[20]/Mu	–	–	–	65/66	–	–	–	–	–	62/61	–
SA-11/G3P[1]/Si	77/85	79/84	–	71/72	–	–	–	–	–	–	94/96
CMP034/G2P[27]/Po	–	–	–	–	–	–	–	–	–	–	**99/100**
YM/G11P[7]/Po	88/94	–	–	85/88	81/90	82/90	–	–	–	–	–
Gottfried/G4P[6]/Po	**92**/96	–	–	61/57	88/98	–	–	–	–	–	–
JL94/G5P[7]/Po	–	–	–	**90**/89	–	–	–	–	–	–	–
rj6906/03/Hu	–	–	–	–	**98**/99	–	–	–	–	–	–
MRC3105/G5P[8]/Hu‡	–	–	–	–	–	**100**/100	–	–	–	–	–
CC117/G5/Po	–	–	–	–	–	91/97	–	–	–	–	–
C134/G5/Po	–	–	–	–	–	90/97	–	–	–	–	–
LL4260/G5P[6]/Hu	–	–	–	–	–	90/94	–	–	–	–	–
KH210/G5P[6]/Hu	–	–	–	–	–	89/93	–	–	–	–	–
IAL-28/G5P[8]/Hu	–	–	–	–	–	85/92	–	–	–	–	–
H-1/G5P[7]/Eq	–	–	–	–	81/90	86/94	–	–	–	85/93	–

*ARN, African Rotavirus Surveillance Network; VP, structural protein; NSP, nonstructural protein; –, not included or not sequenced. High and moderate nucleotide/amino acid percentage identities are in **boldface**.
†Species of origin. Hu, human; Po, porcine; Lp, lapine; Si, simian; Av, avian; Bo, bovine; Ca, canine; Fe, feline; Mu, murine; Eq, equine.
‡VP7 gene of MRC3105 was derived from a porcine rotavirus.

2 G5 isolates with identical VP7 genes in different HRV genetic backgrounds might be independent progenies of a porcine G5 rotavirus that was co-circulating with human DS-1–like and Wa-like strains at the time of identification of the G5 isolate in southwestern Cameroon. Additional sequencing of common porcine and human strains is required to elucidate mechanisms involved in generation of genetic diversity during reassortment of rotaviruses from 2 species.

Although G5P[7] strains might be common in pigs, strain 6784/2000/ARN is a novel representative of this antigen combination in humans. Similarities of some of its gene segments with those of porcine rotavirus strains suggest that ARN G5P[7] is an animal–human reassortant rotavirus in which a few genes are derived from human strains. Introduction of animal rotavirus genes into the genetic background of common HRVs has resulted in global spread of various genotype specificities, including G9 and G12. In these emerging human strains, DS-1 and Wa genogroups served as parental strains to carry the new antigenic variants on the background of old genotype specificities. Further, human G5 strains whose overall genomic composition is Wa-like have a wide geographic distribution and were considered clinically important HRVs in South America during the 1990s. Surveillance is needed to determine if G5P[7] strains on a Wa-like genetic background will spread to other African countries.

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Serotype G12 Rotaviruses, Lilongwe, Malawi

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To assess diversity of rotavirus strains in Lilongwe, Malawi, we conducted a cross-sectional study of children with acute gastroenteritis, July 2005–June 2007. Serotype G12 was identified in 30 (5%) of 546 rotavirus-positive fecal specimens. The G12 strain possessed multiple electropherotypes and P-types, but their viral protein 7 sequences were closely related, indicating that reassortment has occurred.

Rotavirus is the leading cause of severe, acute gastroenteritis, a disease that causes dehydration and death in infants and young children worldwide (*1*); an estimated 527,000 childhood deaths occur annually (who.int/immunization_monitoring/burden/rotavirus_estimates/en/index.htm). Because of the high death rates in children, vaccination to prevent severe disease outcomes after rotavirus infection is an essential public health strategy (*2*,*3*). Currently, 2 live attenuated oral rotavirus vaccines are becoming part of childhood immunization schedules in North America, Latin America, and Europe; Phase III clinical trials are underway in Africa and Asia (*3*).

Rotaviruses are segmented, double-stranded (ds) RNA viruses that possess a triple-layered protein capsid. The 11 dsRNA segments, upon separation by electrophoresis, exhibit profiles that can be broadly categorized into long and short RNA patterns, termed electropherotypes. The rotavirus outer capsid comprises 2 neutralization antigens, VP7 and VP4, which respectively define the G (for glycoprotein) and P (for protease-sensitive) serotypes. The 5 globally most common rotavirus strain types comprise long electropherotype P[8] strains possessing G1, G3, G4, or G9 specificity and short electropherotype G2P[4] strains (*4*). Rotaviruses exhibit considerable diversity, including unusual combinations of electropherotypes and serotypes (which suggests viral reassortment); globally, rare G and P types predominate in some regions (*5*). For example, we have previously described serotype G8 to be a locally prevalent serotype in Blantyre, Malawi (*6*). More recently, rotavirus serotype G12 has emerged in multiple countries (*7*). Our study assesses diversity of rotavirus strains in Lilongwe, Malawi, in anticipation of introduction of a rotavirus vaccine in this country.

The Study

This 2-year, cross-sectional study was undertaken at Kamuzu Central Hospital in Lilongwe from July 2005 through June 2007. Children <5 years of age with acute gastroenteritis who received oral and/or intravenous rehydration therapy were enrolled after parents or guardians gave written, informed consent. Study participants included outpatients and inpatients. A fecal sample was collected from each case-patient and stored at –80°C until rotavirus detection and characterization were undertaken.

Group A rotavirus antigen was detected by a commercial ELISA performed according to the manufacturer's instructions (Rotaclone; Meridian Diagnostics, Cincinnati, OH, USA). Among rotavirus antigen–positive specimens, specimens that exhibited color intensity at least equal to the positive control provided with the Rotaclone kit were selected for further strain characterization. Genotyping by multiplex, heminested reverse transcription-PCR (RT-PCR) was undertaken as previously described (*6*). Specimens that remained G nontypeable were analyzed by using a G12-typing primer (*8*), and those that could not be P-typed were further examined by a degenerate P[8] primer (*9*). Serotype G12 strains were further examined by polyacrylamide gel electrophoresis (PAGE) of rotavirus dsRNA followed by silver staining to determine the rotavirus electropherotype as previously described (*10*).

Full-length VP7 genes of G12 strains representing distinct electropherotypes were obtained by RT-PCR by using primers Beg 9 and End 9 (*11*). Amplification products were purified by using Minispin columns (Amersham, Buckinghamshire, UK) and sequenced by Cogenics Inc. (Hope End, Essex, UK). GenBank accession numbers representing the gene sequence encoding VP7 of each Malawi G12 strain examined are as follows (for each rotavirus strain, the prefix KCH is used to denote Kamuzu Central Hospital): KCH344 (EU573776); KCH1050 (EU573777); KCH1051 (EU573778); KCH1124 (EU573779); KCH569 (EU573780); KCH1074 (EU573781); KCH602 (EU573782).

Rotavirus was detected in 578 (38%) of 1,522 specimens, of which 419 (39%) of 1,070 were from inpatients and 159 (35%) of 452 were from outpatients. A total of

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[1]Deceased.

546 rotavirus- positive specimens were further characterized. The most commonly detected strain types included G1P[8] (47%), G8P[8] (12%), G1P[6] (10%), G8P[6] (7%), G8P[4] (6%), and G12 P[6] (4%) (Table). A total of 48 specimens (9%) could not be assigned a G and/or P type. Overall, G1 was the most common G-type (58%), followed by G8 (29%) and G12 (5%); P[8] was the most common P-type (64%) followed by P[6] (23%) and P[4] (7%).

All but 2 G12 strains were detected in the second year of the study (July 2006 through June 2007). The G12 strains were associated with VP4 types P[6] (n = 22), P[8] (n = 5), P[4] (n = 1), and P[NT] (n = 2) and were investigated further by electropherotyping and nucleotide sequencing. Among the 30 G12 strains examined, 23 (77%) produced an identifiable electropherotype. Among G12P[6] strains, 11 displayed short electropherotypes (2 distinct patterns were recognized, with 1 predominant), and 9 strains had long electropherotypes (with 2 distinct patterns recognized). A single P[8] strain, KCH344, had a recognizable but distinct electropherotype (Figure 1). Two G12P[NT] strains possessed, respectively, short and long RNA profiles (data not shown). The single G12P[4] strain had no visible RNA after PAGE. Full-length VP7 sequences that could be successfully obtained by RT-PCR from G12 strains representing distinct electropherotypes were compared with each other and with published G12 sequences from elsewhere in the world (Figure 2). The VP7 genes of 7 Malawian G12 strains shared >99% nucleotide identity with each other, despite possessing a variety of electropherotypes and P-types, and were most closely related to recently identified G12 strains detected in Nepal, India, and South Africa.

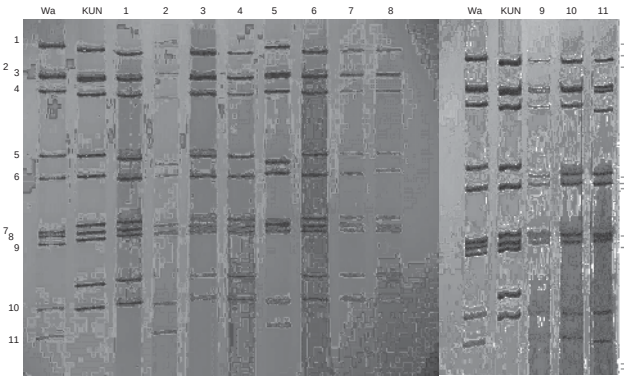

Figure 1. Polyacrylamide gel electrophoresis and silver staining of rotavirus double-stranded RNA of representative serotype G12 strains from Lilongwe, Malawi. RNA segments are indicated to the left. Strains Wa (long electropherotype) and KUN (short electropherotype) are controls. Field strains, designated electropherotype profiles, and P types are as follows: Lane 1, KCH958 short – profile S1, P[6]; lane 2, KCH1120, long – profile L1, P[6]; lane 3, KCH1124, short – profile S1, P[6]; lane 4, KCH1050, short – profile S1, P[6]; lane 5, LOP286, long – profile L2, P[6]; lane 6, LOP523, short – profile S1, P[6]; lane 7, KCH944, short – profile S1, P[6]; lane 8, KCH1074, short – profile S2, P[6]; lane 9, KCH569, long – profile L2, P[6]; lane 10, KCH602, long – profile L2, P[6]; lane 11, KCH344, long – profile L3, P[8].

Conclusions

Rotavirus was identified as a leading cause of gastroenteritis among infants and young children seeking hospital care in Lilongwe, Malawi; the virus was detected in 38% of all case-patients. The G1P[8] strain, globally the most common rotavirus strain type, was also the most commonly identified rotavirus strain in Lilongwe, comprising 47% of all characterized strains. Serotype G8, first identified in Blantyre, Malawi, in the late 1990s in association with the P[6] and P[4] VP4 types and considered to have arisen by viral reassortment from a bovine origin (*6*,*12*), was detected in 29% of strains in association with P-types P[8], P[6], and P[4]. The G8P[8] strain, comprising 41% of all G8 strains in this collection, is gaining increasing global recognition as an emerging strain type (*13*). In this study, we have also identified the globally common serotype G2, in association with VP4 types P[4] and P[6]. Notably, rotaviruses bearing the P[6] VP4 type comprised 23% of all characterized strains, thus providing further evidence of its prominence in Africa (*4*) (Table).

We have identified in Malawi the globally emerging rotavirus serotype G12, which was detected in 5% of all specimens. The single previous description of serotype G12 from the African continent was from Johannesburg, South Africa (*14*). G12 rotaviruses, first identified in the Philippines in 1987, have emerged over the past few years in numerous countries worldwide (*7*).

In our study, G12 was associated predominantly with the P[6] VP4 type and less commonly with P[8] and P[4].

Table. Distribution of rotavirus G and P types in Lilongwe, Malawi, July 2005–June 2007*

Type	G1	G2	G4	G8	G9	G12	G1 + G8	G8 + G9	GNT	Total
P[4]	1	5		32		1				39
P[6]	55	1	1	39	3	22	1		3	125
P[8]	255			64	9	5		2	12	347
P[6] + P[8]	1			1						2
P[NT]	6			20	1	2			4	33
Total	318	6	1	156	13	30	1	2	19	546

*NT, nontypeable.

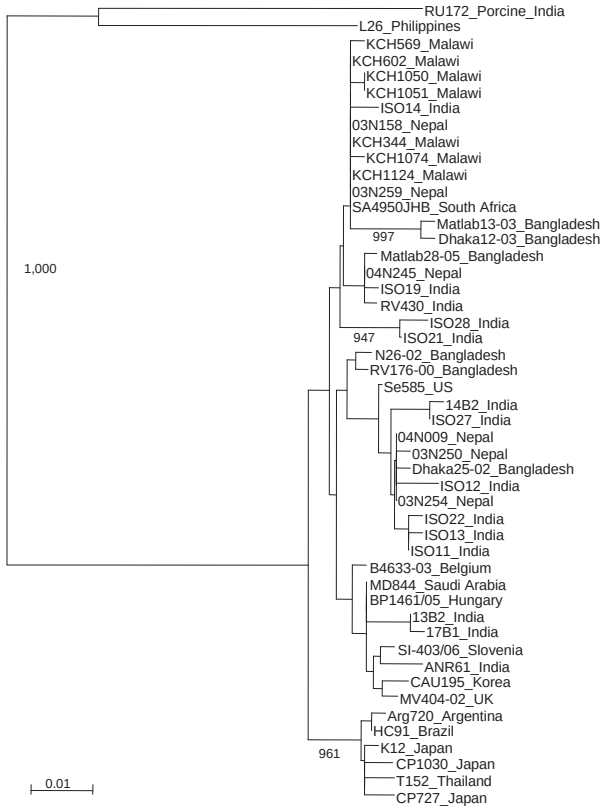

Figure 2. Phylogenetic tree based on VP7 nucleotide sequences from representative Malawi G12 strains and G12 strains deposited in DNA databases. Strain designations are followed by country of origin. Malawi strain KCH1051 is genotype G12P[8] and has an indeterminate RNA profile. Horizontal lengths are proportional to the genetic distance calculated with the Kimura 2-parameter method. Number adjacent to the node represents the bootstrap value of 1,000 replicates and values <80% are not indicated. Scale bar shows genetic distance expressed as nucleotide substitutions per site.

Multiple electropherotypes were demonstrated within P[6] strains, including long and short profiles. In contrast, the VP7 sequence of strains representing each electropherotype and P-type were closely related, sharing >99% nucleotide identity. These findings suggest the introduction of a single G12 VP7 gene into local rotavirus strains, which have subsequently undergone reassortment to generate G12 strains harboring a constellation of electropherotypes and P-types. Similar diversity among G12 strains has recently been described in Nepal (*8*,*15*). The critical role that viral reassortment has played in generating extensive genetic diversity among G12 rotaviruses in Lilongwe mirrors the evolutionary mechanisms that have led to the global emergence of this serotype (*7*). The close relationship of the VP7 genes of Lilongwe G12 strains with G12 strains from Nepal and India suggests an origin in Asia, a hypothesis proposed by Rahman et al. (*7*).

Two current rotavirus vaccines, Rotarix (GSK Biologicals, Rixensart, Belgium) and RotaTeq (Merck & Co., Whitehouse Station, NJ, USA) offer the potential to greatly reduce childhood deaths from rotavirus gastroenteritis (*2*,*3*). The monovalent vaccine Rotarix comprises a human G1P[8] rotavirus strain, whereas the pentavalent vaccine RotaTeq is a human-bovine reassortant vaccine comprising human serotypes G1, G2, G3, G4, and P[8] on a bovine strain background. Although both vaccines are highly effective in preventing severe rotavirus gastroenteritis in North America, Latin America, and Europe, their efficacy in countries harboring a wider diversity of strain types is yet to be fully established (*2*,*3*). Other rotavirus proteins (e.g., VP6 and NSP4) may play a role in the protective immunity against rotavirus infection; G12P[6] strains detected in the current study share neither G- nor P-type with either of the 2 current vaccines and could theoretically challenge vaccine efficacy. Continued surveillance for serotype G12 in Malawi and elsewhere in Africa is needed, given the propensity of this emerging serotype to rapidly spread and establish itself within populations (*7*,*8*).

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G2 Strain of Rotavirus among Infants and Children, Bangladesh

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To determine G and P genotypes, we performed nested PCR on 307 rotavirus specimens collected in Dhaka, Bangladesh, during 2004–2005. G2 (43.3%) was detected at the highest frequency, followed by G4 (19.5%), G9 (13.7%), G1 (12.7%), and G3 (2.6%). P[8] was the most predominant genotype (53.2%), followed by P[4] (42.9%).

Group A rotavirus (RAV) is the leading cause of severe gastroenteritis in infants and young children worldwide and accounts for ≈600,000 deaths in children <5 years of age (*1*). Rotaviruses are members of the *Reoviridae* family (*2*) and are classified into 7 groups (A–G) on the basis of distinct antigenic and genetic properties (*3*). On the basis of neutralization assay and sequence analysis, a total of 15 G and 27 P genotypes of RAV have been documented (*4*). The major human G types are G1, G2, G3, G4, and G9, which, when combined with the P types P[8], P[4], and P[6], account for >80% of rotavirus-associated gastroenteritis (*5*).

The Study

A total of 917 stool specimens were collected from infants and children with acute gastroenteritis in Dhaka Children's Hospital, Bangladesh, during October 2004 to September 2005. Fecal specimens were diluted with distilled water to 10% suspensions and clarified by centrifugation at 10,000 × *g* for 10 min. The supernatant was collected and viral genomes were extracted from fecal specimens by using the QIAamp viral RNA Mini Kit (QIAGEN, Hilden, Germany). Using reverse transcription–PCR (RT-PCR) with specific primers, as previously reported (*6*), resulted in the identification of diarrheal viruses, including group A, B, and C rotaviruses and adenovirus.

RAV-positive samples were then subjected to G and P genotyping by nested PCR with previously published primers (*7*–*9*).The RAV isolate for which the G and P types could not be determined by RT-PCR method was subjected to nucleotide sequence analysis of PCR products positive for VP7 and VP4 genes with the BigDye Terminator Cycle Sequencing Kit and ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). Their VP7 and VP4 nucleotide sequences were compared as well as those of reference rotavirus strains available in GenBank by using BLAST (http://blast.ncbi.nlm.nih.,gov/Blast.cgi). Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 3.2 software (*10*). The sequences of VP4 and VP7 genes of rotaviruses detected in this study had been submitted to GenBank under accession nos. EU855813–EU855822 and EU855823–EU855830, respectively.

Among diarrhea-causing viruses detected, RAV was the most prevalent (33.5%), followed by adenoviruses (1.9%). Rotavirus group B and rotavirus group C could not be detected in this study. Ninety-seven percent of RAV-infected patients were hospitalized. Co-infection between RAV and adenoviruses was identified in 7 of 917 samples. Most of the patients in this study were 1 to 24 months of age; RAV infection was most commonly detected in patients 6 to 23 months of age. Gender distribution of patients with RAV-positive samples was 56% male and 44% female.

We could not initially determine G type for 10 RAV and P type for 8 RAV isolates, even though their VP7 and VP4 genes were successfully amplified by RT-PCR. Sequence analysis showed that all of untypeable RAV were G1 and P[8]. Ten rotavirus G1 sequences were classified into a distinct lineage, lineage1 and sublineage 1a. G1 strains analyzed in this study belonged to the Asian cluster and were most closely related to Dhaka-02, Dhaka-03, and Thai-1602 strains, which had high identities at the nucleotide level with each other (99%–100%). Eight rotavirus P[8] sequences in this study belonged to 1 distinct lineage, lineage P[8]-II, but made a novel sublineage, sublineage P[8]-IIB, which had a high nucleotide sequence identity of 100% within lineage P[8]-II (Figure 1). These untypeable rotavirus P[8] strains contained 2–3 point mutations at the VP4 primer-binding site.

RAV was detected all year round, but 2 peaks in infections occurred: 1 peak apparently lasted 4 months (October 2004 through January 2005) and another peak lasted 3 months (June 2005 through August 2005) (Figure 2). Five different G types, G1–G4 and G9, were detected during the study period. Of these, G2 was the most common (43.3%) followed by G4 (19.5%), G9 (13.7%), G1 (12.7%), and G3 (2.6%). Mixed infections between >1 G genotypes were

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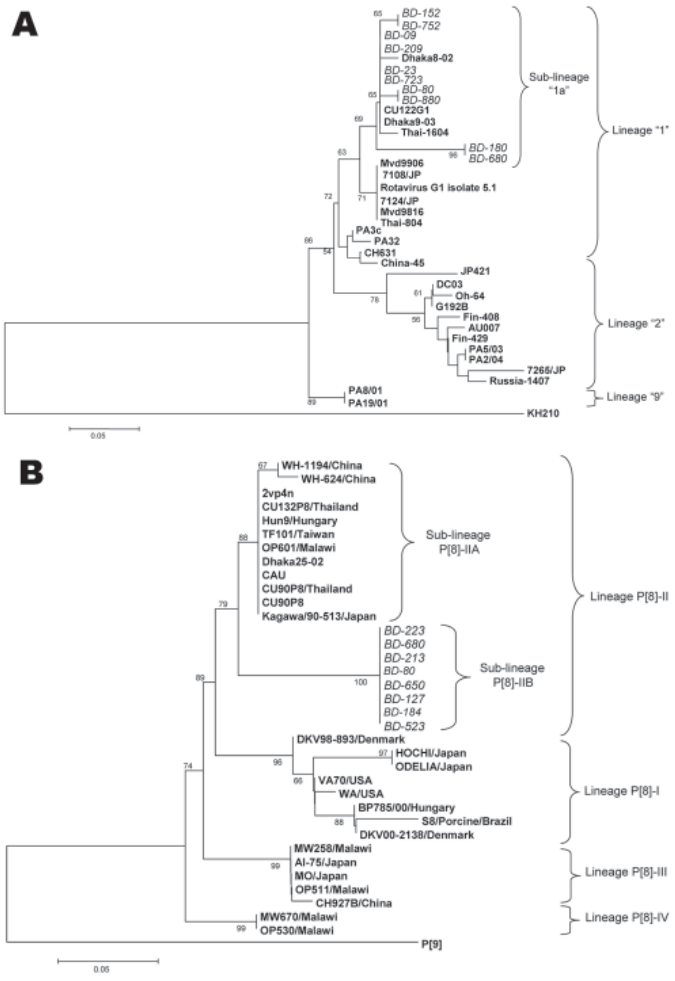

Figure 1. Phylogenetic analysis of the nucleotide sequences of the VP7 and VP4 genes of untypeable group A rotavirus strains (RAV) from Bangladesh. A) Neighbor-joining phylogenetic tree based on nucleotide sequences of the VP7 encoding genes for untypeable RAV strains. B) Neighbor-joining phylogenetic tree based on nucleotide sequences of the VP4 encoding genes for untypeable RAV strains. The numbers in the branches indicate the bootstrap values. Reference strains of RAV G1 and P[8] strains were selected from DNA database of Japan/GenBank under the accession number indicated in **boldface**. G1 strains from Bangladesh are highlighted in *italics*. The scale bars indicate nucleotide substitutions per position. Reference RAV strains used in this study and their accession numbers are as follows: RAV P[8] strains: BP785/00/Hungary (AJ605315), VA70/USA (AJ540229), WA/ USA (L34161), HOCHI/Japan (AB039943), ODELIA/Japan (AB039942), MW670/Malawi (AJ302146), OP530/Malawi (AJ302152), AI-75/Japan (AB008285), MW258/Malawi (AJ302143), OP511/Malawi (AJ302151), CH927B/China (AB008273), MO/Japan (AB008278), Kagawa/90-513/Japan (AB039944), OP601/Malawi (AJ302153), CU132P8/Thailand (DQ235955), DK V98-893/Denmark (AY509908), DK V00- 2138/Denmark (AY509910), S8/Porcine/Brazil (AF052449), CU90P8/Thailand (DQ235978), TF101/Taiwan (AF183870), Hun9/Hungary (AJ605320), WH-1194/China (AY856445), Dhaka25-02 (DQ146652), CU90P8 (DQ235978), 2vp4n (DQ675009), CAU 164 (EU679398) and WH-624/China (AY856444); RAV G1 strains: Dhaka9-03 (DQ482715), CU122G1 (DQ236053), PA5/03 (DQ377596), KH210 (AB303218), 7014/JP (EF079064), rotavirus G1 isolate 5.1(DQ672628), Mvd9906 (AF480278), 7265/JP (EF079066), 7124/JP(EF079069), 7108/JP (EF079068), JP421 (D16326), Fin-408 (Z80303), PA2/04 (DQ377598), Fin-429 (Z80312), AU007 (AB081799), G192B (AF043678), DC03 (AF183859), Oh-64 (U26387), PA3c (DQ377566), PA32 (DQ377574), Thai-1604 (DQ512981), Dhaka8-02 (AY631049), Thai-804 (DQ512979), Mvd9816 (AF480293) CH631 (AF183857), China-45 (U26371), Russia-1407 (S83903), PA8/01 (DQ377592), PA19/01 (DQ377593).

identified in 4% of the specimens (Table). Genotype G2 was detected in every month with a relatively high incidence rate. Among 307 RAV-positive samples, 280 samples were P typed successfully, and P[8] was the most predominant genotype (53.2%), followed by P[4] (42.9%) and mixed infections between different P genotypes (5.0%). G2P[4] combination was the most predominant genotype (39%), followed by G4P[8] (18.2%), G9P[8] (13%), G1P[8] (11.8%), and G3P[8] (2.9%).

Conclusions

Of 917 fecal specimens tested, 307 (33.5%) were positive for RAV. This result was consistent with the previous findings on rotavirus epidemiology in Bangladesh in which its prevalence was ≈29% (*11*,*12*). Our study demonstrated 2 peaks of rotavirus infection. The winter rotavirus peak is usually observed worldwide, but the monsoon peak is not common in settings with temperate climates. Why there was a relation between rainy season and viral infection in this study is not clear. We identified most of the globally common rotavirus types (G1, G2, G4, and G9) in our study. Even though G3 is one of the most prevalent rotavirus types worldwide, the G3 strain has not been detected in Bangladesh since 1993 (*13*). However, we found that 4% of the rotavirus types identified in this study were G3. Results of rotavirus diversity from this study were compared with results of previous studies in Bangladesh (*13*), and we found that G2 was a predominant rotavirus strain among infants and children in Dhaka, Bangladesh.

Rotavirus G4 genotype was the most common genotype in Dhaka from 1992 through 1997 but became a less common rotavirus strain over time; G9 was the leading genotype followed by G2, G4, and G1 in Dhaka (*12*,*14*). The prevalence of G9 strains was nearly the same in our study, but G2 strains showed a dramatic increase. From 2001–2004, the most common rotavirus genotype was P[8] (76%); non-P[8] strains constituted ≈20%. We also found that rotavirus P[8] (53.2%) strain was the most prevalent. We found that the 4 most common strains globally, G1P[8], G2P[4], G3P[8], and G4[8], were found in 83.9% of cas-

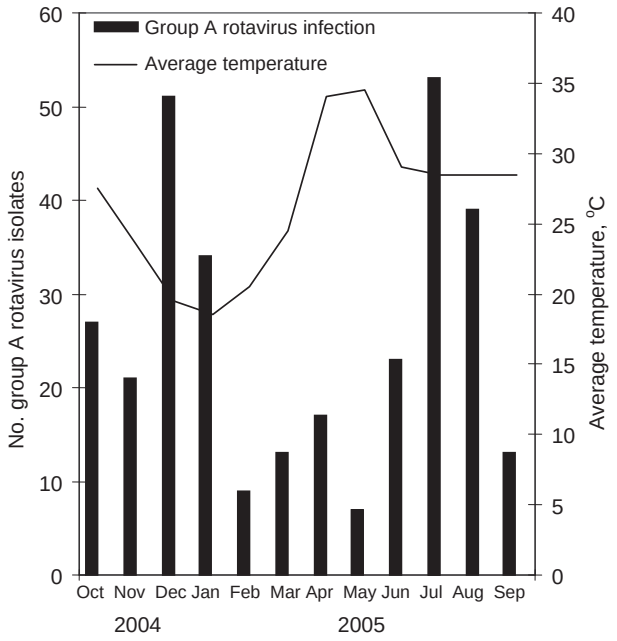

Figure 2. Seasonal pattern of group A rotavirus infection in infants and children with acute gastroenteritis in Dhaka, Bangladesh, October 2004–September 2005.

es. The G1P[8] strains, less common in 2001, became the predominant strains in the following years, but decreased again in 2005–06. Rotaviruses show great genomic diversity, and several studies in different regions of Bangladesh have identified types not targeted by candidate rotavirus vaccines (*11*,*14*). The frequent genomic reassortment among different rotavirus types was accelerated by mixed infection and generated huge genomic diversity (*13*).

RAV has been associated with gastroenteritis outbreaks in infants and children <5 years of age. However, less is known of the age distribution of rotavirus infection in Bangladesh. In this study, infections were most commonly detected in children <2 years of age.

Common clinical symptoms of RAV-infected patients were dehydration (84%), vomiting (69%), abdominal pain (52%), and fever (31%), which are in agreement with previous published reports (*15*). Number of loose stools per day was increased, with most patients (76%) having loose stools 3–5 times per day. Our study is limited because we could not conduct other tests such as enzyme immunoassay or polyacrylamide gel electrophoresis to confirm rotavirus illness. The incidence of rotavirus gastroenteritis identified by RT-PCR could be an overestimate because healthy controls tested by RT-PCR had a 5%–10% general incidence of rotavirus.

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Mr Dey is a PhD student at The University of Tokyo. His research interest focuses on molecular epidemiology of gastroenteritis viruses in humans.

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Table. Distribution of group A rotavirus G and P genotypes among infants and children with acute gastroenteritis in Dhaka City, Bangladesh, 2004–2005*

Genotype	P[8]	P[4]	P[6]	Mixed*	Nontypeable	Total
G1	33	4	0	1	2	40
G2	11	109	0	9	13	142
G3	8	0	0	1	0	9
G4	51	4	0	2	5	62
G9	36	2	1	2	3	44
Mixed*	10	0	0	0	0	10
Total	149	119	1	15	23	307

*>1 G or P genotype was recognized.

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Rotavirus Genotype Distribution after Vaccine Introduction, Rio de Janeiro, Brazil

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Brazil introduced rotavirus vaccination in March 2006. We studied 133 rotavirus-positive fecal samples collected from February 2005 through December 2007. Genotype G2P[4] was found in 1.4% of samples in 2005, in 44% in 2006, and in 96% in 2007. Rotavirus detection rate decreased from 38% in 2005 to 24% in 2007 (p = 0.012).

Group A rotaviruses (RV-A) are the major etiologic agents of acute diarrhea in infants, causing ≈611,000 deaths each year (*1*). The recently developed attenuated G1P[8] vaccine, Rotarix (GlaxoSmithKline, Rixensart, Belgium), was included in the Brazilian Expanded Immunization Program and, after March 2006, became available to the whole birth cohort. Rio de Janeiro is the second largest Brazilian city; vaccine coverage was 43.3% in 2006 and 74.4% in 2007. Although Rotarix was highly efficacious for preventing severe RV gastroenteritis in phase III trials carried out in Latin America and Europe, it appears to be less effective in preventing diarrhea caused by G2P[4] RV-A strains, which do not share either the VP7 or the VP4 surface antigen with the vaccine strain (*2*).

Initial studies carried out in northeastern Brazil after RV-A vaccine introduction demonstrated the predominance of RV-A G2P[4] in vaccinated populations (*3–5*). Also, the apparent extinction of non-G2 rotavirus strains from circulation was associated with a significant reduction in the frequency of RV-A detection in children with gastroenteritis (*5*). This finding suggests that G2P[4] strains could be, to some extent, replacing P[8] genotypes in the postvaccination period.

Sentinel RV-A surveillance, performed in selected pediatric settings as part of the strategies of immunization programs in Latin America, has been recommended to better assess RV-A effects and strain characterization. In this context, studies carried out in Brazil demonstrated that the emergence of G9P[8] RV-A in Rio de Janeiro in the late 1990s was accompanied by the disappearance of common genotypes like G2P[4] and G3P[8] and the continuous detection of RV-A genotype G1P[8] (*6–10*). RV-A G5, an atypical genotype prevalent in the early 1990s, has not been detected in Rio de Janeiro since 1997 (*6–10*). In this study, we estimated the distribution of RV-A genotypes in hospitalized children in Rio de Janeiro before and after the monovalent RV-A vaccine was introduced into the national immunization schedule**.**

The Study

From February 2005 through December 2007, fecal samples were collected from 464 hospitalized children from birth to 5 years of age who exhibited gastroenteritis and dehydration and required intravenous fluid replacement. The study was conducted in Salles Netto Municipal Hospital, a pediatric unit in Rio de Janeiro.

Most children studied (390 [84%]) were not eligible for full vaccination; they either were born before January 1, 2006, or were <4 months of age. Nevertheless, 39 (8.4%) had been vaccinated with 2 doses of Rotarix, and 35 (7.5%) did not receive the vaccine.

Samples were collected after written consent was given by the parents. This study was approved by the Oswaldo Cruz Foundation Ethical Research Committee (protocol no. 311/06).

Polyacrylamide gel electrophoresis and a combined enzyme immunoassay for RV-A strains and adenoviruses were used to detect RV-A. Most samples were G- and P-typed through seminested reverse transcription–PCR, as described (*11*). Seventeen RV-A–positive samples were P-typed through partial genome sequencing. This method was also used to G-type 1 sample. These strains could not be typed through PCR. All samples that were P-typed through sequencing were P[8]. The only sample G-typed through sequencing was G9.

RV-A strains were detected in 133 (29%) of 464 samples. Genotype distribution showed a different profile for each year: 45% G9P[8], 30% G3P[8], 14% G1P[8], and 1.4% G2P[4] in 2005; 41% G2P[4], 18% G3P[8], and 15% G9P[8] in 2006; and 96% G2P[4] in 2007 (Table).

In the 18 months from July 2006 through December 2007, almost all RV-A–positive samples (35/36, 97%) showed G2P[4] specificity, which suggests a shift in geno-

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type distribution, characterized by an increase in G2P[4] detection since 2006. When the pre- and postvaccination periods were compared, these changes in genotype distribution were found to be accompanied by a significant reduction in the detection rate of RV-A from 38% (73/193) in 2005 to 24% (26/109) in 2007 ($p = 0.012$ by χ^2 test). Vaccination rates in the RV-A–positive and –negative groups (considering only children eligible for full vaccination) were 29% (4/14) and 58% (35/60), respectively (odds ratio 0.29; 95% confidence interval 0.07–1.15; $p = 0.043$ by Fisher exact test, 1-sided. The 4 RV-A–positive vaccinated children were infected with G2P[4] genotype.

Conclusions

The first studies that assessed the RV-A genotype distribution after the introduction of Rotarix were carried out in northeastern Brazil (*3*–*5*). They offered the hypothesis that vaccination with the monovalent G1P[8] vaccine possibly created conditions in which RV-A G2P[4] could acquire selective advantage over P[8] genotypes (*5*). Nevertheless, a temporal periodicity, within the ≈10-year cyclic pattern of G2P[4] occurrence in Brazil, should be considered to explain the increased detection of this genotype since 2006. This periodicity could coincide with RV-A vaccine introduction and the consequent reduction of circulation of non-G2 strains.

G2P[4] RV-A was not detected from 2000 to 2004 in Rio de Janeiro (*2*–*6*); it was identified in 2005 (1.4%) and reemerged in 2006 (41%). Similarly, in northern Brazil, RV-A G2P[4] was detected in 2005 after a period of absence (A. Linhares, pers. comm.). When other Latin American countries are considered, an outbreak of RV-A gastroenteritis with a high rate of G2P[4] detection was recently described in Honduras (*12*). According to Patel et al. (*13*), ongoing surveillance in El Salvador, Guatemala, and Honduras showed that G2P[4] was the predominant circulating strain in 2006 (68%–81%). In Argentina, this genotype was also circulating in 2006 (J. Stupka, pers. comm.). RV-A with short electropherotype, characterized as G2P[4], was detected at high frequency in 2005 in Paraguay, after a 6-year absence (*14*). In these South American countries that border Brazil, there are no RV-A immunization campaigns, and G2P[4] was detected before introduction of Rotarix in Brazil.

In Bangladesh, the rate of G2P[4] detection increased recently (43% in 2005–2006) (*15*). The detection of G2P[4] since 2005 in countries where RV-A vaccination is not implemented reinforces the possibility of natural reemergence of this genotype.

Our data also suggest a significant reduction in the rate of RV-A detection between the pre- and postvaccination periods. The comparison of vaccination rates between RV-A–positive and –negative children, even with a small sample size, suggests that vaccinated children have a reduced risk for severe RV-A diarrhea.

This survey is among the first to evaluate the effects of Rotarix in Brazil, the first Latin American country to introduce universal rotavirus vaccination. We believe that the emergence of strains that may escape protection can be a challenge to the RV-A immunization program in Brazil and needs to be continuously monitored.

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Table. Frequency of rotavirus A infection and distribution of G and P genotypes from February 2005 through December 2007, Rio de Janeiro, Brazil*

Vaccination status and year	No. samples	No. (%) rotavirus positive	No. (%) G1P[8]	No. (%) G2P[4]	No. (%) G3P[8]	No. (%) G9P[8]	No. (%) other genotypes, mixed or not typeable
Ineligible for full vaccination†							
2005	193	73 (38)	10 (14)	1 (1.4)	22 (30)	33 (45)	7 (9.5)
2006	148	34 (23)	1 (2.9)	14 (41)	6 (8)	5 (5)	8 (23)
2007	49	12 (24)	1 (8)	11 (92)	–	–	–
Vaccinated‡							
2006	6	Negative§					
2007	33	4 (12)	–	4 (100)	–	–	–
Not vaccinated							
2006	8	Negative§					
2007	27	10 (37)	–	10 (100)	–	–	–
Total	464	133 (29)	12 (9)	40 (30)	28 (21)	38 (29)	15 (11)

*–, absence of genotypes.
†Born before January 1, 2006, or <4 months of age.
‡Children were considered vaccinated if they had received 2 doses of vaccine.
§All stool samples were negative for rotavirus.

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Rickettsia helvetica in *Dermacentor reticulatus* Ticks

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We report on the molecular evidence that *Dermacentor reticulatus* ticks in Croatia are infected with *Rickettsia helvetica* (10%) or *Rickettsia slovaca* (2%) or co-infected with both species (1%). These findings expand the knowledge of the geographic distribution of *R. helvetica* and *D. reticulatus* ticks.

Rickettsia helvetica organisms were first isolated from *Ixodes ricinus* ticks in Switzerland and were considered to be a new nonpathogenic species of the spotted fever group (SFG) rickettsiae (*1*,*2*). Recently, *R. helvetica* was linked to acute perimyocarditis, unexplained febrile illness, and sarcoidosis in humans in Europe (*3*–*5*). It is generally accepted that *Dermacentor marginatus* is the main vector of *R. slovaca* and that *I. ricinus* is the main vector of *R. helvetica* (*1*,*2*,*6*).

Until now, only in the southern (Mediterranean) part of Croatia have *R. conorii*, *R. slovaca,* and *R. aeschlimannii* been detected in *Rhipicephalus sanguineus*, *D. marginatus,* and *Hyalomma marginatum* ticks, respectively (*7*). Human disease caused by *R. conorii* (Mediterranean spotted fever) has also been described in this region (*8*). No published reports of *R. helvetica* in Croatia are available. In a previous study, antibodies to SFG rickettsiae were found in dogs in the northwestern continental part of the country, where *D. marginatus* and *I. ricinus* ticks are common (*9*). Given the importance of this finding, we set up this study to provide the molecular evidence of the presence of *R. helvetica* and *R. slovaca* in Croatia. Prior to the study, *D. reticulatus* ticks had not been found in Croatia, although they were prevalent in neighboring Hungary (*10*). We used molecular methods to establish whether *D. reticulatus* ticks are also present in Croatia.

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The Study

Using the cloth-dragging method, during March–May 2007 we collected 100 adult *Dermacentor* spp. ticks from meadows in 2 different locations near Cakovec, between the Drava and Mura rivers in the central part of Medjimurje County. This area is situated in the northwestern part of Croatia, at 46″38′N, 16″43′E, and has a continental climate with an average annual air temperature of 10.4°C at an altitude of 164 m.

To isolate DNA from ticks, we modified the method used by Nilsson et al. (*11*). Before DNA isolation, ticks were disinfected in 70% ethanol and dried. Each tick was mechanically crushed in a Dispomix 25 tube with lysis buffer by using the Dispomix (Medic Tools, Zug, Switzerland). Lysis of each of the crushed tick samples was carried out in a solution of 6.7% sucrose, 0.2% proteinase K, 20 mg/mL lysozyme, and 10 ng/ml RNase A for 16 h at 37°C; 0.5 molar EDTA, and 20% sodium dodecyl sulfate was added and further incubated for 1 h at 37°C. Extraction was performed twice with 80% phenol (1:1, vol:vol) and methylenchloride/ isoamylalcohol (24:1, vol:vol). DNA was precipitated with isopropanol. The DNA-pellet was washed with 70% ethanol and centrifuged at 16.000 × *g* for 15 min. After the ethanol was removed, the pellet was dried at 50°C and dissolved in 50 μL distilled water.

For the detection of *Dermacentor* spp., we used conventional PCR based on ribosomal internal transcribed spacer 2 (ITS2) sequences (*12*). In addition, we developed quantitative real-time PCR for the detection of *R. helvetica* based on the *ompB* gene and *R. slovaca* based on the *ompA* gene. Primer and probe sequences are shown in Table 1. *Borrelia* DNA was investigated with real-time PCR described by Schwaiger et al. (*13*).

All PCR-derived products generated from ticks (11 *ompB*, 3 *ompA,* and 13 ITS2) were sequenced. Sequencing reactions were performed by using a modified Sanger method and the BigDye Terminator Cycle Sequencing Kit version 1.2 (Applied Biosystems, Carlsbad, CA, USA) on an ABI 3730 capillary DNA Analyzer (Applied Biosystems), employing the same primer pairs as for amplification of the PCR products. Sequencing was performed by both Microsynth AG (Balgach, Switzerland) and our laboratory. All sequences were aligned with known sequences by using BLAST (http://blast.ncbi.nim.nih.gov/Blast.cgi).

The sequences of the ITS2 spacer regions obtained from 13 *Dermacentor* spp. ticks infected with *R. helvetica* and *R. slovaca* were 99.8% identical to the corresponding *D. reticulatus* ITS2 sequence (S83080) and 85% identical to the corresponding *D. marginatus* sequence (S83081). The 646-bp ITS2 spacer fragment is sufficient to discriminate between the *Dermacentor* spp. The consensus sequence of the 13 *D. reticulatus* ITS2 spacer regions determined in this study was deposited at the European

Table 1. Primers and probes designed for real-time PCR*

Species	Primers and probe	Sequence (5′ → 3′)
Rickettsia helvetica	*ompB*_forward	GATTTCGACGGTAAAATTACC
	*ompB*_reverse	GCTACCGATATTACCTACAG
	*ompB*_probe†	ACTCTACTGCTACAAGTATGGTTGCTACAG
R. slovaca	*ompA*_forward	GTGATAATGTTGGCAATAATAATTGG
	*ompA*_reverse	CTCTCGTTATAATCGAACCAAC
	*ompA*_probe†	CAGCAGGAGTACCATTAGCTACCCCTCC
Dermacentor spp.	ITS_forward	GTGCGTCCGTCGACTCGTTTTGA
	ITS_reverse	ACGGCGGACTACGACGGAATGC

*Amplified products: 162 bp (*R. helvetica*); 228 bp (*R. slovaca)*; 646 bp *(D. reticulatus)*; standard curve: slope; Y-intercept and correlation coefficient: –3.634, 40.129, 0.9937 (*R. helvetica*); –0.23, 42.82, 0.9942 (*R. slovaca*); ITS, internal transcribed spacer.
†The TaqMan probes were labeled with the fluorescent dyes FAM at the 5' end and TAMRA as quencher at the 3' end.

Molecular Biology Laboratory database under accession no. FM212280.

Results of the identification of *R. helvetica* and *R. slovaca* are shown in Table 2. The amplified *ompB* sequences of *R. helvetica* were 100% identical to the corresponding *ompB* gene of the *R. helvetica* strain C9P9 (AF123725), 92% identical to "Candidatus R. hoogstraalii" (EF629536), 89% identical to *R. asiatica* (DQ110870), 84% identical to *R. rhipicephali* (AF123719), and 83.3 % identical to *R. raoultii* (EU036984, DQ365798, DQ365797). The amplified *ompA* sequences were 100% identical to the corresponding *ompA* gene of *R. slovaca* and showed 2- to 12-bp differences to the corresponding *ompA* sequences of other *Rickettsia* spp.

In summary, *R. helvetica* DNA was detected in 10 of 100 *D. reticulatus* ticks, and the pathogen loads ranged from 380 to 1,700 copies per tick. *R. slovaca* DNA was found in 2 of 100 *D. reticulatus* ticks with copy numbers of 400 and 460. One *D. reticulatus* tick was co-infected with *R. helvetica* (410 copies) and *R. slovaca* (20,000 copies). No *Borrelia burgdorferi* DNA was found in *D. reticulatus* ticks.

Conclusions

Scientific literature supports the premise that *D. marginatus* ticks are the main vector of *R. slovaca* and that *I. ricinus* ticks are the main vector of *R. helvetica* (*1*,*2*,*6*). *R. slovaca* was also detected in *D. reticulatus* ticks (*6*,*14*). Additionally, the DNA of *R. raoultii* strain Marne, which is well separated from *R. helvetica* according to phylogenetic analyses of 16S rDNA sequences, was also detected in *D. reticulatus* ticks (*14*,*15*).

Previous studies showed that *D. marginatus* ticks are common in Croatia, and *R. slovaca* was identified in 36.8% of *D. marginatus* ticks collected in the southern part of the country (*7*). Further, *R. helvetica* as well as *D. reticulatus* ticks have never been detected in Croatia. In our study, 2% of *D. reticulatus* ticks were infected by *R. slovaca*, 10% were positive for *R. helvetica*, and 1% (1 tick) was co-infected by both pathogens. Our findings may explain the high seroprevalence (20.7%) of SFG antibodies in dogs detected in a previous study in the continental part of Croatia that is *R. conorii* free (*9*). This study suggests that these antibodies to SFG rickettsiae are presumably related to *R. helvetica* and *R. slovaca* infections, which can be transmitted by the same tick vector.

Because *D. reticulatus* is the second most common tick species occurring in all 16 counties of neighboring Hungary, we believe our findings point to an enlargement of its distribution area (*10*). Visual identification of *Dermacentor* spp. ticks has traditionally been confirmed on the basis of morphologic features. Because *D. marginatus* and *D. reticulatus* exhibit overlapping phenotypes, this means of identification can be very difficult (*12*). Therefore, we cannot exclude the possibility that *D. reticulatus* ticks were frequently misinterpreted as *D. marginatus.* Our study

Table 2. Identification of *Dermacentor, Rickettsia*, and *Borrelia* species by molecular methods

Species	Identification method	Targeting sequence	Confirmation	Results
Dermacentor spp.	PCR	ITS2 (646 bp)	Sequencing	13/13 (100%) positive;* 99.8% identical to *D. reticulatus* ITS2 sequence (S83080)
R. helvetica	Real-time PCR	*ompB* (162 bp)	Sequencing	11/100 (11%) positive;† 100% identical to *R. helvetica* strain C9P9 (AF123725)
R. slovaca	Real-time PCR	*ompA* (228 bp)	Sequencing	3/100 (3%) positive;† 100% identical to *R. slovaca* strains‡
B. burgdorferi	Real-time PCR	*flaB* (p41)	Not done (PCR negative)	0/100 positive; not detected in *Dermacentor* spp. ticks§

*Only infected ticks (13 ticks) were identified by molecular methods (PCR and sequencing).
†100 ticks were analyzed; 10 were positive for *R. helvetica* only, 2 for *R. slovaca*; 1 was co-infected with *R. helvetica* and *R. slovaca*.
‡ GenBank accession nos. EU622810, DQ649052, DQ649051, DQ649050, DQ649049, DQ649048, DQ649047, DQ649046, DQ649045, DQ649030, DQ649029, Q649027, DQ649054, DQ649053, and U43808.
§DNA of *B. burgdorferi* DSM 4680 and *B. afzelii* DSM 10508 were used as positive controls.

shows that the identification problem can be solved through use of molecular biology techniques.

We provide molecular evidence of the existence of *D. reticulatus* ticks in Croatia. Our results expand the knowledge of *R. helvetica* hosts. *D. reticulatus* ticks occur at far more sites than previously known and thus have probably expanded their habitats. Our data point out the need for further studies on the epidemiology of *R. helvetica* and other SFG rickettsiae in Croatia as well as their association with infections in humans and animals.

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Variation in Antimicrobial Resistance in Sporadic and Outbreak-related *Salmonella enterica* Serovar Typhimurium

Eva Møller Nielsen, Mia Torpdahl, Steen Ethelberg, and Anette M. Hammerum

The prevalence of different antimicrobial resistance profiles and variants of the *Salmonella* genomic island 1 (SGI1) was reported for *Salmonella enterica* serovar Typhimurium DT104 strains isolated from patients in Denmark. Variation in antimicrobial resistance and corresponding changes of SGI1 were shown among isolates from a foodborne outbreak.

Phenotypic and genotypic typing methods are important for surveillance and outbreak detection of *Salmonella* infections. The stability of the markers assessed by the typing method helps identify clusters and the source of outbreaks (*1*). The phenotypic tests, including antimicrobial-drug resistance profiles and phage typing, are usually considered very stable and therefore often used for surveillance of *S. enterica* serovar Typhimurium. To obtain the discrimination needed for outbreak investigations, high-discriminatory molecular methods like pulsed-field gel electrophoresis (PFGE) and multiple-locus variable number of tandem repeats analysis (MLVA) are favorable. However, band shifts in PFGE and changes in number of repeat units in MLVA can occur for epidemiologically linked isolates. DT104 is the most prevalent phage type in Denmark, accounting for 21% of the *S. enterica* ser. Typhimurium cases in 2006. Most DT104 isolates are resistant to at least ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamides (Su), and tetracycline (T) (ACSSuT). The 5 resistance genes are found in a multidrug resistance (MDR) region that is located on the chromosome in a region termed *Salmonella* genomic island 1 (SGI1) (*2*). Recently, SGI1 has also been found in other *Salmonella* serovars (*3*), and several variants of the SGI1 MDR regions have been described (*4*).

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The Study

We report on the prevalence of different antimicrobial-drug resistance profiles among Danish *Salmonella* ser. Typhimurium DT104 strains from human infections. Furthermore, we show results indicating that a DT104 outbreak strain lost resistance genes by shifting to the SGI1-B variant during the outbreak. Consequently, the outbreak included isolates with both the ACSSuT and the ASu phenotype.

During 2003–2006, 307 isolates of *Salmonella* ser. Typhimurium DT104 and 16 DT104b were obtained from persons with clinical disease in Denmark. Several outbreaks and clusters of DT104 were detected; some have previously been reported (*5*–*8*). The 323 isolates were characterized by MLVA (97% of isolates), PFGE (78%), and susceptibility testing (100%). Susceptibility to a standard panel of antimicrobial agents (www.danmap.org) was determined by microbroth dilution and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (*9*), except for ciprofloxacin (≥0.125 μg/mL was used as breakpoint). The 5-locus MLVA described by Lindstedt et al. (*10*) and PFGE, according to the PulseNet protocol (*11*), were performed and analyzed as previously described (*5*). To obtain a cluster-independent data set, we reduced the 323 isolates to 146 unique strains on the basis of the MLVA and resistance profile (i.e., with different MLVA and/or resistance profile) (Table 1).

Strains with phenotypes lacking resistance to ≥1 of the antimicrobial agents (ACSSuT) typical for SGI1 were further investigated for the presence of SGI1 variants. PCR was performed for detection of the SGI1 left and right junctions by using primers U7-L12, LJ-R1, 104-RJ, and C9-L2 (*2*); detection of the integron conserved segments (5′-CS and 3′-CS) was done by using primers L1 and R1 (*3*); and detection of the antimicrobial resistance genes *aad*A2, *qac*/*sul1*, *flo*R, *tet*(G), and *bla*P1 was performed by using the primers described by Boyd et al. (*12*).

Most DT104 and DT104b isolates displayed the phenotype ACSSuT (Table 1), which is compatible with the possession of SGI1; 122 (84%) of the 146 strains were resistant to ACSSuT with or without additional antimicrobial drug resistance that is regarded as independent of the SGI1 (e.g., flouroquinolones). Three percent of the strains were additionally resistant to trimethoprim (Tm) and therefore likely to possess the SGI1-A variant, and 6% of the strains were fully sensitive (all of these were DT104). The remaining strains had a variety of resistance profiles, including profiles that comply with the descriptions for variants SGI1-B and SGI1-C (Table 1).

Forty isolates were related to a restaurant foodborne outbreak in 2005. This outbreak was shown to be caused by raw beef, served as *carpaccio* at a restaurant in a limited period of 4 weeks (*6*). The isolates displayed variation with all applied typing methods: phage typing, MLVA, PFGE,

Table 1. Antimicrobial resistance profile of 323 clinical *Salmonella enterica* serovar Typhimurium DT104 isolates, Denmark, 2003–2006*

Phenotype	SGI1 genotype†	Unique types, no. strains (%)	All isolates, no. (%)
ACSSuT‡	SGI1§	122 (84)	251 (78)
ACSSuTTm¶	SGI1-A	5 (3)	9 (3)
ASu	SGI1-B	4 (3)	24 (7)
SSu	SGI1-C	3 (2)	11 (3)
ASSuTTm	SGI1-C	1 (0.7)	2 (0.6)
ASSu	SGI1-B	1 (0.7)	2 (0.6)
ACST#	SGI1	1 (0.7)	1 (0.3)
SSuT	Not present	1 (0.7)	3 (0.9)
Sensitive	Not present	8 (6)	20 (6)
Total		146 (100)	323 (100)

*One isolate per multilocus variable number of tandem repeats analysis (MLVA) and antimicrobial resistance profile is represented in the data set of 146 strains with unique types. A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfonamides; T, tetracycline; Tm, trimethoprim.
†SGI, *Salmonella* genomic island. Genotype determined by the SGI1-related PCR assays described in the text (1 isolate per "unique type"); see Table 2 for selected details.
‡Additional antimicrobial-drug resistances: nalidixic acid and ciprofloxacin (22 strains), amoxicillin/clavulanic acid (3 strains), gentamicin (1 strain).
§Only a small subset of 10 ACSSuT strains was tested by PCR for the presence of SGI1 genes.
¶Additional antimicrobial-drug resistances: nalidixic acid, ciprofloxacin, and gentamicin (1 strain).
#Additional antimicrobial-drug resistances: nalidixic acid and ciprofloxacin.

and resistance profile; 33 isolates were typical MDR DT104 (ACSSuT) with 1 PFGE and MLVA profile. One isolate differed in phage type (nontypeable) and had a variant of the PFGE profile that differed by 1 band. Three isolates had different MLVA types (variation in 1 of the 5 loci) (*6*). Three other isolates were resistant to only ampicillin and sulfonamide. Detection of SGI1-related genes and regions by PCR showed that the ACSSuT isolates were positive in all PCRs as expected for SGI1 (Table 2), whereas the 3 ASu isolates were positive for the left/right junction, but only 1 integron and *pse*-1 and *qac/sul1*, in accordance with the phenotype and the SGI1-B variant, were positive (*3,12*). The most likely mechanism for the creation of variants of SGI1 is considered to be homologue recombination between identical DNA segments (*12*). SGI1-B can thus be generated from SGI1 by a single crossover at *intI*1. Recombination could have taken place in the intestine of the infected animal, during processing or storage of the beef, or during infection of the patients. It is less likely that the outbreak was caused by multiple unrelated strains of DT104 because both the PFGE and MLVA types, including the variants, were distinctive and unique to this outbreak.

In addition to the 3 isolates related to the *carpaccio* outbreak, 21 human *Salmonella* DT104 isolates in 2003–2006 had the phenotype ASu. All of these had the same PFGE profile (worldwide, the typical DT104 PFGE profile) (*13,14*) but 3 different MLVA profiles (represented by 18 isolates, 2 isolates, and 1 isolate each). The cluster of 18 isolates had the phenotype ASu and the MLVA profile 133 (Table 2), and most of these isolates were likely part of an outbreak related to pork from Denmark (December 2003–March 2004). Another cluster of 3 isolates was received in the laboratory within a 3-day period in June 2003 from patients living in the same region. These isolates belonged to a MLVA profile that has not been detected since. The phenotype of 2 of these isolates was ASu, and 1 was ACSSuT (Table 2). No outbreak investigation was carried out at that time, and we can only speculate that this is a similar

Table 2. Characterization of *Salmonella* genomic island 1 in isolates with atypical antimicrobial-drug phenotype and isolates likely to be related (possible clusters or outbreaks)*

Outbreak (no. isolates)	MLVA profile	PFGE pattern	Phenotype	SGI1 variant	Left/right junction	*intI1* L1-R1 1.0/1.2 kb	*pse-1*	*florR*	*aadA2*	*qac/sul1*	*tet*(G)
Carpaccio outbreak 1 (33)	253	205	ACSSuT	SGI1	+/+	+/+	+	+	+	+	+
Carpaccio outbreak 2 (3)	253	205	ASu	SGI1-B	+/+	–/+	+	–	–	+	–
Pork outbreak 2003–2004 (18)	133	14	ASu	SGI1-B	+/+	–/+	+	–	–	+	–
Jun 2003 "cluster" (2)	437	14	ASu	SGI1-B	+/+	–/+	+	–	–	+	–
Jun 2003 (1)	437	14	ACSSuT	SGI1	+/+	+/+	+	+	+	+	+
Oct 2005–Jan 2006 (8)	443	14	SSu	SGI1-C	+/+	+/–	–	–	+	+	–
Jan 2006 (1)	443	14	ASSuTTm†	SGI1-C	+/+	+/–	–	–	+	+	–
Dec 2003 (1)	309	14	ASSu‡	SGI1-B	+/+	–/+	+	–	–	+	–
Sep 2006 (1)	270	nd	ACST	(SGI1)	+/+	+/+	+	+	+	+	+
Aug 2003 (3)	681	14	SSuT§	–	–	–	–	–	–	–	–
2003–2006 (8)	Various	Various	Sensitive	–	–	–	–	–	–	–	–

*MLVA, multilocus variable number of tandem repeats analysis; PFGE, pulsed-field gel electrophoresis.
†Other genes detected by PCR: *bla*, *str*AB, and *tet*(B).
‡Other genes detected by PCR: *str*AB and *sul*2.
§Other genes detected by PCR: *str*AB *sul*2, and *tet*(A).

occurrence of shift in antimicrobial-drug resistance during an outbreak. A cluster of 9 isolates with MLVA 443 occurred late in 2005 and in January 2006; most patients lived in a specific geographic area. Eight of these isolates had the phenotype SSu (equivalent to SGI1-C), and 1 isolate, which appeared late in the cluster, was ASSuTTm. PCR confirmed the presence in all 9 MLVA 443 isolates of the left and right junction, the typical SGI1 genes conferring resistance to SSu, and 1 cassette of class 1 integrons (lacking the *pse*1-associated cassette) (Table 2). The ASSuTTm- isolate was additionally positive for the non–SGI1-related genes *bla*, *str*AB, and *tet*B. In this cluster, it seems likely that the variation in phenotype was caused not by variation in SGI1 but rather by acquisition of antimicrobial-drug resistance genes independent of SGI1 (e.g., on plasmids).

Conclusions

Most of the *Salmonella* DT104 isolates from patients in Denmark had the typical ACSSuT phenotype; however, a small fraction (6%) of DT104 isolates were fully sensitive and correspondingly negative for all SGI1-related genes and regions tested for by PCR. Approximately 10% of the isolates had a different phenotype, and most of these had an antimicrobial-drug resistance profile corresponding to SGI1-A, -B, or –C, which could be verified by PCR assays mapping parts of SGI1. A number of clusters and outbreaks occurred in the study period, and shifts in antimicrobial-drug resistance phenotype and genotype seem to have taken place in several outbreaks. These shifts were most clearly seen in the *carpaccio* outbreak (6), in which all evidence pointed at a single source outbreak, though the applied typing methods showed some variation among isolates, including a shift from SGI1 to SGI1-B in 3 of 40 isolates. Two other clusters of isolates showed variation in antimicrobial-drug resistance. We therefore conclude that variability of both genotypic and phenotypic characteristics can be expected during a foodborne outbreak; this variability should be taken into consideration when defining outbreak-related cases.

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Predicting High Risk for Human Hantavirus Infections, Sweden

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An increased risk for hemorrhagic fever with renal syndrome caused by Puumala hantavirus was forecast for Sweden in 2007. The forecast was based on a predicted increase in the number of *Myodes glareolus* rodents (reservoir hosts). Despite raised awareness and preparedness, the number of human cases during July 2007–June 2008 was 1,483, a new high.

Puumala virus (PUUV) is the etiologic agent of nephropathia epidemica, a mild form of hemorrhagic fever with renal syndrome (HFRS). PUUV is likely to be the most prevalent hantavirus in Europe (*1*); the bank vole *Myodes* (*Clethrionomys*) *glareolus*, one of the most widespread and abundant mammal species on the continent, is a natural reservoir (*2*–*4*). PUUV is excreted from bank voles by saliva, urine, and feces, and transmission to humans often occurs by inhalation of aerosolized excreta. An average of 10–40 HFRS cases are serologically confirmed per 100,000 population every calendar year in HFRS-endemic northern Sweden (*5*,*6*). However, in 2007 an all-time high of 2,195 cases were reported in Sweden, almost 4 times the previous record (n = 564 in 1998). A total of 807 cases, or 313 cases/100,000 population, were reported from Västerbotten County, a HFRS-endemic area. Approximately 90% of all HFRS cases are found within the HFRS-endemic northern region of Sweden (*5*), but cases outside this region most often originate from the HFRS-endemic areas and are found during the summer season as a consequence of residents spending their holidays in the north (*7*). The all-time high in HFRS diagnoses during the winter of 2006–2007 may have been affected not only by high bank vole numbers but also indirectly by extreme winter conditions (*7*).

We monitored data on small mammals from northern Sweden and used these data to predict and subsequently verify high bank vole numbers in the fall of 2007, which indicated an increased risk of PUUV exposure to humans. We discuss additional factors that may alter bank vole–human encounter rates and influence the occurrence of HFRS in Sweden.

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The Study

HFRS has been a reportable disease in Sweden since 1989; most cases occur during fall and winter, correlating with bank vole abundance in fall (*5*,*8*–*10*). In our study, all unique HFRS cases in Sweden from July 1989 through June 2007 were grouped into HFRS seasons (July–June) and used for regression analysis and prediction. We also used reported cases in the July 2007 through June 2008 season to confirm our risk prediction.

Data on bank vole abundance in spring and fall within the HFRS-endemic region have been available since fall 1971 through monitoring by Umeå University. Collection of these data is part of the National Environmental Monitoring Programme under the administration of the Swedish Environmental Protection Agency (*11*–*13*).

The number of HFRS cases increased during the 18 twelve-month study periods but with considerable interannual variation coupled with the 3- to 4-year population cycles of the bank vole (Figure 1). Before fall sampling in 2007, we forecast bank vole abundance (*7*) by multiplying the spring index (3.16 bank voles/100 trap-nights) with the expected population growth rate from spring to fall. Previous observations showed that the growth rate decreases gradually throughout the cycle. During the 10 previous bank vole cycles studied in the same phase during fall of 2007, i.e., year 2 in the cycle, the population increased by a factor ranging from 3.4 to 13.1; median was >5 (*11*–*13*). Trapping indices of fall 2006 and spring 2007 show a striking resemblance to those in 1972–73, when the bank vole population subsequently increased 4-fold during the summer of 1973 (*11*–*13*). A 4-fold increase was thus conceived as a reasonable assumption of the growth rate during the summer of 2007, giving an estimated fall trapping index of 12.64 in 2007. A more conservative assumption of a 3-fold increase gave an index of 9.48, and a bolder estimate based on a 5-fold increase gave an index of 15.80. Thus, the fall trapping index of 2007 would most likely range from 9.48 to 15.80 bank vole specimens per 100 trap-nights, which is 20%–95% higher than 7.64, the index in fall of 2006 (*7*). The trappings showed that the fall index attained in 2007 was 10.76, i.e., ≈41% higher than in the previous year (*13*).

Linear regression analysis showed a positive correlation between incidence of HFRS cases in Sweden and fall bank vole indices (Figure 2). Using the obtained regression equation including all seasons from 1989–90 through 2006–07 and considering an observed fall index of 10.76,

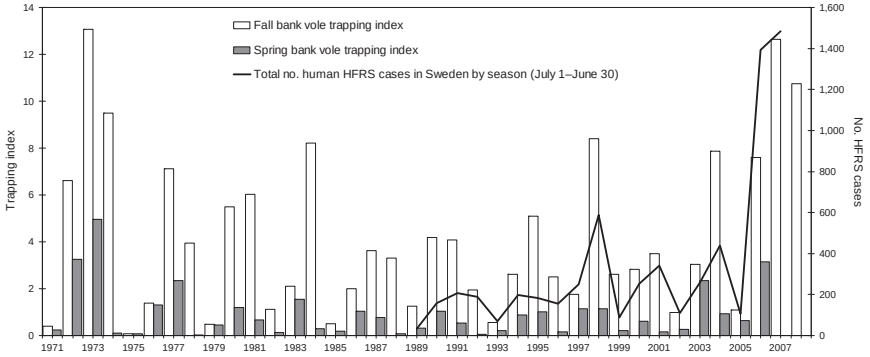

Figure 1. Human cases of hemorrhagic fever with renal syndrome (HFRS) by 12-month periods from July 1 through June 30 (black line), starting July 1989, when HFRS became reportable in Sweden, and ending June 2008. Bank vole trapping index in fall (white bars) and spring (gray bars) are shown from fall 1971 through 2007. Bar for fall 2007 represents predicted (*7*) trapping index. Bar on far right represents subsequently obtained actual trapping index.

we estimated that the number of HFRS cases during 2007–08 would reach 1,274. Based on the a priori predicted indices of 9.48, 12.64, and 15.80, the number of HFRS cases during 2007–08 was estimated to reach 1,152, 1,451, and 1,741, respectively. The total number of reported unique HFRS cases in Sweden during this period reached 1,483 (Figure 2).

The generally higher peaks and seemingly also higher trough levels in numbers of HFRS cases in Sweden during the past decade, compared with the preceding one (Figure 1), may partly be explained by a better recognition of clinical symptoms and improved diagnostic tools. However, we believe that the increase is real, and we advance 2 hypotheses, which are not mutually exclusive, to explain the general increase of HFRS cases and the unexpected high numbers of notified HFRS cases in 2006–07 and 2007–08.

First, there seems to have been an increase of low-phase abundance and spatial distribution of bank voles and a shorter duration of the low phase in later years (*12*). These 3 factors may in turn, acting alone or in concordance, have increased the rate of PUUV transmission and increased the successive build-up of infection rates among bank voles during the population cycles relative to the situation in the early 1980s (*8*), resulting in increased rates of human encounters with infectious voles. Second, as we recently suggested (*7*), milder winters (*14*) with a less persistent and less protective snow cover, either part of normal weather variation or caused by global warming, may trigger the behavior of bank voles to more frequently leave unfavorable natural habitats and enter human dwellings for shelter. Thereby, bank vole–human encounter rates and PUUV transmission to humans would increase. Also, among HFRS patients in Sweden who were confident about time and place of PUUV exposure, >80% claimed that they were exposed inside or adjacent to a human dwelling (*5*). Thus, we suggest that a combination of high reservoir numbers (Figure 1) and a sudden extensive loss of protective snow cover likely caused the abrupt increase of HFRS cases in midwinter 2006/07 (online Appendix Figure, available from www.cdc/gov/EID/content/15/1/104-appF.htm) because of the abundant infestations of human dwellings by bank voles. Under such circumstances, the virus may also circulate more successfully between aggregated conspecifics and thus further increase the risk to cohabiting humans, as observed in North America (*15*). The still elevated number of cases in 2007–08 was likely caused by excessive bank vole numbers in late 2007 but also seemed related to the loss of snow cover (in coastal areas only), which led to a less pronounced midwinter peak than in 2006–07.

Conclusions

We found a strong positive correlation and a repeated temporal pattern between bank vole abundance and HFRS cases in Sweden. We used monitoring data to first predict and later verify bank vole abundance in fall and also to

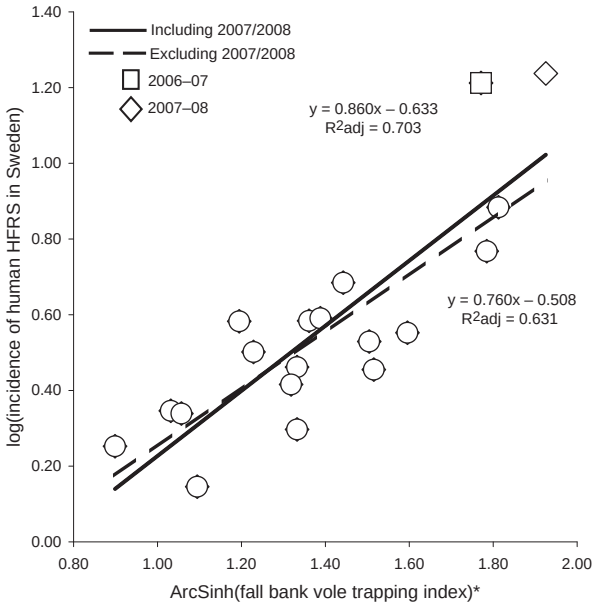

Figure 2. Linear regressions of incidence of human cases of hemorrhagic fever with renal syndrome in Sweden per 12-month period (July–June) from 1989 through 2007, based on fall bank vole trapping indexes from late September of the concurrent season ($p< 0.001$). *Because trapping indices are proportions, the arcsine transformation was used.

predict the risk to humans of acquiring HFRS during the fall/winter of 2007–08. Results obtained were used to raise public awareness and clinical preparedness, yet during July 2007 through June 2008, a total of 1,483 HFRS diagnoses were reported, a new high.

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Polyomaviruses KI and WU in Immunocompromised Patients with Respiratory Disease

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Polyomaviruses KI (KIPyV) and WU (WUPyV) were recently identified, mainly in respiratory specimens from children. Among 200 patients with respiratory disorders admitted to Saint Louis Hospital, Paris, France, KIPyV was detected in 8% and WUPyV in 1%. KIPyV was significantly more frequent among human stem cell transplant patients (17.8% vs. 5.1%; p = 0.01).

Recently, 2 new, distinct polyomaviruses (PyVs), KI (KIPyV) and WU (WUPyV), were identified in respiratory specimens, mainly from children <5 years of age with respiratory tract infections. The first retrospective studies of respiratory specimens in Sweden and Australia showed a KIPyV prevalence of 1% and 2.5%, respectively (*1*,*2*). Studies conducted in Australia and the United States showed a WUPyV prevalence in respiratory specimens of 3% and 0.7%, respectively (*3*). Further studies conducted in Canada and South Korea have shown similar frequencies (*4*,*5*). In this study, we examined the prevalence of KIPyV and WUPyV in immunocompromised patients with suspected respiratory tract infections.

The Study

From January through June 2007, 265 respiratory samples were received in the laboratory of Saint Louis Hospital, Paris: 154 nasal aspirates (NA) and 111 bronchoalveolar lavage (BAL) specimens collected from 200 patients with suspected upper or lower respiratory tract infections. This hospital specializes in the management of immunocompromised patients. Respiratory samples were collected for the diagnosis of acute respiratory illness; 89% of samples were from immunocompromised patients. Their median age was 46 years (range 3.6–85.3 years). Given the observational nature of the study, French law did not require ethical approval or informed consent.

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The specimens were routinely tested for influenza A and B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, and 3 by immunofluorescence assay (Imagen; DakoCytomation, Trappes, France). Specimens positive for KIPyV or WUPyV were tested for adenoviruses; human bocavirus; human rhinoviruses; human metapneumovirus; human coronaviruses OC43, 229E, NL63, HKU1; and human PyVs BK and JC by using PCR methods (*6*–*11*). Total nucleic acid was extracted from 200 μL of NA, BAL, or stool specimens by using the EasyMag System (bioMérieux, Marcy l'Etoile, France). KIPyV was detected with an in-house real-time PCR assay targeting the *VP1* gene. The primers and hydrolysis probe were designed by using Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). The final reaction volume was 25 μL and contained 12.5 pmol of SLKI-VP1s (5′-GGAAATACAGCTGCTCAGGAT-3′) and SLKI-VP1as (5′-CTTTGATACTTGAACCGCTTTCCTT-3′), 6.25 pmol of corresponding probe SLKI-VP1PR (5′-6FAM-C GTGACCCCACCCCTCATTACTGGTC-TAMRA-3′), 12.5 μL of TaqMan Universal Master Mix (Applied Biosystems), and 5 μL of DNA extract. The reaction was run on a 7500 Real-Time PCR System (Applied Biosystems). The specificity of positive specimens was confirmed by using PCR and nested PCR with primers POLVP1–39F/POLVP1–363R and POLVP1–118F/POLVP1–324R, as described (*1*). The PCR products were then sequenced and compared with the previously described sequences from Sweden and Australia (GenBank accession nos. EF127906, EF127907, EF127908, EF520287, EF520288, and EF520289). WUPyV was detected by PCR as described (*3*). PCR products with the expected molecular weights were sequenced by using primers AG0044 and AG0045 and compared to published sequences (GenBank accession nos. EF444550, EF444551, EF444552, EF444553, and EF444554) (*3*).

KIPyV was detected in 17 (6.5%) of the 265 respiratory samples and in 16 (8.0%) of the 200 patients. All cases were confirmed by a nested PCR targeting another region of the VP1 gene. Twelve of the 17 PCR products were successfully sequenced, and all shared 100% homology with published sequences. WUPyV was detected in only 2 patients (1.0%). Genome sequencing showed 98% homology with reported WUPyV sequences.

Six KIPyV-positive patients (37.5%) had co-infections with other respiratory viruses, and 2 of them (12.5%) had a pulmonary bacterial infection (online Appendix Table, available from www.cdc.gov/EID/content/15/1/107-appT.htm). One WUPyV-infected patient who exhibited acute respiratory failure had concomitant pneumonia caused by *Pseudomonas aeruginosa* infection. None of the 15 patients who were positive for KIPyV or WUPyV and tested for fungi had respiratory or blood samples positive for *Aspergillus* spp.

The clinical characteristics of the patients with KIPyV or WUPyV infection and their general outcome until December 2007 are given in the online Appendix Table. All but 3 had well-identified systemic immunosuppression. However, all had severe coexisting conditions. All KIPyV- and WUPyV-positive patients had acute respiratory disorders.

Eight KIPyV-positive patients had received allogeneic stem cell transplants; 5 of them had exhibited profound neutropenia in the 2 weeks before the respiratory sample was found to be positive. The detection of KIPyV was significantly more frequent among hematopoietic stem cell transplant (HSCT) recipients than among other patients (17.8% [8/45] vs. 5.1% [8/155], p = 0.01) (Table). Lung or sinus imaging was assessed by computed tomography scan for 12 KIPyV-positive patients. Lung parenchyma abnormalities were noted in 9 patients, and sinusitis was diagnosed for 2 patients.

Taking into consideration both the frequency of digestive symptoms in our patients and the former published detection of KIPyV in a stool sample, we looked for KIPyV infection in the available stool samples while the respiratory samples were being assessed for KIPyV (*1*). Strikingly, 3 of 4 samples tested were positive for KIPyV (with cycle threshold values of 35.0, 38.3, and 40.3). All were collected from HSCT recipients. In addition, 1 HSCT patient who experienced diarrhea demonstrated persistent excretion of KIPyV in 9 consecutive stool specimens collected between June and the end of November 2007 (data not shown).

Table. Frequency of KI polyomavirus detection in respiratory samples*

Sample source	KIV detection in respiratory samples, no. (%)		Total
	Positive	Negative	
HSCT patients	8 (17.8)†	37 (82.2)	45
Other patients	8 (5.2)	147 (94.8)	155
Total	16	184	200

*HSCT, hematopoietic stem cell transplantation.
†χ^2 test with Yates correction; p = 0.01.

Conclusions

This study shows the prevalence of KIPyV and WUPyV among immunocompromised patients with respiratory disorders. Previously, these 2 viruses had been observed mainly in young children (*1*–*3*). Of the few adult patients with KIPyV or WUPyV infection mentioned in these studies, most were immunocompromised (*3*,*12*).

Considering the seemingly higher prevalence of KIPyV in our population (8%), immunocompromised patients may be more susceptible to this PyV, as they are to JC and BK viruses (*13*–*15*). Results from previous reports suggest a similar frequency of both KIPyV and WUPyV infections being found in respiratory specimens, ranging from 1% to 3%. In contrast, in our series, we found a likely difference between the prevalence of KIPyV (8%) and WUPyV (1%), which suggests that the replication or reactivation of the 2 viruses in the respiratory tract may differ between immunocompromised and immunocompetent patients. However, this difference requires further investigation, in particular, by using similar real-time PCR assays. Notably, a significantly higher prevalence of KIPyV infection was found among HSCT patients, which suggests that a profound T-cell deficiency may be a factor in facilitating KIPyV replication.

As reported in other populations, our patients who yielded positive specimens for KIPyV or WUPyV had conditions ranging from a common cold to acute respiratory distress that required invasive ventilation. Respiratory coinfections, observed in other studies, had likely accounted for at least some clinical features. In the 7 of our patients in whom KIPyV was the sole pathogen detected in the respiratory tract, despite comprehensive screening for viruses, bacteria, parasites, and fungi, clinical and radiographic patterns were varied. Some of the patients had only upper respiratory tract infections, notably sinusitis, whereas others had lung parenchyma abnormalities as defined by computed tomographic scan imaging. However, due to the retrospective nature of the study, and therefore the lack of a control group of immunocompromised patients without respiratory symptoms, the association of KIPyV infection with the occurrence of respiratory disease cannot be stated definitively.

In conclusion, the seemingly higher frequency of KIPyV shedding in immunocompromised patients (as observed with other PyVs) and the detection of KIPyV as a single pathogen in respiratory disease (e.g., as cytomegalovirus recurrence can lead to pneumonia in immunocompromised patients) together support a reactivation hypothesis. Nevertheless, a reinfection hypothesis cannot be excluded due to immunocompromised patients' increased risk of acquiring viral infection from exogenous sources

Controlled prospective studies of KIPyV shedding before and during immunosuppression will help determine the pathogenic role of this virus. The clinical implication of KIPyV detection in stools and the mechanisms underlying the concomitant presence in gastrointestinal and respiratory tracts also deserve further analysis.

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Dr Mourez is a virologist in the Laboratory of Microbiology, Saint Louis University Hospital, Paris. His research interests include the development of tests for the diagnosis of emerging respiratory viruses and the study of the circulation and molecular analysis of human respiratory viruses in pediatric and immunocompromised patients.

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Hepatitis E Virus Genotype 3 Diversity, France

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We characterized 42 hepatitis E virus (HEV) genotype 3 strains from infected patients in France in 3 parts of the genome and sequenced the full-length HEV genotype 3f genome found in Europe. These strains are closely related to swine strains in Europe, which suggests zoonotic transmission of HEV in France.

Hepatitis E is a water-borne infection in developing countries and is believed to spread zoonotically in industrialized countries (*1*). Hepatitis E virus (HEV) is a positive-sense RNA virus that belongs to the family *Hepeviridae* (*2*). The coding region consists of 3 discontinuous open reading frames (ORFs). One region within ORF1, the hypervariable region, displays substantial genetic diversity (*2*). HEV strains are classified into 4 genotypes, and it was recently proposed that HEV genotypes are divided into 24 subtypes (*3*). Although genotypes 1 and 2 have been found exclusively in humans, genotypes 3 and 4 have been found in humans and animals such as pigs, boars, and deer. Genotypes 1 and 2 have been isolated in tropical and subtropical countries in Asia, Africa, and America; genotype 4 has been found only in Asia. Genotype 3 has been identified almost worldwide, but the distribution of its 10 subtypes varies greatly. Subtypes 3a and 3j strains have been mainly identified in North America; 3b, 3d, and 3g strains in Asia; and 3c, 3e, 3f, 3h, and 3i strains in Europe (*3*). The finding of several cases of HEV infection in southern France (*4*,*5*) prompted us to characterize the diversity of the strains and to determine the full-length sequence of the most prevalent genotype in this area.

The Study

We studied 42 HEV strains from patients at Toulouse University Hospital, France. None of the patients had traveled abroad during the previous 6 months or reported any contact with pigs before the onset of the disease. HEV infection was determined by detecting HEV RNA by using molecular tools (*5*). We sequenced 2 fragments within ORF1, the hypervariable region, and the RNA-dependent RNA polymerase (RdRp) genes as previously described (*6*,*7*). A 348-nt fragment of ORF2 was amplified according to the protocol of Inoue et al. (*8*). The whole genome was amplified with 6 overlapping reverse transcription–PCRs. The primers are listed in Table 1. The reverse transcription conditions were 50°C for 30 min and 85°C for 5 min. The PCR cycling conditions were initial denaturation at 94°C for 2 min, then 50 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec, and elongation at 68°C for 3 min.

The phylogenetic tree obtained for the ORF2 region showed that all strains belonged to genotype 3 (Figure 1, panel A). A total of 37 strains (TLS1–TLS37) segregated as a distinct clade among genotype-3 reference sequences, whereas 5 other strains (TLS38–TLS42) formed distinct branches in the tree. TLS1–TLS37 could be subtyped as 3f, according to the classification proposed by Lu et al. (*3*). The nucleotide sequences of our local 3f strains and the strains from Spain and the Netherlands, which had been identified in humans, pigs, and sewage of animal origin, had 90.2%–95.3% identity. The 5 remaining strains were also genetically related to swine strains. Strains TLS38 and TLS42 were more closely related to 3e strains from swine in Great Britain (88.5%–92.1% nucleotide sequence identity). These strains were also closely related to Japanese strains AB248520 and AB248522, which may have a British origin (87.5%–93.4% identity) (*9*). Strains TLS40 and TLS41 were located on the same branch as swine HEV isolates identified in the Netherlands and classified as subtype 3c. The nucleotide sequence of TLS41 had 91.7%–92.7% identity with that of strains from pigs in the Netherlands, whereas that of TLS40 was only 85.5%–86.1% identical to that of pig strains from the Netherlands. Strain TLS39 was similar to the 3b strains identified in Japan in human patients or animals (86.8%–90.1% nucleotide sequence identity).

The topology of the phylogenetic tree obtained for the RdRp region was similar to that of ORF2 (Figure 1, panel B). All the strains clustered with genotype 3 strains. The same 37 strains (TLS1–TLS37) clustered together (87.9%–99.1% nucleotide sequence identity) and the same 5 strains (TLS38–TLS42) appeared to be more divergent.

The hypervariable region gave the same clade of 37 strains in the phylogenetic tree, whereas the 5 remaining strains were more divergent (Figure 2, panel A). Strains TLS1–TLS37 exhibited only 70.1%–86.8% nucleotide sequence identity in this part of the genome, which shows the great diversity of this region. According to the primers position (*6*), a fragment of 345 nt was expected for all the

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Table 1. Primers used to amplify the whole hepatitis E virus genotype 3f genome

Fragment size, bp	Nucleotide position*	Sense primers (5′ → 3′)	Antisense primers (5′ → 3′)
990	1–990	TAGGCAGACCACGTATGTGGTCGATGCCATGGA	GCCGGTCCCAGATRTGSACCGGRA
1,294	878–2172	ACAGAGGTGTATGTTAGATCCATATTTGGC	GGGGAGAAGTCGCTAGAGAAACCTGATGT
2568	2001–4569	CCCAGCGSCWTTCGCTGACCGG	CGGATAAGCCACTGGGGCATGCCRCACT
736	4542–5278	AGTGYGGCATGCCCCAGTGGCTTATCCG	GCCGGTGGCGCGGGCAGCATAGGCA
1,480	5003–6484	ACGAATGTYGCGCAGGTYTGTGT	CCCTTRTCCTGCTGNGCATTCTCGACAGA
964	6363–7327	GACAGAATTRATTTCGTCGGC	TTTCCMGGGRGCGCGGAACCCCGAA

*Nucleotide position refers to Burma strain, M73218.

strains. Actually, the fragments obtained after amplification of the hypervariable region of the genotype 3f strains varied from 435 nt for 29 samples, to 412 nt for 3 samples, and to 348 nt for 5 samples. For the 3e strains, the fragments were 387 nt and 412 nt. The PCR product from the hypervariable region was 345 nt for only the 3c and 3b strains.

The complete genome from TLS25 strain (genotype 3f) was amplified. The genomic length was 7,321 nt with the 3 major ORFs. Comparison with full-length reference sequences showed an insertion of ≈90 nt in the hypervariable region. Phylogenetic analysis of the sequences of HEV genotypes 1–4 strains confirmed that TLS25 belonged to

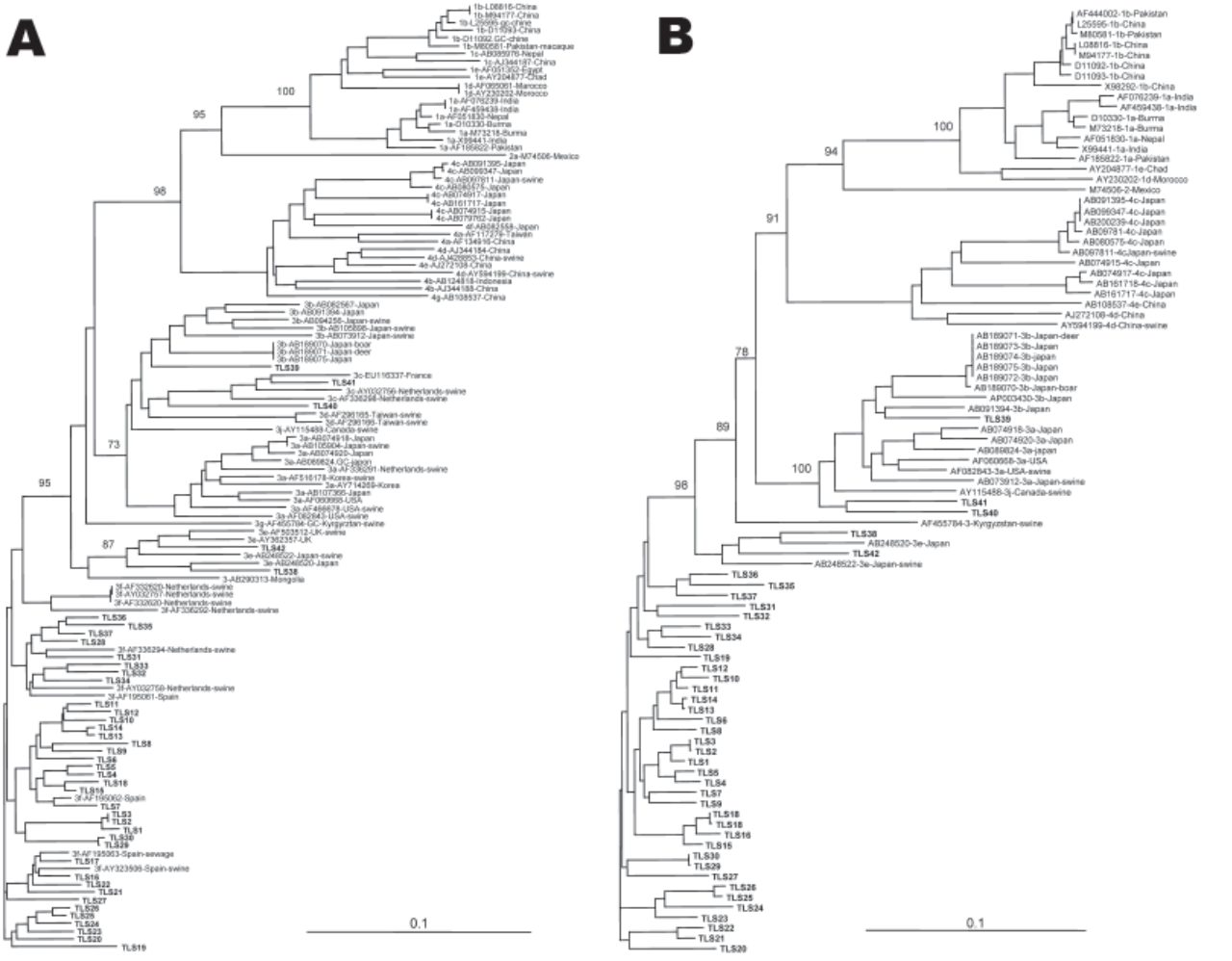

Figure 1. Phylogenetic relationship among hepatitis E virus (HEV) strains from southwestern France and reference strains available in GenBank based on a 348-nt sequence in the open reading frame 2 (A) and on a 383-nt sequence of HEV RNA–dependent RNA polymerase (B). Genetic distances were calculated by using the Kimura 2-parameter method; phylogenetic trees were plotted by the neighbor-joining method. The reproducibility of the branching pattern was tested by bootstrap analysis (1,000 replicates). Each branch was labeled with the GenBank accession number of the strain, the geographic area, and the host, if nonhuman, and the geographic area where the sequence was isolated. The genotype and subtype were identified according to Lu et al. (*3*). Scale bars represent nucleotide substitutions per site. **Boldface** indicates the G3 French strains.

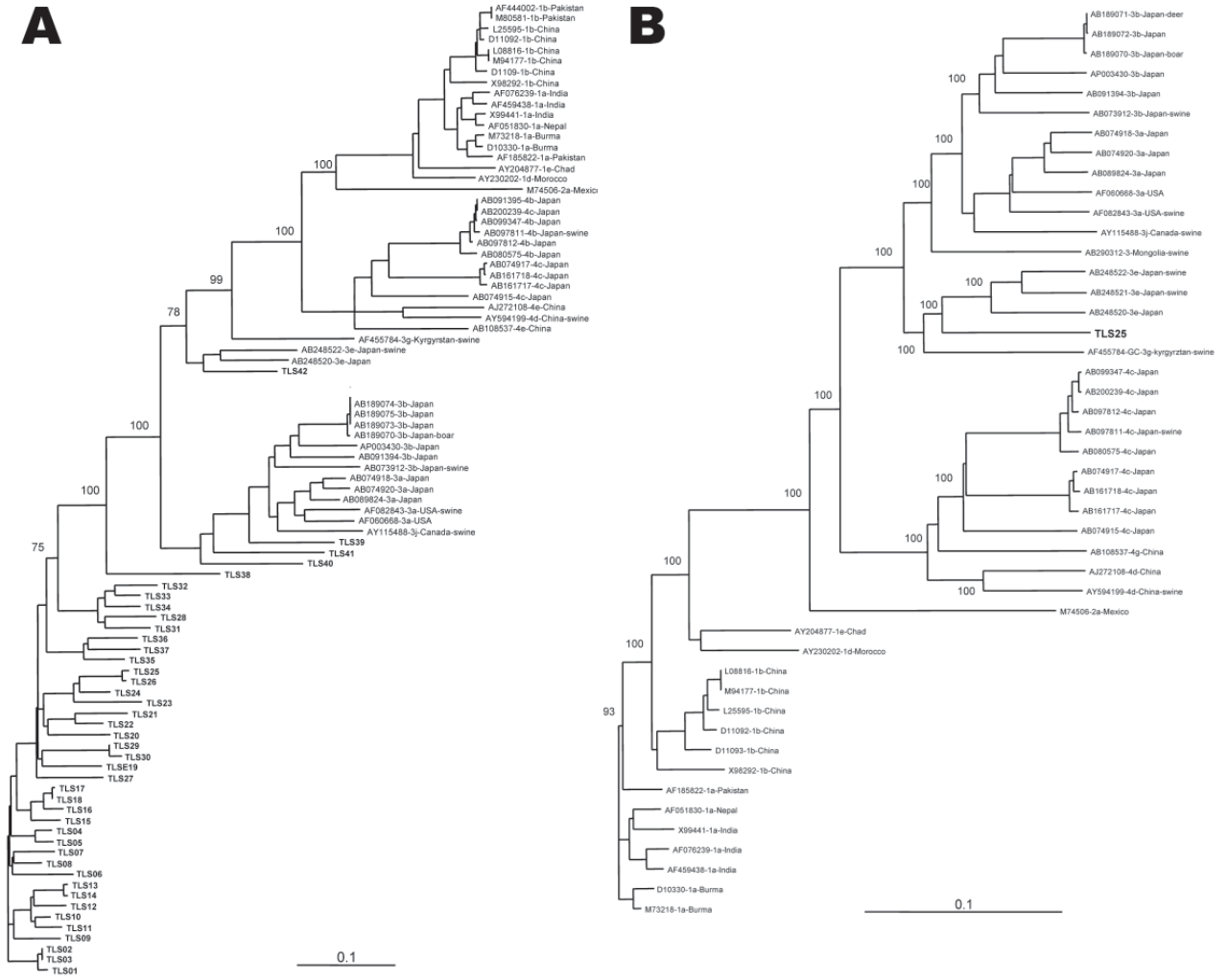

Figure 2. Phylogenetic relationship among hepatitis E virus (HEV) strains from southwestern France based on a 345-nt sequence of HEV hypervariable region (A) and on the full-length sequence of TLS25 and HEV strains whose entire sequence is known (B). Genetic distances were calculated by using the Kimura 2-parameter method; phylogenetic trees were plotted by the neighbor-joining method. The reproducibility of the branching pattern was tested by bootstrap analysis (1,000 replicates). Each branch was labeled with the GenBank accession number of the strain, the geographic area, and the host, if nonhuman, and the geographic area where the sequence was isolated. The genotype and subtype were identified according to Lu et al. (*3*). Scale bars represent nucleotide substitutions per site. **Boldface** indicates the G3 French strains.

genotype 3 and was genetically distinct from the genotype 3 strains found in Asia and in the United States (Figure 2, panel B). Comparisons with the complete genome sequences of HEV indicated that strain TLS25 shared 72.0%–72.9% nucleotide sequence identity with genotype 1 strains, 72% nucleotide sequence identity with genotype 2 strain, and 73.7%–74.5% nucleotide sequence identity with genotype 4 strains (Table 2). Strain TLS25 shows 83.1%–84.3% nucleotide sequence identity with HEV genotype 3e, 81.8% identity with the HEV strain 3g, 79.7%–80.3% identity with HEV genotype 3b, and 79.5%–81.8% identity with HEV genotype 3a over the entire genome. No recombination event was detected within the TLS25 strain by using the Recombinant Detection Program (http://darwin.uvigo.es/rdp/rdp.html). The sequences were deposited in GenBank under accession nos. EU495106–EU495232.

Conclusions

Most (88%) of the HEV strains in France belonged to 3f subtype, but 3c, 3e, and 3b strains were also identified. Phylogenetic analyses indicated that HEV strains in France were related to swine strains previously identified in Europe. This full-length sequencing of an HEV genotype 3 strain from Europe showed it to be distinct from the genotype 3 strains found on other continents, which illustrates the great diversity of this genotype. Due to an insertion in

Table 2. Nucleotide sequence identity between TLS25 (hepatitis virus E genotype 3f) and available full-length genomes

GenBank accession no.	Country	Origin	Genotype	Sequence homology, %
M73218	Burma	Human	1a	72.7
D10330	Burma	Human	1a	72.8
AF051830	Nepal	Human	1a	72.7
AF076239	India	Human	1a	72.3
X99441	India	Human	1a	72.6
AF459438	India	Human	1a	72.5
AF185822	Pakistan	Human	1a	72.3
L08816	China	Human	1b	72.5
M94177	China	Human	1b	72.7
L25595	China	Human	1b	72.7
D11093	China	Human	1b	72.5
D11092	China	Human	1b	72.8
X98292	China	Human	1b	73.0
AY230202	Morocco	Human	1d	72.9
AY204877	Chad	Human	1e	72.1
M74506	Mexico	Human	2a	72.0
AB200239	Japan	Human	4c	74.2
AB099347	Japan	Human	4c	74.2
AB097811	Japan	Swine	4c	74.3
AB097812	Japan	Human	4c	74.2
AB080575	Japan	Human	4c	73.6
AB074915	Japan	Human	4c	74.5
AB074917	Japan	Human	4c	74.4
AB161717	Japan	Human	4c	74.1
AB161718	Japan	Human	4c	74.1
AB108537	China	Human	4g	74.4
AJ272108	China	Human	4e	73.7
AY594199	China	Swine	4d	74.1
AF455784	Kirgizstan	Swine	3g	81.8
AB074918	Japan	Human	3a	80.4
AB074920	Japan	Human	3a	80.1
AB089824	Japan	Human	3a	80.6
AF082843	USA	Swine	3a	80.5
AF060668	USA	Human	3a	79.5
AB189070	Japan	Boar	3b	80.1
AB189071	Japan	Deer	3b	79.9
AB189072	Japan	human	3b	79.7
AP003430	Japan	human	3b	80.1
AB073912	Japan	Swine	3b	80.2
AB091394	Japan	Human	3b	80.3
AY115488	Canada	Swine	3j	80.2
AB248522	Japan	Swine	3e	84.2
AB248521	Japan	Swine	3e	83.1
AB248520	Japan	Human	3e	84.3
AB290312	Mongolia	Swine	3	79.7

the hypervariable region, the TLS25 sequence is longer than other HEV sequences available in GenBank. A variation in the length of the hypervariable region in 1 human strain of genotype 3e was previously reported (*9*). Because the function of this region of the genome is still unknown, the effect of such insertions on virus biology has yet to be elucidated. Zhai et al. reported that phylogenetic analyses within the RdRp region correlated well with the results from the phylogenetic analyses of the complete genome (*7*), whereas Lu et al. found that the ORF2 region was the region that determined most accurately genotypes and subtypes (*3*). Our data indicate that both regions can be used to determine the genotype and subtype. The source of autochthonous hepatitis E infection in industrialized countries is unknown. One hypothesis, supported by molecular epidemiologic studies, is that it is an emerging zoonotic infection (*10*,*11*). Contamination with HEV may be linked to occupational exposure (*12*,*13*), consumption of undercooked meat (*14*,*15*), or exposure to a contaminated environment.

Our study characterized several human HEV strains and sequenced the full-length HEV 3f genome. These strains were closely related to European swine strains. Pro-

spective in-depth epidemiologic studies based on structured interviews are ongoing to clarify the routes of transmission in southwest France.

Dr Legrand-Abravanel is a researcher in the Virology Department at Tououse University Hospital. Her primary research interest is the genetic variability of hepatitis viruses.

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Falciparum Malaria in Patient 9 Years after Leaving Malaria-Endemic Area

To the Editor: A 30-year-old African man, without any specific medical history, came to the emergency department of Brugmann University Hospital in Brussels, Belgium, on March 18, 2008, because of malaise, profuse transpiration, and dizziness that day. He also reported a 3-day history of muscle pain and pain while urinating, for which his general practitioner prescribed ciprofloxacin. Originally from Guinea-Conakry, the patient reported no travel outside Belgium after his arrival >9 years earlier. No recurrent fever episodes were noted during this period. Two weeks before becoming ill, a friend visiting from Guinea-Conakry stayed at his home for 7 days.

Results of a physical examination were normal, except for a temperature of 37.5°C. Blood analysis showed moderate anemia (hemoglobin 12.9 g/dL) and thrombocytopenia (platelet count 73,000/μL) with total bilirubin and C-reactive protein levels of 1.5 mg/dL and 7.0 mg/dL, respectively. Because we suspected subfebrile malaria in this patient, a blood smear was prepared. It showed ring-shaped trophozoites of *Plasmodium falciparum* with a parasite density of 0.1%. Serologic tests showed an antibody titer to *Plasmodium* spp. of 3,200. A blood sample was sent to the national reference laboratory at the Institute of Tropical Medicine in Antwerp. The diagnosis of *P. falciparum* malaria was confirmed by microscopy and real-time PCR. Follow-up was uneventful because the patient responded to a 7-day course of oral quinine and doxycycline.

Malaria, a potential life-threatening disease caused by *P. falciparum*, usually occurs within 2 months after the bite of an infective mosquito. A few reports mention a delay of ≥1 year between exposure and initial clinical symptoms, probably related to the disappearance of residual protective immunity in immigrants (*1*,*2*). Impaired immunity has also been linked to late malaria, implicating a chronic low-grade *P. falciparum* infection that becomes clinically evident in an immunocompromised person (*3*). In addition, several cases of malaria without any travel history to a malaria-endemic region have been described. A possible explanation for this type of malaria is exposure to an imported *Anopheles* spp. mosquito, referred to as airport, luggage, or container malaria (*4*). Transmission by indigenous anopheline mosquitoes when weather conditions are favorable has been reported in some European countries (*5*,*6*). Cases of *P. falciparum* malaria without any evidence of a mosquito bite have been reported and related to transfusion of parasitized erythrocytes, intravenous drug use, or accidental needlestick injuries (*4*).

We report a clinically atypical case of late *P. falciparum* malaria that may have been contracted by the bite of an anopheline mosquito captured in the luggage of the patient's visiting friend (*7*). Unreported travel to a malaria-endemic region was possible but unlikely because our patient stayed illegally in Belgium and leaving the country would risk being repatriated to Guinea-Conakry. Indigenous malaria was excluded because he became ill during the winter, a time when proliferation of local *Anopheles* spp. in Belgium is difficult. The patient did not receive any recent blood transfusions and denied being an intravenous drug user, although this possibility cannot be excluded.

This type of malaria, also known as luggage or suitcase malaria, makes adequate and timely diagnosis difficult because a history of exposure to a possibly malaria-infected mosquito is apparently absent. Moreover, our patient had few classic symptoms or signs, such as high-grade fever, chills, or headaches; this pattern complicates diagnosis. This lack of typical malaria symptoms may be related to the fact that before coming to the hospital, the patient took ciprofloxacin, which has in vitro activity against *P. falciparum*. Another possible reason is residual immunity to malaria, which was no longer protective but still capable of attenuating symptoms or signs of malaria. This finding implicates recrudescence of disease after a long period of asymptomatic infection with *P. falciparum*. Serologic analysis detected high levels of antibodies to *Plasmodium* spp., which suggests a chronic infection rather than a new one.

It is generally accepted that protective immunity wanes after several months of nonexposure. Support for this thesis is the frequency of clinical malaria in African adults who visit their families after a long stay in a country where the disease is not endemic. However, these cases might be caused by antigenic variation of *P. falciparum* in the area visited, which would enable the parasite to evade the host's immune response (*8*). Residual immunologic memory against *P. falciparum* has been suggested, which would link persistent immunity with late recrudescence or with less severe or complicated disease in immigrants (*2*,*9*). Moreover, *P. falciparum* has been transmitted through blood transfusions from donors from malaria-endemic regions several years after exposure, which suggests long subpatent periods (*10*).

This case highlights the problem of diagnosing *P. falciparum* malaria in patients without a recent travel history to malaria-endemic areas. In such cases, autochthonous malaria, whether transmitted by an imported or an indigenous mosquito or by infected blood cells or needles, should be excluded. Residual protective immunity, even after several years of nonexposure to *P. falciparum*, may explain persistent asymptomatic infection and late recrudescence of disease.

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Linezolid-Resistant *Staphylococcus cohnii*, Greece

To the Editor: Since 2003, linezolid has typically been used to treat infections caused by multidrug-resistant gram-positive cocci such as vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* (*1*). In Greece, a major problem is nosocomial dissemination of vancomycin-resistant enterococci. Use of linezolid for the treatment of such infections led to the emergence of linezolid–vancomycin resistant *E. faecium*; however, linezolid resistance of staphylococci is still relatively low in this country (*2*). We describe an outbreak caused by a linezolid-resistant *S. cohnii* in an intensive care unit (ICU) in Greece.

From July through October 2007, nonrepetitive coagulase-negative staphylococci that exhibited resistance to linezolid, were isolated from blood cultures from 4 separate patients hospitalized in the ICU at Sismanoglion General Hospital of Athens, a 450-bed tertiary care hospital. The ICU is a 10-bed, level II unit, comprising 2 rooms with 1 bed each and 2 rooms with 4 beds each. Each isolate was recovered in 2 of 2 blood culture sets per patient, indicating true bacteremia. The demographic and clinical information for the patients is described in the Table. The mean duration of time preceding linezolid therapy was 22 days.

Isolates were first identified to the species level by using an API Staph system (bioMérieux, la Balme les Grottes, France) and a molecular method based on the *tuf* gene followed by sequencing analysis (*3*). Susceptibility testing for various antimicrobial agents was performed by disk diffusion and using Clinical Laboratory Standards Institute criteria; susceptibilities were interpreted according to Institute guidelines (*4*). In addition, MICs to oxacillin, vancomycin, teicoplanin, quinupristin-dalfopristin, linezolid, daptomycin, and tigecycline were determined by Etest (AB Biodisk, Solna, Sweden) according to manufacturer's instructions. Resistance genes *mecA*, *vat*, *vga*, *erm*, *aac(6′)-Ie+aph(2″)*, *ant(4′)-Ia*, and *aph(3′)-IIIa*, as markers for resistance to β-lactams, dalfopristin, macrolides, and aminoglycosides, were identified by PCR as previously reported (*5*,*6*). The presence of G2576T in domain V of the 23S rRNA, which is mainly associated with linezolid resistance in clinical isolates, was detected by using PCR and digestion of the product with *NheI* (*2*). The number of mutated versus nonmutated alleles was determined as described by Pillai et al. (*7*). In addition, isolates were examined for the presence of the *cfr* gene, which was found to be correlated with linezolid resistance in some coagulase-negative staphylococci and for mutations of ribosomal protein L4, L22 genes (*8*,*9*). Clonality of isolates was assessed by pulsed-field gel electrophoresis (PFGE) after digestion of chromosomal DNA with *SmaI* (*2*).

The molecular method identified the isolates as *S. cohnii* subsp. *ureolyticus*. The API Staph system has correctly identified 2 of them; the re-

Table. Clinical characteristics of 4 patients from whom linezolid-resistant *Staphylococcus cohnii* was isolated, Greece, 2007*

Patient no.	Gender/ age, y	Medical history	Reason for hospitalization	Previous treatment	Date of hospital admission	Date of sample collection	Outcome	Date of death or discharge
1	F/82	No relevant history	Acute cholecystitis, necrotizing pancreatitis	CIP, CAZ, IMP, TZP, LIN	May 10	Jul 20	Death	Aug 21
2	M/38	Alcoholism	Lung abscess	CIP, CAZ, LIN	Jul 14	Aug 21	Recovery	Oct 20
3	M/52	COPD, melitensus diabetes	Necrotizing pneumonia	TZP, CLI, IMP, LIN	Aug 21	Sep 25	Recovery	Oct 30
4	M/65	No relevant history	Septic shock	IMP, CIP, TZP, LIN	Sep 17	Oct 28	Recovery	Dec 10

*CIP, ciprofloxacin; CAZ, ceftazidime; IMP, imipenem; TZP, piperacillin-tazobactam; LIN, linezolid; CLI, clindamycin; COPD, chronic obstructive pulmonary disease.

maining 2 isolates were falsely characterized as *S. xylosus*. According to disc diffusion test results, the isolates were resistant to cefoxitin, oxacillin, penicillin, rifampin, quinupristin-dalfopristin, erythromycin, clindamycin, fusidic acid, tobramycin, gentamicin, and linezolid. MICs were linezolid 32, oxacillin 256, quinupristin-dalfopristin 8, vancomycin 2, teicoplanin 2, tigecycline 0.125, and daptomycin 0.5 mg/L. Molecular methods detected the following resistance genes: *mecA, ermA, aac(6´)-Ie+aph(2″),* and *aph(3´)-IIIa.* The isolates, despite their resistance to streptogramins, were negative for *vat* and *vgaA* genes. In addition, all isolates carried the G2576T mutation and had 4 of 5 mutated alleles. No isolate carried the *cfr* gene or any mutation on ribosomal protein L4 and L22 genes. PFGE results indicated that all isolates were clonally related, belonging to the same clone.

Outbreaks caused by linezolid-resistant staphylococci are rare worldwide (*10*); in Sismanoglion Hospital, before the outbreak period, no linezolid-resistant staphylococci and enterococci had been isolated. However, in the ICU, a statistically significant increase in the use of linezolid was observed in 2004 and in 2007 (0.58 vs. 1.34 defined daily doses/100 patient-days, respectively); heavy use of linezolid may have created substantial selection pressure in favor of the linezolid-resistant isolates.

The 4 patients were treated in the same room by the same personnel; thus, a potential explanation for this outbreak is patient-to-patient transmission of linezolid-resistant strains on the hands of healthcare personnel. However, cultures of ICU personnel (nasal cavity and hands) grew only methicillin-resistant *S. aureus* and methicilllin-resistant *S. epidermidis*. In addition, environmental samples taken from the beds and the equipment of these patients were negative for *S. cohnii*. Strict control measures were taken (e.g., isolation of infected patients, increased environmental cleaning, and reinforcement of proper glove and gown use and hand disinfection with alcohol gel), and the outbreak strain was not recovered from other patients in the ICU or in other departments of the hospital after the initial outbreak. In conclusion, to avoid spread of staphylococcal resistance in ICUs, measures such as hand hygiene and adequate central venous catheter handling should be taken, and policies regarding antimicrobial drug use must be applied.

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Buruli Ulcer in Long-Term Traveler to Senegal

To the Editor: Buruli ulcer (BU) is caused by infection of subcutaneous fat with the environmental pathogen *Mycobacterium ulcerans*. BU has been reported or suspected in more than 30 countries. It has never been reported in Senegal and Guinea-Bissau (*1*). We report a case of travel-associated BU in a French traveler to Senegal.

The patient was a 24-year-old Caucasian man who came to the University Hospital of Bordeaux, France, with a nonhealing lesion on the anterior left leg that had been present for ≈12 weeks. The patient had traveled in Senegal to the border of Guinea-Bissau from September 2006 through August 2007. His trip had begun in Dakar and proceeded south to the districts of Kaolack, Toubacouta, and Casamance. The patient stayed in Casamance during the rainy season from June 2007 through August 2007. He had been working on construction of wood dugouts, had been bare-legged regularly, and had been in contact with stagnant water.

He first noticed a lesion during June 2007, which had gradually increased to a small, centrally crusted ulcer. By the end of August 2007 (week 8 of the lesion), skin examination showed a 3 × 6-cm necrotizing ulcer with central crusting and an erythematous border (Figure). The lesion was not warm or tender but generated a seropurulent discharge. Concurrently, palpable left inguinal lymph nodes were observed.

Bacteriologic swabs identified *Staphylococcus aureus* and group A *Streptoccocus pyogenes*. Two punch-biopsy specimens were taken from the border of the lesion. Histologic analysis showed nonspecific acute and chronic dermal inflammation with necrotizing granulomas that extended into the subcutaneous tissues, suggestive of infection with atypical *Mycobacterium* spp. Bacteriologic examination did not identify acid-fast bacilli (negative direct smear result after Ziehl-Neelsen staining) or other specific microorganisms (negative direct smear results after periodic acid–Schiff, Giemsa, and Gram staining). Tissue specimens were placed into BACTEC 12B broth (Becton Dickinson, Franklin Lakes, NJ, USA) (incubated at 35°C) and onto Löwenstein-Jensen slants (incubated at 30°C). No growth was detected after 42 days. On the basis of clinical findings, we suspected a diagnosis of BU.

Taq-Man real-time quantitative PCR that used primers for 2 *M. ulcerans*–specific genes (insertion sequence *2404* and ketoreductase B gene) (*2*,*3*) and negative controls showed positive results for DNA from both biopsy specimens. A normalized standard curve was constructed, which indicated a bacterial load of ≈6×10^3 organisms/g of tissue.

Laboratory investigations indicated a total leukocyte count of 16,400 cells/μL (reference range 3,600–10,000 cells/μL) and a C-reactive protein level of 0.59 mg/mL (reference value <0.01 mg/mL). Results of radiologic investigations were normal. The

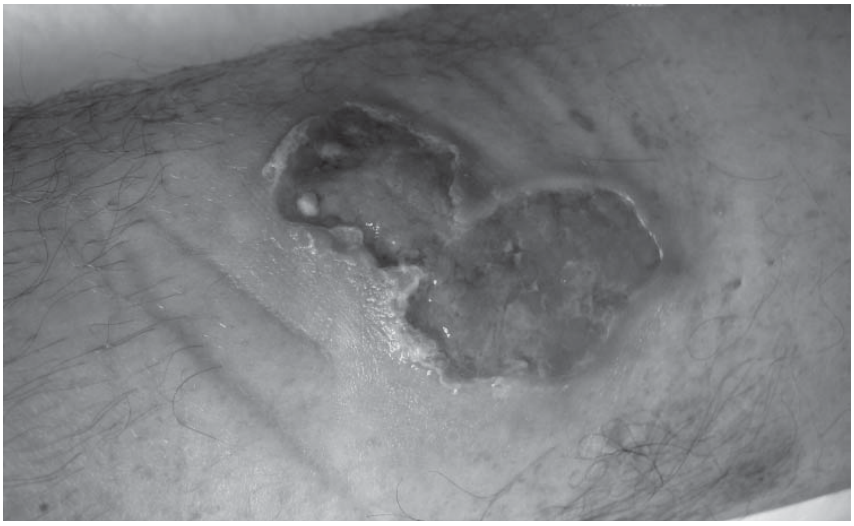

Figure. Ulcer (3 × 6 cm) on anterior side of the left leg of the patient, showing an erythematous border.

patient was treated with rifampin (600 mg/day) and moxifloxacin (400 mg/day) for 12 weeks. Additional surgical excision was planned 4 weeks after treatment was begun. Unfortunately, 15 days later, the patient was lost to follow-up.

BU has been reported in many West African countries, with Guinea being the northern limit of reported cases. Detection of this case of BU suggests that the region in West Africa endemic for this disease has been underestimated or is expanding. Infection in the traveler may have occurred in Casamance, if one assumes an incubation period of 6 weeks to 3 months. Further cases should be actively sought in this region and adjoining districts visited to evaluate the geographic extent of the disease.

The environmental reservoir and mode of transmission of BU in our patient are unknown. Exposure of unprotected skin with stagnant or slow-flowing water is linked with BU. Our patient reported prolonged contact with water during his occupation. Recent studies implicating aquatic predator insects (*4*,*5*) and mosquitoes (*6*) in transmission of BU suggest that use of insect repellents and protective clothing may help prevent infection.

The diagnosis of BU in this patient relied on the PCR detection of 2 *M. ulcerans*–specific genes; this procedure is considered adequate (*7*,*8*). The relatively low number of organisms detected may explain the negative acid-fast bacilli smear and culture results (*9*). Our report of *M. ulcerans* infection from Senegal is not surprising because southern Senegal shares similar ecologic features with neighboring affected countries, especially during the heavy rainy season.

Although BU is a disease that affects mainly persons in recognized disease-endemic areas, this case emphasizes that tropical skin ulcers should be considered in differential diagnosis of BU in travelers returning from disease-endemic countries (*1*,*10*). Diagnostic delays can be avoided by use of *M. ulcerans*–specific PCR, a test available from World Health Organization collaborating laboratories, which enables rapid confirmation of diagnosis of BU.

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Evidence of Maternal–Fetal Transmission of *Parachlamydia acanthamoebae*

To the Editor: *Parachlamydia acanthamoebae* is a recently identified agent of pneumonia (*1*–*3*) and has been linked to adverse pregnancy outcomes, including human miscarriage and bovine abortion (*4*,*5*). Parachlamydial sequences have also been detected in human cervical smears (*4*) and in guinea pig inclusion conjunctivitis (*6*). We present direct evidence of maternal–fetal transmission of *P. acanthamoebae*.

We tested 78 amniotic fluid samples from patients who delivered prematurely (defined as spontaneous delivery before 37 weeks of gestation) at the Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, from 2003 to 2006. DNA was extracted by using the QIAampDNA Mini kit (QIAGEN, Hilden, Germany) and was tested by using a specific *Parachlamydia* real-time PCR (*7*). One positive sample (threshold cycle of 34.2) was confirmed by using the 16SigF-Rp2Chlam PCR, which targets a large DNA segment of the 16S rRNA gene (*8*). Because these 2 PCRs target different DNA segments, the positive PCR results were not due to PCR contamination with amplicons. The sequence exhibited 99.6% similarity with *P. acanthamoebae* strain Hall's coccus (*1*) (GenBank accession no. AF366365).

The sample was obtained from a 29-year-old woman during her second pregnancy, at 16 weeks of gestation. Amniocentesis was performed because a first-trimester test suggested a Down syndrome risk of 1/100. At the time of amniocentesis, the patient had cough and flu-like symptoms of 3 weeks' duration, which resolved spontaneously in a few weeks. Cytogenetic analysis showed a normal 46XX karyotype; amniotic fluid culture remained sterile. The pregnancy ended prematurely at 35 weeks and 6 days with the vaginal delivery of a 2,060-g newborn (<5th percentile). The mother and child had an uneventful hospital course.

The role of *Parachlamydia* as the etiologic agent of premature labor and intrauterine growth retardation is likely because 1) all vaginal, placental, and urinary cultures were negative; 2) results of routine serologic tests were negative; and 3) only *Parachlamydia* was detected in the amniotic fluid. Intrauterine infection caused by *Parachlamydia* spp. may be chronic and asymptomatic until adverse pregnancy outcomes occur (*4*).

The infection of this pregnant woman might have occurred through zoonotic contact through her work as a butcher in a rural area known for cattle breeding. Of interest, a recent study of 482 healthy Swiss men found 3 who were seropositive for *Parachlamydia* sp., and all 3 came from the same rural area near Lausanne (within <20-km radius) (*9*). Moreover, the patient owns 2 guinea pigs, potential vectors of the bacterium (*6*). Other modes of transmission are possible, e.g., contaminated water (free-living amebae may serve as hosts for *Parachlamydia* spp. and are widespread in water networks) and ingestion of undercooked meat or contaminated cow milk.

A plausible pathogenic scenario for this case of possible maternal–fetal transmission of *P. acanthamoebae* might include bacteremia in the context of a lung infection. This could have resulted in intrauterine infection and intrauterine growth restriction.

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Emerging *Mycobacteria* spp. in Cooling Towers

To the Editor: The importance of nontuberculous mycobacteria (NTM) in various clinical situations recently has increased. Members of the *Mycobacterium avium* complex (MAC) cause a high percentage of infections in persons with acquired immunodeficiency syndrome. Some species are considered emerging pathogens, particularly those of the *M. chelonae–M. abscessus* group. They occur not only in immunocompromised persons but also in persons without predisposing conditions. Although sources of infection are considered to originate from the human environment, until now cooling towers were not clearly demonstrated to be one of these contamination sources, despite being suspected (*1*). Natural streams, groundwater, brook waters, and swamps already were reported to contain different species of NTM. Constructed environments, such as hospital hot water systems, aerosols from showers, ice machines, swimming pools, dental unit water, endoscopes, and bronchoscopes are other sources (*2*). Other studies have shown members of MAC in drinking-water distribution systems (*3*).

A 1999 study in South Africa reported the presence of mycobacteria in cooling towers but reported no details about species (*4*). In 2003, cooling towers were reported to be a potential source of slow-growing mycobacterial species (*1*). Some mycobacterial species recently were shown to survive within amebae (*5*). We aimed to study the population of ameba-associated mycobacterial species in 3 cooling towers using a co-coculture method.

These cooling towers (E, H, and O), located in downtown Paris, France, were sampled in May 2006. Water was taken in a sample point of the cooling circuit located just before the entrance of the tower basins. Because these cooling towers are regularly treated by oxidizing biocide BCDMH (1-bromo-3-chloro-5, 5-dimethylhydantoin) to prevent development of *Legionella* spp., no *Legionella* spp. or *Legionella*-like amebal pathogens were isolated.

Two liters of water samples were filtrated through 0.22-µm pore-sized filters that were injected onto amebal microplates as previously described (*6*) and incubated at 32°C. We screened amebal microplates by examination under inverted microscope and by Ziehl-Nielsen, Gram, and Gimenez staining. Positive wells were subcultured on axenic medium and incubated at 32°C for 10 days. Acid-fast isolates were identified by using partial *rpo*B gene amplification and sequencing with Myco-F and Myco-R primers (*7*). These primers did not allow amplification of partial *rpo*B gene for the 6 *M. phocaicum* strains. We thus used Myco-F/Myco-Rbon (5′-AGCGGCTGCTGGGTGATCAT-3′) primer pair for *M. phocaicum* (*8*). We compared these sequences with the *rpo*B gene sequence of *Mycobacteria* type strains available in the GeneBank database by using BLASTn on the NCBI website (www.ncbi.nlm.nih.gov).

We observed bacteria growing in amebae. Subculture on axenic media led to polymicrobial cultures and allowed isolation of 33 mycobacterial strains (Table). All these strains were submitted to molecular identification. The 33 isolates corresponded to 5 mycobacterial species: *M. fortuitum, M. conceptionense, M. chelonae, M. chimaera,* and *M. phocaicum* (Table). Some of these mycobacteria already had been shown to survive in free-living amebae and to be implicated in human diseases, such as *M. chelonae* (*5*). The same author demonstrated recently that 26 environmental mycobacteria survived in the trophozoites and cysts of *Acanthamoeba polyphaga* (*5*). The recently described species *M. phocaicum* was isolated only in samples from humans and was associated with chronic pneumonia (*9*). However, the natural source of this species is still unknown. *M. fortuitum* was described as an opportunistic *Mycobacterium* associated with disseminated infections in a leukemia patient or in furunculosis after footbath in nail

Table. Identification of isolated nontuberculous mycobacteria according to their *rpoB* gene sequence similarity and GenBank accession numbers

Closest officially described species (*rpoB*)	Isolates (no.), N = 33	Range in % gene similarity to type strain (GenBank accession no.)	Accession numbers of sequences of isolates
Mycobacterium chelonae	O (2), E (2)	99.7 (AY147163)	EU770577
M. conceptionense	O (12)	99.2–99.9 (AY859695)	EU770583, EU770584
M. fortuitum	E (2), H (5), O (3)	99.5–99.7 (AY147165)	EU770578, EU770579, EU770580
M. chimaera	H (1)	99.7 (EF521908)	EU770576
M. phocaicum	E (5), O (1)	98.3–98.6 (AY859693)	EU770581, EU770582

salons. *M. conceptionense* belongs to the *M. fortuitum* group and was isolated in a posttraumatic osteitis inflammation (*10*). *M. chimaera* belongs to MAC and has been isolated only in patients with pulmonary disorders but not from immunocompromised patients. The authors reported that the isolate showed unusually high virulence. We isolated this species for the first time in an environmental sample. It could belong to the transitory flora of the H cooling tower because it was isolated only once in our samples. Cooling towers already had been investigated for slow-growing mycobacteria (*1*), and our study showed they also can be an environmental source of rapidly growing NTM pathogens. In our procedure for amebal co-cultures, incubation cannot last >10 days because amebae do not survive longer in Page's amebal saline buffer. Moreover, agar plates were incubated for 10 days only, which explains why we could recover only rapidly growing mycobacteria. The cooling towers, already known as a source of dissemination of *Legionella* spp., may disseminate mycobacteria in aerosols and be a previously nonrecognized source of infection.

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Clostridium difficile–related Hospitalizations among US Adults, 2006

To the Editor: The threat to public health posed by *Clostridium difficile*-associated disease (CDAD) continues to increase within and outside the United States. In a recent analysis, we detected a 23% annual increase in CDAD-related hospitalizations from 2000 through 2005 and a near-doubling in the associated age-adjusted case-fatality rate from 2000 through 2004 (*1*). In view of the aging US population, this rapid growth, along with the increased virulence and diminished susceptibility to antimicrobial drug treatments, if sustained, will not only strain the US healthcare system (*2*,*3*) but also will cause significant illness and death. For this reason, understanding up-to-date trends in CDAD-related hospitalizations is critical. Since the Agency for Healthcare Research and Quality recently made available its 2006 update to the National Inpatient Sample (NIS) data (*4*) on the Healthcare Costs and Utilization Project Net (HCUPNet) website (*5*), I explored the trends in CDAD hospitalizations beyond our 2005 estimates.

From the HCUPNet website (*5*), I identified CDAD-related hospitalizations for 2000–2006 in the NIS data (*4*). The NIS is a stratified 20% sample of US community hospitals, and data are weighted to provide national estimates (*4*). CDAD was identified by presence of the International Classification of Diseases, 9th revision, Clinical Modification (ICD-9-CM) diagnosis code 8.45 (intestinal infection with *Clostridium difficile*), and the numbers of discharges per year were age-stratified. To benchmark CDAD incidence against the general growth in hospitalizations over time, I obtained age-stratified numbers of total hospitalizations from HCUPNet

for each year and derived CDAD-related hospitalization incidence as a function of total annual US hospitalization volume. I also obtained censal and intercensal data on the numbers and demographic characteristics of the US population during 2000–2006 from the US Census Bureau (*6*). On the basis of these data, I calculated age-specific hospitalization incidence rates and repeated these analyses by census region (Northeast, Midwest, South, and West). Finally, I explored trends in CDAD principal diagnosis hospitalizations as a proportion of all CDAD-related hospitalizations.

Overall, the rate of growth in CDAD-related hospitalizations slowed in 2006, with a crude volume increase of 6.7% over 2005; the difference in the raw volume from 2005 through 2006 did not reach statistical significance ($p = 0.33$). When hospitalization incidence was stratified by age, the greatest increase from 2005 through 2006 occurred in the 45- to 64-year age group (7.2%, from 8.48 to 9.09 cases per 10,000 population) and the smallest increase occurred in the ≥85-year group (0.9%, from 110.71 to 111.70 per 10,000 population); the 18- to 44-year (5.7%, from 2.26 to 3.39 per 10,000 population) and 65- to 84-year groups (5.1%, from 46.57 to 48.96 per 10,000 population) had results between the other 2 groups (Table). Although consistently higher in women than men, the population incidence of CDAD-related hospitalizations in men (4.0%) and women (5.0%) rose similarly (Table). In addition, although the volume of CDAD principal diagnosis had not previously exceeded 25% of total CDAD-related hospitalizations (*1*), in 2006 this proportion increased to 28% (Table). In examining CDAD hospitalization trends regionally, in the Northeast I found a directional reduction in incidence at both the total hospitalizations level and population level. CDAD-related hospitalizations in the Midwest, South, and West continued to increase (Table).

While previously growing at the unsustainable rate of 23% per year, from 2005 through 2006, CDAD-related hospitalizations in adults increased 6.7%, representing a potential considerable slowing of the epidemic. Whereas the number and incidence of CDAD-related hospitalizations decreased in the Northeast, such hospitalizations in the remaining regions had sustained, albeit smaller than in prior years, increases. Additionally, the proportion of all CDAD-related hospitalizations for which CDAD was the principal diagnosis for the hospitalization rose to 28% overall. Given the definition of a "principal diagnosis" in the NIS database as "that condition established after study to be chiefly responsible for occasioning the admission of the patient to the hospital for care; the principal diagnosis is

Table. Incidence of *Clostridium difficile*–related hospitalizations among US adults, 2000–2006, by age group and region*

Hospitalization variable	Year						
	2000	2001	2002	2003	2004	2005	2006
All US adults per 10,000 population							
18–44 y	1.31	1.33	1.66	1.71	1.96	2.26	2.39
45–64 y	4.53	4.58	5.92	6.31	7.20	8.48	9.09
65–84 y	22.41	23.94	31.61	33.74	39.06	46.57	48.96
≥85 y	52.09	57.03	70.15	74.99	89.39	110.71	111.70
Principal diagnosis CDAD as a proportion of total CDAD, %							
18–44 y	26.3	26.8	26.9	26.1	28.1	29.1	30.7
45–64 y	24.4	25.2	24.4	23.2	24.3	25.5	27.4
65–84 y	22.8	23.7	23.3	22.8	23.8	24.9	27.8
≥85 y	22.1	24.4	23.6	23.2	24.0	25.0	29.8
By sex per 10,000 population							
M	4.10	6.30	5.71	6.03	7.03	8.59	9.00
F	5.72	6.83	7.71	8.32	9.71	11.72	12.29
By sex per 1,000 hospitalizations							
M	3.83	5.81	5.24	5.54	6.43	7.79	8.13
F	3.81	4.51	5.04	5.45	6.36	7.66	8.02
Regionally per 10,000 population							
Northeast	7.05	7.59	9.30	10.40	10.73	14.46	14.28
Midwest	5.70	5.32	7.96	8.51	9.86	11.32	12.27
South	4.05	4.98	6.26	6.49	8.19	9.50	9.64
West	3.72	3.52	4.10	4.39	5.39	6.73	7.32
Regionally per 1,000 hospitalizations							
Northeast	5.15	5.52	6.74	7.47	7.64	10.17	10.00
Midwest	4.36	3.98	5.90	6.31	7.26	8.26	8.97
South	2.97	3.59	4.49	4.66	5.89	6.83	7.06
West	3.41	3.24	3.76	4.00	4.92	6.16	6.85

*CDAD, *C. difficile*–associated disease.

always the reason for admission," this rise could represent altered disease manifestation, severity, or virulence.

In summary, the previously noted rate of growth in CDAD-related hospitalizations in US adults appears to have slowed somewhat in 2006. The encouraging downward trend in CDAD in the Northeast requires further exploration. Of concern is the increasing proportion of CDAD-related hospitalizations coded as the primary reason for admission because this may signal a change in characteristics of the disease. Given that the new data represent only 1 year, and the difference between the raw numbers from 2005 through 2006 did not reach statistical significance, these findings need to be interpreted with caution and monitored annually. In general, although helpful, most nationwide data have a considerable lag time. In this and other resistant diseases sweeping the US hospitals, real-time surveillance data are needed for more prompt and actionable policy development.

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Pulmonary Tuberculosis and *Mycobacterium bovis,* Uganda

To the Editor: In 2005, prevalence of human tuberculosis (TB) in Uganda was 559 cases/100,000 population (*1*). In 2002, the average number of extrapulmonary TB cases in humans, considered a crude indicator of the level of bovine TB, was 7.5% of TB cases for Uganda and 6% for Mbarara district, the main Ugandan milk basin (*2*). Worldwide, the proportion of human cases caused by *Mycobacterium bovis* has accounted for 3.1% of all forms of TB (*3*). Although zoonotic TB is more often reported as an extrapulmonary disease, recent publications report that 0.4%–10% of sputum isolates from patients in African countries are *M. bovis* (*3*). These studies, however, give little information about the cattle environment. A 2002 survey of dairy cattle in Mbarara district reported that 74% of herds and 6% of individual animals were reactive to the single tuberculin test (*4*). However, this test does not differentiate between *Mycobacterium* species involved. We therefore explored whether *M. bovis* might be a major threat to human health in this region.

From September 2004 through January 2005, we surveyed 658 patients who had been admitted to the Mbarara University Teaching Hospital TB ward after positive bacterial findings for at least 3 sputum smears or positive chest radiographs for smear-negative patients. Of 90 randomly selected patients, only 70 samples were available for analysis to differentiate the species in the *M. tuberculosis* complex; the other samples were excluded because of contamination, lack of mycobacteria growth on culture, or postal delay in transportation of sample. The questionnaire asked about patients' demographic data (including occupation), association with cattle, and milk consumption habits. Genomic DNA was extracted from the pellet culture of Middlebrook 7H9 broth (Difco; Cergy, France) as described previously (*5*). DNA samples were used to carry out PCRs and hybridization processes; we used the GenoType MTBC kit (Hain Lifescience GmbH; Nehren, Germany) for differentiation in the *M. tuberculosis* complex, especially between *M. tuberculosis* and *M. bovis* species (*6*).

Questionnaire responses showed that 27/64 (42.2%; 6 did not answer) patients had a history of raw milk consumption; nevertheless, 20/24 (83%; 3 did not answer) reported that they boiled fresh milk before consuming it, as did 54/60 (90% of all patients; 10 did not answer). Eating undercooked or raw meat was reported by 91% of the patients. Most patients were adult males (ratio 2.14:0.97 male:female for the district population); 8.6% were <18 years old (56% in the district); and average number of persons in household was 5.7 vs. 4.8 for the district (*7*). Of the samples, 8.6% were from extrapulmonary sites.

After amplification and hybridization of sample DNA, 69 samples were found to be *M. tuberculosis,* and 1 was not a *Mycobacterium* species. Our sampling method would detect at least 1 case of *M. bovis* in *n* patient specimens if the prevalence of bovine TB was $\geq p(0.033\%)$ according to the

formula in which *a* is the first order error (5%):

$$n = \frac{\log a}{\log(1 - p)}$$

Because of the change in sample size, the limit prevalence was redetermined by using the inverse of the formula above:

$$p = 1 - \sqrt[n]{a}$$

If at least 1 sample was positive for *M. bovis*, the prevalence of bovine TB among patients would be ≥4.2%. However, the prevalence of *M. bovis* was <4.2% and confirmed the low-level involvement of *M. bovis* in human TB in Mbarara district. These findings are consistent with previous work in Uganda's capital, Kampala, and in other African or Asian countries (*2*,*8*,*9*). The estimation of extrapulmonary cases among all TB cases (95% confidence interval 2%–15.2%) did not differ from the official estimate. We can add, using the second formula shown above, that among the 6 extrapulmonary TB cases, the prevalence of *M. bovis* is <39.3%. Our results come from a population in a highly rural area (91.5% of the population in Mbarara district) (*7*), where the high prevalence of animal TB has been reported.

These results could be explained by the patients' consumption habits, which reduce the risk for contamination. Even if bovine TB could also be found in other farm or wild animals, it seems to have a minor effect on public health. Zoonotic TB appeared to not be a major public health problem in Mbarara district. However, this finding could also result from underdiagnosis of extrapulmonary TB, from prevalence of *M. tuberculosis* being so high that in proportion *M. bovis* is a minor problem, or from rural populations' difficult access to TB diagnosis (directly observed therapy case detection rate in Uganda in 2005 was 37%) (*1*).

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Vertical Transmission of *Pneumocystis jirovecii* in Humans

To the Editor: Currently, animal and human studies favor an airborne transmission pattern for *Pneumocystis* pneumonia (*1*). However, the early age of acquisition of *Pneumocystis* spp. in different mammals, including humans, warrants study of vertical/transplacental transmission as an additional route of transmission of this stenoxenic microorganism.

Available studies on transplacental transmission of *Pneumocystis* spp. suggest that it varies among mammal species on the basis of the type of placenta (*2*). Transplacental transmission of *Pneumocystis* spp. has been demonstrated in rabbits (*2*,*3*), but it

seems not to occur in rats and mice that have severe combined immunodeficiency (*1*). In humans, transplacental transmission was first suggested by a few reports of *Pneumocystis* spp. pneumonia in neonates published before the AIDS epidemic (*4*). Recently, a controversial case of vertical transmission of *P. jirovecii* was reported: an infection in the lungs of a fetus of an HIV-positive mother with *Pneumocystis* spp. pneumonia (*5*). However, the study did not identify the organisms as *Pneumocystis*, and a subsequent fluorescein-labeled monoclonal antibody test yielded negative results (*6*).

The present study was conducted to evaluate transplacental transmission of *P. jirovecii* by molecular techniques. Placentas and lung tissues of aborted fetuses from immunocompetent women who had miscarriages were studied. To enhance specificity of the study, we used 2 genetic loci in *Pneumocystis* spp. DNA: the mitochondrial large subunit rRNA (mtLSU-rRNA) gene and the gene encoding for dihydropteroate synthase (DHPS). We analyzed 40 paraffin-embedded tissue blocks from the placentas and lungs of 20 fetuses at 28 ± 8 weeks of gestation. The study was reviewed and approved by the ethical committee of University Hospital, Seville, Spain.

DNA was extracted from a mixture of five 10-μm sections of each tissue block. Histologic sections were processed by using xylene and ethanol for paraffin removal and were then rehydrated. DNA was extracted by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions.

DNA amplification at the mtLSU-rRNA locus was conducted by using nested PCR as described (*7*). Samples identified as positive by this PCR were amplified by using primers DHPS-3 and DHPS-4 to detect the DHPS gene (*7*). To prevent false-positive results caused by contamination, pipettes with filters were used at all stages. DNA extraction, preparation of the reaction mixture, PCR amplification, and detection were conducted in different areas under a laminar flow hood. Positive and negative controls were included in each reaction. All experiments were repeated at least twice.

P. jirovecii genotypes can be characterized by identifying polymorphisms at the mtLSU-rRNA locus (positions 85 and 248) and at the DHPS locus (positions 55 and 57). Amplicons from all samples that yielded positive PCR results for the 2 loci were sequenced directly from both ends by using a model ABI 377 automated sequencer and an ABI prism Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The derived mtLSU-rRNA and DHPS gene sequences were compared with sequences available in databases by using the National Center for Biotechnology Information (Bethesda, MD, USA) BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The mtLSU-rRNA fragment was amplified from 11 lung and 8 placenta samples. Simultaneous DNA amplification of 2 loci of *P. jirovecii* was observed in lung tissue samples from 7 (35%) of 20 fetuses and from 1 (5%) of 20 placenta samples. Sequencing of the mtLSU-rRNA gene showed 3 polymorphisms, and DHPS gene analysis showed only wild-type genotype in all samples (Table).

Our results provide molecular evidence of *P. jirovecii* transplacental transmission in humans. No available data on the development of *Pneumocystis* organisms in female genital organs was provided (*8*). In contrast, morphologic and molecular evidence of hematogenous dissemination of *P. jirovecii* from infected lungs has been provided by many authors (*8*). *Pneumocystis* DNA has been documented in blood or amniotic fluid samples from pregnant rabbit does (*3*), in which transplacental transmission of *Pneumocystis* spp. occurred. In humans, *P. jirovecii* colonization was observed in 5 (15.5%) of 33 pregnant women in their third trimester (*9*). These data suggest that physiologic immunodepression associated with pregnancy may favor *Pneumocystis* spp. colonization and mother-to-fetus transmission of the fungus by the hematogenous route. The transplacental route could enhance transmission of

Table. *Pneumocystis jirovecii* genotypes in samples identified as positive by nested PCR for 2 loci from fetal lung tissues and placenta samples*

	Lung tissues		Placenta tissues	
Case	mtLSU-rRNA genotype	DHPS genotype	mtLSU-rRNA genotype	DHPS genotype
C1	3	1	–	–
C2	1	1	1	–
C3	3	1	–	–
C4	3	1	–	–
C5	2	1	1	–
C6	1	1	3	–
C7	1	1	–	–
C8	–	–	1	1
C9	3	–	–	–
C10	–	–	3	–
C11	3	–	1 and 3	–
C12	1 and 3	–	1	–
C13	–	–	1	–
C14	3	–	–	–
C15-C20	–	–	–	–

*Genotype 1, polymorphism 85C/248C in mitochondrial large subunit (mtLSU)–rRNA gene and 55 Trh/57 Pro inihydropteroate synthase (DHPS) gene; genotype 2, polymorphism in 85A/248C; genotype 3, polymorphism in 85T/248C.

P. jirovecii independent of environmental hazards.

Isolation of pathogens from an aborted fetus does not necessarily mean that they have caused the death of the fetus because many agents appear to pass through the fetal-placental unit and cause little damage. However, fungal infection is a major worldwide cause of abortion in cattle (*10*), and the surprising high prevalence of *P. jirovecii* infection found in dead fetuses in our study emphasizes the need to study the possible role of this fungal organism in human abortion.

Our findings could be of potential clinical importance and could open a new field of research, which should be explored. Further research should assess the scope of the problem and design rational preventive strategies, if necessary.

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Avian Influenza Virus (H5N1) in Human, Laos

To the Editor: The first avian influenza (H5N1) outbreak in poultry in Laos occurred in 2003 and subsided in March 2004 after massive killing of poultry to contain the disease. Extensive surveillance from July 2005 through January 2006 did not detect any influenza virus subtypes in chicken, ducks, quails, and pigs in live bird markets in the Vientiane, Champasak, and Savannakhet Provinces (*1*). Avian influenza virus (H5N1) was reintroduced into Laos in February 2006 but showed a lower incidence. Viruses isolated in this country in 2004 belonged to genotype Z, clade 1, and 2006 isolates belonged to clade 2.3.4 (online Appendix Figure, panel A, available from www.cdc.gov/EID/content/15/1/127-appF.htm) (*1*).

Avian influenza (H5N1) had not been reported in humans in Laos until February 27, 2007 (*2*). Our patient was a 15-year-old adolescent girl who lived in a suburb of Vientiane where an outbreak of influenza (H5N1) in poultry had been confirmed on February 7, 2007. Influenza-like symptoms developed in the patient on February 10. She was hospitalized in Vientiane with fever and respiratory symptoms on February 15. On February 17, her parents brought her to a private hospital in Nong Khai Province, Thailand. Oseltamivir was prescribed on February 19. On February 20, she was transferred to the Nong Khai Provincial Hospital because of rapid, progressive, severe pneumonia with acute respiratory distress syndrome. When we suspected avian influenza in this patient, clinical specimens were tested.

A diagnosis of infection with avian influenza (H5N1) was based on positive results obtained by reverse transcription–PCR (RT-PCR), viral isolation in MDCK cells inoculated with an endotracheal suction specimen

collected on February 22, and a 4-fold increase in neutralizing antibody titers from 80 to 320 in paired blood specimens collected on February 25 and March 1, as assayed against autologous virus. This virus isolate was named A/Laos/Nong Khai 1/07(H5N1). Subsequent samples were collected on February 25 and March 7 (day of death). Results of RT-PCR were positive for the sample collected on February 25 only; virus isolation results were negative for both samples.

The virus was screened for a novel reassorted gene by a multiplex RT-PCR and 8 primer pairs specific for each genomic segment of genotype Z, clade 1 virus (*3*). All segments except the polymerase A (PA) segment were amplified, which indicated that the new virus was different from genotype Z viruses. The viral genome was sequenced and submitted to GenBank (accession nos. EU499372–EU499379 for hemagglutinin, nonstructural protein, matrix protein, nucleoprotein, PB1, PB2, neuraminidase, and PA genes, respectively). Phylogenetic analysis showed that this virus belonged to genotype V (online Appendix Figure, panel B) (*4*); phylogenetic analysis of the hemagglutinin gene (www.who.int/csr/disease/avian_influenza/smaltree.pdf) showed that it belonged to clade 2.3.4 (online Appendix Figure, panel A).

Protein sequence at the hemagglutinin cleavage site harbored many basic amino acids (RERR_RKR). One amino acid deletion and 1 amino acid change were found when compared with RERRRKKR, which is present in most avian influenza viruses (H5N1). There was no change in receptor binding site. This virus had glutamic acid at aa 627 in the PB2 protein, aspartic acid at aa position 92 in nonstructural protein 1, and 5 aa deletions at positions 80–84 in the nonstructural protein 1. Analysis of the neuraminidase gene showed a 20-aa deletion in the stalk protein; there was no mutation of histidine to tyrosine at aa position 274, a position shown to be the oseltamivir resistance marker in the neuraminidase 1 viral genome (*5*). Mutations in the matrix 2 gene showed that amantadine resistance was not present in our virus (*6*). Our in vitro assay (*7*) showed that this virus was sensitive to oseltamivir and amantadine.

Since 2003, genotype V influenza viruses (H5N1) have been reported in some East Asian countries. Genetic diversity in the hemagglutinin gene has classified those genotype V viruses into distinct clades. Viruses from avian species in South Korea in 2003 and Japan in 2004 (*8*,*9*) belong to clade 2.5. A/chicken/Shanxi/2/2006 isolate belonged to clade 7. Human cases in People's Republic of China, i.e., A/China/GD01/06, A/Shenzhen/406H/06, A/Jiangsu/1/2007, and A/Jiangsu/2/2007, belong to clade 2.3.4, the same clade as A/chicken/Thailand/NP-172/2006 and the virus from our study.

Highly pathogenic avian influenza viruses (H5N1) that caused outbreaks in Thailand since 2004 belong to genotype Z, clade 1. Introduction of genotype V clade 2.3.4 virus, A/chicken/Thailand/NP-172/2006, to Nakhon Phanom Province occurred in November 2006 (*10*), the same year that clade 2.3.4 virus was introduced into Laos (online Appendix Figure, panel A). On the basis of hemagglutinin gene phylogeny, A/Laos/Nong Khai 1/2007 is closely related to A/chicken/Nongkhai/NIAH 400802/2007 and A/chicken/Thailand/NP-172/2006. Phylogenetic analysis suggested that viruses from these 2 countries shared the same origin. There was extensive movement across the Mekong River even before the bridge linking Nong Khai from Vientiane was opened. However, the route of transmission of genotype V viruses from east Asian to Southeast Asian countries could not be elucidated.

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Fatal HIV Encephalitis in HIV-Seronegative Patients

To the Editor: Acute encephalitis is rarely seen in patients infected with HIV (*1*). In addition, HIV in patients who are seronegative is extremely rare, particularly in the setting of current screening ELISAs (*2*). We report a case of encephalitis and HIV in the same patient, which resulted in death.

A 44-year-old Caucasian woman sought treatment at our hospital with a 1-week history of fever, unsteady gait, and progressive confusion. Her medical history included hypothyroidism, depression, and chronic alcohol abuse. The patient's first tests for HIV were negative at 19 and 12 months prior to admission during routine intake screening for jail inmates (Abbott HIV AB HIV-1/HIV-2 [rDNA] enzyme immunoassay [EIA] kit; Abbott Laboratories, Abbott Park, IL, USA). Six months before admission, the patient had a viral exanthem of blistering rash on her lips, palate, and chest. Two weeks later, she had oral thrush and a leukocyte count of 1,700 cells/μL. An HIV ELISA result was negative. Three months before admission, she was admitted to a different hospital for weakness, abdominal pain, intermittent fever, diarrhea, persistent oral candidiasis, and ethanol withdrawal. She had leukopenia and thrombocytopenia. A fourth HIV ELISA result was negative. The patient had been admitted to our hospital one week before the current admission with symptoms of fever, confusion, and urinary tract infection. Lumbar puncture showed an elevated protein level (106 mg/dL). A fifth HIV test result 6 days before most recent admission was negative. Five days before admission, she had been discharged to a rehabilitation facility.

On this hospitalization, she had fatigue, headache, disequilibrium, dysarthria, and blurred vision. Initial examination showed fever of 101.3°F, poor word recall, and a wide-based gait. Laboratory tests showed mild anemia and a leukocyte count of 2×10^3 cells/μL.

Over the next few days the patient's fever persisted and her mental status fluctuated. Tests on hospital day 2 showed a CD4 count of 101/mL (16.9%). Magnetic resonance imaging (MRI) of the brain showed diffuse symmetric white matter disease (Figure, panel A). Samples sent on hospital day 9 eventually showed wild-type HIV with a viral load >500,000 copies/mL. Repeat cerebrospinal fluid (CSF) test results were negative for cryptoccocus antigen, and PCR results were negative for cytomegalovirus, herpes simplex virus (HSV), and JC polyoma virus. The next day, a sixth HIV ELISA result was negative. The serum level of HIV p24 antigen was 202 pg/mL.

On hospital day 13, the patient began treatment with zidovidine, lamuvidine, didanosine, and nevirapine. Within 24 hours, seizures and catatonia developed in the patient. An electroencephalogram showed diffuse wave form slowing. A repeat MRI showed worsened white matter disease (Figure, panel B). The result of a seventh HIV screening ELISA performed on hospital day 15 was negative. Two days later, the HIV viral load was 241,789 copies/mL. On hospital day 19, her serum levels were within normal limits: immunoglobulin (Ig) M level (164 mg/dL), IgG level (1,440 mg/dL), a 3× normal IgA level (1,060 mg/dL), and no oligoproteins. The CSF had an IgG level >10× normal (72 mg/dL), elevated IgG levels for HSV1 (1:160) and HSV2 (1:40), was negative for virus culture, and showed a negative PCR result for JC polyoma virus. On hospital day 23, the eighth HIV ELISA result was negative. The Abbott HIVAB HIV-1/HIV-2 (rDNA) EIA was used throughout the hospitalization. On hospital day 24, supportive care was withdrawn and the patient

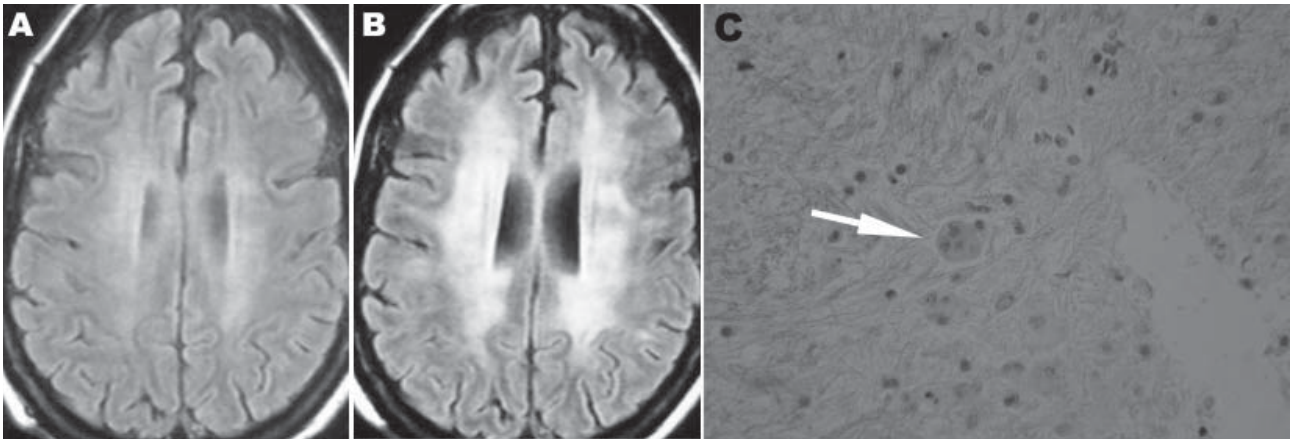

Figure. Brain magnetic resonance imaging (MRI) and autopsy findings for the patient. Brain MRI showed diffuse white matter disease on hospital day 2 (A) and marked progression on hospital day 14 (B). Autopsy histopathologic analysis showed microglial nodules, multinucleated giant cells (arrow), perivascular inflammatory cells, vasculopathy with mural fibrosis, perivascular hemosiderin deposition, degeneraton of the central white matter, and neuronal apoptosis (C).

died. Throughout her hospitalization, blood, urine, and CSF cultures remained sterile.

Autopsy showed acute HIV encephalopathy and cerebral vasculopathy. The findings included multifocal microglial nodules, perivascular inflammatory cells, vasculopathy with mural fibrosis and perivascular hemosiderin deposition, degeneration of the central white matter, and neuronal apoptosis (Figure, panel C). She also had *Pneumocytis jiroveci* pneumonia and hepatosteatosis without cirrhosis.

There are several possible explanations for the patient's HIV seroconversion failure. The first explanation is that the patient was subacutely infected but had a retarded humoral response. Delayed seroconversion has been documented up to 42 months after infection (*3*), but this seems unlikely with current ultrasensitive assays. Another possibility is that she was infected with a strain undetectable by screening ELISAs, such as HIV-1 Group N or a rare Group M subtype recombinant variant. This hypothesis also seems unlikely because of the rarity and geographic distribution of these strains (*4*). A third possibility is transient seroconversion with reversion to seronegative status (*5*,*6*). However, given the number and frequency of screening tests in this case, even transient seroconversion would probably have been detected. Another hypothesis, one consistent with the patient's rapid demise, is infection with a particularly virulent HIV variant, which led to rapid immunocompromise and failure of seroconversion. Such infections have been observed in rapid progressors, in which CD4+ T-cell depletion is so swift that B cells receive no T-cell help and are therefore not able to mount an effective immune response (*7*). In addition, chronic alcoholism may have contributed to immune failure and a rapidly progressive disease course (*8*–*10*).

This case raises several disturbing and interesting questions and possible avenues for future research. The diagnosis of acute HIV encephalopathy with a CD4 count of 100 cells/μL raises the likelihood that this patient was infected with at least 1 strain containing particularly neurotropic properties, possibly with X4 or R5X4 tropism, or that her brain was particularly primed for HIV-induced damage. Understanding the neurotropic properties of different strains of HIV may help prevent similar adverse outcomes in other patients.

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Classical *ctxB* in *Vibrio cholerae* O1, Kolkata, India

To the Editor: Among the 206 serogroups of *Vibrio cholerae,* O1 and O139 are associated with epidemic cholera. Serogroup O1 is classified into 2 biotypes, classical and El Tor. Conventionally, the 2 biotypes can be differentiated on the basis of a set of phenotypic traits. Comparative genomic analysis has shown variations in different genes between these biotypes (*1*). Cholera toxin (CT), the major toxin responsible for the disease cholera, has 2 epitypes or immunologic forms, CT1 and CT2 (*2*). Another classification recognizes 3 genotypes on the basis of the *ctxB* gene sequence variation (*3*). In the past few years, a new emerging form of *V. cholerae* O1, which possesses traits of both classical and El Tor biotypes, has been isolated in Bangladesh (*4*,*5*), Mozambique (*6*), Vietnam, Hong Kong, Japan, and Zambia (*7*). These strains were variously labeled as Matlab variants, hybrids, or altered El Tor strains.

Our study analyzed, in chronological order, strains of *V. cholerae* O1 that were isolated over 17 years (1989–2005). We used strains isolated during diarrhea surveillance conducted at the Infectious Diseases Hospital, Kolkata (Calcutta), to determine precisely when the hybrid strains appeared in this region. A total of 171 strains of *V. cholerae* O1, which were selected to cover different months of each year, were included in this study, along with 2 reference strains for classical and El Tor biotypes. The *V. cholerae* strains were confirmed serologically by slide agglutination using a specific polyvalent antiserum to *V. cholerae* O1.

We focused on the *ctxB* gene. The strains were examined by mismatch amplification mutation assay (MAMA)–based PCR for detecting the *ctxB* allele; a common forward primer was used for 2 alleles, FW-Com (5′-ACTATCTTCAGCATATGCACATGG-3′); and 2 allele-specific primers, Re-cla (5′-CCTGGTACTTCTACTTGAAACG-3′) and Re-elt (5′-CCTGGTACTTCTACTTGAAACA-3′), were used for classical and El Tor biotypes, respectively (*8*). Results of the MAMA-PCR are summarized in the Table. All of the 123 *V. cholerae* O1 strains from 1995 through 2005 yielded only the classical type of *ctxB,* which indicates that since 1995 the classical type has completely replaced the El Tor type *ctxB* (Table). To reconfirm our PCR-based results, we selected 25 representative strains for DNA sequencing of the *ctxB* gene. The deduced amino acid sequences were aligned with the CtxB sequences of reference strains N16961 (El Tor) and O395 (classical). The deduced amino acid sequences of all 25 strains were identical to those of the classical reference strain; histidine was at position 39 and threonine was at position 68. Thus, the results from DNA sequencing of the *ctxB* gene confirmed the MAMA-PCR results.

Table. Prevalence of different types of *ctxB* alleles among *Vibrio cholerae* O1 strains, Kolkata, India, 1989–2005

Year isolated	No. strains tested	No. alleles		
		Classical *ctxB*	El Tor *ctxB*	Classical + El Tor *ctxB*
1989	6	0	6	0
1990	7	4	3	0
1991	10	8	0	2*
1992	10	4	5	1*
1993	6	4	2	0
1994	9	8	1	0
1995	23	23	0	0
1996	10	10	0	0
1997	10	10	0	0
1998	10	10	0	0
1999	10	10	0	0
2000	10	10	0	0
2001	10	10	0	0
2002	10	10	0	0
2003	10	10	0	0
2004	10	10	0	0
2005	10	10	0	0

*These strains carry the *ctxB* gene for El Tor, as well as classical strains.

Our results highlight a noteworthy event in the evolution of recent *V. cholerae* strains. Analysis of type *ctxB* that had been circulating in Kolkata for 17 years (1989–2005) showed that in 1989 only the El Tor allele of *ctxB* was present. Our results further indicate that classical type *ctxB* emerged in 1990, although El Tor type *ctxB* was still present in almost equal numbers during that year. During 1991, a unique event took place when the classical type became predominant, along with strains having both classical and El Tor type *ctxB*. In 1994, isolation of strains with El Tor *ctxB* became rare, and the major *ctxB* allele was of the classical type. *V. cholerae* O1 strains from 1995 onward were found to carry classical type *ctxB*, which totally replaced the El Tor type *ctxB* allele.

Replacement of El Tor type *ctxB* by the classical allele has been reported in Bangladesh since 2001 (*5*), but this event seems to have occurred earlier in Kolkata. Perhaps the new type of El Tor strains arose when *V. cholerae* strains with typical seventh pandemic El Tor genetic background were replaced with strains having the *ctxB* gene, possibly driven by selective pressure to survive and adapt better in host intestines. Considering the increase in the global prevalence of cholera (*9*), the origin and spread of these new variants of *V. cholerae* strains should be tracked in the population by genome analysis. Finally, this study has described a brief period from February 1991 through December 1992 when El Tor strains had CTX prophages of both classical and El Tor types (data not shown), along with the *ctxB* of both biotypes. Notably, this period coincided with an unprecedented event in the history of cholera—the genesis of the O139 serogroup. After this serogroup reemerged in 1996, it harbored 2 types of CTX prophages, namely, El Tor and Calcutta (*10*). Further, these strains with *ctxB* of both biotypes might also have had a pivotal role behind the emergence of El Tor strains with classical *ctxB*. Further studies are warranted to determine whether any distinct relationship exists between these overlapping events.

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Letters

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

Sphingomonas mucosissima Bacteremia in Patient with Sickle Cell Disease

To the Editor: The genus *Sphingomonas* was proposed by Yabuuchi et al. in 1990 (*1*) and amended by Takeuchi et al. in 1993 (*2*). It now has been subdivided into 4 separate genera: *Sphingomonas sensu stricto*, *Sphingobium*, *Novosphingobium,* and *Sphingopyxis*. The bacteria of the genus *Sphingomonas* are yellow-pigmented, nonfermenting, gram-negative bacilli with a single polar flagellum; they are widely distributed in the natural environment, especially in water and soil (*3*). These bacteria are characterized by the presence of a unique sphingoglycolipid with the long-chain base—dihydrosphingosin, ubiquinone 10 (Q-10), and 2-hydroxymyristic acid (2-OH C14:0)—and the absence of 3-hydroxy fatty acids (*4*). *S. mucosissima* was isolated and identified in 2007 by Reddy and Garcia-Pichel from biologic soil crust samples collected from sandy arid soil in the US Colorado Plateau (*5*). *Sphingomonas* spp. are opportunistic pathogens and have recently been implicated in a variety of community-acquired and nosocomial infections, considered to originate from contaminated hospital equipment or manipulation of some medical devices (*3*). The survival of *Sphingomonas* spp. in indoor dust particles as aerosols and their resistance to many disinfecting and toxic chemicals may explain their ability to colonize medical devices such as mechanical ventilators, catheters, and bronchofiberoscopes (*6*). In the past few years, these organisms, in particular *S. paucimobilis*, have been implicated in a variety of community-acquired and nosocomial infections.

We report a case of *S. mucosissima* bacteremia in a patient with sickle cell disease. In February 2008, a 17-year-old woman with homozygous sickle cell anemia was hospitalized when her condition suddenly became worse. The patient had undergone a splenectomy in 1992 and a cholecystectomy in February 2007. Four days after admission, she had a fever of 38.7°C. Two aerobic blood specimens, drawn on the fifth day of her hospitalization, yielded gram-negative bacilli after a 24-hour incubation. The gram-negative bacilli were positive for catalase and oxidase but remained unidentified by API 20NE strip (bioMérieux, Marcy l'Etoile, France). MICs of antimicrobial drugs were determined for the gram-negative bacilli by using an Etest assay (AB BIODISK, Solna, Sweden) on Mueller-Hinton medium. MICs were 1 μg/mL for cefotaxime, 1 μg/mL for amoxicillin–clavulanic acid, 2–3 μg/mL for vancomycin, 0.064 μg/mL for imipenem, 4–5 μg/mL for ceftazidime, 1 μg/mL for amikacin, 3 μg/mL for ciprofloxacin, and 0.047 μg/mL for trimethoprim-sulfamethoxazole.

DNA was extracted from 1 colony by using a QIAamp Tissue kit (QIAGEN, Hilden, Germany) as described by the manufacturer. A 16S rDNA sequence was obtained (1,410 bp) by using the fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (5′-ACGGCTACCTTGTTAC GACTT-3′) primer pair (*7*,*8*). Using BLAST version 2.2.9 software (www.ncbi.nlm.nig.gov/BLAST), we determined that this sequence showed 98% similarity with the 16S rDNA sequence of *S. mucosissima* (GenBank accession no. AM229669). A phylogenetic neighbor-joining tree resulting from comparison of sequences of the 16S rDNA genes of *Sphingomonas* spp. was made with the MEGA 3.1 software (www.megasoftware.net). This analysis confirmed that the isolate belonged to *S. mucosissima*.

Initial treatment of intravenous administration of ceftriaxone was begun. The fever resolved after 1 day and the patient's condition improved. Treatment was stopped after 5 days, and the patient remained apyretic. Two *S. mucosissima* isolates were recovered from 2 different blood-culture samples drawn 24 hours apart, which suggests that *S. mucosissima* was not just a transient organism but indeed was responsible for the patient's septicemia. Phenotypic identification of the gram-negative bacilli failed because the definite bacterial species *S. mucosissima* was not included in the API database (http://industry.biomerieux-usa.com/industry/food/api/apiweb.htm) used for the phenotypic identification. However, the isolates' biochemical characteristics were consistent with those previously reported for *S. mucosissima* (*5*) (Table). Final identification was achieved by comparing the almost complete 16S rDNA sequence with homologous sequences deposited in GenBank.

Table. Biochemical characteristics of the previously reported *Sphingomonas mucosissima* isolate (AM229669) and the isolate from this study

Characteristic	*S. mucosissima*	Isolate from this study
Biochemical characteristics		
Oxidase	+	+
Catalase	+	+
Phosphatase	+	+
β-galactosidase	–	–
Gelatinase	–	–
Nitrate reduction	–	–
Assimilation of carbon compounds		
Alanine	+	+
Glucose	+	+
Glutamic acid	–	–
Mannitol	–	–
Sucrose	+	+

We believe that the patient's intravenous catheter was the source of the infection because she did not have wound infections, and cultures of her urine were negative for infectious agents. Antimicrobial drug treatment, selected on the basis of an in vitro *S. mucosissima* susceptibility profile, facilitated the patient's recovery. This case report illustrates that the pathogenic potential of *S. mucosissima* should be considered in diagnosis in such cases because the organism can cause bacteremia in patients, primarily in those with underlying debilitating conditions and those who have undergone medical interventions.

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WU Polyomavirus in Fecal Specimens of Children with Acute Gastroenteritis, China

To the Editor: WU polyomavirus (WUPyV) is a recently described PyV found in patients with acute respiratory tract infections (*1*). The role of the virus in disease pathogenesis remains unclear. The ability to detect it in clinical specimens would help in the determination of its replication sites and its routes of transmission and dissemination. WUPyV has been found in specimens from the respiratory tract only (*1*).

Previous studies of other PyVs, including BK virus, JC virus, and the newly identified KIPyV, demonstrated their presence in fecal specimens (*2*,*3*), which suggests their potential for transmission through the gastrointestinal (GI) tract (*2*). Because some children (6.8%–27.7%) who had WUPyV results in previous studies (*1*,*4*,*5*) displayed respiratory and GI clinical signs, we speculated that WUPyV might also be transmitted through the GI tract.

In this study, we tested for the presence of WUPyV in children with acute gastroenteritis. A total of 377 fecal specimens were collected from children with acute nonbacterial gastroenteritis at the Outpatient Clinic Department of the Beijing Children's Hospital from March 2006 through November 2007. Patients with nonbacterial gastroenteritis were defined as 1) those who had acute, watery, but not bloody, diarrhea, accompanied by other clinical signs and symptoms such as fever, abdominal cramps, nausea, vomiting, and headache; and 2) those who had negative test results for any known bacteria that might cause gastroenteritis, such as *Salmonella* spp., *Shigella* spp., *Staphylococcus* spp., *Campylobacter jejuni*, *Clostridium* spp., *Escherichia coli*, and *Yersinia* spp.

All patients, whose ages ranged from 1 month to 13 years (mean age 11.7 months, median age 9 months), did not exhibit apparent clinical respiratory signs. Fecal specimens from patients were diluted in phosphate-buffered saline (pH 7.2) by using a 10% wt/vol ratio and were cleared of cell debris by centrifugation (2,500 × *g*, 5 min). Virus nucleic acids were extracted by using the NucliSens miniMAG and isolation reagents according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France). Samples were subsequently screened for group A rotavirus (RVA) by using the rotavirus ELISA diagnostic kit (Lanzhou Institute for

Biologic Products, Lanzhou, People's Republic of China). In addition, samples were screened for enteric adenovirus, astrovirus, norovirus, and human bocavirus by PCR (*6*,*7*). WUPyV DNA was detected by PCR with the primer pair AG0048 and AG0049, which generated a 250-bp amplicon as described previously (*1*). Positive PCR amplicons were then verified by sequencing. Confirmed sequencing results demonstrated WUPyV DNA in 2 (0.5%) of 377 fecal specimens. These 2 positive samples were obtained from 2 patients, ages 6 months and 2 years, who experienced acute diarrhea but had no respiratory or other clinical signs and symptoms. RVA was also detected in both samples.

Nucleotide sequences of WUPyV obtained from this study were submitted to the GenBank database (accession nos. EU684312 and EU684313). To investigate whether these nucleotide sequences had any unique features, we analyzed the 2 WUPyV isolates to determine the extent of homology between these genes and those documented in the GenBank database by using MEGA software version 4.0 (www.megasoftware.net) and the neighbor-joining method. The nucleotide sequences of the VP2 gene from the WUPyV strains found in this study showed 99%–100% homology with the strains described previously for WUPyV (GenBank accession nos. EU754877, EU754878, EF444557, EF444562, EF444593, EF655819, EU296475, EU395815, EU678910, EU693905, EU358752).

Our observations indicate that a candidate respiratory pathogen, WUPyV, can also be detected in specimens from the GI tract. In addition, the codetection of human RVA, a major cause of viral gastroenteritis in children, in both WUPyV-positive specimens underscores the need for further investigations to clarify the precise role of WUPyV in the pathogenesis of acute gastroenteritis. The reason for the presence of WUPyV in the GI tract is unclear. Our findings were unlikely to have been caused by cross-contamination because samples were prepared and analyzed in 2 laboratories independently, and strict controls were used during the process of nucleic acid extraction and PCR analysis to monitor contamination.

WUPyV may act as an opportunistic pathogen in the GI tract, colonize the GI tract without causing any disease, or be a part of the endogenous viral flora that are reactivated by other viral infections (*1*). However, although positive samples were obtained from patients who had acute gastroenteritis without any apparent clinical respiratory symptoms, we cannot exclude the possibility that the detection of WUPyV in fecal specimens might result from its transient presence in patients who have swallowed virus-containing sputum or nasal secretions. It is also possible that WUPyV persists in the respiratory tract without inducing symptoms (*8*,*9*). Thus, the study of asymptomatic control groups of patients with diarrhea would be of particular interest because these patients may provide critical insight into the pathogenesis of WUPyV.

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SCC*mec* Typing in Methicillin-Resistant *Staphylococcus aureus* Strains of Animal Origin

To the Editor: Van Loo et al. described the presence of staphylococcal cassette chromosome *mec* (SCC*mec*) type III in some methicillin-resistant *Staphylococcus aureus* sequence type (ST) 398 isolates related to pig farming (*1*). SCC*mec* types are based on the allotype of *ccr* genes and the *mec* gene complex. Class A *mec* has intact *mecI/R* regulator genes. Type III SCC*mec* has type 3 *ccr* genes and class A *mec* complex, whereas type V SCC*mec* contains *ccrC* and class C *mec* (*2*,*3*). The authors typed SCC*mec* of the isolates by the method of Zhang et al. (*4*), in which type III is defined by amplification of a 280-bp fragment located in the junkyard region. This fragment is found in SCC*mer* that is associated with SCC*mec* type III.

We have typed SCC*mec* of the same 4 isolates that were reported to be SCC*mec* type III positive by using the primer sets defined by Ito et al. (*2*,*3*) and Lim et al. (*5*) for *ccr* types 1–3 and *ccrC* and 4 additional primers developed at our institute (Table) in single PCRs. All ST398 isolates were PCR negative when primers specific for SCC*mec* type III were used, but PCR positive with the *ccrC*-specific primers. DNA sequencing confirmed the product as *ccrC*. Further, the isolates did not have a class A *mec* complex, a requisite for SCC*mec* type III, because a *mecI*-specific PCR was negative for these isolates. In addition, Southern hybridizations with digoxigenin-dUTP–labeled PCR fragments obtained with our primer pair specific for *ccr3* and primers for *ccrC* (*3*) showed no hybridization with the *ccrA/B3* probe (except for the positive control). All of the ST398 isolates hybridized with the *ccrC*-specific probe.

We conclude that on the basis of generally accepted definitions SCC*mec* type V is present in these ST398 pig-farming–related isolates, not SCC*mec* type III. Therefore, researchers should be aware that some typing methods may lead to inadequate results.

This research was supported by the Department of Medical Microbiology, University Medical Center, Utrecht, the Netherlands.

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Table. Primers used to type SCC*mec* of MRSA ST398 isolates*

Genes	Primer name	Primer sequence (5′ → 3′)
*ccrA/B*1	ccr1B-for	CTT TCA CGA TAG ACA CAG
	ccr1B-rev	TAA AAG AAG TTC ATA GCC GTT AAA TTG G
*ccrA/B*2	ccr2B-for	GCA TTC ATC ATC AAT CAA AAT G
	ccr2B-rev	CTA TAA CCT TCT GTG CTT TGC A
*ccrA/B*3	ccr3B-for	TCC GTA ATA AGA AGC AAC TTC AC
	ccr3B-rev	ACT ATA GCC TTC AGT ACT TTG GA
*ccrA/B*4	ccr4B-for	TGA AGA AGC ACA AGA GCG GC
	ccr4B-rev	CTG CAC CAC ATT TTG GGC AC

*SCC*mec*, staphylococcal cassette chromosome *mec*; MRSA, methicillin-resistant *Staphylococcus aureus*; ST, sequence type.

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In Response: We thank Jansen et al. for their comments about the SCC*mec* types of sequence type (ST) 398 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates (*1*). For SCC*mec* typing of MRSA, several different PCR methods have been published. We originally chose the SCC*mec* PCR developed by Zhang et al. (*2*) because at that time it was the method of choice in many published papers. Fluit et al. questioned whether the SCC*mec* type III isolates were correctly typed (*1*). To prove that the results of typing these 4 isolates were incorrect, these researchers performed several different SCC*mec* PCRs, including a PCR with

primers they developed themselves. In addition, Southern hybridization was done. The results showed that SCC*mec* III ST398 MRSA isolates should be typed as SCC*mec* type V. In this conclusion we agree with the authors. It seems clear that Zhang's method incorrectly identified 4 of the animal-related ST398 isolates as SCC*mec* type III instead of SCC*mec* type V. Whether all ST398 MRSA are SCC*mec* type IV or V remains unclear. Recently, an article by Nemati et al. was published in which ST398 MRSA was also typed as SCC*mec* III (*3*). However, in that study the SCC*mec* typing method of Zhang was also used.

In conclusion, the choice of SCC*mec* typing method is directly related to obtaining accurate SCC*mec* results for ST398 isolates. To date, almost all animal-related ST398 MRSA isolates are SCC*mec* types IV and V.

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School Closure to Reduce Influenza Transmission

To the Editor: Cowling et al. reported on the effects of school closure in Hong Kong, People's Republic of China, during March 2008 in response to influenza-related deaths of children (*1*). The influenza epidemic started in January 2008 and peaked in late February, but the 2-week school closure did not begin until March 12. Consequently, the school-based epidemic was on the decline by the time officials closed schools. Other studies have suggested that early school closures can help reduce influenza illness in the community and among school children, especially during a pandemic (*2–6*). However, surveillance systems that rely on school absenteeism or deaths would likely provide information too late during the outbreak for school closure to effectively reduce influenza transmission.

The Centers for Disease Control and Prevention (CDC) has recommended early closure of schools as a community mitigation measure in the event of a severe pandemic (*7*). Specifically, CDC recommends rapidly initiating activities such as advising sick persons to stay home, dismissing children from schools, closing childcare facilities, and initiating further social distancing measures within a state or a community at the beginning of the upslope of a pandemic wave (acceleration interval), i.e., when cases are initially identified and community transmission begins to occur (*8*). We concur with the authors that the 2007–08 influenza season was already waning by the time the decision was made to close schools (deceleration interval).

School closure used as a single pandemic control measure is predicted to be less effective than early, concurrent use of multiple measures. Socially disruptive measures like early school closure and keeping children from congregating in the community would likely reduce community transmission of pandemic disease, but would also create secondary challenges (*9*,*10*). Therefore, to ensure maximal benefit for reducing disease transmission, interventions should be implemented early and concomitantly with other nonpharmaceutical and pharmaceutical measures, accompanied by public education, and used judiciously based on pandemic severity.

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In Response: We agree with Koonin and Cetron (*1*) that early application of any intervention during an influenza epidemic or pandemic is critical in maximizing population health benefits. Further, the longer an intervention is sustained, the greater the likely benefit.

Whether surveillance data can inform public health interventions may depend on the timeliness of the data as well as the length of the epidemic. In tropical and subtropical settings, influenza tends to circulate longer. Although duration of the epidemic could enable delayed interventions a chance of success, social distancing interventions may need to be sustained to ensure that the epidemic does not revive when the intervention period ends.

One important study not mentioned by Koonin and Cetron is a natural experiment in France where the staggering of school holiday periods in different regions enabled Cauchemez et al. to estimate that school holidays prevent 16%–18% of seasonal influenza cases (*2*). In contrast to our study of a single school closure event in response to 1 seasonal outbreak, the French study considered preplanned holiday periods spanning many years.

Although pandemic plans often describe action to be taken depending on features in the epidemic curve (e.g., the acceleration interval as the upslope of the epidemic curve), we would argue that more focus should be given to underlying transmission dynamics. In our analysis of the effect of school closures in Hong Kong, we used a simple statistical technique (*3*) to estimate the underlying reproductive number. Changes in the epidemic curve may lag behind changes in the underlying transmission dynamics by at least 1 serial interval, as has previously been shown for severe acute respiratory syndrome (*3*–*5*). Public health practitioners must be encouraged to use these methods routinely.

Finally, we concur that a multipronged, targeted, layered approach will likely provide the best mitigation strategy in the event of a pandemic. However, we caution against conflating good public health practice of "pulling out all the stops" in the event of a pandemic with good scientific practice of evaluating the independent effect of school closures, which was the object of our article.

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Legionella: Molecular Microbiology

Klaus Heuner and Michele Swanson, editors

Caister Academic Press, Norwich, UK, 2008
ISBN: 978-1-904455-26-4
Pages: 249; Price: US $300

The first 3 chapters of *Legionella*: Molecular Microbiology deal with clinical aspects of legionellosis. Paul Edelstein provides a fascinating glimpse into the history of Legionnaires' disease, revealing little-known facts from the perspective of one who participated in the investigation of the 1979 outbreak at Wadsworth Veterans Administration Hospital in Los Angeles, California. Although the chapter on epidemiology focuses on Legionnaires' disease in Europe, it provides new information from the European Working Group for *Legionella* Infections.

The remaining 9 chapters cover various aspects of the molecular biology of *Legionella*. These chapters provide up-to-date information for basic scientists, but they are also of value to clinicians. However, the chapter on genetics and immunology of host resistance to *Legionella* infections is illuminating, yet disappointing—illuminating because it shows that much has been learned but disappointing in that we still do not understand much about the biology of *Legionella* infections. It is also disappointing that the efforts of clinicians and basic scientists are not coordinated at the time of outbreaks, so material could be collected that would enable more in-depth study of the genetics and immunology of host resistance.

The book provides a glimpse into the challenges of studying an intracellular pathogen that is found in human macrophages and in amebae. For anyone who studies intracellular pathogens, the chapter on mechanisms of intracellular survival and replication of *L. pneumophila* will be instructive.

One minor distracting feature is that the introduction to most chapters tends to repeat the same material about Legionnaires' disease. Also, a background in molecular biology would be helpful in understanding some of the more technical chapters, although it is not absolutely necessary. Overall, not only will this book be pertinent to all who study *Legionella* spp. and other intracellular microorganisms in the laboratory, but it will also be a valuable reference for infectious disease clinicians, microbiologists, and public health professionals.

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ANOTHER DIMENSION

It Can't Happen Here

Marcela Natiello

A banal virosis, no doubt
Fever fades away, back at work
I feel weary, I'll be all right, I'm sure
What, now? My body itches
Could this be an allergy?
A pill to halt the itching
Again ill, what does this nausea mean?
Could this be dengue?
It's on the borders, the global warming …
And yet, I didn't leave the city
In Buenos Aires you can't …
Just in case, I have a test done
I can't believe it, the test is positive
Spread the news, could be more cases,
— Needs confirmation, it can't happen here
Why not? Imported cases, *Aedes* thriving …
There must be others, we must warn people
— We are not sure, can't happen here
At last, new test confirms. You must warn people
Not even now? What about the others?
Those who don't know, they are at risk
— It won't spread, can't happen here
As a physician I feel responsible
Please spread the news
It did happen here!

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Acknowledgement

I thank Dr. Viviana Ritacco and Mrs. Liliana Palumbo for reviewing the manuscript.

Dr Natiello is the patient referred to in this poem and a pneumonologist at the Muñiz Hospital, Buenos Aires, Argentina. Her primary research interest is the epidemiology of tuberculosis.

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Cameron Hayes (b. 1969) The Russians knew perfectly well that the happiness of the African animals was that they had such low expectations—before the pets were introduced (detail) (2008). Oil on linen (203.2 cm × 254 cm) Copyright Cameron Hayes. Courtesy Ronald Feldman Fine Arts, New York

Traveling Light and the Tyranny of Higher Expectations

Polyxeni Potter

"Three tribes of Babylonians," Herodotus wrote, "eat nothing but fish, which they catch and dry in the sun. They pound the dried fish in a mortar with a pestle and sift through a cloth then mix with liquid and bake like bread." Such are their customs, he reported, "Having no physicians, they bring the sick to the agora to receive advice from passers-by who have similar ailments."

Travel anecdotes fill Herodotus' histories. He recorded them so that "happenings will not be lost to human memory nor great and fantastic deeds … fade." Mocked for his accounts of outlandish behavior, Herodotus got no respect until centuries later, when similar unlikely behavior was seen elsewhere, and its anthropological and ethnographic roots were verified. Human fascination with travel to mysterious lands has occupied artists as well as writers throughout the ages. Australian painter Cameron Hayes, whose work graces this month's cover, offers his own narrative version of travel.

Hayes, whose interests in human behavior are reflected in all his work, traces his roots far from today's art centers, even if he exhibits in galleries all over the world. Born in Sydney and now based in Melbourne, he has explored the effects of European settlement on the Aboriginal population in Milikapiti on Melville Island off the northern coast of Australia. He has articulated in his art the loss of cultural identity and health to often well-intentioned outside influences. This journey inward sharpened his vision of today's global scene, which he views with suspicion and satirizes without mercy in his paintings.

Hayes' style, resistant to prevailing art trends, is narrative. His work tells a story, in the tradition of Hieronymus Bosch (c. 1450–1516), who painted fantastic images derived from biblical and folkloric sources to address the moral conflicts of his day. Hayes also monitors human behavior and evaluates its effects. In complex scenes packed with minute detail, he projects the absurdity of human interaction in a globalized world gone mad. His acid humor is reminiscent of Pieter Bruegel the Elder's, only he takes on not just the country yokel but humanity at large.

Unlike many contemporary artists whose work often relies on theory and explanation, Hayes says little about his paintings, allowing the viewer to draw conclusions directly from his densely populated, multifocal, fictitious scenes and their hidden messages. "Far out to sea and west of

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Spain,/There is a country named Cokaygne," goes the poetic description of medieval utopia that could be describing Hayes' destinations, "No place on earth compares to this/ For sheer delightfulness and bliss." At first glance, his colorful paintings appear playful and lyrical, full of movement and intrigue. "There's no fly or flea or louse/In clothes, in village, bed, or house;/There's no thunder, sleet, or hail,/Or any nasty worm or snail." But on closer inspection, a story unfolds that is often disturbing as much as captivating, dark as well as enlightening.

"They bury their Dead with their Heads directly downwards; because they hold an Opinion, that in eleven Thousand Moons they are all to rise again; in which Period, the Earth (which they conceive to be flat) will turn upside down, and by this Means they shall, at their Resurrection, be found ready standing on their Feet," wrote Jonathan Swift about the inhabitants of Lilliput, in Gulliver's Travels. Swift, continuing in the tradition of Herodotus, wrote about travel adventures. But, an inveterate satirist, he spiced them liberally with biting wit intended to upset and reform a malfunctioning society. "The chief end I propose to myself in all my labours is to vex the world rather than divert it."

In The Russians knew perfectly well …, Hayes' travel report seems to marry the wide-eyed astonishment of Herodotus with the edginess of Swift. Strange things happen in far off lands. But not even Hieronymus Bosch could have anticipated an angle as original and frightful as Hayes'. This time it is not the natives who demonstrate outlandish behavior but the visitors. Animals, he suggests, once lived happily in the wild, munching and frolicking in a potent state of anarchic freedom, living and dying their natural lives and deaths. Then humans arrived in their iron birds bringing their traps, their needs, their greed, their haplessness, and their neuroses.

The scene unfolds inside and outside the airplane and in some vacuous unreal landscape beneath. The panoramic view, a carnival of shape and color, yields a diminutive cosmos of stunning complexity. Animals, moved away from their natural habitat and become domesticated, have turned into caricatures of themselves, mindlessly engaged in meaningless tasks for no reason. The cartoonlike elephant on the upper left corner covers the eyes in dismay; the giraffe is clearly distressed. Awash in human fashions, the animals exhibit bizarre symptoms, biting themselves and each other or perched weirdly on floating vegetation.

Human behavior, in ancient Babylon, Lilliput, or Milikapiti, has cultural, economic, and public health consequences. Ecotourism has attracted people to remote animal habitats, and commerce has moved animals to new environments. Despite evidence of disease risks, demand for exotic pets is high. Despite inherent hazards (Buruli ulcer, malaria, dengue, avian flu, norovirus infection), humans move freely around the globe. "People," Hayes says, "invariably find creative and elaborate ways of maintaining their perception, against all the available evidence, rather than questioning their perception of reality."

In the wild, animals had no expectations. They did not travel far, nor did they carry luggage. Their happiness was guaranteed. Now, part and parcel of public transportation, they have lost not just their innocence and wildness but also the natural quarantine rendered by the borders of their habitat. And their bags are packed with more than human expectations. They have joined the growing zoonoses network, unknowingly moving microorganisms around the globe and expanding the scope and span of disease.

Acknowledgment

The author thanks Louise E. Shaw for her help in obtaining permission to use The Russians knew perfectly well that the happiness of the African animals was that they had such low expectations—before the pets were introduced.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

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Kupe Virus, a New Virus in the Genus *Nairovirus*

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Biochemical and Molecular Characterization of 263K Scrapie Agent in Hamster Species

Spread of Oseltamivir-Resistant Influenza A Virus (H1N1), Norway, 2007–2008

Medical Procedures and Risk for Sporadic Creutzfeldt-Jakob Disease

Shifting Epidemiology of *Vibrio parahaemolyticus* Outbreaks in Southern Chile

Surveillance of Invasive Group A Streptococcal Disease in Fiji, 2005–2007

Outbreak of Skin and Soft Tissue Infections Caused by *Staphylococcus aureus*, Northern Italy

Rapid Identification of Melioidosis Outbreak

Simian T-Lymphotropic Virus in Hunter-Collected Nonhuman Primates, Cameroon

Evidence for Seoul Virus as Cause of Hantavirus Disease Outbreak, Shenyang, China

Retrospective Analysis of Pediatric Malaria Imported to Industrialized Countries

Highly Pathogenic Avian Influenza Virus (H5N1) from Frozen Duck Carcasses, Germany, 2007

Complete list of articles in the February issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

February 12–13, 2009
The International Symposium on the Asian Tiger Mosquito
Rutgers University
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Article Title

Sphingomonas paucimobilis Bloodstream Infections Associated with Contaminated Intravenous Fentanyl

CME Questions

1. Which of the following organisms is most likely to have been reported as a contaminant associated with betamethasone injection?

A. *Sphingomonas paucimobilis*
B. *Serratia marcescens*
C. *Pseudomonas putida*
D. *Exophiala* spp.

2. Which of the following is least likely to be an accurate description of *S. paucimobilis*?

A. Gram-positive
B. Glucose-nonfermenting
C. Yellow-pigmented
D. Found in soil and water

3. In this case series, which of the following was investigated as a source of exposure to fentanyl that resulted in *S. paucimobilis* bacteremia infection in patients?

A. Intravenous infusions
B. Contrast agents
C. Medications
D. All of the above

4. Which of the following strategies was recommended by the study authors to reduce the incidence of bacterial contamination by compounding pharmacies?

A. Inspect source of drugs before preparation
B. End-product sterility testing
C. Elimination of compounding pharmacies
D. None of the above

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

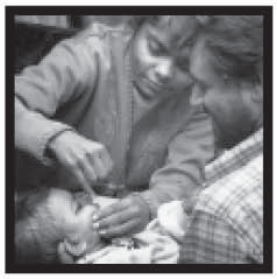
   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

Fighting the obesity epidemic

Screening Women for Breast Cancer



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December 2008

Zoonotic Infections

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Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (www.cdc. gov/ncidod/EID/trans.htm).

Instructions to Authors

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, figure legends, appendixes, and figures. Each figure should be in a separate file.

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. Provide figures as separate files, not embedded in MS Word. Use Arial font for text content. Place keys within figure area. Provide footnotes and other information (e.g., source/copyright data, explanation of boldface) in figure legend. Submit figures with text content in native, editable, PC file formats (e.g., MS Excel/PowerPoint). Submit image files (e.g., electromicrographs) without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files. Submit separate files for multiple figure panels (e.g., A, B, C). EPS files are admissible but should be saved with fonts embedded (not converted to lines). No PNG or BMP files are admissible. For additional guidance, contact fue7@cdc.gov or 404-639-1250.

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief 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 be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

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

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.