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July 2014

Antimicrobial Resistance

Eileen Pestorius (b. 1939) The Alamo (2014) Watercolor on paper (12 3/4 x 24 in/32.385 cm x 60.96 cm) Courtesy of the Artist

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On the Cover

Eileen Pestorius (b. 1939)

The Alamo (2014)

Watercolor on paper (12 3/4 × 24 in/ 32.385 cm × 60.96 cm)

Courtesy of the Artist

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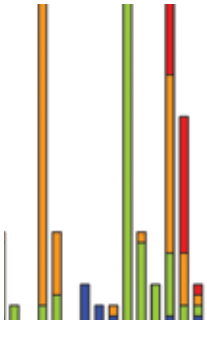

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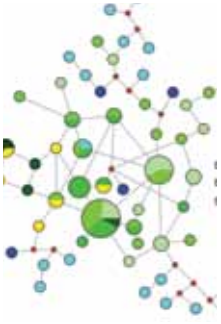

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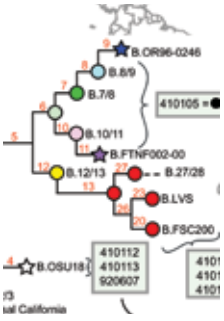

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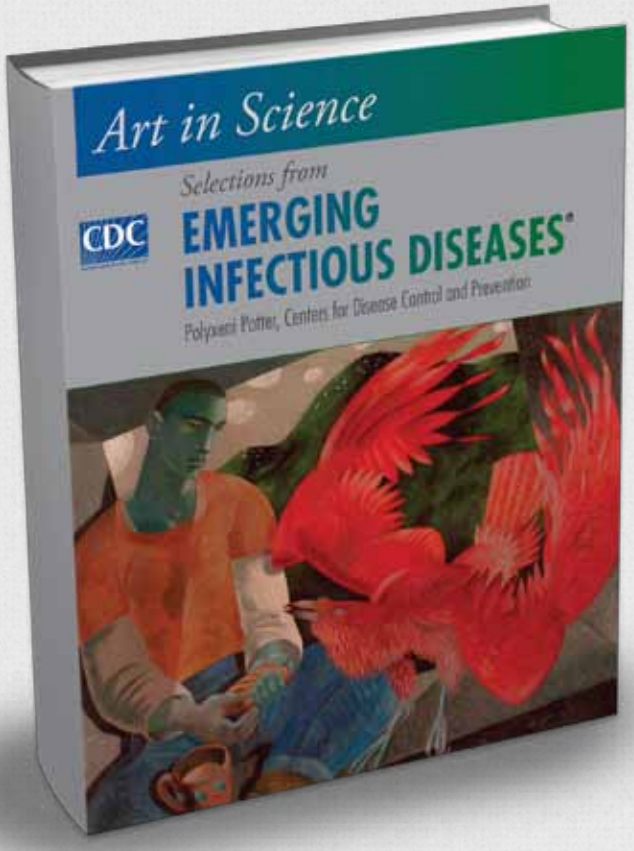

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This collection of 92 excerpts and covers from **Emerging Infectious Diseases** will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.

Salmonella enterica Serovar Enteritidis, England and Wales, 1945–2011

Christopher R. Lane, Susan LeBaigue, Oluwaseun B. Esan, Adedoyin A. Awofisyo, Natalie L. Adams, Ian S.T. Fisher, Kathie A. Grant, Tansy M. Peters, Lesley Larkin, Robert H. Davies, and Goutam K. Adak

In England and Wales, the emergence of *Salmonella enterica* serovar Enteritidis resulted in the largest and most persistent epidemic of foodborne infection attributable to a single subtype of any pathogen since systematic national microbiological surveillance was established. We reviewed 67 years of surveillance data to examine the features, underlying causes, and overall effects of *S. enterica* ser. Enteritidis. The epidemic was associated with the consumption of contaminated chicken meat and eggs, and a decline in the number of infections began after the adoption of vaccination and other measures in production and distribution of chicken meat and eggs. We estimate that >525,000 persons became ill during the course of the epidemic, which caused a total of 6,750,000 days of illness, 27,000 hospitalizations, and 2,000 deaths. Measures undertaken to control the epidemic have resulted in a major reduction in foodborne disease in England and Wales.

A pandemic of *Salmonella enterica* serovar Enteritidis infection was recognized by epidemiologists in the United States in the late 1970s; a 6-fold rise in these infections was observed in northeastern United States during 1976–1986 (*1*). A review of outbreak investigations revealed that 27 (77%) of 35 outbreaks were associated with the consumption of foods containing grade A eggs (*1*). The most commonly reported phage types were SE8, SE13, and SE13a. In 1990, the World Health Organization reviewed *Salmonella* surveillance data for 1979–1987 and found that isolation rates for *S. enterica* ser. Enteritidis had increased in 24 of the 35 nations that provided data. Increases were recorded in countries from every continent except Asia (*2*). Evidence from outbreak investigations in Spain, Hungary, France, Norway, and the United States implicated eggs (*3*). Microbiologicical investigations conducted in the United Kingdom also showed the presence of phage type SE4 in chicken meat (*4*) and raw shell eggs (*5*,*6*). In 1988, the UK Public Health Laboratory Service Communicable Disease Surveillance Centre conducted a case–control study of primary sporadic SE4 infections in England. The investigators demonstrated associations between human infection and the consumption of chicken and raw egg dishes (*7*). We reviewed national surveillance and research data to examine the factors underlying the epidemic of *S. enterica* ser. Enteritidis and to estimate its overall impact on the population of England and Wales.

Author affiliations: Public Health England, London, UK (C.R. Lane, S. LeBaigue, O.B. Esan, A.A. Awofisyo, N.L. Adams, I.S.T. Fisher, K.A. Grant, T.M. Peters, G.K. Adak); and Animal Health and Veterinary Laboratories Agency, London (L. Larkin, R.H. Davies)

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Methods

Surveillance of *S. enterica* Infections and Other Intestinal Diseases in England and Wales

Systematic national surveillance of laboratory-confirmed salmonellosis in humans in England and Wales has been in continuous operation since 1945. Diagnostic laboratories refer all *Salmonella* isolates to the national reference laboratory for confirmation and characterization, and data on all first confirmations are entered into a national surveillance database (*8*).

We extracted data from this database to provide annual totals for human infection with *S. enterica* by serotype and phage type. Multipliers derived from previous studies (*9*–*11*) were applied to the number of laboratory reports received to produce estimates of the numbers of community cases, days of illness, hospitalizations, hospital bed-days occupied, and deaths for 1982–1987, 1988–1998, and 1999–2011 that were attributable to SE4.

Multipliers published in 1996 (*9*) were used for the emergence and epidemic stages and those from 2008 (*10*) for the decline stage.

In addition, local health protection units return standardized data (i.e., etiology, outbreak location, morbidity/mortality rates, vehicles of infection, and evidence of association) on all detected general outbreaks of infectious intestinal diseases to national surveillance (*12*). These data are also stored in a dedicated database.

Surveillance of *S. enterica* in Poultry

Data on *Salmonella* spp. in poultry in Great Britain (England, Wales, and Scotland) are reported by the Animal Health and Veterinary Laboratory Agency (*13*). A *Salmonella* incident is defined as the first isolation of a given serovar from a particular animal, group of animals, or their environment on a single premises within a defined period (usually 30 days) (*13*).

Data Analyses

Data were abstracted from the national surveillance databases described above. Descriptive analyses were done in Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA); 95% CIs of the estimates of the burden of disease in the community were calculated from the upper and lower confidence limits reported in previous studies (*9,10*). All statistical analyses were performed by using Stata version 12 (StataCorp LP, College Station, TX, USA).

Results

Trends in Human Salmonellosis in England and Wales

Figure 1 shows the contribution of *S. enterica* ser. Enteritidis to the overall scope of human salmonellosis in England and Wales during 1945–2011. During this period, >740,000 laboratory reports of *S. enterica* infection were received; almost 330,000 (43%) were for *S. enterica* ser. Enteritidis. The reporting patterns show that the epidemiology of this pathogen can be divided into 4 stages: pre-epidemic (1945–1981); emergence (1982–1987); epidemic (1988–1998); and decline (1999 onwards).

The surveillance trends for *S. enterica* for the years 1945–1981 mainly reflect the reporting patterns for serotype Typhimurium; for most of this period, this serotype was the most commonly reported, whereas serotype Enteritidis accounted for <10% of cases of salmonellosis in all but 5 of the 37 years of the pre-epidemic stage. During the emergence stage, the percentage of salmonellosis cases caused by serotype Enteritidis rose from 9% (1,099 reports) to 33% (6,746 reports). In 1988, serotype Enteritidis supplanted serotype Typhimurium as the most commonly reported serotype.

S. enterica ser. Enteritidis accounted for more than half of all salmonellosis cases for all of the epidemic stage (1988–1998). In 1997, reporting of serotype Enteritidis accounted for 70% (23,231 reports) of all salmonellosis cases. During the decline stage, the share of salmonellosis attributable to serotype Enteritidis fell from 60% (10,827 reports) to 28% (2,566 reports in 2011). Despite its sharp decline during the final years of the surveillance period, however, reporting of serotype Enteritidis has remained above the levels observed during the pre-epidemic stage.

Surveillance of *S. enterica* ser. Enteritidis, 1982–2011

We examined trends in the reporting of *S. enterica* ser. Enteritidis during 1982–2011 in more detail. During this period, 312,719 laboratory reports for serotype Enteritidis were received. After reports of travel-associated infection were excluded, 269,779 reports remained. In 1982 and 1983, SE8 was the most commonly identified phage type, accounting for ≈60% of all cases. Indigenously acquired infection with SE4 was reported at a crude rate of

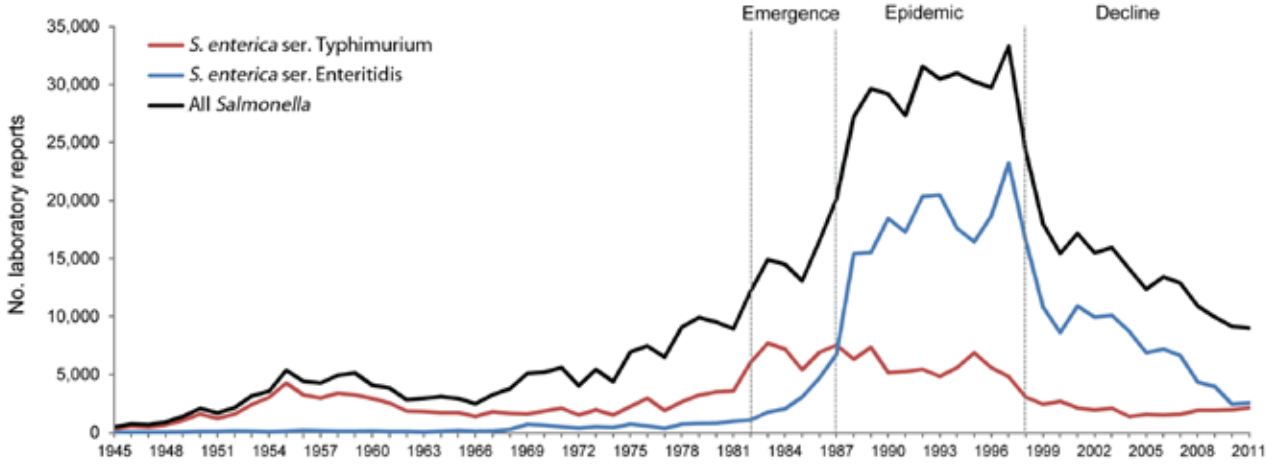

Figure 1. Laboratory reporting of *Salmonella enterica* infections in England and Wales, 1945–2011. Emergence stage, 1982–1987; epidemic stage, 1988–1998; decline stage, 1999–2011. Ser., serovar.

0.5 cases/100,000 population in 1982. However, in 1984, SE4 became the dominant phage type, contributing 57% of all indigenously acquired infections (crude rate 1.4 cases/100,000 population). Figure 2 shows that the emergence stage marked an accelerating rise in indigenously acquired SE4 infection in England and Wales. During this period, the incidence of indigenously acquired SE4 infection was sustained at or above a crude rate of 30 cases/100,000 population. The reporting of SE4 infections reached its peak in 1993 at 16,127 laboratory reports (i.e., 86% of all indigenously acquired *S. enterica* ser. Enteritidis infections).

The decline stage was characterized by absolute and relative reductions in the contribution of SE4 to the overall scope of *S. enterica* ser. Enteritidis infection. By 2011, the crude rate of reporting had fallen to 0.4 cases/100,000 population. This stage also represents a period when other phage types came into prominence. Even so, for every year from 1984 to 2001, SE4 accounted for more than half of all indigenously acquired *S. enterica* ser. Enteritidis infections.

During its emergence, 7,481 reports of indigenous SE4 infection were received. This compares with 143,767 reports received during the epidemic stage and 29,522 during the decline. Estimates for the burden of indigenous disease attributable to SE4 infection during the emergence, epidemic, and decline stages are shown in the Table.

Demographically, we found no significant regional or gender differences in the reporting rates for SE4 infection. Children ≤14 years of age consistently accounted for one quarter of all cases (crude rates: 1982, 0.4 cases/100,000 population; 1984, 1.1 cases/100,000; 1992, 45 cases/100,000; 2011, 0.6 cases/100,000).

Surveillance of General Outbreaks of Infection in England and Wales, 1992–2011

Standardized surveillance reports were returned for 2,667 general outbreaks of foodborne infection in England and Wales during 1992–2011. *S. enterica* was the causative agent in 1,195 (45%) outbreaks; 914 (34%) cases were attributable to *S. enterica* ser. Enteritidis, of which 585 (22%) were attributable to SE4. In the portion of the *S. enterica* ser. Enteritidis epidemic stage during which general outbreak surveillance was in operation (1992–1998), SE4 infections accounted for 474 (30%) of the 1,576 outbreak reports received, compared with 7% for other *S. enterica* ser. Enteritidis (non-SE4). However, during the decline stage, the proportion of foodborne outbreaks caused by SE4 infections fell to 10% (111/1,082), and during the last 5 years of surveillance (2007–2011), SE4 accounted for only 3% of outbreaks (10/330).

During 1992–2011, the trends in the reporting of foodborne outbreaks in England and Wales were partially driven by outbreaks of SE4 infections (Figure 3). During 1992–2011, a total of 9% (12,647/133,959) of all SE4 laboratory reports received were linked to general outbreaks. By 2011, the numbers of SE4 laboratory reports and general outbreaks had fallen to 1% of the 1992 reporting levels.

Vehicles of infection were identified in 471 (80%) of 585 SE4 outbreaks reported during 1992–2011. Chicken meat accounted for 76 (16%) outbreaks, but chicken-associated outbreaks of SE4 declined sharply during the surveillance period. During 1992–1993, a total of 31 (16%) of 192 SE4 outbreaks were attributable to chicken meat, but during 1994, the proportion of SE4 infections attributable to chicken meat fell to 10% (4/39), where it remained through 2011. By contrast, 195 (41%) of the SE4 outbreaks were attributable to egg consumption. During the epidemic stage, SE4 accounted for 159 (79%) of 201 egg-associated *S. enterica* ser. Enteritidis outbreaks (Figure 4). The decline stage was marked by sharp falls in the number and proportion (36/95 [38%]) of egg-associated *S. enterica* ser. Enteritidis outbreaks attributable to SE4. Only 5 egg-associated outbreaks of SE4 infection were reported during 2007–2011. By contrast, the contribution of non-SE4 isolates rose from 21% (42/201) during 1992–1998 to 62% (59/95) during 1999–2011.

Lightly cooked desserts were the most commonly reported egg-based vehicles of infection implicated in

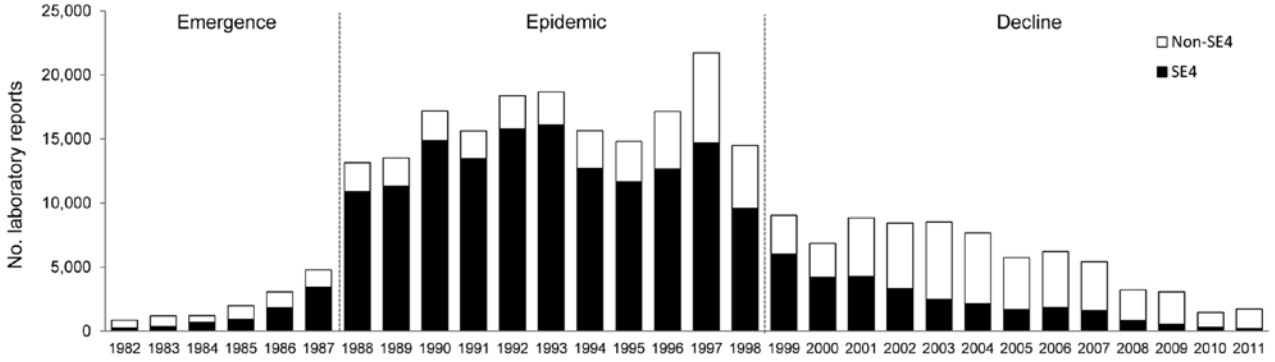

Figure 2. Laboratory reporting of indigenously acquired *Salmonella enterica* serovar Enteritidis infections in England and Wales, 1982–2011. Emergence stage, 1982–1987; epidemic stage, 1988–1998; decline stage, 1999–2011. SE4, *S. enterica* ser. Enteritidis phage type 4.

Table. Estimated rates of disease attributable to *Salmonella enterica* serovar Enteritidis phage type 4 during 3 periods, England and Wales, 1982–2011

Stage	No. laboratory-confirmed cases	No. community cases (95% CI)	No. days of illness	No. hospital admissions	No. hospital bed-days	No. deaths
Emergence, 1982–1987	7,481	16,458 (8,379–71,817)	270,000	1,000	6,000	90
Epidemic, 1988–1998*	143,767	374,516 (161,019–1,380,163)	5,000,000	21,000	122,000	1,630
Decline, 1999–2011†	29,522	135,801 (41,331–661,292)	1,300,000	5,000	30,000	410
Total	180,770	526,766	6,570,000	27,000	158,000	2,130

*Multiplier from (*9*).
†Multiplier from (*10*).

S. enterica ser. Enteritidis outbreaks during the epidemic stage. This group excludes cakes but includes custard-based desserts such as tiramisu and zabaglione; mousses; meringues; and custom-made ice creams and sorbets. This category accounted for 109 (54%) of the 201 egg-associated *S. enterica* ser. Enteritidis outbreaks reported during 1992–1998; of these outbreaks, 80 (40%) were attributable to SE4. In the 13 following years, the proportion of egg-associated outbreaks associated with these desserts fell to 33% (31/95); half of these (16) were caused by SE4.

Lightly cooked/uncooked sauces made from raw eggs (e.g., hollandaise sauce, mayonnaise) were implicated in 24 (12%) of the 201 egg-associated *S. enterica* ser. Enteritidis outbreaks during the epidemic stage; 22 (92%) of these were caused by SE4. Thirteen sauce-associated outbreaks were reported in the following 13 years; 3 (23%) were caused by SE4.

In contrast to other food vehicles, the number of outbreaks associated with simple egg dishes (i.e., fried eggs, boiled eggs, scrambled eggs, omelets, egg fried rice) increased during the decline stage. During 1992–1998, simple egg dishes were implicated in 51 (25%) of 201 outbreaks; the number rose to 49 (52%) of 95 outbreaks during 1999–2011. The proportion of outbreaks associated with simple egg dishes that were attributable to non-SE4 rose from 12% (6/51) during the epidemic stage to 67% (33/49) during the decline stage.

Only 7 outbreaks linked to eggs served in Chinese restaurants were reported during 1992–1998; all were caused by SE4. A total of 21 outbreaks linked to Chinese restaurants were reported during 1999–2011, and 4 were caused by SE4. The dish most commonly implicated was egg fried rice (22/28 outbreaks [79%]).

Surveillance of *S. enterica* Infection in Livestock

We found few national surveillance reports of *S. enterica* ser. Enteritidis in nonpoultry livestock. For the few incidents in which the pathogen was identified in cattle, sheep, pigs, and turkeys, SE4 was the predominant phage type isolated.

We compared trends in national surveillance data for *S. enterica* in chickens in Great Britain during 1985–2011(Figure 5) with those for human infection. Post-1991 data showed that a high proportion of the outbreaks from 1985–1990 were likely to be the result of SE4 infection.

The trends in the reporting of *S. enterica* ser. Enteritidis in chickens and cases of human infection were in general agreement during the emergence stage and the first 6 years of the human epidemic stage (1988–1993). The contribution of *S. enterica* ser. Enteritidis to reported incidents of salmonellosis rose from 3% (15/553) in 1985 to 66% (881/1,342) in 1993, the year in which vaccination of breeder chicken flocks against this pathogen was introduced. A 2-stage decline followed; the first stage was marked by a 70% (618/881) decrease in reports of *S. enterica* ser. Enteritidis infections in chickens during the 1994 calendar year, corresponding with wide uptake of

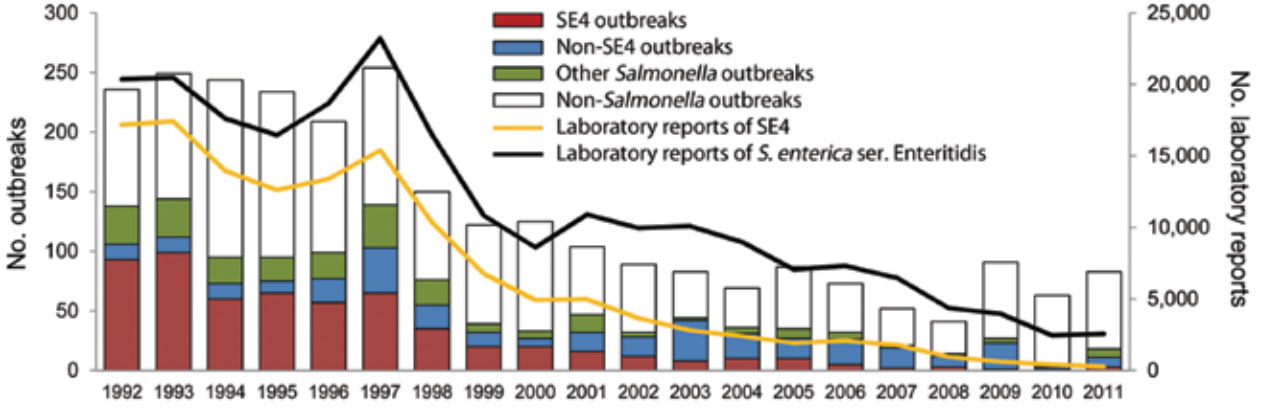

Figure 3. Trends in the pathogens associated with general outbreaks of foodborne infection in England and Wales, 1992–2011. SE4, *Salmonella enterica* serovar Enteritidis phage type 4.

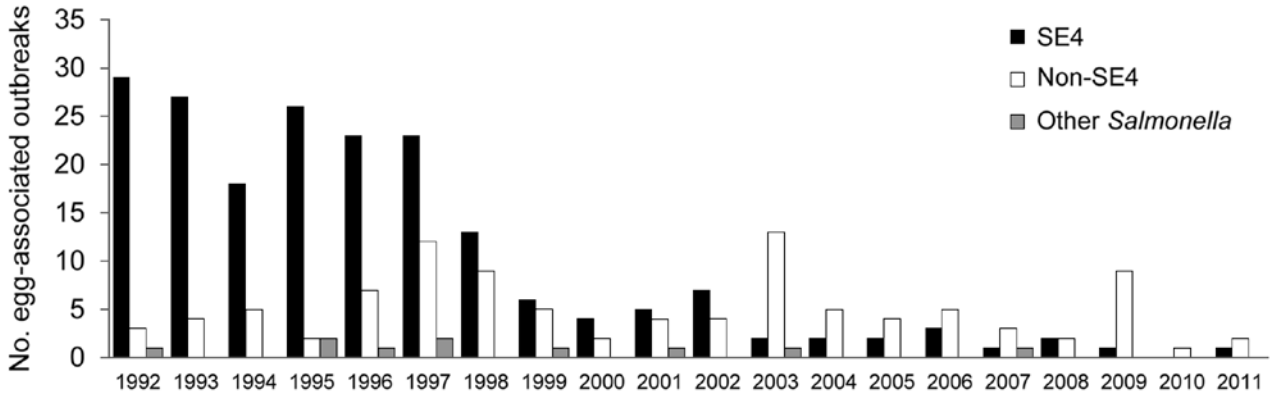

Figure 4. Trends in the reporting of general outbreaks of salmonellosis associated with the consumption of eggs in England and Wales 1992–2011. SE4, *Salmonella enterica* serovar Enteritidis phage type 4.

vaccination among breeding flocks. A plateau in reporting was then observed for the remainder of the human epidemic (1994–1998); case levels were maintained at 20%–30% of the 1993 value.

The second stage of decline followed the introduction and subsequent extension of the vaccination program (R.H. Davies, pers. comm.), enhanced farm hygiene, and management standards implemented through a farm assurance scheme for major egg layer flocks in 1997 (*14*). This decline lasted for 2 years. Since 1999, incident reporting has remained below 5% of 1993 levels for all but 2 of 12 years. Reporting has shown an ongoing decline that corresponds with extension of vaccination and improved control measures to smaller-scale egg producers; industry preparations for the implementation of the *Salmonella* National Control Programme in commercial laying chicken flocks in 2008; and application of harmonized European Union–wide restrictions on sale of fresh eggs from flocks infected with *S. enterica* ser. Enteritidis or Typhimurium, which began in 2009 (Figure 5). In 2001, attenuated vaccines were replaced by live vaccines, and in 2003, improved *S. enterica* ser. Gallinarum rough mutant 9R auxotrophic live vaccines were adopted.

During the 27-year period, *S. enterica* ser. Enteritidis accounted for 24% (6,074/25,049) of reported *S. enterica* incidents in chickens. However, 94% (5,690/6,074) of these incidents were reported during 1987–1998, the height of the human epidemic.

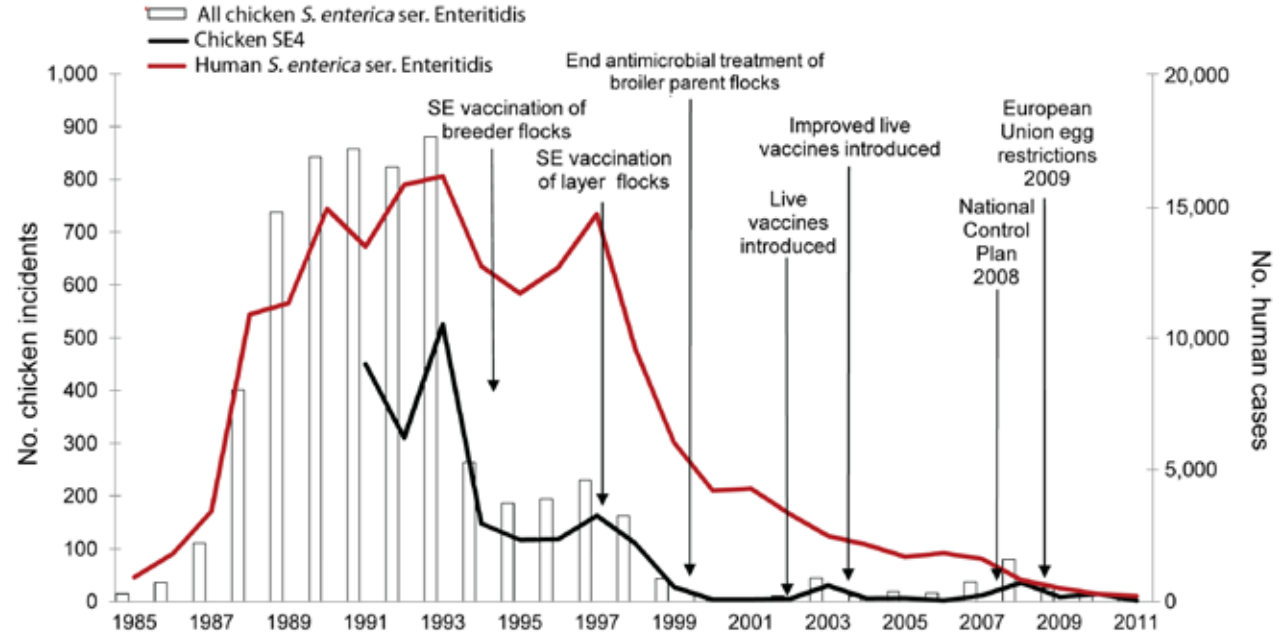

Figure 5. Trends in the reporting of incidents of *Salmonella enterica* in chickens in Great Britain versus laboratory reporting of human *S. enterica* serovar Enteritidis infection, England and Wales, 1985–2011. SE4, *S. enterica* ser. Enteritidis phage type 4.

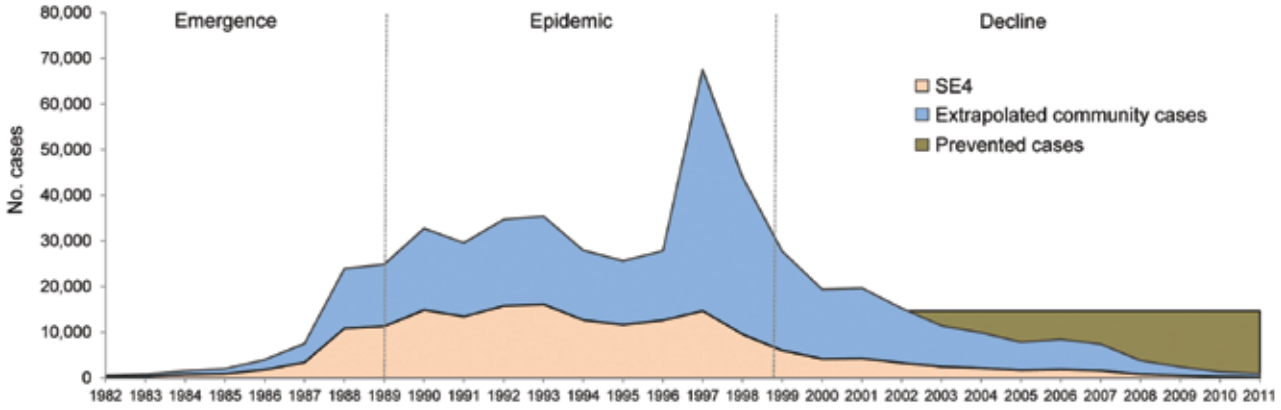

Figure 6. Trends in reporting of *Salmonella enterica* serovar Enteritidis phage type 4 (SE4), extrapolated burden of disease, and estimated number of cases prevented by SE4 elimination programs, England and Wales, 1982–2011.

Discussion

Our examination of almost 7 decades of national surveillance data leads us to the conclusion that the emergence of *S. enterica* ser. Enteritidis infection in 1982 resulted in the largest, most persistent epidemic of foodborne infection attributable to a single subtype of any pathogen since systematic national microbiologic surveillance of disease was established in England and Wales. The national *Salmonella* surveillance dataset provides an uninterrupted, 67-year record of the epidemiology of human *S. enterica* infection in England and Wales. Our analyses of the serotype and phage typing dataset enabled us to examine the size and duration of epidemics of foodborne infection caused by subtypes belonging to a range of serovars of *S. enterica* that have occurred since 1945. These epidemics included several sustained, high-impact outbreaks: *S. enterica* ser. Typhimurium during 1949–1961(*15*); *S. enterica* ser. Agona during the late 1960s/early 1970s; *S. enterica* ser. Hadar during the late 1970s; *S. enterica* ser. Typhimurium DT204 during the early 1980s (*14*); and *S. enterica* ser. Typhimurium DT104 during the 1990s (*16*).

The *S. enterica* ser. Typhimurium epidemic of the 1950s gave rise to ≈20,000 excess laboratory reports in 12 years, a mean of 1,667 per year. By comparison, our estimates indicate that the SE4 epidemic gave rise to an excess of ≈160,000 laboratory reports of indigenous infection over 30 years, a mean of 5,333 per year.

The underlying causes that lay behind the rise and fall of earlier epidemics of salmonellosis are poorly understood (*15*). The scale and geographic reach of the rise of *S. enterica* ser. Enteritidis were recognized at an early stage, which led to the development of concerted national and international initiatives. These efforts have enabled scientists to gain better insight into the factors that mediated the course of what is now recognized as a sustained and continuing foodborne pandemic. The work of scientists from many countries has shown that *S. enterica* ser. Enteritidis emerged and quickly became established in much of the global poultry flock (*2*). An ecologic niche may have been created after the introduction of eradication programs targeted against *S. enterica* serovars Pullorum and Gallinarum and as a result of international trade in infected breeding stock (*17*), before the importance of *S. enterica* ser. Enteritidis infection was recognized and minimal monitoring was put in place (*18,19*).

Surveillance data demonstrate that the rates of human *S. enterica* ser. Enteritidis infection in England and Wales remained high during 1988–1998 despite national guidance aimed at the public and industry (*20*). From 1997 to 2011, disease incidence decreased 99%. This decrease cannot be explained by changes in the performance of surveillance resulting from the behavior of patients, clinicians, or laboratories. The results of 2 studies of intestinal disease (*9,10*) demonstrate that relatively small changes in the ascertainment of salmonellosis by laboratory report surveillance occurred during this period.

Comparison of trends in the reporting of incidents in the chicken flock in Great Britain with human surveillance data showed that the rise in human *S. enterica* ser. Enteritidis infection matched the rise in disease in chicken farms. Reporting of incidents in chickens started to decrease in 1994, after the introduction of a voluntary national vaccination and flock hygiene program targeted at breeder chicken flocks (*21*) (Figure 5). The vaccine was not specific to SE4. Although the program was not mandatory, anecdotal stakeholder information indicates that it was adopted by a large proportion of the industry. The reduction of reported infection in chickens appears to have had a limited effect on the trend in human infection as measured by laboratory report surveillance (Figure 5). However, the reporting of outbreaks of *S. enterica* ser. Enteritidis associated with the consumption of chicken also showed a sharp decline

dating from 1994. By contrast, the reporting of egg-associated outbreaks did not start to decline until 1997, after the introduction of *S. enterica* ser. Enteritidis vaccination and flock hygiene program aimed at laying chicken flocks (*22*). This program included improved rodent control; feed monitoring; biohazard control; microbiological monitoring throughout all stages of production; and industry quality assurance schemes (*23*). This point also marks the start of the sharp decline in the human *S. enterica* ser. Enteritidis epidemic. Therefore, after considering the trends in the human and veterinary surveillance data and the findings of the 1988 case–control study (*7*), we infer that the epidemic in humans was associated with the consumption of both chicken and eggs. However, because control of *S. enterica* ser. Enteritidis in the production of chicken meat had much less effect on the course of the epidemic than control in eggs, we further conclude that the epidemic was largely attributable to the contamination of eggs. Persons became infected through the consumption of contaminated foods in commercial catering and home settings. The improvements in hygienic practice from egg production and distribution through the main supermarket chains has resulted in major improvements in the microbiologic quality of eggs bought by consumers in the United Kingdom (*20*).

Well-designed and -maintained national programs using hygiene control strategies to control *S. enterica* in primary production and distribution have also been successful in reducing the occurrence of *S. enterica* ser. Enteritidis in the food chain in the United States (*24*) and Denmark (*25*). However, data from harmonized surveillance of layer flocks in Europe indicate that *S. enterica* ser. Enteritidis infection remains a problem in egg production in many European Union member states (*26*). International surveillance data (*27*) and recently reported outbreaks also demonstrate that contamination of eggs remains a problem in many parts of Europe and the United States (*28*). This knowledge adds weight to our conclusion that the reduction in *S. enterica* ser. Enteritidis in chicken flocks in Great Britain stemmed from the introduction and maintenance of a suite of carefully designed and regulated interventions. In addition, accumulating evidence indicates that cross-sectoral national control strategies designed according to national needs and conditions can be extremely effective in reducing the risk to human populations worldwide. Our analyses of outbreak data show that the risks associated with the use of eggs in uncooked or lightly cooked desserts and sauces highlighted in previous studies (*4*–*7*) continued in England and Wales until *S. enterica* ser. Enteritidis had effectively been eradicated from egg production.

Analyses of the laboratory report and outbreak surveillance show that the overall impact of SE4 in England and Wales has been greatly reduced. However, control of non-SE4 has been less successful, a finding reflected in data from other countries in Europe (*29*). Investigation of outbreaks indicates that infection is mainly transmitted through the consumption of imported eggs in commercial catering (*12,30,31*). Outbreaks linked to desserts and sauces served in the catering sector have declined markedly, but Italian restaurants were commonly associated with egg-associated outbreaks during the epidemic stage (*31*). However, since 1997, only 4 non-SE4 and 1 SE4 outbreaks were associated with Italian restaurants (*31*). This change is thought to be because many restaurants switched to liquid pasteurized eggs or Great Britain–produced eggs for sauces and desserts. We performed an informal review of restaurant menus and found these desserts and sauces are still widely available in restaurants in the United Kingdom but that simple egg-based dishes served in Chinese restaurants still tend to be made using raw shell eggs (*31*). Previous research indicates that use of imported raw shell eggs and poor hygiene practice are more common in this sector (*31*). Therefore, a need exists for establishment of safer practices across the catering sector.

Our estimates indicate that the *S. enterica* ser. Enteritidis epidemic in England and Wales had serious effects on the population and on the health care system. Had epidemic-stage infection levels been maintained from 1999 onward, we estimate that the introduction of effective interventions by the egg and poultry industries in Great Britain probably would have prevented ≈904,000 cases of illness in the community (225,973 20,247,145) (Figure 6), ≈6,300,000 days of illness, ≈26,000 hospitalizations, and ≈2,000 deaths since 1998. These figures should be treated with caution, but we suggest that a robust cost-benefit analysis of the epidemic and the interventions that led to its control would have great value for the development of improved food safety policies.

Acknowledgments

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Epidemiology, Clinical Manifestations, and Outcomes of *Streptococcus suis* Infection in Humans

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Streptococcus suis, a bacterium that affects pigs, is a neglected pathogen that causes systemic disease in humans. We conducted a systematic review and meta-analysis to summarize global estimates of the epidemiology, clinical characteristics, and outcomes of this zoonosis. We searched main literature databases for all studies through December 2012 using the search term "streptococcus suis." The prevalence of *S. suis* infection is highest in Asia; the primary risk factors are occupational exposure and eating of contaminated food. The pooled proportions of case-patients with pig-related occupations and history of eating high-risk food were 38.1% and 37.3%, respectively. The main clinical syndrome was meningitis (pooled rate 68.0%), followed by sepsis, arthritis, endocarditis, and endophthalmitis. The pooled case-fatality rate was 12.8%. Sequelae included hearing loss (39.1%) and vestibular dysfunction (22.7%). Our analysis identified gaps in the literature, particularly in assessing risk factors and sequelae of this infection.

Streptococcus suis is a neglected zoonotic pathogen that has caused large outbreaks of sepsis in China (*1*,*2*) and has been identified as the most common and the third leading cause of bacterial meningitis in adults in Vietnam and Hong Kong, respectively (*3*–*5*). *S. suis* infection is acquired from pigs, either during slaughtering or by handling and eating undercooked pork products. It is potentially preventable (*3*,*6*). Epidemiology of the infection differs between Western and Asian regions (*7*), and the role of high-risk eating habits (i.e., ingesting raw or undercooked pig parts, including pig blood, organs, and meat) in some Asian communities recently has been recognized (*6*,*8*,*9*). Rates of *S. suis* infection are low in the general populations of Europe and North America, and cases are concentrated among occupationally exposed groups, including abattoir workers, butchers, and pig breeders (*10*,*11*).

In a 2009 review, ≈700 *S. suis* infections were reported worldwide by 2009, mostly from China and Vietnam (*12*). Clinical characteristics of this infection have been reviewed (*12*,*13*) and include meningitis, sepsis, endocarditis, arthritis, hearing loss, and skin lesions. Treatment of *S. suis* infection requires ≈2 weeks of intravenous antimicrobial drugs (*12*). The death rate varies, and deafness is a common sequela in survivors.

Although substantial new data on the incidence, clinical and microbiological characteristics, and risk factors for *S. suis* infection have accumulated during recent years, the prevalence of this infection has not measurably decreased. We conducted a systematic review and meta-analysis to update the evidence and summarize the estimates of epidemiologic and clinical parameters to support practitioners' and policy makers' efforts to prevent and control this infection.

Methods

We conducted the review in accordance with recommendations of the PRISMA statement (*14*). The protocol

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for this review has been registered at PROSPERO International prospective register of systematic reviews (no. CRD42011001742).

Search Strategy and Selection Criteria

We systematically and inclusively searched 5 main electronic databases (PubMed, Scopus, ISI Web of Science, Science Direct, and Google Scholar) for all studies published until the end of December 2012 (Figure 1). The following search term was used as a text word in each database, as follows: PubMed—"streptococcus suis" in all fields, limited to humans; Scopus—"streptococcus suis" in all fields, excluding veterinary medicine articles; ISI Web of Science—"streptococcus suis" in topic with exclusion of veterinary science areas; Science Direct—"streptococcus suis" in all fields, with articles in veterinary medicine journals excluded; and Google Scholar—"all in title: 'streptococcus suis.'"

We also searched using the same search term "streptococcus suis" in other databases, including Virtual Health Library, SIGLE, WHOLIS, LILACS, IMSEAR-HELLIS, and China Academic Journals Full-text Database and checked the reference lists of retrieved articles. We did not restrict the types of studies and publication languages, and non-English papers were translated for review. Publications were excluded if they did not report any human cases of *S. suis* infection, reported data that overlapped with already included articles and provided no additional information, reported cases without information indicating the location of the patients, or reported data that could not be reliably extracted.

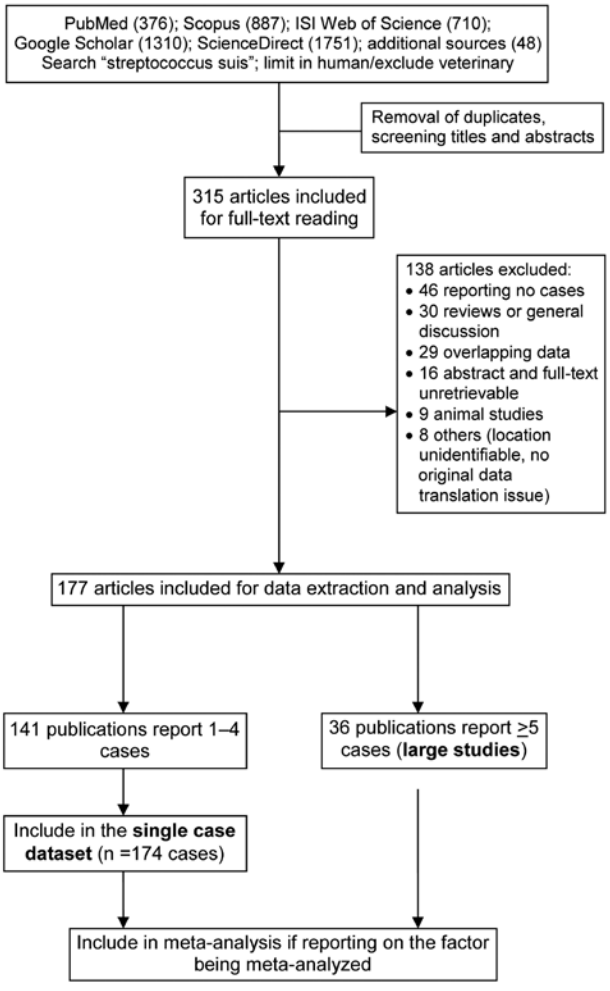

Figure 1. Flow diagram of the search and review process for this review of *Streptococcus suis* infection.

Data Extraction

Two reviewers (N.H. and V.T.L.H.) independently screened the titles and abstracts, and examined the full-text publications by using identical selection criteria and data abstraction forms. The results of data extraction showed a high degree of agreement between the reviewers (κ>0.90 for the main variables). Any disagreements were resolved by discussion and consensus between the reviewers and other authors (N.T. Huy, H.W., P.H., K.H.). We emailed the original authors of the articles that contained ambiguous data (1 email attempt per author) for clarification, and the ambiguous data were excluded from analyses if we did not receive a response.

Data extracted included year of publication, year of data collection, study design, data collection approach, country of origin, hospital where the patients were recruited, patient characteristics, clinical manifestations, methods of diagnosis, clinical and laboratory parameters, outcomes, and histories.

Analyses

We described the relevant epidemiologic and clinical factors using count for number of cases, proportions with 95% CIs for categorical factors (sex, occupation, exposure, history), and mean with SD for continuous factors (age, duration, and laboratory parameters). Event rates are presented as proportions with 95% CIs for signs, symptoms, and outcomes. We defined an event rate as the ratio of number of events to the number of all patients assessed in each study.

We pooled all single cases from the publications that reported <5 cases into 1 dataset and produced summary outputs, which were then meta-analyzed with other large studies (reporting ≥5 cases). We report the values of reviewed factors in 3 sets: summary values from the single-case dataset, median values (range) of the large studies, and pooled values from the meta-analysis as appropriate.

Meta-analysis was conducted by using Comprehensive Meta-analysis software version 2 (Biostat, Englewood, NJ, USA; http://www.Meta-Analysis.com) when >2 studies reported the reviewed factor. We tested heterogeneity using the Q statistic and I^2 test (*15*). Pooled values and 95% CIs were generated from a fixed-effects model or from a random-effects model, and each was study weighted

by the inverse of that study's variance. We used the fix-effects model when heterogeneity was not significant and a random-effects model when heterogeneity was evident (*16*). Median (range) was converted to mean (SD) by using proposed formulas (*17*), and interquartile ranges to SDs and subgroup values to total values by Cochrane suggested methods (*18*).

We assessed publication bias using funnel plots and the Egger's regression test (if ≥10 studies were included in the meta-analysis). If publication bias was found, the Duvall and Tweedie trim and fill method was performed to add possible missing studies to improve the symmetry and calculate the adjusted pooled values (*19*). We used subgroup analyses (when >10 studies were included) and bivariate meta-regression (when >20 studies were included) to examine the effect of study-level variables, including year of publication (2005 and earlier vs. after 2005 [because the Sichuan outbreak occurred in 2005]), study design (case series, outbreak investigation, cross-sectional), location (China mainland, Hong Kong, Thailand, Vietnam, and others), data collection (retrospective vs. prospective) and meningitis rate (<50%, 50%–90%, and >90%) on the main outcomes. The general linear model was used for meta-regression, with adjustment for multiple comparisons by using the Bonferroni method and weighting by study sample size.

Results

Systematic Review

We used 177 publications that met inclusion and exclusion criteria for data extraction and final analyses (Figure 1; online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/20/7/13-1594-Techapp1.pdf). The studies were diverse in terms of design, data collection, and reporting approaches. We identified 20 case series (8 from South-East Asia region, 8 from the Western Pacific region, and 4 from Europe) and 21 cross-sectional studies (9 SouthEast Asia, 8 Western Pacific, and 4 Europe). Five articles about 3 outbreaks (in Sichuan and Jiangsu, China; and Phayao, Thailand) were classified as outbreak investigation reports. The only prospective case–control study was conducted in Vietnam (Table 1).

Epidemiology

By the end of 2012, a total of 1,584 cases had been reported in the literature (including 189 probable cases identified in 3 outbreaks), mainly from Thailand (36%), Vietnam (30%), and China (22%). More than half (53%) were in the Western Pacific region; 36% were in the South East Asia region, 10.5% in the European region, and 0.5% in the Americas. The highest cumulative prevalence rate was in Thailand (8.21 cases/million population), followed by Vietnam (5.40) and the Netherlands (2.52) (country population data for 2008–2012 by World Bank [*20*]) (Figure 2).

The pooled mean age of the patients was 51.4 years, and 76.6% were men (Table 2). All case-patients were adults, except 1 female infant reported in Thailand (*21*). The pooled proportion of case-patients with occupational exposure was 38.1% (95% CI 24.4%–53.9%); this proportion was higher for industrialized countries than for other countries (83.8% [95% CI 73.4%–90.7%] for the United Kingdom, Netherlands, and Japan together). Recent

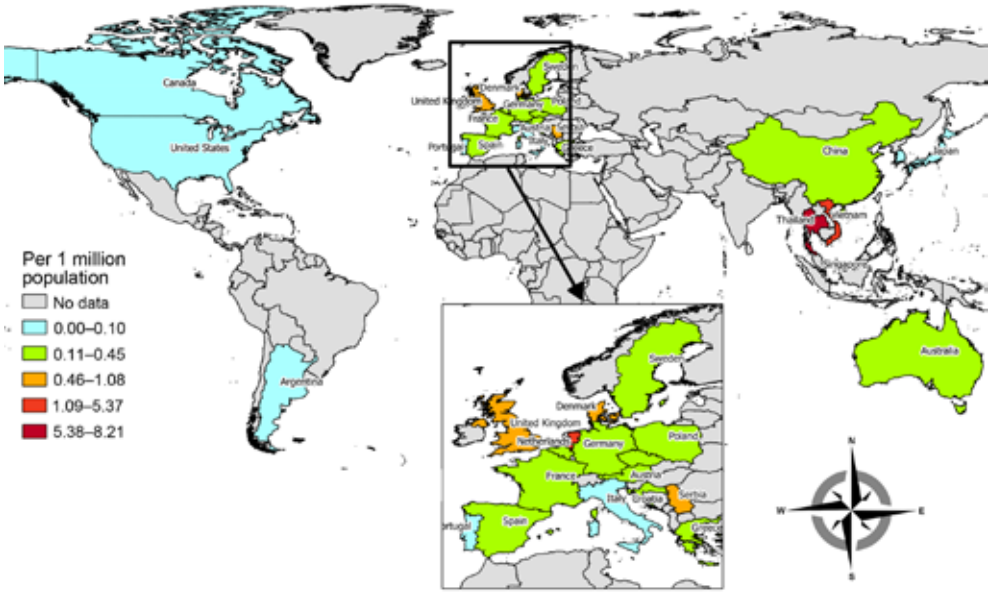

Figure 2. Global cumulative prevalence of *Streptococcus suis* infection through 2012.

Table 1. Characteristics of 177 articles in a systematic review of *Streptococcus suis* infection

Characteristic	Articles, no. (%)	Cases reported, no. (%)*
Geographic region†		
Europe	98 (55)	168 (11)
Western Pacific	47 (27)	836 (53)
SouthEast Asia	24 (14)	572 (36)
Americas	8 (5)	8 (0.5)
Type of study design		
Case report	130 (73)	151 (7)
Case series	20 (11)	511 (25)
Cross-sectional	21 (12)	761 (37)
Outbreak investigation	5 (3)‡	532 (26)
Case–control	1 (1)	101 (5)
Data collection approach		
Retrospective	159 (90)	1299 (63)
Prospective	15 (9)	697 (34)
Both§	3 (1)	60 (3)
Language of publication¶		
English	130 (74)	1947 (95)
Spanish	13 (7)	15 (1)
French	12 (7)	13 (1)
Other#	22 (12)	81 (4)
Year of publication		
1968–1980	13 (7.5)	18 (1)
1981–1990	27 (15)	95 (5)
1991–2000	32 (18)	119 (6)
2001–2005	28 (16)	115 (6)
2006–2010	55 (31)	1052 (51)
2011–2012	22 (12.5)	659 (32)

*Case duplicates were removed in the counts for the geographic region subheading (totaling 1,584 cases, no duplicates). Duplicates were not removed in the counts for other subheadings (totaling 2,056 cases, with duplicates).
†Geographic regions as defined by the World Health Organization.
‡Includes 3 articles reporting about the patients in the Sichuan outbreak in China; each was included for analysis of different factors.
§Included in the prospective groups in subsequent analyses.
¶Almost all large studies were published in English. Most reports in languages other than English were case reports.
#German (7 articles); Dutch (4); Czech, Italian, and Japanese (2 each); Chinese, Polish, Serbian, Swedish, and Thai (1 each).

contact with pigs or pork was reported for 15.5% of single cases but for 33.9% (95% CI 21.1%–49.5%) in the meta-analysis. History of eating meals containing pork was reported mainly in Asia (Thailand and Vietnam); the pooled estimate was 37.3% (95% CI 20.2%–58.3%). For Thailand only, the proportion was 55.8% (95% CI 33.7%–75.9%). In other countries, only 1 patient in France was reported eating artisanal dry sausage (*22*), and 1 patient in the United States ate raw pork while traveling in the Philippines (*23*) before the infection.

Skin injury was shown for one fourth of case-patients, and alcohol use was evident in approximately one third of case-patients. However, a case–control study in Vietnam did not identify alcohol use as an independent risk factor after adjustment for other risk factors and confounders (*6*). The most commonly reported preexisting condition was diabetes. Other conditions included underlying heart disease, hypertension, cirrhosis, and cancer (online Technical Appendix Table 3). Smoking was mentioned in 5.2% of patients in the single-case dataset.

Microbiological Diagnosis

Blood and/or cerebrospinal fluid culture were the most common reported diagnostic methods among the case reports (online Technical Appendix Table 4). Molecular diagnosis was more common in the large studies (11 studies) than in case reports. The most prevalent strain was serotype 2 (86.5%), followed by serotype 14 (2.3%), and serotype 1 (0.6%) of all 1,156 patients with serotype information mentioned in the articles. Serotypes 4, 5, 16, and 24 also were reported (1 patient per serotype).

Misdiagnosis of *S. suis* infection was not uncommon, either by conventional biochemical tests or commercial identification systems. The bacteria were often reported as viridans streptococci in initial cultures. In fact, up to 70% of all viridans streptococci cases in Thailand were confirmed as *S. suis* infections in the follow-up investigations (*24*). A total of 62.5% of *S. suis*–infected patients in another study in Thailand (*25*) and 20% in a study inthe Netherlands (*10*) were initially reported to be infected with viridans streptococci. Misidentification of the infectious agent as *S. bovis* (2 patients), *S. pneumoniae* (1 patient), and *S. faecalis* (1 patient) also was reported in the Netherlands series. Tsai et al. (*26*) showed large variations between the 2 commercial systems (Phoenix Identification System, Beckon Dickinson, Sparks, MD, USA; and Vitek II GPI Card, bioMérieux Vitek, Hazelwood, MO, USA), and misidentification of *S. suis* as *S. acidominimus* was common when the Phoenix system was used.

Table 2. Epidemiologic factors of patients with *Streptococcus suis* infection included in a systematic review

Variable	Single-case dataset, %*	Large studies, median (range), %	Meta-analysis, pooled mean (95% CI), %†	No. studies meta-analyzed, %‡
Mean age, y, n = 156	49.4	50.5 (37.0–61.2)	51.4 (49.5–53.2)	25
Male sex, n = 155	83.2	77.5 (37.5–100)	76.6 (72.2–80.6)	26
Pig-related occupation	58.6	25.0 (0–100)	38.1 (24.4–53.9)	21
Contact with pig/pork	15.5	33.3 (2.4–100)	33.9 (21.1–49.5)	14
Eating of high-risk food	4.0	53.3 (5.9–88.7)	37.3 (20.2–58.3)	9
Skin injury	19.5	16.0 (9.5–71.4)	25.1 (15.1–38.7)	8
Drinking of alcohol	8.6	23.0 (4.8–83.9)	29.7 (17.2–46.3)	13
Concurrent diabetes§	2.9	7.2 (3.2–25.0)	8.0 (4.6–13.7)	9

*N = 174 unless otherwise indicated.
†Random-effects model unless otherwise specified.
‡Include the single-case dataset and the large studies (online Technical Appendix Table 2, http://wwwnc.cdc.gov/EID/article/20/7/13-1594-Techapp1.pdf).
§Other less common underlying conditions are listed in online Technical Appendix Table 3.

S. suis is mostly sensitive to penicillin; resistance was reported in only 2 patients (*21*,*27*). After cessation of antimicrobial drug treatment, infection relapsed in a small proportion of patients, including those for whom the organism tested highly sensitive to penicillin (*28*,*29*). The pooled relapse rate was 4.4% (Table 3).

Clinical Syndromes

Meningitis was the most common clinical syndrome (pooled rate 68.0% [95% CI 58.9%–75.8%]), followed by sepsis (25.0% [95% CI 20.5%–30.2%]), arthritis (12.9% [95% CI 6.0%–25.6%]), endocarditis (12.4% [95% CI 6.7%–21.9%]), and endophthalmitis (4.6% [95% CI 2.8%–7.4%]) (Table 3). Toxic shock syndrome also was reported as a distinct severe clinical feature at high rates in 2 outbreaks in China (64.0% and 28.9% of patients) (*2*,*30*) and in Thailand (37.7%) (*24*) but at a rate of only 2.9% among the case reports.

We found evidence of publication bias in the meta-analysis of meningitis rates (Figure 3) (significant Egger's test result). The adjusted rate, based on the Duvall and Tweedie trim and fill method, was 56.0% (95% CI 45.2%–66.2%). Our meta-regression analysis showed that meningitis rate was significantly associated with country of publication, study design, and data collection approach (online Technical Appendix Table 5), although only country of publication remained significant in a multivariable model. All patients in the 4 studies conducted in Vietnam had meningitis. When we excluded these studies, the pooled rate of meningitis was reduced to 59.4% (95% CI 51.1%–67.1%), and the adjusted value after we used the trim and fill method was 54.8% (95% CI 46.0%–63.4%). In contrast, if we excluded the 2 outbreak investigations in China, because sepsis dominated these outbreaks, the pooled meningitis rate increased slightly to 72.2% (95% CI 62.4%–80.2%).

Table 3. Main clinical and laboratory parameters at admission of the patients with *Streptococcussuis* infection in a systematic review*

Variable	Single-case dataset	Large studies, median value (range)	Meta-analysis, pooled mean (95% CI)†	No. studies meta-analyzed‡
Clinical syndrome, %§				
Meningitis	69.5	64.5 (30.2–100)	68.0 (58.9–75.8)	26
Sepsis¶	19.5	23.8 (11.8–39.4)	25.0 (20.5–30.2)	12
Arthritis	2.87	16.7 (1.5–50.0)	12.9 (6.0–25.6)	12
Endocarditis	8.6	14.3 (1.9–39.0)	12.4 (6.7–21.9)	10
Endophthamiltis	2.9	4.5(1.5–28.6)	4.6 (2.8–7.4)#	9
Spondylodiscitis	4.6	1.9 (1.5-2.4)	3.7 (2.1-6.6)	4
Toxic shock syndrome	2.9**	37.7 (28.9–64.0)	25.7 (9.8-52.6)	4††
Mean duration, d				
Onset to admission, n = 90	7.3	3.5 (2.0–11.4)	4.1 (2.7–5.4)	7
Hospitalization, n = 68	20.5	17.4 (13.0–19.2)	17.2 (15.6–18.9)#	5
Symptoms, %				
Meningeal sign‡‡	49.4	66.7 (12.5–95.1)	67.1 (54.9–77.4)	18
Skin rash	10.9	12.5 (0–52.0)	15.4 (8.6–25.9)	10
Shock	8.6	11.8 (1.3–64.0)	11.9 (6.3–21.5)	12
Respiratory failure	5.2	20.0 (8.3–35.8)	16.7 (8.6–29.9)	6
Acute renal failure	5.2	8.3 (1.3–28.0)	7.1 (2.2–20.5)	5
Disseminated intravascular coagulation	10.3	6.0 (2.4–57.1)	10.3 (5.4–18.8)	9
Relapse	2.9	7.3 (2.9–8.3)	4.4 (2.4–7.8)#	5
Laboratory values (mean)§§				
Leukocytes, 10^9 cells/L, n = 98	17.4	15.1 (13.9–18.2)	15.8 (14.6–16.9)	9
Hemoglobin, g/L, n = 22	106.7	–	–	–
Platelets, 10^9/L, n = 41	121.0	182.4 (115–241.5)	164.9 (132.9–197)	7
Blood glucose, mg/dL, n = 32	147.8	–	–	–
C-reactive protein, mg/L, n = 36	349.7	–	–	–
Cerebrospinal fluid				
Leukocytes, cells/mm^3, n = 88	3,166	2029 (450–3253)	2330 (1721–2939)#	7
Protein, g/L, n = 74	3.20	2.35 (1.7–4.18)	2.45 (1.91–2.99)	7
Glucose, mg/dL, n = 70	20.9	8.60 (1.7–25.6)	12.6 (3.5–21.7)	6

*N = 174 unless otherwise indicated. –, not applicable because no large study reported these data.
†Random-effects model unless otherwise indicated.
‡Includes the single-case dataset and the large studies (online Technical Appendix Table 2, http://wwwnc.cdc.gov/EID/article/20/7/13-1594-Techapp1.pdf).
§Other less common syndromes included peritonitis, myositis, pneumonia, sacroiliitis, abdominal aortic aneurysm, hemorrhagic labyrinthitis, gastroenteritis, vertebral osteomyelitis, lymphadenopathy, cellulitis, and vertigo.
¶Case-patients with toxic shock syndrome in China and in Thailand not included in this sepsis category.
#Mixed-effects model.
**Counted if the author described the case as toxic shock syndrome.
††Include 3 large studies reporting toxic shock syndrome, including 2 outbreaks in China (*2,30*) and 1 prospective study in Thailand (*24*).
‡‡Mainly reported with neck stiffness.
§§Reference values may differ among laboratories. Commonly used reference values for presented laboratory blood tests are as follows: leukocytes 4.0–10 × 10^9 cells/L; hemoglobin 140–170 g/L (for male patients) and 120–160 g/L (for female patients); platelets 150–350 × 10^9/L; blood glucose (fasting) 70–100 mg/dL; C-reactive protein 0–8.0 mg/L. Reference ranges for cerebrospinal fluid are as follows: leucocytes 0–5 cells/mm^3; protein 0.15–0.60 g/L; glucose 40–80 mg/dL. (Source: http://im2014.acponline.org/for-meeting-attendees/normal-lab-values-reference-table/)

Table 4. Summary rates of the main clinical outcomes among patients with *Streptococcus suis* infection included in a systematic review

Variable	Single-case dataset, n = 174	Large studies, median (range)	Meta-analysis, pooled mean (95% CI)	No. studies meta-analyzed*
Death	10.3	8.9 (0.0–56.0)	12.8 (9.0–18.0)	25
Hearing loss†	44.8	38.7 (6.0–100)	39.1 (31.0–47.8)	26
Recovery from hearing loss	‡	5.0 (0.0–52.3)	15.4 (5.3–37.3)	8
Vestibular dysfunction§	16.7	25.0 (3.3–60.0)	22.7 (15.6–32.0)	13
Visual impairment	4.0	–	–¶	–

*Includes the single-case dataset and the large studies (online Technical Appendix Table 2,http://wwwnc.cdc.gov/EID/article/20/7/13-1594-Techapp1.pdf).
†Studies included if case-patients were reported to have any degree of hearing impairment (unilateral or bilateral, temporary or permanent).
‡Reliable data could not be extracted for the majority of the case reports.
§Studies included if case-patients were reported to have ataxia, vertigo, loss of balance, or vestibular dysfunction.
¶Dashes indicate not applicable because no large study reported these data.

Case-Fatality Rates

The pooled case-fatality rate (CFR) for *S. suis*–infected patients was 12.8% (95% CI 9.0%–18.0%) (Table 4). This rate varied by country; reported rates were lowest in Vietnam (Figure 4). However, country of publication was not significant in the bivariate meta-regression after adjustment for multiple comparisons (online Technical Appendix Table 5). Instead, only meningitis rates remained significant in explaining between-study variations in CFR. Meningitis rates correlated negatively with CFRs among the included studies (Figure 5). Studies with meningitis rates <50% had significantly higher CFRs than did studies with meningitis rates >90% (mean CFR difference 20.3%, p = 0.001). The pooled CFR was 4.0% (95% CI 2.2%–7.0%), estimated for the studies in which all patients had meningitis (*3*,*4*,*9*,*10*,*31*–*33*), whereas the pooled rate for the other studies was 17.1% (95% CI 12.3%–23.4%). CFRs were higher for outbreaks than for nonoutbreaks (21.6% [95% CI 6.4%–52.5%] vs. 11.5% [95% CI 7.9%–16.7%]).

Clinical Outcomes

Among the survivors, hearing loss (pooled rate 39.1% [95% CI 31.0%–47.8%]) and vestibular dysfunction (22.7% [95% CI 15.6%–32.0%]) were the most common sequelae (Table 4). Reported rates for both sequelae varied widely, even within a country such as Thailand, (online Technical Appendix Figures 1–4). Similar to CFRs, there was a marginally positive correlation between hearing loss and meningitis rates (p = 0.05) (online Technical Appendix Table 5). The pooled hearing loss rate for studies in which all patients had meningitis was 55.3% (95% CI 36.2%–72.9%), compared with 34.0% (95% CI 26.0%–43.1%) for the remaining studies. For the vestibular dysfunction, none of the investigated study-level factors were significant. Among the Asian countries, the reported rate of vestibular sequelae was lowest in Vietnam (4.0%).

Limited information described how hearing loss and vestibular dysfunction were evaluated during and after infection. Eight of 25 large studies reporting hearing loss indicated whether hearing loss was permanent after hospital discharge. Only 4 described their follow-up processes; follow-up time ranged from 2 months to 30 months (*4*,*8*,*28*,*31*). On the basis of these limited data, we estimated a comparatively low median rate of recovery from hearing loss of 5.0% (range 0%–52.3%) and the pooled rate of 15.4% (95% CI 5.3%–37.3% (Table 4). Hearing loss might be mediated by adjunctive corticosteroid treatments, as was shown in a trial in Vietnam (*34*). Of the *S. suis* patients, 12.3% had deafness in at least 1 ear in the dexamethasone treatment group (n = 57), compared with 37.7% in the placebo group (n = 53).

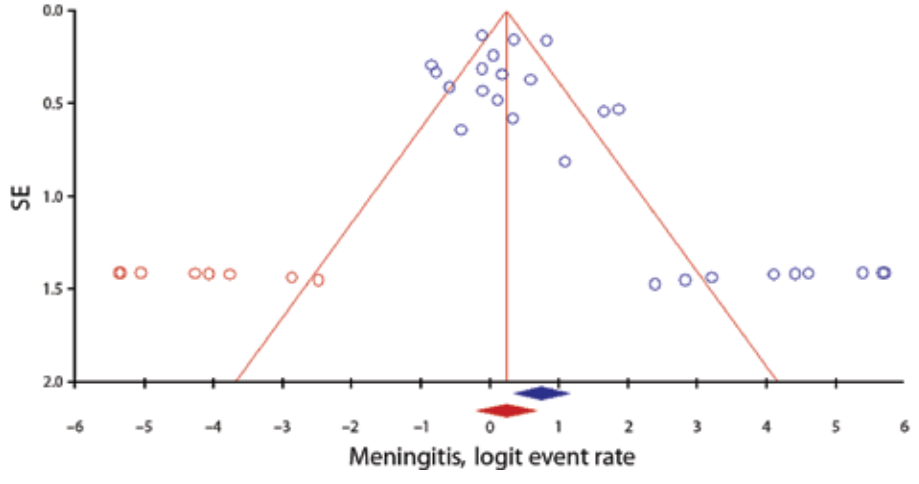

Figure 3. Funnel plot showing evidence of publication bias among 26 studies in a meta-analysis of meningitis rates in *Streptococcus suis* infection. Each blue circle represents each study in the meta-analysis, forming an asymmetric funnel plot with a pooled log event rate (gray rhombus). Eight missing studies (red circles) added in the left side through the trim and fill method to make the plot more symmetric and gave an adjusted log event rate (red rhombus), which was lower than the original one.

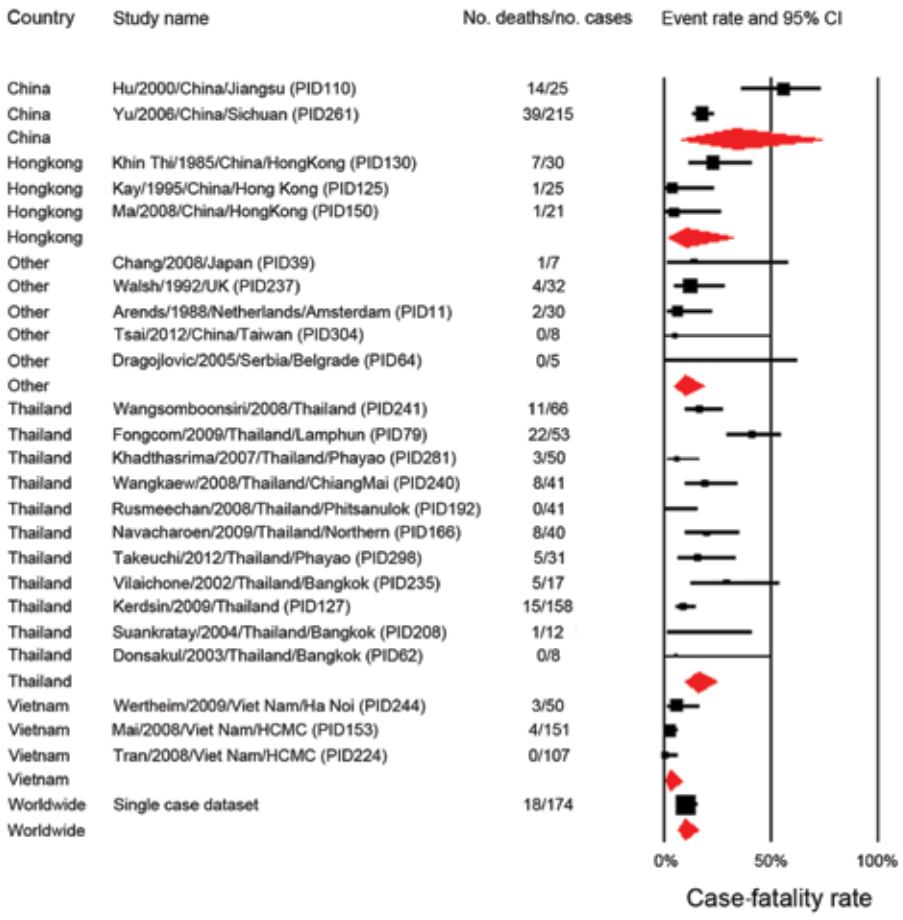

Figure 4. Forest plot of subgroup meta-analysis (random effects) for the case-fatality rates by country reported in the 25 studies included in a review of *Streptococcus suis* infection. For each study, the event rate of the death outcome and 95% CI are presented, with size proportional to study weight. The red rhombus indicates the pooled event rate for each country group.

Discussion

We have updated estimates of the global prevalence, epidemiology, and clinical characteristics of *S. suis* infections in humans. After possible duplicates were removed, the total number of *S. suis* infections by 2012 was close to 1,600 cases, doubling the figure published in 2009 (*12*). Most of the increase comprised cases from Thailand and Vietnam, placing both countries in the highest disease prevalence stratum in the world. In contrast, only a few cases have been reported from the Americas, particularly the United States, the second largest producer of pigs worldwide (*35*). This low number might be attributable to the high industrialization of pig farming systems in the region. Nevertheless, we saw far more cases in Europe, a region where modern farming operations are presumably similarto those in the Americas. Other plausible explanations include the lower virulence of North American bacterial strains (*36*) or different slaughtering practices.

We counted only published cases; the actual number of cases would be considerably higher, particularly in areas to which *S. suis* is endemic, such as Asian countries with extensive pig rearing. The problem of underestimation is further exacerbated by the fact that *S. suis* infection is not a notifiable disease in many countries. In addition, lack of diagnostic capacities or limited disease awareness in local laboratories can result in undiagnosed or misdiagnosed cases.

Meningitis is the main syndrome in approximately two thirds of *S. suis*–infected patients, although this finding varied by country. The syndromic distribution of the reported cases may depend on study design and case ascertainment methods. All major studies in Vietnam ascertained *S. suis* cases from the population of patients with central nervous system diseases, which could lead to underrepresentation of *S. suis* patients with clinical syndromes other than meningitis. Only 1 patient without meningitis (diagnosed as spontaneous bacterial peritonitis with serotype 16 infection) has been reported in this country (*37*). Nevertheless, whether the existing strains infecting humans in Vietnam mainly cause meningitis remains unclear. In fact, lumbar puncture is performed regularly for all *S. suis*–infected patients, including those with severe sepsis, at a hospital for tropical diseases in Vietnam, and almost all had exhibited typical characteristics of bacterial meningitis in cerebrospinal fluid. On the other hand, meningitis might not be diagnosed or reported from other countries, therefore reducing the global *S. suis* meningitis estimate.

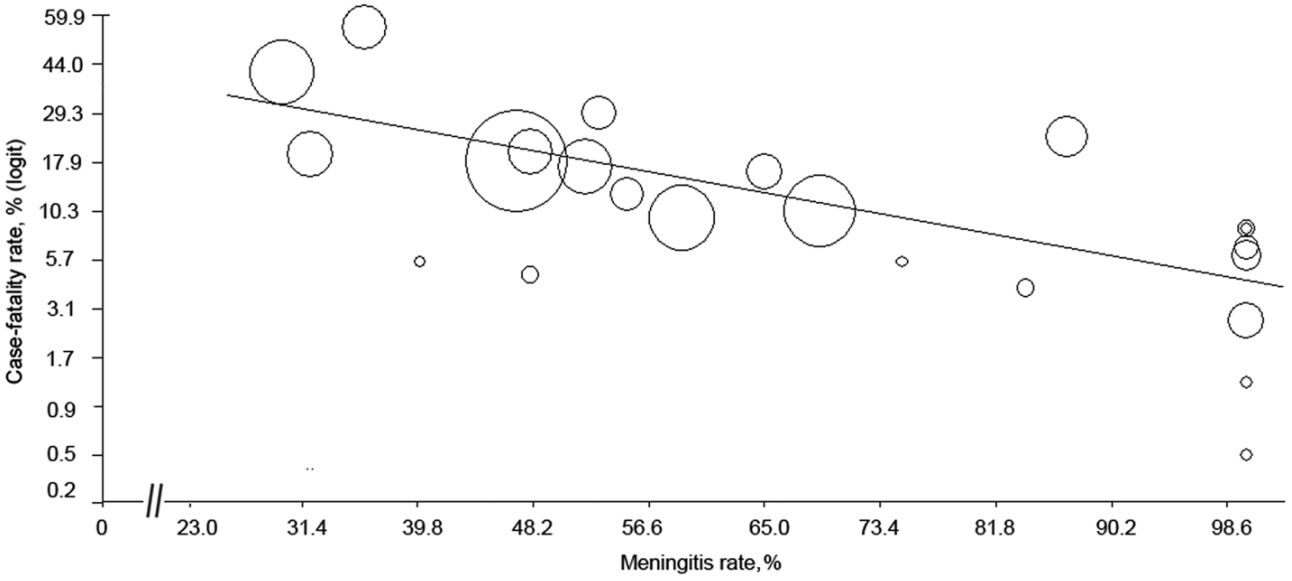

Figure 5. Meta-regression scatter plot showing the correlation between case-fatality rate and meningitis rate in a review of *Streptococcus suis* infection. The logit event rate was calculated for case-fatality rate as follows: logit event rate = ln[event rate/(1 – event rate)]. Each circle represents a study in the meta-analysis, and the size of the circle is proportional to study weighting. Studies with higher meningitis rates tended to report lower death rates.

The difference in CFR between case-patients with meningitis and case-patients with severe sepsis has been documented in both outbreak and nonoutbreak situations in China and Thailand (*1*,*2*,*24*). Significantly more deaths were reported among *S. suis* patients with systemic infection, including hypotension, septic shock, multiorgan failure, and disseminated intravascular coagulation in these studies. In the Sichuan outbreak in 2005, the CFR reached 62% for patients classified as having streptococcal toxic shock syndrome (*1*). Several hypotheses have been suggested; however, the pathologic mechanisms underlying this high CFR remain to be elucidated (*7*,*12*). Regarding meningitis cases, the pooled CFR is lower than that for other common causes of adult bacterial meningitis, such as *S. pneumoniae* (19%–37%) (*38*) and *Neisseria meningitidis* (10%) (*39*). However, the rates of sequelae caused by *S. suis* tend to be higher than those caused by other agents reported in a recent meta-analysis (*40*).

We were unable to establish pooled risk estimates for different risk factors because of a lack of studies with appropriate designs. In the Netherlands, the annual risk for *S. suis* meningitis among abattoir workers and pig breeders was 1,500 times higher than that in the general population (*10*). In Vietnam, *S. suis*–infected patients were more likely to have eaten high-risk foods (odds ratio [OR] 4.38), to have pig-related occupations (OR 5.52), and to have pig exposure while having skin injuries (OR 15.96) than community controls (*6*). The lower proportions of patients with occupational exposure in Thailand and Vietnam than in Europe shown in our meta-analysis supports the hypothesis that other risk factors, including food consumption practices, may play a major role in the epidemiology of *S. suis* infection in Asia.

This review is not without limitations. The included studies were highly heterogeneous in quality and in the factors reported, which reduced the number of studies included in each meta-analysis. The summary values of the single-case dataset should be interpreted with caution because the patients in this merged "sample" were heterogeneously "recruited" from different populations, with different assessment protocols. In addition, the studies were mainly retrospective; data could have been easily missed on recall or by re-collecting from the existing data records. We were unable to assess the extent to which this misinformation could affect the overall estimates. However, data collection approach was not significantly associated with the main outcomes examined under this review in our meta-regression analyses.

This review helps to highlight areas in which additional research is needed. Geographic gaps obviously exist in the data on *S. suis* cases, especially in the pig rearing countries in the Americas, Eastern Europe, and Asia, such as Mexico and Brazil, Russia, and the Philippines, respectively. Second, much uncertainty remains in understanding sequelae of *S. suis* infection and recovery from these conditions over time. Careful prospective assessments of these debilitating outcomes and associated social and economic impacts are essential for understanding and reducing the effects of *S. suis* infection. More studies also are needed to assess the treatment effects of adjunctive corticosteroid on hearing loss or other neurologic sequelae.

The effects of *S. suis* infection are mainly in Asia; occupational exposure and eating possibly contaminated foods containing undercooked pig tissues are prime risk factors. Further research in Asia should focus on the factors pertinent to local risk for infection, including the practices of unsafe handling and consumption of pork. Prevention of human infections needs to be tailored for different risk groups, and studies are needed to assess the feasibility and effectiveness of those tailored programs. Additional work is needed to assess the fraction of *S. suis* cases that can be attributed to different risk factors (the population-attributable fraction) and the proportion of *S. suis* cases that might be preventable if specific risk factors were reduced.

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Lessons for Control of Heroin-Associated Anthrax in Europe from 2009–2010 Outbreak Case Studies, London, UK

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Release date: June 11, 2014; Expiration date: June 11, 2015

Learning Objectives

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

1. Describe clinical manifestations of heroin-associated anthrax in the 2009–2010 outbreak in the United Kingdom, based on a study report
2. Discuss the epidemiology of heroin-associated anthrax in the 2009–2010 UK outbreak
3. Identify lessons learned from the 2009–2010 UK outbreak of heroin-associated anthrax

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Outbreaks of serious infections associated with heroin use in persons who inject drugs (PWIDs) occur intermittently and require vigilance and rapid reporting of individual cases. Here, we give a firsthand account of the cases in London during an outbreak of heroin-associated anthrax during 2009–2010 in the United Kingdom. This new manifestation

of anthrax has resulted in a clinical manifestation distinct from already recognized forms. During 2012–13, additional cases of heroin-associated anthrax among PWIDs in England and other European countries were reported, suggesting that anthrax-contaminated heroin remains in circulation. Antibacterial drugs used for serious soft tissue infection are effective against anthrax, which may lead to substantial underrecognition of this novel illness. The outbreak in London provides a strong case for ongoing vigilance and the use of serologic testing in diagnosis and serologic surveillance schemes to determine and monitor the prevalence of anthrax exposure in the PWID community.

Heroin-associated anthrax resulting from direct injection or injection under the skin, or "skin popping," among persons who inject drugs (PWIDs) is a distinct form of anthrax seen during a 2009–2010 outbreak in Scotland and England and again during 2012–2013 in northern Europe and Germany. There are an estimated 281,000 heroin users in England and >50,000 in Scotland (1), suggesting that the cases recognized and diagnosed during the outbreaks are the tip of the iceberg.

The first 2 cases of heroin-associated anthrax occurred in the Greater Glasgow and Clyde area of Scotland and were reported on December 10, 2009. By July 2010, there were 47 confirmed case-patients in Scotland, of whom 13 (28%) died (*2*). In January 2010, the first cases outside Scotland were described in England and Germany (*3*); the final outbreak total in England was 6, with 4 deaths (*4*). The last case from this outbreak occurred in October 2010, and Health Protection Scotland declared the UK outbreak over on December 23, 2010; no active surveillance was established afterward (*4*,*5*).

In June 2012, 21 months after the last reported case in the United Kingdom, a fatal case was reported in Regensburg, Bavaria, Germany. As of March 2013, 7 more cases had occurred in the United Kingdom, including 5 in England; 4 patients died during this outbreak: 2 in England, 1 in Scotland, and 1 in Wales. Another 6 cases occurred in Germany, Denmark, and France, bringing the total to 14 as of (March 2013) (*6*–*8*), which suggests that contaminated heroin remains in circulation and that vigilance should be maintained.

The only reported case of heroin-associated anthrax before this outbreak was during 2000 in an injecting drug user in Norway in whom fatal hemorrhagic encephalitis developed. Although anticipated, no outbreak emerged (*9*).

Bacillus anthracis

Bacillus anthracis is a spore-forming, gram–positive zoonosis which causes infection in humans through contact with contaminated animals or animal products (*10*). It occurs naturally in soil and mainly affects hoofed animals including goats, cattle, and sheep that ingest endospores (*11*–*13*). *B. anthracis* endospores are hardy and resistant to drying, heat, ultraviolet light, and many disinfectants, and can lie dormant in soil for many years (*10*). The endospores can be ingested, inhaled, or enter through skin abrasions after which they are phagocytosed by macrophages, where they germinate, resulting in activation and recruitment of other immune cells (*10*,*11*). In some cases, the bacteria are not destroyed and can activate a program of antigen-presenting cells and migrate toward lymph nodes (*11*); replication in the lymphatic system can then lead to septicemia. *B. anthracis* secretes 3 polypeptides called protective antigen, lethal factor, and edema factor, which combine to form exotoxins (*10*,*11*). These toxins have numerous effects on phagocytes, including impairment of maturation, impairment of chemotaxis of different phagocytes, and inhibition of phagocyte function (*11*). Edema toxin (protective antigen and edema factor) inhibits neutrophil function and lethal toxin (protective antigen and lethal factor) stimulates macrophages to release tumor necrosis factor α and interleukin-1α (*10*). It is possible that the host immune response contributes to the virulence of the pathogen, stimulating greater inflammatory cytokine release and recruitment of cells, including neutrophils, which have an essential role in controlling anthrax infection (*11*).

Typically, infection occurs through entry of the spores through the skin, by ingestion of contaminated meat, or by inhalation; contamination via these routes commonly results in cutaneous, gastrointestinal, or pulmonary anthrax, or may manifest as hemorrhagic meningitis (*10*,*12*,*13*). The intravenous route of exposure has added a new complexity to the immunopathogenic picture and has resulted in novel, severe, and highly variable patterns of manifestation which are described in the clinical cases section of this article. Anthrax has been used as a biowarfare select agent, and there is concern regarding potential future use. In 2001, the greater New York City metropolitan area in the United States was the scene of an attack (*14*) which resulted in at least 22 cases of anthrax, leading to 5 deaths. These cases resulted from a mix of inhalational and cutaneous exposures, affecting mainly persons who had contact with contaminated items sent through mail. This was a deliberate bioterrorist attack in which envelopes containing anthrax spores were delivered to victims. Early recognition of the first case resulted in a prompt public health response and epidemiologic investigations in the United States and internationally (*15*).

International Heroin Production and Transportation

Afghanistan is now the largest exporter of heroin, accounting for 93% of the world's supply (*16*). The United Kingdom National Anthrax Outbreak Control Team hypothesized that the heroin responsible for the 2009

outbreak had been contaminated at its likely source in Afghanistan or entered the supply chain by introduction of cutting or dissolving agents or through animal hides used for transport (*17*).

Phylogeographic analysis demonstrated that the anthrax strains responsible for the 2009–2010 outbreak in Scotland were closely related to strains from Turkey and not to prior isolates from Scotland or Afghanistan (*18*). Anthrax is endemic in Turkey, and heroin passing through Turkey may have been contaminated while being transported in contaminated goatskins (*18*). Isolates from the 2009–2010 and 2012–2013 outbreaks and the isolate from the case-patient from Norway in 2000 were subjected to comparative molecular typing by using a 31-marker multilocus variable number tandem repeat analysis and a broad single-nucleotide-polymorphism analysis. Results demonstrated that these strains were almost identical (*8*).

Clinical Cases

Here we give a firsthand account of 3 persons with anthrax associated with subcutaneous or intraarterial injection of heroin who sought care at different hospitals in the National Hospital Service trust. These cases highlight the spectrum of disease, specific management challenges, and the importance of serologic testing.

The manifestations of disease, treatments, and outcomes are summarized in the Table. Patient 1 manifested extensive, painless edema in the left thigh at the subcutaneous injection site. (Figure 1, panels A and B.) Despite remaining lucid and appearing comfortable, she was hemodynamically unstable and initially managed for severe soft tissue infection with septicemia; *B. anthracis* was identified from blood cultures the next day. Patient 2 also had extensive tissue involvement at the site of subcutaneous injection to the right buttock; he required extensive tissue debridement (Figure 2, panels A and B). Patient 3 had a pseudoaneurysm of the femoral artery after he injected heroin into that vessel; he was systemically well, although febrile, on arrival to the acute care facility. Samples taken at debridement and cultured were negative for anthrax; however, positive serology indicated recent infection.

These cases demonstrate the range of clinical manifestations, from relative hemodynamic stability (patient 3) to multiorgan failure requiring intensive care support (patients 1 and 2.) Clinical features of anthrax-associated soft tissue infection that differentiate it from that caused by other bacteria include the degree of edema affecting surrounding tissue (*18*,*19*), the excessive bleeding at the time of surgery, and the lack of a clear demarcation between affected and unaffected tissue.

In patients that have predominantly soft tissue manifestations, there is a notable absence of the eschar that is typical of cutaneous anthrax, marking this manifestation as a distinct form. Despite extensive tissue involvement as seen in patients 1 and 2, the degree of discomfort displayed by the patients was markedly less than would be expected, and they appeared deceptively comfortable despite the clinical features and laboratory tests that indicated severe infection. Both of these patients were coagulopathic with substantial renal failure requiring hemofiltration. The leukocyte count and C-reactive protein level were not as elevated as might be expected, given the degree of tissue involvement and the degree of organ failure evident; this was particularly evident in patient 2.

Patients 1 and 2, as seen in the Scottish anthrax case-patients, exhibited a biphasic illness with an initial recovery, then further deterioration (*20,21,22*). Radiologic features of pulmonary anthrax include pleural effusions, mediastinal widening, paratracheal or hilar fullness, and parenchymal infiltrates. Patients 1 and 2 showed chest x-ray evidence of severe pulmonary edema shortly after arrival.

Patients 1 and 2 were managed by a multidisciplinary team that included plastic surgeons, a gynecologist (for patient 1), intensive care specialists, microbiologists, and infectious diseases physicians. Patient 1 was the first case documented in London; the team drew on experience from the US Centers for Disease Control and Prevention, Health Protection Scotland, and the Rare and Imported Pathogens Laboratory at Porton Down, England. Anthrax immune globulin intravenous (human) (AIGIV) was approved for patient 1, but was not given because she had improved by the time of its arrival. Patient 2 received AIGIV within 24 hours of arrival at the hospital.

Microbiological Diagnosis

In test results for patients 1 and 2, gram-positive rods were seen on Gram stain from blood culture and *Bacillus anthracis* grew. Gram stain of tissue culture from debridement from patient 2 also showed gram-positive bacilli. Serologic testing and PCR on EDTA blood samples were also positive for both of these patients.

Patient 3 was referred to the vascular surgeons with concern regarding his pseudoaneurysm. He was febrile on admission, and an infectious diseases consultation was requested to evaluate antibiotic drugs in view of skin and tissue cultures which had grown anaerobic organisms. He had injected heroin before onset of illness, although he had not injected drugs for 10 years before this instance. Blood cultures and tissue taken at the time of his pseudoaneurysm repair were negative on culture and by PCR for *B. anthracis*; however, his strongly positive serologic test results were indicative of recent infection.

Discussion

The death rate early in the 2009–2010 anthrax outbreak in Scotland was ≈50% and dropped to 28% toward the end of the outbreak (*23*). These results are likely to be a reflection of increased vigilance, earlier identification of cases, and an increase in experience in managing the cases. There is speculation that, rather than destroying the contaminated heroin, dealers in Scotland may have "cut," or diluted, the batch with other supplies; this may have resulted in a reduction in the size of the inoculum. The death rate was also high in the 2012–2013 outbreak, although this outbreak was more limited in extent than the one in 2009–2010.

Role of Surgery

Regarding soft tissue infections related to heroin-associated anthrax, the role of surgery was initially unclear, and the suggestion was made that disturbing a lesion may result in clinical deterioration, although this hypothesis remains contentious (*20*,*21*,*24*). Based on experiences in Scotland and Health Protection Scotland advice, guidelines from Public Health England (PHE) advocated timely debridement of affected tissue with the aim of reducing the toxin load. Patients 1 and 2 had extensive debridement, but in patient 1 in particular, the extent of involvement and poor demarcation of affected tissue made this difficult. Toward

Table. Details of 3 heroin-associated anthrax patients from the 2009–2010 anthrax outbreak, London, United Kingdom*

Characteristic	Patient 1	Patient 2	Patient 3
Age, y/sex	43/F	30/M	60/M
Comorbidities	HIV, hepatitis C	Hepatitis B, hepatitis C, thromboembolic disease	Hepatitis C, left femoral artery pseudoaneurysm
Route of infection	Subcutaneous injection to left thigh 3 d before admission	Subcutaneous injection to right buttock 1 wk before admission	Injected into left femoral artery
Site affected when patient sought treatment	Extensive involvement: painless edema and blistering of the left thigh, lower abdomen, genitals	Right buttock erythematous, swollen, edematous, and painful; edema extended to genitals	Pulsatile mass at left groin area; no edema or swelling evident
Surgery	Extensive debridement by general surgery and gynecology performed on 2 occasions; skin graft applied later	Early, limited debridement performed on d 1 of hospitalization. Skin graft applied later	On hospital d 1, surgery performed to repair left femoral artery pseudoaneurysm and debridement; further debridement performed at d 19
Anthrax testing results			
Culture	Blood culture of specimen drawn on admission positive in <24 h	Blood and tissue cultured on admission positive 24 h after admission	Blood and tissue cultured on admission negative
Serologic	Positive	Positive	Positive
PCR	Positive	Positive	Negative
Initial antibiotic drugs	Ceftriaxone, clindamycin, vancomycin	Clindamycin, ciprofloxacin, flucloxacillin, vancomycin, gentamicin	Clindamycin, ciprofloxacin, flucloxacillin, benzylpenicillin, metronidazole
Outcome	Initially lucid and comfortable but hemodynamically unstable. Debridement on 2 occasions. Anthrax PCR post–antibiotic drug treatment negative; coagulopathy resolved by day 29 with normal platelets and clotting studies. On day 31, brain stem ischemia developed; died on d 50 after airway complications.	After initial debridement, electively intubated to treat edema causing respiratory compromise. Received AIGIV within 24 h of admission. Vacuum-assisted therapy pump was used, then skin graft, with good outcome. Recovered and was discharged to complete 60 d of ciprofloxacin and clindamycin.	After first surgery on hospital d 1, continued broad-spectrum antibiotic drugs for 10 d. Received a further 14 d of broad-spectrum antibiotic drugs after debridement on d 19. Made a good recovery and was discharged home. Strongly positive serologic results subsequently received.
Test results for blood samples taken at admission (reference range)†			
Leukocyte count ($4.2–11.2 \times 10^9$ L)	23.1	16.8	10.1
Neutrophils ($2.0–7.1 \times 10^9$/L)	14.6	14.6	4.9
CRP (0–4 mg/L)	179	71	230
Hemoglobin (13.0–16.8 g/dL)	15.7	6.7	9.8
INR (1.0)	4.4	1.5	1.0
Platelets ($130–370 \times 10^9$/L)	374	30	238
Creatinine (60–125 μmol/L)	385	488	137
Albumin (30–45 g/L)	24	23	30

*Patients 1, 2, and 3 represent the diversity of the cases seen and the spectrum of manifestation caused by heroin-associated anthrax. Clinical features associated with this condition include the degree of edema present, the absence of the eschar associated with cutaneous anthrax, and the biphasic nature of the illness; in the severe cases, Patients 1 and 2 experienced multiorgan dysfunction and coagulopathy. AIGIV, anthrax immune globulin intravenous; CRP, C-reactive protein; INR, international normalized ratio.
†Reference ranges from Imperial College Healthcare (http://www.imperial.nhs.uk/services/pathology/index.htm).

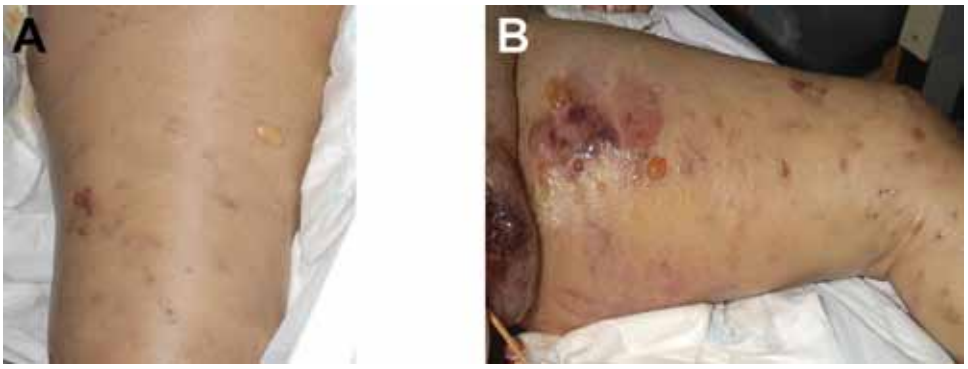

Figure 1. Manifestation of heroin-associated anthrax in patient 1, who injected heroin under the skin of her left thigh. Panel A demonstrates substantial edema and blistering of skin. Manifestation is more pronounced in Panel B, which demonstrates more blistering and bruising.

the end of the 2012–2013 outbreak, a more conservative approach was being considered by clinical specialists.

These 3 cases demonstrate the clinical spectrum of anthrax among heroin users from minimal evidence of overt sepsis to soft tissue involvement and multiorgan failure. Several factors are potential contributors to this; these may include delayed manifestation; delays in recognition and subsequently, in initiating therapy; the size and route of entry of the inoculum; comorbidities; and immunologic factors.

The Tip of the Iceberg

Patient 3 sought treatment mainly for his femoral artery pseudoaneurysm after injecting heroin, although after admission, it was found that he was febrile, had elevated inflammatory markers, and there was evidence of necrosis at debridement. Culture and molecular tests of tissue collected at debridement were negative, possibly caused by early antibiotic drug therapy before that procedure. His manifestation of illness was not initially counted as a case, but this was revised considering the strength of his serologic response which suggested recent infection. This patient's lack of physical compromise despite active infection is notable. A case report from Glasgow described a patient who had chronic sinuses in the left groin after injecting heroin; there was evidence of local infection and sepsis with *B. anthracis,* but he was was uncompromised (*25*). This raises the possibility that the cases seen in Scotland and England may be the tip of the iceberg, particularly if some patients who are relatively asymptomatic and not overtly septic are treated for soft tissue infections with antibiotic drugs that are effective against anthrax, and those infected persons are not admitted to a hospital.

The seroprevalance of infection in PWIDs who had potentially used heroin from infected batches is unknown and, given the number of heroin users in the United Kingdom, this knowledge would shed further light on our understanding of the immune response to anthrax. This is particularly the case given that some affected individuals may be asymptomatic or minimally symptomatic. Patient 1's partner injected from the same batch at the same time but showed no evidence of anthrax during follow-up.

Information was disseminated through the press and directly to at risk groups to ensure heightened public awareness; this led several PWIDs to present to the trust concerned regarding possible infection however they proved serologically negative. Community testing of PWIDs may be an important initiative to determine the seroprevalence of anthrax, identify subclinical cases and guide further research into anthrax and immunity.

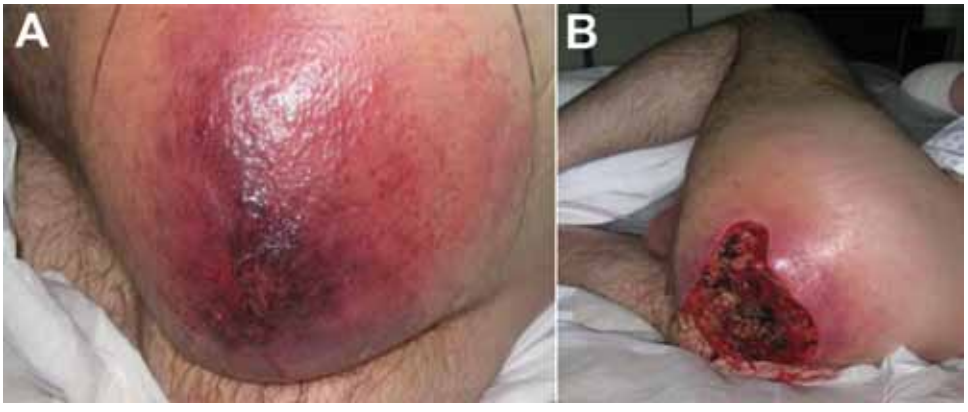

Figure 1. Manifestation of heroin-associated anthrax in patient 2, who injected heroin under the skin of his left buttock 1 week before seeking treatment. Panel A demonstrates severe edema with poorly demarcated erythema. He had debridement within hours of arrival. Panel B demonstrates the extent of debridement required to reach healthy tissue and to reduce the toxin burden; there was copious drainage of fluid and pus as well as bleeding to the area. The tissue between affected and unaffected areas was poorly demarcated.

Lessons from the 2009–2010 Outbreak

This outbreak reinforces the importance of vigilance in the early identification of emerging infections to ensure rapid identification of cases, containment of an outbreak, and effective management. Early cooperation and dissemination of information to at-risk groups and health care professionals are also vital.

The case-patients in the 2009–2010 outbreak most commonly sought treatment with localized abscesses or inflammatory lesions at the site of injection or skin popping with a breadth of presentation from subclinical to hemorrhagic meningitis and peritonitis (*12*,*20*,*25*–*27*). The novelty of this outbreak is the mode of transmission with endospores injected (in most cases) directly into the blood supply, resulting in novel soft tissue manifestations and severe systemic manifestations. The recurrence of cases in 2012–2013 has reinforced the awareness that vigilance should continue to be exercised in medical practice, looking for links among cases with early reporting and automatic collating of cases so that outbreaks can be identified early.

Severe Skin and Soft Tissue Infection

The Infectious Diseases Society of America guidelines for skin and soft tissue infections make special mention of the treatment for cutaneous anthrax, given the possibility of anthrax use as a bioterrorism agent (*28*). The recommendations for necrotizing fasciitis include a broad spectrum of antibiotics which are similar to those recommended for the anthrax outbreak; these include ciprofloxacin for suspected anthrax and a combination of a β-lactam antibiotic, ciprofloxacin, and clindamycin for suspected necrotizing fasciitis (*29*). These recommendations are similar to the PHE recommendations for antibiotics to be used in suspected cases of soft tissue anthrax, specifically, ciprofloxacin, clindamycin, and penicillin plus additional cover for necrotizing fasciitis with flucloxacillin and metronidazole (*30*,*31*). The use of antibiotics that have anti-anthrax activity, particularly in severe soft tissue infections in PWIDs, could possibly mask overt cases.

Anthrax immunoglobulin is available on a named patient basis with prior agreement from the US Food and Drug Administration and the Centers for Disease Control and Prevention for patients who fulfill the particular clinical features and who have confirmed laboratory evidence of *B. anthracis* infection in a normally sterile site with epidemiologic evidence of possible infection (*28*). It is derived from plasma of humans vaccinated with BioThrax (adsorbed anthrax vaccine) (Emergent BioSolutions Inc, Bracknell, UK) and contains polyclonal toxin-neutralizing antibodies against protective antigens; it is intended for use as an adjunct to antibiotics to counter the toxin-mediated immune activations (*32*).

Waste Management

Human-to-human transmission of anthrax has not been reported. However, the clinical waste from patients is potentially hazardous; hence, early recognition and coordination are essential. In 2010, PHE produced updated guidance that highlighted the need to address the threat of potential contamination. This included guidance related to the collection of samples and guidance related to any sharps and contaminated waste that should be incinerated or autoclaved. Anthrax is a Hazard Group 3 pathogen and should be handled in a Biosafety Level 3 facility by using a Class 1 protective safety cabinet (*30*,*31*). All sharps and any waste contaminated by blood or bodily fluids and should be incinerated or autoclaved to interrupt transmission (*31*).

The Need for Vigilance

Given the novel nature of the clinical manifestation, clinicians may not consider anthrax in the differential diagnosis of severe infections in PWIDs, resulting in undiagnosed cases. Clinical awareness of the associated risk for injection-related infection by rare pathogens in this population is therefore crucial, particularly because the range of clinical manifestation is broad. It is therefore possible that the incidence among PWIDs may be significantly higher for each overt case than that reported for several subclinical cases. The re-emergence of anthrax in 2012–2013, albeit at a smaller scale than in 2009–2010, suggests anthrax-contaminated heroin remains in circulation.

Other Considerations

For the cases from June 2012, the European Monitoring Centre for Drugs and Drug Addiction in association with the European Centre for Disease Prevention and Control issued a rapid risk assessment highlighting the presence of contaminated heroin in northern Europe and disseminated information to relevant parties (*33*). Of note, there was a delay in diagnosis of 1 fatal case in Germany because of the initial identification of the bacillus as *B. cereus*.

This new form of anthrax associated with PWID reinforces the importance of emerging infections in clinical practice and the potential global impact of such outbreaks. In addition, it highlights how globalization, international travel, and the impact of global conflicts on drug production and supply routes can change the landscapes by which infectious diseases originate and spread. The new cases during 2012 suggest that contaminated heroin remains in circulation.

Conclusion

Here we describe 3 cases from the 2009–2010 outbreak of heroin-associated anthrax in the United Kingdom. Our interest lies in the way that the anthrax spores were

introduced into the body, their novel effects, and breadth of manifestation. Lessons learned from the detection and management of these cases is of renewed interest given the cases seen during June 2012.

Clinicians may not consider anthrax in the differential diagnoses of sepsis and soft tissue infection among PWIDs, leading to an underestimate of the incidence of anthrax in this novel manifestation. The reemergence of anthrax makes a strong argument for serologic surveillance to determine the prevalence of anthrax exposure in PWIDs and to further our understanding of the nature of anthrax immunity and pathogenesis in this cohort. Results of this surveillance would have major implications for health policy in managing an infection for which there is heightened public concern.

Outbreaks among heroin users occur intermittently (*34*), hence vigilance should be exercised for these patients. Ongoing cases in Europe suggest that affected batches of heroin remain in circulation, therefore efficient reporting and noting the association between heroin use and illness are vital in ensuring early awareness of outbreaks.

The possibility that there may be a reservoir of undiagnosed disease, particularly in those with subclinical or mild symptoms, makes a strong case for serologic surveillance among PWIDs seeking treatment for sepsis or soft tissue infections. This will help determine the serologic prevalence in the community and may confirm the possibility that some heroin users possess immunity to anthrax through prior low-level exposure to contaminated drugs. Greater understanding of the nature of anthrax immunity and pathogenesis in this cohort is necessary for the development of health policies targeting this infection for which there is heightened public concern.

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Norovirus Epidemiology in Community and Health Care Settings and Association with Patient Age, Denmark

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Norovirus (NoV) is a major cause of gastroenteritis. NoV genotype II.4 (GII.4) is the predominant genotype in health care settings but the reason for this finding is unknown. Stool samples containing isolates with a known NoV genotype from 2,109 patients in Denmark (patients consulting a general practitioner or outpatient clinic, inpatients, and patients from foodborne outbreaks) were used to determine genotype distribution in relation to age and setting. NoV GII.4 was more prevalent among inpatients than among patients in community settings or those who became infected during foodborne outbreaks. In community and health care settings, we found an association between infection with GII.4 and increasing age. Norovirus GII.4 predominated in patients ≥60 years of age and in health care settings. A larger proportion of children than adults were infected with NoV GII.3 or GII.P21. Susceptibility to NoV infection might depend on patient age and infecting NoV genotype. Cohort studies are warranted to test this hypothesis.

Norovirus (NoV) is a major cause of viral gastroenteritis (*1*) and a common cause of outbreaks of acute gastroenteritis in institutional settings, such as hospitals, nursing homes, and schools. Foodborne outbreaks of NoV infection are also common (*2,3*).

NoVs are positive-sense, single-stranded, non-enveloped RNA viruses (*4*). On the basis of amino acid or nucleotide sequencing of the polymerase and capsid regions, NoV can be divided into 6 genogroups (GI–GVI) and several genotypes. GI, GII, and GIV are human pathogens (*5–7*). Recombination events within a genogroup are common (*8*). Thus, genotyping of NoV should ideally be based on sequencing of the capsid and polymerase regions of the viral genome (*9*).

NoV sequences reported to the Foodborne Viruses in Europe Network come from mainly foodborne outbreaks or outbreaks in health care settings (*2*). Outbreaks in health care settings are most often caused by NoV genogroup II genotype 4 (GII.4) (*10–13*). The proportion of outbreaks caused by GII.4 is lower in non–health care settings (*2,3,12,14*). Elderly persons seem to be more susceptible to NoV infection (*15,16*). This susceptibility has been suggested to be genotype dependent (*3*).

The purpose of this study was to describe the distribution of NoV genotypes among infections in patients consulting a general practitioner (GP) or outpatient clinic, patients in health care settings, and patients in foodborne outbreaks. The association between NoV GII.4 and age of the patients in community and health care settings was also determined.

Materials and Methods

Patient Samples

The study included patients who had stool samples test positive for NoV during routine diagnostic virus analyses at the Department of Virology at Statens Serum Institut, Copenhagen, Denmark, during 2006–2010. This department serves as a reference laboratory and, throughout the study period, also served as the primary virus diagnostic laboratory for most GPs, outpatient clinics, and hospitals in Denmark. Information about sampling date, setting (i.e., hospital, GP, or outpatient clinic), age, and sex of the patients was obtained from the laboratory database. Samples from patients infected during suspected foodborne outbreaks of gastroenteritis were accompanied by special

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request forms at submission to the laboratory. Information regarding hospital admissions (dates and wards) during the study period was obtained from the Danish Health and Medicines Authority. Patients registered in the laboratory database as inpatients were excluded if hospitalization at the time of sampling could not be verified. Collection and registration of patient data were approved by the Danish Data Protection Agency (record nos. 2012–54–0046 and 2010–54–1076).

Using the personal identification numbers mandatory for all Danish citizens, we obtained postal addresses for patients ≥60 years of age who were positive for NoV and had a sample with an assigned genotype submitted from an outpatient clinic or GP. The addresses were used to determine if these patients were nursing home residents as of July 2013. Patients who had died before July 2013 were excluded because it was not possible to determine if they had been nursing home residents (n = 21).

Patient NoV samples were obtained from 3 settings. The first group consisted of inpatients and nursing home residents (referred to as health care settings), the second group consisted of patients consulting a GP or outpatient clinic (referred to as community settings), and the third group consisted of patients from foodborne outbreaks. The patients were from all 5 regions of Denmark.

Sampling and admission dates were used to estimate whether infections were nosocomial or community acquired. An infection was classified as community acquired if stool samples were obtained on the day of admission or the following day, nosocomial if samples were obtained on day 5 or afterwards, and indeterminate if samples were obtained between these 2 periods. Multiple samples were submitted from 1,060 patients. To avoid overrepresentation of patients chronically infected with NoV, only the first NoV-positive sample from each patient was included. During the study period, samples were continuously selected for genotyping. The intention was to type all samples from community settings, ≥1 sample from every hospital ward per month, and 1 sample from each foodborne outbreak, respectively, which yielded 2,231 samples.

RNA Extraction

Stool samples were processed as 10% (wt/vol) suspensions in phosphate buffer solution, centrifuged at 4°C for 30 min at 3,400 *g*, and analyzed within 72 h of arrival. Nucleic acids were extracted by using MagNa Pure LC (Roche Diagnostics, Hvidovre, Denmark) and the Viral NA Small Volume Kit (Roche Diagnostics) according to the manufacturer's instructions.

Real-time Reverse Transcription PCR

NoV GI and GII were detected by real-time reverse transcription PCR (RT-PCR) by using the OneStep RT-PCR Kit (QIAGEN, Aarhus, Denmark) and primers and probes, as previously described (*17*). PCR conditions are shown in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/20/7/13-0781-Techapp1.pdf).

NoV Genotyping

Polymerase RT-PCR

Polymerase gene sequences were obtained by using primers JV12Y-JV13 (*18*) or JV12BH-NVp110 (*18,19*) in 1 round of amplification. If PCR results were negative, a nested PCR was performed (*20*). Using the abovementioned primers, we performed an RT-PCR with the OneStep RT-PCR Kit (QIAGEN) for the first-round PCR and AmpliTaq 360 DNA Polymerase (Applied Biosystems, Naerum, Denmark) for second-round PCR according to the manufacturers' instructions. PCR conditions are shown in the online Technical Appendix.

Capsid RT-PCR

Capsid gene sequences were obtained by using a semi-nested GI-specific primer set (GIFF-1, GIFF-2, and GIFF-3 for a first-round PCR and GISKR [GIFFN and GISKR] for a second-round PCR), which amplified 305 bp of the GI capsid gene; or a semi-nested GII-specific primer set (G2FB-1, G2FB-2, and G2FB-3 for a first-round PCR and G2FBN [COG2F and G2SKR] for a second-round PCR), which amplified 299 bp of the GII capsid gene (*17,21,22*). Using these primers, we performed an RT-PCR by using the OneStep RT-PCR Kit (QIAGEN) for a first-round PCR and AmpliTaq 360 DNA Polymerase (Applied Biosystems) for a second-round PCR according to the manufacturers' instructions. PCR conditions are shown in the online Technical Appendix.

Sequencing

PCR products were prepared for sequencing by using Exo-SAP (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. Both strands of DNA were sequenced by using an ABI 377 DNA Sequencer (Applied Biosystems) with the same primers used for RT-PCR and the Big Dye Terminator Kit 1.1 (Applied Biosystems).

Sequence Analysis and Identification of Genotype

Sequence analysis and assembly were performed by using BioNumerics version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). Genotypes were assigned by using phylogenetic analyses (http://www.rivm.nl/mpf/norovirus/typingtool) (*6*). Genotyping was primarily based on the polymerase sequence. If this procedure was not successful, sequencing of the capsid genome was attempted. For some sample gene products, both regions were sequenced. If divergent genotypes were detected in the capsid and polymerase genes, the capsid genotype was used.

Descriptive Analyses

Distribution of patients with respect to age and setting was initially determined by using all 3,848 samples. To obtain a representative picture of the distribution of circulating NoV genotypes and to avoid including several patients from the same outbreak, we included only the first sample from each clinic and ward within a calendar month (n = 1,612). The difference in age between patients with and without an assigned genotype was obtained for community and health care settings separately by using the Wilcoxon-Mann-Whitney test. The association between an assigned genotype (as the outcome) and age and sex (separately) was evaluated by using univariable logistic regression analysis. The association between genotype and age group was tested by using the Pearson χ^2 test.

Association between NoV GII.4 and Patient Age

The association between age and infection with NoV GII.4 was measured by using multilevel logistic regression analysis. Patients grouped within the same cluster (ward or clinic) are often more similar than randomly selected patients from different clusters. To account for this lack of independence between patients in clusters, a multilevel model was used that assumed a normal distribution of random effects. A total of 523 clusters (212 wards and 311 clinics) were included. The outcome was NoV genotype as the binary variable (GII.4 or non-GII.4). Three covariates were included in the analysis as fixed effects: age (<3, 3–19, 20–39, 40–59, and ≥60 years), setting (community or health care), and sex. Two interactions were considered of interest and were included in the analyses; these were the interactions between setting and age and between setting and sex. Backward elimination was used to exclude non-significant interactions by first removing the most non-significant interaction.

The mean cluster size was 3.73 (range 1–55). To evaluate the effect of a small cluster size, the analysis was repeated by including only clusters (i.e., wards and clinics) with ≥5 patients in the analysis. The analysis was also repeated by using logistic regression without any random effect on the descriptive dataset shown in Table 1 (i.e., first patient with an assigned genotype from each clinic and ward within a calendar month).

Stata software version 11.2 (StataCorpLP, College Station, TX, USA) and SAS version 9.3 (SAS Institute, Cary, NC, USA) were used for analyses. Significance was determined at $p<0.05$ and by using 2-sided tests.

Results

During the 5-year study period, stool samples from 18,796 patients were submitted to the Department of Virology at Statens Serum Institut. A total of 4,056 patients were positive for NoV. After exclusion of patients with uncertain hospitalization status, 3,848 patients were included for further analysis (Table 1). These patients were from 230 wards in 60 hospitals in Denmark, 356 general practices or outpatient clinics, and 46 suspected foodborne outbreaks. A NoV genotype was identified for 2,109 patients. Of these patients, 1,713 had samples initially selected for genotyping. In 223 of the selected samples, a genotype was not obtained because of lack of sensitivity or sample material; genotyping was not attempted for 295 other samples.

A genotype based on sequence information from the polymerase and the capsid genes was obtained for NoVs in 349 (17%) samples. NoVs from 1,496 (71%) samples were genotyped by partial sequencing of the polymerase gene and NoVs from 264 (13%) samples were genotyped by partial sequencing of the capsid gene. Thus, a genotype was established for NoVs in samples from 204 (89%) wards, 59 (98%) hospitals, and 313 (88%) clinics. A genotype was established for NoVs in ≥1 sample from all foodborne outbreaks. The age distribution differed significantly between patients for whom an NoV genotype was identified and those for whom it was not (community settings: $p = 0.002$; health care settings: $p<0.001$. However, when we compared patients ≥60 years of age in community settings with patients <3 years of age, the proportion of genotyped NoVs in samples did not differ significantly (odds ratio 0.6, 95% CI 0.4–1.1, $p = 0.1$).

Among the 2,109 patients for whom the infectious agent had an assigned NoV genotype, 882 patients were

Table 1. Age and setting for 3,848 patients with stool samples positive for norovirus, Denmark, 2006–2010*

	All patients, no. (%)			Descriptive analysis,† no. (%) patients positive for GII.4)‡		
Age group, y	Community settings	Health care settings	Foodborne outbreaks	Community settings	Health care settings	Foodborne outbreaks
<3	680 (61)	36 (1)	NR	490 (51)	23 (57)	NR
3–19	113 (10)	15 (1)	NR	71 (27)	10 (50)	NR
20–39	145 (13)	76 (3)	NR	94 (62)	33 (64)	NR
40–59	93 (8)	240 (9)	NR	64 (64)	90 (89)	NR
≥60	86 (8)	2,196 (86)	NR	62 (82)	629 94)	NR
Total	1,117 (100)	2,563 (100)	168 (100)	781 (54)	785 (91)	46 (41)

*Only 1 sample per patient was included. GII, genogroup II; NR, not relevant.
†Only 1 patient per clinic or ward per month was included.
‡Proportion of patients infected with novovirus GII.P4 or G.II.4 in each age group.

from community settings, 1,070 were from health care settings, and 157 were from foodborne outbreaks. Patients from health care settings were further grouped into nosocomially infected patients (n = 539), patients with community-acquired infections (n = 248), patients with an indeterminate source of infection (n = 274), and nursing home residents (n = 9).

A total of 22 NoV capsid and 15 polymerase genotypes were detected among the genotyped samples. In patients from community settings, 20 capsid and 12 polymerase genotypes were detected, and 14 capsid and 8 polymerase genotypes were detected in NoVs from patients in health care settings. With the exception of GII.21, all NoV genotypes detected in health care settings were also detected in community settings. Among the samples from the 46 foodborne outbreaks, 15 NoV capsid and 11 polymerase genotypes were detected. The 2 most prevalent genotype combinations were GII.P21_GII.3 and GII.P7_GII.6, and the 6 most common genotypes were GII.P4, GII.4, GII.6, GII.3, GII.P21, and GII.7 (Figure 1). Clinics (n = 60) and wards (n = 63) that were represented with ≥5 patients with an assigned genotype had median proportions of NoV GII.4 of 57% (range 14%–100%) and 96% (range 17%–100%), respectively.

The age distribution differed considerably between patients from community and health care settings (Table 1). A significantly larger proportion of patients from health care settings were ≥60 years of age (2,196/2,563, 86%) (p<0.001). In contrast, patients from community settings were mainly children <3 years of age (680/1,117, 61%) (p<0.001).

Descriptive Analyses

In these analyses, only 1 patient per calendar month from each GP, outpatient clinic, and ward was included (Table 1). Foodborne outbreaks were described on an outbreak level with only 1 sample representing each outbreak (n = 46 outbreaks).

The distribution of NoV genotypes according to age and setting is shown in Figure 2. The distribution differed between community and health care settings. Although most patients from health care settings were infected with GII.4 (712/785, 91%), this genotype was detected in a significantly lower proportion of patients from community settings (421/781, 54%) (p<0.001). The proportion infected with GI was significantly higher in foodborne outbreaks (22%) than in community settings (6%) and health care settings (2%) (p<0.001). When samples positive for NoV GII.4 and GII.P4 were excluded, the proportion of GI was similar for those infected in community (13%) and health care settings (16%) but significantly higher for those infected in foodborne outbreaks (37%) (p = 0.001).

The proportion of children <3 years of age infected with NoV GII.3 or GII.P21 ranged from 11% to 25% during the study period. However, ≤3% of adults ≥60 years of age were positive for these genotypes. This difference was significant for each year studied (each year tested: p<0.001).

Association between NoV GII.4 and Patient Age

When we compared with younger and older infected persons, we found a strong association between infection with NoV GII.4 and patient age ≥60 years in community and health care settings. This association was greater in health care settings than in community settings (Table 2) (p<0.001 for effect of age and setting). The mean

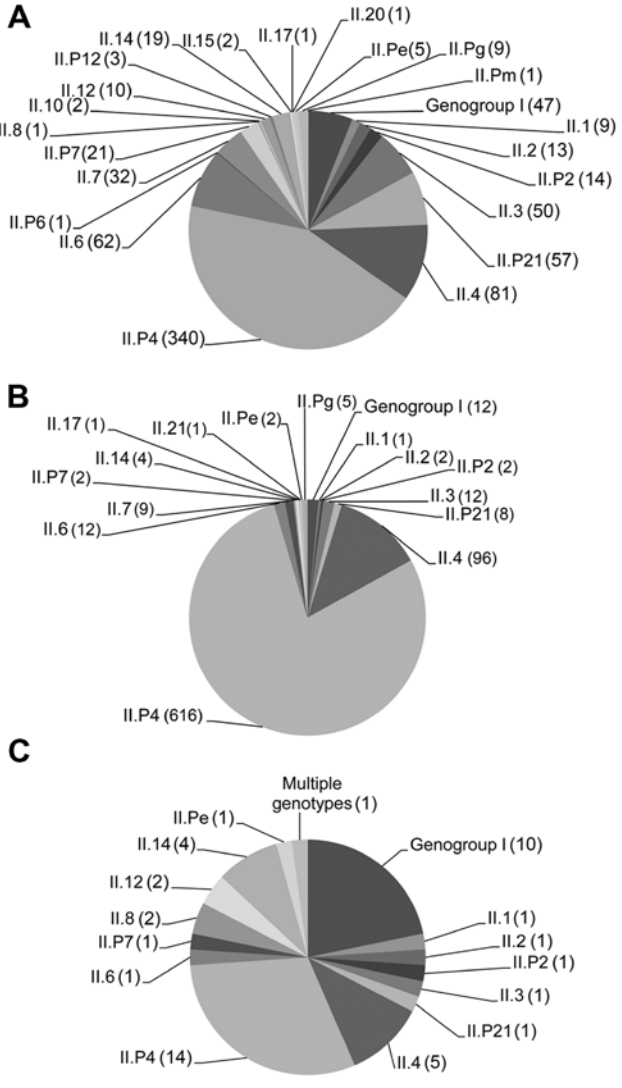

Figure 1. Distribution of norovirus genotypes of isolates from stool samples of A) patients in community settings (n = 781 samples), B) patients in health care settings (n = 785 samples), and C) patients in foodborne outbreaks (n = 46 samples), Denmark, 2006–2010. From each clinic and hospital ward, only the first sample with an assigned genotype per calendar month is included. Values in parentheses are numbers of isolates with a specific genotype or genogroup.

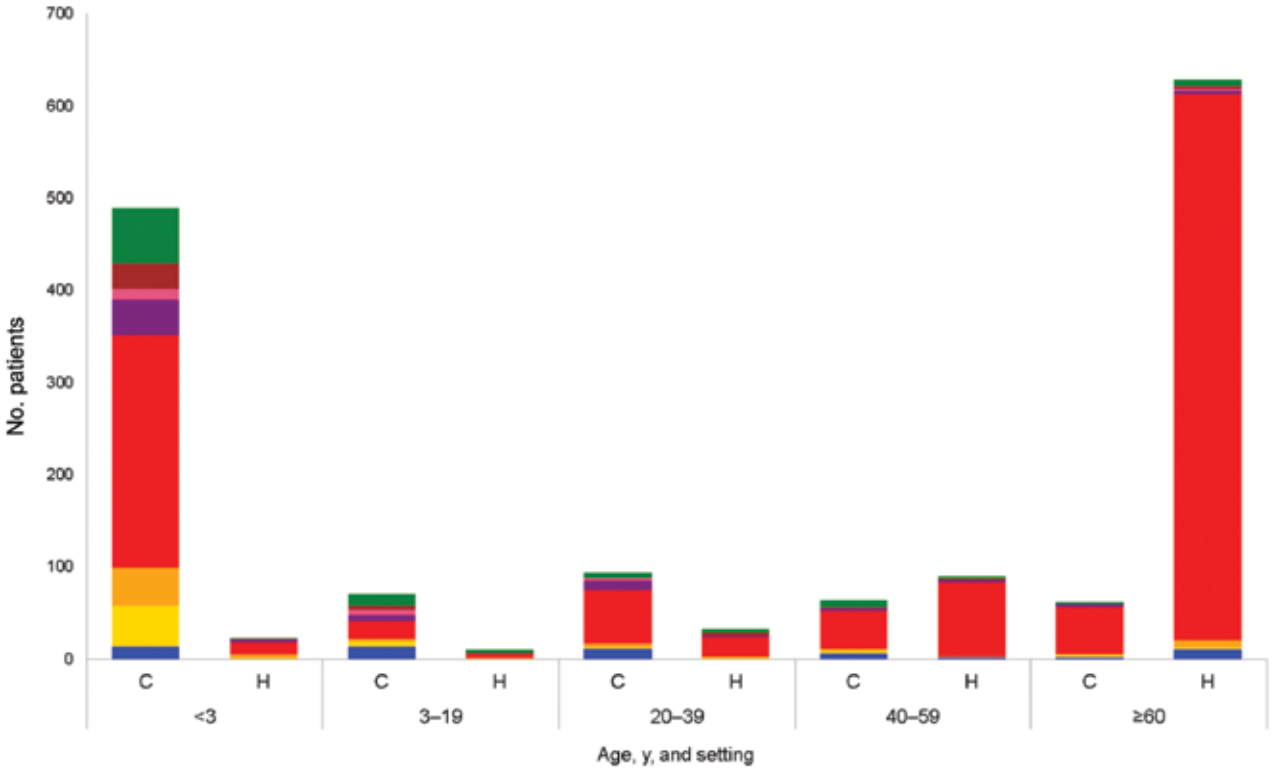

Figure 2. Distribution of norovirus genotypes by patient age and setting for 1,566 patients positive for norovirus, Denmark, 2006–2010. Only 1 patient from each general practice or outpatient clinic (n = 781 patients) and ward (n = 785 patients) within a calendar month were included (foodborne outbreak patients were not included). Inpatients and patients from nursing homes were grouped as patients from health care settings. Genogroup I (GI), blue; GII.P21, gold; GII.3, orange; GII.4 or GII.P4, red; GII.6, purple; GII.P7, pink; GII.7, brown; all other genogroup II, green. C, community; H, health care.

proportion of NoV GII.4 within each ward or clinic with respect to the mean patient age is shown in Figure 3. The sensitivity analysis showed similar results regarding odds ratios (Table 2).

Discussion

In this study of 3,846 patients who were positive for NoV by routine diagnostic procedures for gastroenteritis in Denmark during 2006–2010, we detected an association between an age ≥60 years and infection with NoV GII.4 in patients from community and health care settings. We also found that NoV GII.P21/GII.3 was more prevalent in children than in adults, and NoV GI was more frequently detected in patients from foodborne outbreaks than in patients from community and health care settings.

NoV GII.4 was the most prevalent genotype among patients in health care settings (in 91%). However, only 54% of patients from community settings were infected with this genotype. This finding is consistent with findings in a recent study from the United States, which reported that 84%–87% of outbreaks in hospitals and long-term care facilities were caused by GII.4 compared with 17%–75% in other settings (*13*). The reason for the predominance of GII.4 in health care settings has been debated. Patient characteristics, such as increased susceptibility caused by concurrent illnesses or older age, have been suggested (*3*). Virus characteristics, such as greater inherent virulence or increased virus shedding, thereby facilitating transmission in settings with a high concentration of persons, have also been suggested as contributing factors (*2,23*). A study by Vega et al. reported that older age was associated with GII.4 outbreaks in diverse settings, such as schools, restaurants, and hospitals (*13*). Our study confirmed this association, which was present in community and health care settings and could partly explain the predominance of GII.4 in hospital settings. The association was stronger for patients in health care settings than for patients in community settings, but the reason for this difference is unknown. Once introduced into a hospital setting, NoV GII.4 might be more easily transmitted than other genotypes, thus infecting elderly patients already hospitalized for other reasons (*2*).

Other studies compared the clinical manifestations of infection with NoV GII.4 with those of infection with other NoV genotypes. Two studies of persons in nursing homes showed that symptoms were more severe in persons infected with GII.4 than in persons infected with other NoV

Table 2. Association between age and infection with norovirus GII.4, Denmark, 2006–2010*

Variable	All patients with an assigned genotype, n = 1,952				Sensitivity analysis 1, n = 1,299 patients		Sensitivity analysis 2, n = 1,566 patients	
	Total no. (%)†	No. (%) positive for GII.4‡	OR	95% CI	OR	95% CI	OR	95% CI
Community settings								
Age, y								
<3	558 (63)	291 (52)	0.60	0.39–0.91	0.46	0.25–0.86	0.63	0.40–1.00
3–19	80 (9)	21 (26)	0.19	0.10–0.37	0.09	0.03–0.24	0.22	0.11–0.43
20–39	114 (13)	73 (64)	Ref	NA	Ref	NA	Ref	NA
40–59	66 (7)	43 (65)	1.03	0.54–1.95	0.86	0.30–2.42	1.07	0.55–2.08
≥60	64 (7)	53 (83)	2.71	1.27–5.78	2.23	0.73–6.88	2.86	1.32–6.19
Health care settings								
Age, y								
<3	25 (2)	13 (52)	0.46	0.16–1.29	0.50	0.13–1.98	0.75	0.25–2.22
3–19	12 (1)	6 (50)	0.41	0.11–1.52	0.23	0.04–1.19	0.58	0.14–2.41
20–39	44 (4)	31 (71)	1 (Ref)	NA	1 (Ref)	NA	1 (Ref)	NA
40–59	119 (11)	107 (90)	3.73	1.54–9.05	3.42	1.20–9.72	4.59	1.75–12.09
≥60	870 (81)	828 (95)	8.39	4.07–17.29	8.37	3.55–19.78	9.62	4.38–21.12
Sex								
M	965 (49)	716 (74)	1.23	0.96–1.56	1.26	0.92–1.74	1.22	0.95–1.58
F	987 (51)	750 (76)	Ref	NA	Ref	NA	Ref	NA
			Estimate	SE	Estimate	SE	Estimate	SE
Variation between clusters§	NA	NA	0.053	0.089	0.062	0.101	NA	NA
Intraclass correlation coefficient	NA	NA	2.6%	NA	1.95%	NA	NA	NA
Dispersion parameter	NA	NA	0.99	NA	0.99	NA	1.01	NA

*Sensitivity analysis 1 was a multilevel logistic regression model that included only wards and clinics with ≥5 patients. Sensitivity analysis 2 was a logistic regression model (without random effect) that included only the first patient per month within each ward and clinic. GII, genogroup II; OR, odds ratio; Ref, referent; NA, not applicable. An OR>1 indicates an increased risk for norovirus GII.4 infection compared with patients 20–39 y of age.
†Proportions for community settings do not add up to 100% because of rounding.
‡Proportion of patients infected with norovirus GII.P4 or GII.4 in each age or sex group.
§Estimated to be 1.12 (SE 0.18) in the random intercept-only model (i.e., without the effect of age, sex, and setting).

genotypes (*24,25*). This finding could be caused by the age of the participants, a finding consistent with the results of our study. A study of NoV infections in newborns, whom the authors presumed to have no pre-existing immunity, showed that the length of symptomatic NoV infection was longer when newborns were infected with NoV GII.4 than with other genotypes (*23*). This finding differs from the hypothesis that only elderly persons have more severe infections when infected with NoV GII.4 than with other genotypes. Because an association between persons ≥60 years of age and infection with NoV GII.4 was also observed in patients from community settings in our study, the increased duration of symptomatic NoV GII.4 infection in infants could be caused by immaturity of their immune systems. Desai et al. conducted a literature review of published NoV outbreaks and concluded that in community settings and long-term care facilities, incidence of hospitalization and death was increased during infection with NoV GII.4 compared with non–GII.4 NoV (*26*). These findings support the theory that the high proportion of GII.4 in hospital settings is caused by viral characteristics rather than host characteristics. However, Desai et al. did not control for age, which might have biased the results toward an increased risk for hospitalization during infection with NoV GII.4.

We determined that NoV GI was more prevalent in foodborne outbreaks than in outbreaks in health care and community settings, which is consistent with previous studies that reported that GI is more frequently observed in foodborne NoV outbreaks than in outbreaks involving person-to-person transmission (*2,3*). Excluding GII.4, the proportion of GI was similar in health care and community settings.

When we grouped GII.3 and GII.P21, we observed a higher proportion of these genotypes in young children than among patients ≥60 years of age. This finding is consistent with those of other studies, which indicate that GII.P21 (formerly classified as GII.b) and GII.3 infect mainly young children (*10,27–29*). It has been hypothesized that genotypes that preferentially infect young children, such as GII.3, require less antigenic variation because of the constant renewal of the host population with persons who do not have established immune-associated protection from previous infections (*30*). This situation is in contrast to that for GII.4, which infects a large proportion of the adult population and thus requires a constant change in host-binding receptors to evade the immune response.

Our study had several limitations. First, sampling bias was caused by inclusion of samples collected for routine diagnostic virus analyses, rather than a cohort encompassing all cases of acute gastroenteritis. Generally, only a few patients in hospital outbreaks of infectious gastroenteritis in Denmark are diagnosed by laboratory testing. We assume that most hospitalized patients with community-acquired

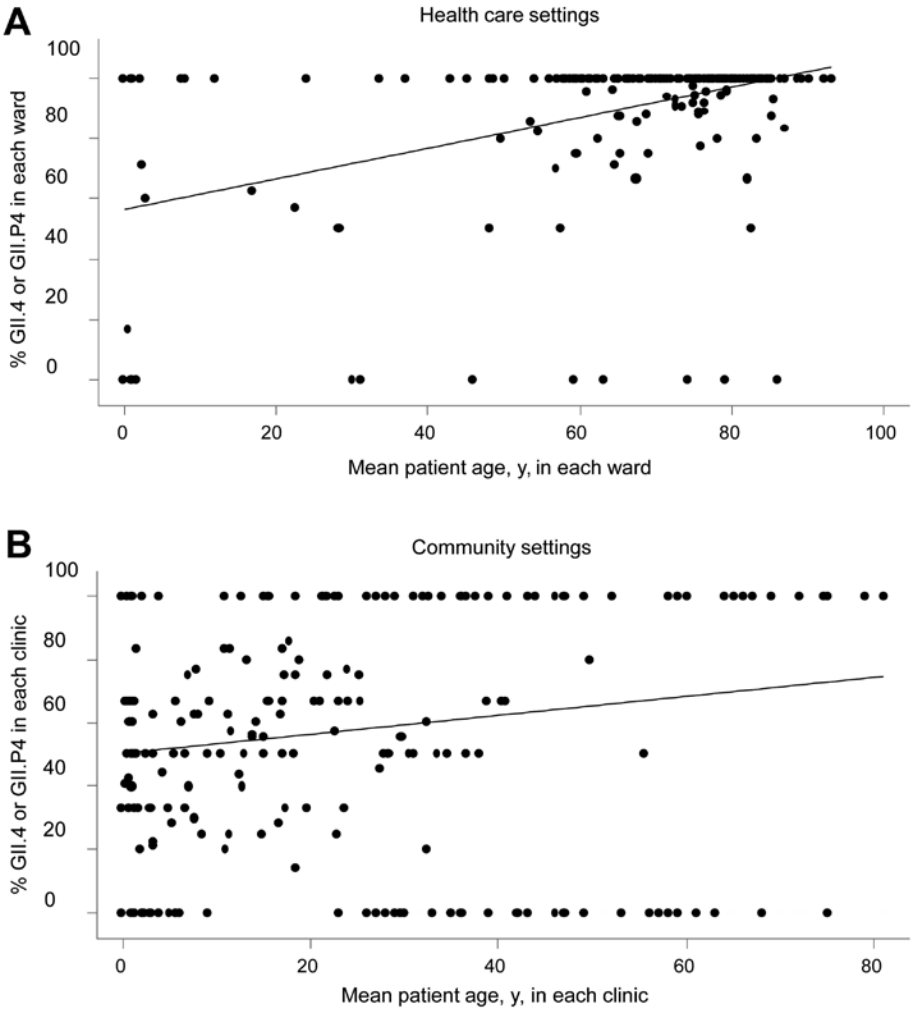

Figure 3. Proportions of norovirus genogroups GII.4 or GII.P4 with respect to mean age of patients with an assigned genotype in each hospital ward (A) (n = 212 wards, 1,070 patients) or clinic (B) (n = 311 clinics, 882 patients), Denmark, 2006–2010. Regression lines are indicated.

NoV infection are tested for NoV as a differential diagnosis to bacterial causes of gastroenteritis, but this assumption might not be correct. As in another study (*31*), our study had an overrepresentation of young and old persons. This overrepresentation could have affected the outcome because it is likely that severity of disease, concurrent illnesses, and young age increase the probability of seeking medical attention. If elderly persons are more likely to be tested for NoV and GII.4 is more virulent than other NoV genotypes, the statistical results could be biased toward an increased effect of age on the odds of infection with GII.4. The best way of testing this hypothesis would be to conduct a cohort study that included all patients with gastroenteritis.

Second, genotyping of NoV isolates was only performed for selected number of NoV-positive patients, but these samples represented almost 90% of all wards, GPs, and outpatient clinics submitting samples to the laboratory. The distribution of age differed between patients whose sample isolates had an assigned genotype. However, a notable finding was the association between NoV GII.4 and age ≥60 years old in community settings. The proportions of genotyped samples did not differ between patients ≥60 and <3 years of age in community settings. Furthermore, we detected 22 NoV capsid and 15 polymerase genotypes, which made it unlikely that problems with laboratory methods systematically biased the results.

Third, we did not have epidemiologic data to compare the distribution of NoV genotypes at an outbreak level. Alternatively, the association between age and NoV GII.4 was examined by including the random effect of clinics and wards. This feature was possible because of the large number of available clinics and wards (*32*). The association between NoV GII.4 and age was also observed in sensitivity analyses that included 1 patient per clinic or hospital ward in each calendar month or included clinics and wards with ≥5 patients.

Fourth, we estimated that most hospitalized patients were nosocomially infected with NoV. This estimate was based on admission and sampling dates because clinical data were not available. For some patients, sampling

may have been performed >1 day after onset of symptoms. Therefore, the proportion of nosocomially infected patients might have been overestimated.

In this retrospective study of NoV gastroenteritis in Denmark, we compared infections in patients from foodborne outbreaks, community settings, and health care settings. Our results confirmed that most NoV genotypes circulating in health care settings were GII.4 and that infection with NoV GII.P21 or II.3 was more prevalent in children than adults. We observed an association between older age and infection with NoV GII.4, which could partly explain why most NoV infections in health care settings are caused by this genotype. Cohort studies testing this hypothesis would be of value.

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Changes in Capsule and Drug Resistance of Pneumococci after Introduction of PCV7, Japan, 2010–2013

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We aimed to clarify changes in serotypes and genotypes mediating β-lactam and macrolide resistance in *Streptococcus pneumoniae* isolates from Japanese children who had invasive pneumococcal disease (IPD) after the 7-valent pneumococcal conjugate vaccine (PCV7) was introduced into Japan; 341 participating general hospitals conducted IPD surveillance during April 2010–March 2013. A total of 300 pneumococcal isolates were collected in 2010, 146 in 2011, and 156 in 2012. The proportion of vaccine serotypes in infectious isolates decreased from 73.3% to 54.8% to 14.7% during the 3 years. Among vaccine serotype strains, genotypic penicillin-resistant *S. pneumoniae* strains also declined each year. Among nonvaccine serotype strains, 19A, 15A, 15B, 15C, and 24 increased in 2012. Increases were noted especially in genotypic penicillin-resistant *S. pneumoniae* isolates of serotypes 15A and 35B, as well as macrolide resistance mediated by the *erm*(B) gene in 15A, 15B, 15C, and 24.

Invasive pneumococcal disease (IPD), such as meningitis, sepsis, and empyema, substantially contributes to illness and death in children (*1*,*2*). After increasing numbers of cases caused by penicillin (PEN)–resistant *Streptococcus pneumoniae* (PRSP) emerged and rapidly spread worldwide during the 1990s (*3*,*4*), the need for a vaccine effective in infants became clear. In the United States, a 7-valent pneumococcal conjugate vaccine (PCV7) was introduced in 2000 and made available for routine use in all children 2–23 months of age and in children 24–59 months of age at risk for pneumococcal infection (*5*). Subsequent surveillance studies demonstrated a marked decrease in prevalence of pneumococcal infection caused by vaccine serotypes, including PRSP (*6–8*). In particular, PCV7 appears to have decreased incidence of meningitis caused by vaccine serotypes (*9*), and cases caused by non-PCV7 serotype strains, such as PRSP with serotype 19A, have increased in the United States (*8*,*10*,*11*). Such changes suggest that nonvaccine serotypes are replacing vaccine serotypes in some countries (*12–14*).

A next-generation 13-valent pneumococcal conjugate vaccine (PCV13) was licensed for use in the United States in 2010 (*15*). PCV13 has been approved in 128 countries, and children in 83 countries have undergone routine PCV13 vaccination. Recently, Richter et al. reported that an increase of type 19A was halted by introduction of PCV13, whereas serotype 35B increased; coverage provided by PCV13 was effective in only 41.4% of children ≤5 years of age in 2010 and 2011 (*16*). Furthermore, Kaplan et al. reported a slight increase in serotype 33F (*17*).

In Japan, PRSP has increased rapidly as a cause of respiratory tract infections, acute otitis media, and IPD in children since the late 1990s (*18*,*19*). PCV7 received final approval in October 2009 and has been used clinically in infants on a voluntary basis since February 2010. Since November 2010, PCV7 use has been encouraged for children <5 years of age throughout Japan by an official program, the Provisional Special Fund for the Urgent Promotion of Vaccination. As a result, estimated rates of PCV7 vaccination for such children were <10% in 2010, 50%–60% in 2011, and 80%–90% in 2012.

PCV7 was incorporated into the routine vaccination schedule for children in Japan beginning in April 2013; before then, however, its coverage rate against IPD had decreased rapidly from 71.8% in 2006 to 51.6% in 2011 (*20*).

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Most recently, PCV13 was approved by the government in June 2013, later replacing PCV7 as a routine vaccination in November 2013. The purpose of our study was to clarify changes during April 2010–March 2013 of serotypes and genotypes mediating β-lactam and macrolide resistance in *S. pneumoniae* isolates from children <18 years of age who had IPD before and after PCV7 introduction.

Methods

Patients and Pneumococcal Strains

All study participants were children <18 years of age who had IPD. Pneumococcal isolates from normally sterile clinical samples, such as blood, cerebrospinal fluid, pleural effusions, and joint fluid, were collected and examined.

Medical institutions that had a microbiology laboratory and a pediatric department with hospitalization facilities were permitted to participate actively in surveillance. An estimated 25% of all general hospitals in Japan participated. Participating hospitals were distributed nearly uniformly throughout Japan (Figure 1). These hospitals took part in the surveillance project after the laboratory director or hospital director granted permission in writing. A questionnaire collecting information for every case-patient was completed anonymously in accordance with the ethical guidelines for conducting epidemiologic studies in Japan.

Pneumococcal isolates were collected nationwide during 3 periods. The first surveillance period was April 2010–March 2011, when voluntary vaccination with PCV7 was estimated to be <10% (vol-PCV7: 2010). The second period was April 2011–March 2012, when the estimated vaccination rate was 50%–60% because of the Urgent Promotion of Vaccination incentive (post-PCV7: 2011). The third period was April 2012–March 2013, when the vaccination rate was 80%–90% just before introduction of PCV13 (pre-PCV13: 2012). We collected a total of 602 isolates from 341 general hospitals: 300 in 2010, 146 in 2011, and 156 in 2012.

Serotypes and Antimicrobial Drug–Resistant Genotypes

Serotypes of all isolates were determined by the capsular quellung reaction using antiserum purchased from the Statens Serum Institute (Copenhagen, Denmark). Alterations in 3 penicillin-binding protein (PBP) genes mediating β-lactam resistance in *S. pneumoniae—pbp1a* (PBP1A), *pbp2x* (PBP2X), and *pbp2b* (PBP2B)—were identified by real-time PCR methods that we developed and reported previously (*21*). The PCR system detected the presence of amino acid substitution(s) in conserved amino acid motif(s), such as serine-threonine-methionine-lysine, in each PBP. The genes *mef* (A) and *erm* (B), which confer resistance to macrolide antimicrobial drugs, also were identified by real-time PCR (*21*).

Genotype (g) based on molecular analysis is represented here as penicillin-susceptible *S. pneumoniae* (gPSSP) possessing 3 normal *pbp* genes; penicillin-intermediate *S. pneumoniae* (gPISP), further classified as gPISP (*pbp2x*), gPISP (*pbp1a*+*pbp2x*), or gPISP (*pbp2x*+*pbp2b*); or penicillin-resistant *S. pneumoniae* (gPRSP), possessing all 3 abnormal *pbp* genes (*22*). Relationships between susceptibilities to β-lactam agents among phenotypes of *S. pneumoniae* and resistance genotype were described previously (*21*). Genotypes involving macrolide resistance are represented here as the following: macrolide-susceptible *S. pneumoniae* not possessing any genes (MLS); intermediate macrolide resistance to 14- or 15-membered macrolides mediated by the *mef*(A) gene (MLR-*mef*[A]); high macrolide resistance to all macrolides mediated by the *erm*(B) gene (MLR-*erm*[B]); and high macrolide resistance possessing both genes (MLR-*mef*[A]+*erm*[B]).

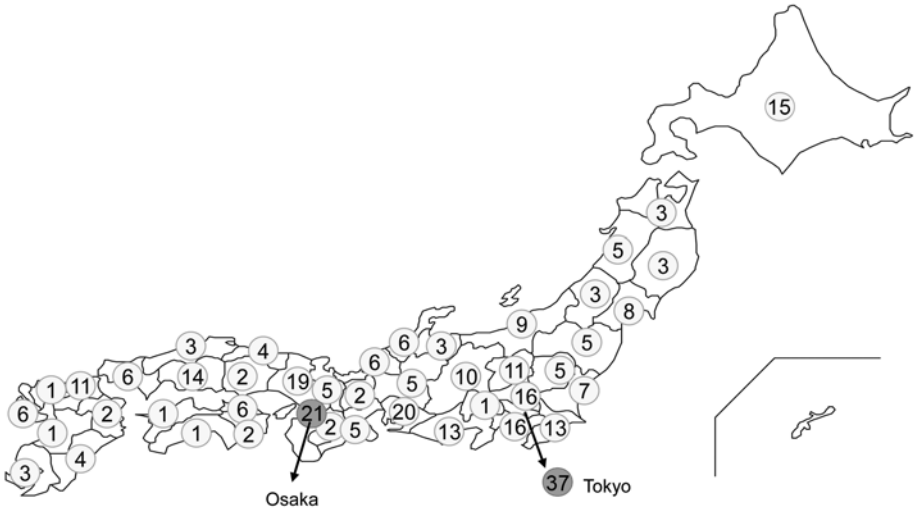

Figure 1. Distribution of general hospitals participating in surveillance for invasive pneumococcal disease, Japan, April 2010–March 2013. Numbers in gray shaded circles show the number of hospitals in Tokyo and Osaka; other numbers show the number of the hospitals in each prefecture.

For each strain transferred to our laboratory, we promptly identified capsular type by quellung reaction, and resistance genotype was determined by real-time PCR. Results were returned immediately to medical staff at the referring hospital. Medical personnel caring for the patients considered this surveillance and results reporting system very helpful, and it was continued for 3 years.

Multilocus Sequence Typing

Multilocus sequence typing for the strains collected was performed according to previously described methods with slight modifications (*23*). Primers listed on the website of the Centers for Disease Control and Prevention (http://www.cdc.gov/ncidod/biotech/strep/alt-MLST-primers.htm) were used. Multilocus sequence typing and eBURST analyses were performed according to published methods (http://spneumoniae.mlst.net).

Statistical Analysis

We assessed statistical significance of differences in serotype distribution between the 3 periods, age groups, and specific infectious diseases. We used χ^2 tests or the Fisher exact test, using Ekuseru-Toukei 2012 software for statistics (Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results

Patient Age and Coverage Rate by PCV7

Estimated rates of vaccination with PCV7 were <10% in 2010 (voluntary-PCV7) but rose to 50%–60% with funding in 2011 (post-PCV7) and to 80%–90% with enhanced implementation of PCV7 just before the transition from PCV7 to PCV13 in 2012 (pre-PCV13). *S. pneumoniae* isolates from patients with IPD, collected every year, decreased by half in 2011 and 2012 from those in 2010. In particular, vaccine serotypes decreased significantly among patients ≤4 years of age (p<0.001) but not among those ≥5 years of age (p = 0.733) (Table 1). For all patients, coverage rate by PCV7 decreased rapidly from 73.3% in 2010 to 54.8% in 2011 to 14.7% in 2012, and prevalence proportion of nonvaccine serotypes increased for 3 years (p<0.001).

Year-to-Year Changes in Vaccine and Nonvaccine Serotype Prevalence Proportion by Disease

We compared year-to-year changes in prevalence of vaccine and nonvaccine serotypes of *S. pneumoniae* in patients who had meningitis; sepsis and bacteremia; pneumonia; and other invasive infections, including cellulitis, arthritis, endocarditis, and empyema during each of the 3 years studied (Table 2). Pneumonia cases were included only when *S. pneumoniae* was isolated from blood cultures. Vaccine serotype strains decreased significantly in patients with meningitis (p = 0.006), sepsis and bacteremia (p<0.001), and pneumonia (p<0.001).

Table 1. Vaccine and nonvaccine serotypes of *Streptococcus pneumonia*e in children after introduction of PCV7, Japan, April 2010–March 2013*

Age group, y, and serotype	Isolates, no. (%) 2010, n = 300†	2011, n = 146‡	2012, n = 156§	p value
<1				<0.001
VT	51 (17.0)‡	26 (17.8)	3 (1.9)	
NVT	19 (6.3)	14 (9.6)	25 (16.0)	
1				<0.001
VT	103 (34.3)	25 (17.1)	9 (5.8)	
NVT	31 (10.3)	28 (19.2)	62 (39.7)	
2–4				<0.001
VT	55 (18.3)	23 (15.8)	6 (3.8)	
NVT	19 (6.3)	14 (9.6)	39 (25.0)	
≥5				0.733
VT	11 (3.7)	6 (4.1)	5 (3.2)	
NVT	11 (3.7)	10 (6.8)	7 (4.5)	
Total				<0.001
VT	220 (73.3)	80 (54.8)	23 (14.7)	
NVT	80 (26.7)	66 (45.2)	133 (85.3)	

*PCV7, 7-valent pneumococcal conjugate vaccine; VT, vaccine serotype (serotypes 4, 6B, 9V, 14, 18C, 19F, 23F); NVT, serotypes not included in PCV7.
†Voluntary vaccination with PCV7.
‡Implementation of Urgent Promotion of Vaccination incentive.
§Just before the introduction of 13-valent PCV.

Correlation between Serotype and Genotype

We also determined correlations between changes of serotypes and resistance genotypes in all 602 isolates for each period (Figure 2). These resistance genotypes were classified according to real-time PCR results concerning 3 PBP genes: *pbp1a*, *pbp2x*, and *pbp2b*.

Table 2. Year-to-year changes in prevalence of vaccine and nonvaccine serotypes of *Streptococcus pneumoniae* in children after introduction of PCV7, Japan, April 2010–March 2013*

Disease and serotype	Isolates, no. (%) 2010, n = 300†	2011, n = 146‡	2012, n = 156§	p value
Meningitis				0.006
VT	42 (14.0)	17 (11.6)	6 (3.8)	
NVT	20 (6.7)	17 (11.6)	15 (9.6)	
Sepsis and bacteremia				<0.001
VT	111 (37.0)	42 (28.8)	13 (8.3)	
NVT	48 (16.0)	39 (26.7)	94 (60.3)	
Pneumonia				<0.001
VT	54 (18.0)	14 (9.6)	3 (1.9)	
NVT	8 (2.7)	6 (4.1)	21 (13.5)	
Other				0.147
VT	13 (4.3)	7 (4.8)	1 (0.6)	
NVT	4 (1.3)	4 (2.7)	3 (1.9)	
Total				<0.001
VT	220 (73.3)	80 (54.8)	23 (14.7)	
NVT	80 (26.7)	66 (45.2)	133 (85.3)	

*PCV7, 7-valent pneumoccal conjugate vaccine; VT, vaccine serotype (serotypes 4, 6B, 9V, 14, 18C, 19F, 23F); NVT, serotypes not included in PCV7.
†Voluntary vaccination with PCV7.
‡Implementation of Urgent Promotion of Vaccination incentive.
§Just before the introduction of 13-valent PCV.

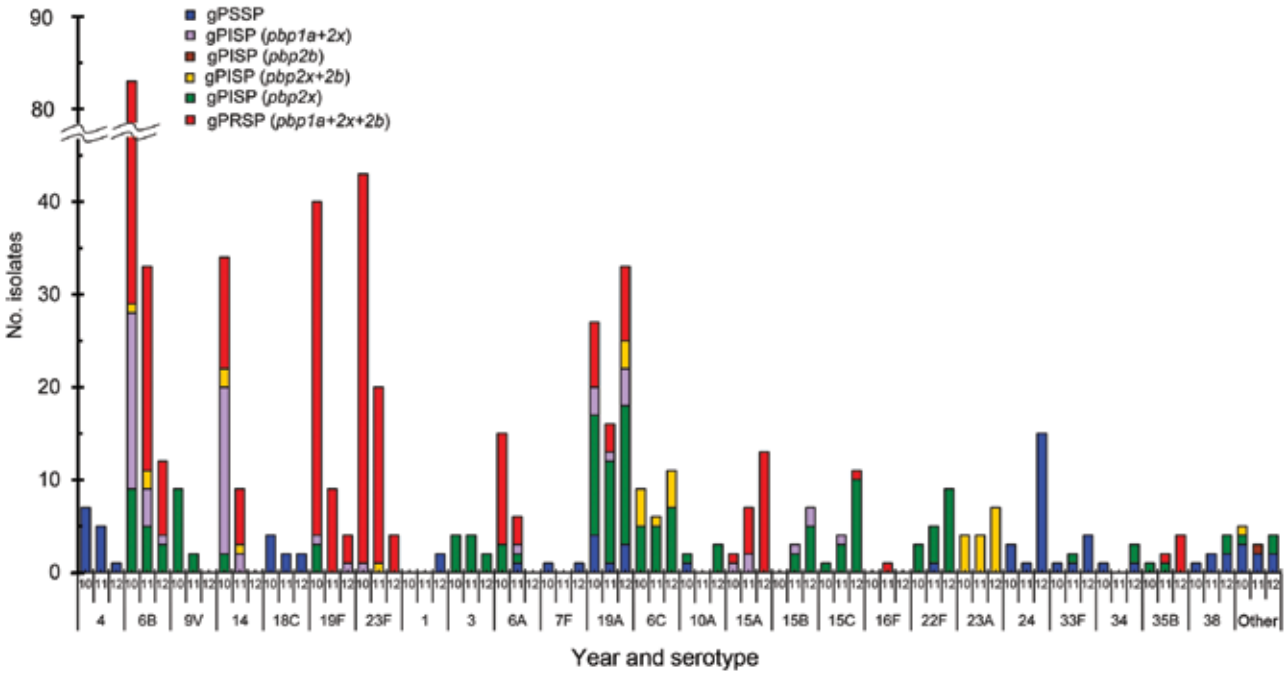

Figure 2. Changes in serotype number and penicillin resistance according to genotype, Japan, April 2010–March 2013. gPSSP, genotypic penicillin-susceptible *Streptococcus pneumoniae*; gPISP, genotypic penicillin-intermediate resistant *S. pneumoniae;* gPRSP, penicillin-resistant *S. pneumoniae*. The parentheses express abnormal *pbp* gene mediating penicillin resistance. 2010 indicates first surveillance period (April 2010–March 2011); 2011 indicates second surveillance period (April 2011–March 2012); 2012 indicates third surveillance period (April 2012–March 2013).

Overall, gPRSP and gPISP (*pbp1a*+*pbp2x*), respectively, declined from 54.7% and 14.3% in 2010, to 47.3% and 8.2% in 2011, to 26.3% and 5.1% in 2012. Among them, vaccine serotypes 6B, 14, 19F, and 23F, including gPRSP and gPISP (*pbp1a*+*pbp2x*), which showed high frequency in 2010, decreased significantly during the 2 subsequent periods ($p<0.001$). Of serotypes contained in PCV13, serotype 6A, showing cross-protective immunity with 6B, also decreased markedly in 2012, but prevalence of serotype 19A increased. Serotypes 1, 3, and 7F included a very small number of isolates.

Coverage rate of PCV13 decreased rapidly from 89.0% in 2010 to 72.6% in 2011 to 39.1% in 2012 ($p<0.001$). Nonvaccine serotypes increased, especially serotypes 24 and 33F, identified as gPSSP; 15B, 15C; and 22F, gPISP (*pbp2x*); and 15A and 35B, gPRSP.

Main clonalities within major nonvaccine serotypes were identified as sequence type (ST) 3111 and ST2331 in serotype 19A, ST63 in serotype 15A, ST199 in serotypes 15B and 15C, ST433 in serotype 22F, ST338 of clonal complex (CC) 156 in serotype 23A, ST5496 of CC2572 in serotype 24, ST717 in serotype 33F, and ST558 in serotype 35B. STs in gPRSP among nonvaccine serotypes were as follows: major strains of serotype 15A belonged to ST63; serotype 15C belonged to ST83 of CC81; serotype 35B belonged to ST558; and serotype 16F belonged to ST8351 of CC3117.

Yearly Changes in Macrolide Resistance

Macrolide resistance was classified into 4 groups: MLS, MLR-*mef*(A), MLR-*erm*(B), and MLR-*mef*(A)+*erm*(B). Comparing results between 2010 and 2012, proportions of resistance types of MLR-*mef*(A), MLR-*erm*(B), and MLR-*mef*(A)+*erm*(B) in vaccine serotypes decreased, whereas that of MLR-*erm*(B) in nonvaccine serotypes increased significantly ($p<0.001$) (Figure 3).

Relationships between serotypes and macrolide resistance genes changed from year to year (Figure 4). In contrast to decreases in vaccine serotype strains possessing *erm*(B) or *mef*(A) genes, nonvaccine serotypes 15A, 15B, 15C, and 24, which possess the *erm*(B) gene mediating high macrolide resistance, increased. Almost all strains of serotype 19A possessed both genes *erm*(B) and *mef*(A).

Changes of Relative Ratios of Every Serotype

As for increases and decreases in the proportion of each serotype isolated from patients <5 years of age from 2010 (vol-PCV7) to 2012 (pre-PCV13), vaccine serotype strains 6B, 14, 19F, and 23F, and 6A (which shows cross-protective

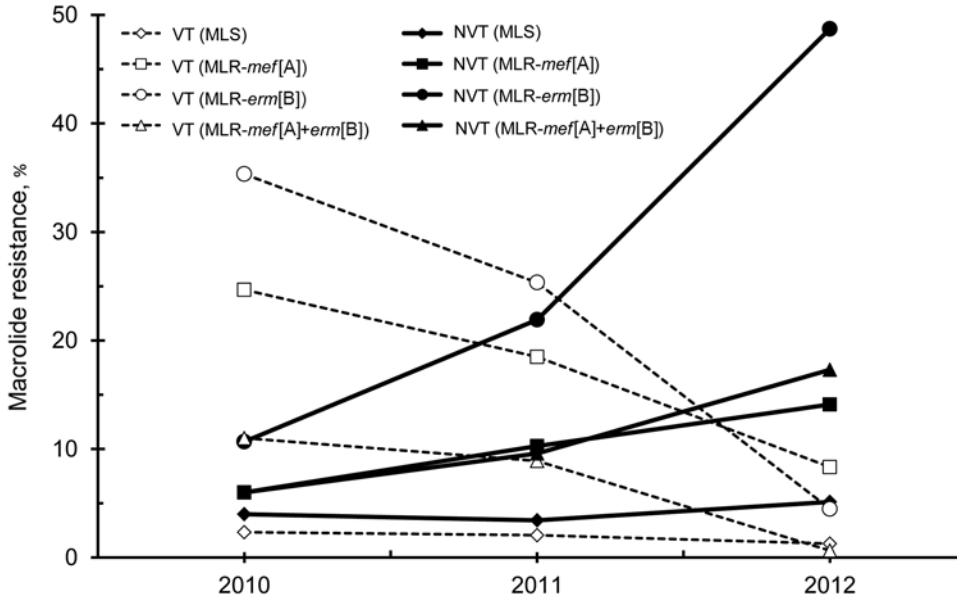

Figure 3. Proportional yearly changes in macrolide resistance according to resistance genes *erm*(B) and *mef*(A) identified by real-time PCR, Japan, April 2010–March 2013. The percentage of each resistance gene was calculated from the number of *Streptococcus pneumoniae* strains for each year. VT, vaccine serotype (serotypes 4, 9V, 18C, 6B, 14, 19F, 23F) included in the 7-valent pneumococcal vaccine; NVT, serotypes not included in the 7-valent pneumococcal conjugate vaccine; MLS, macrolide-susceptible strains not possessing any resistance gene; MLR-*mef*(A), macrolide-resistant strain possessing the *mef*(A) gene; MLR-*erm*(B), macrolide-resistant strain possessing the *erm*(B) gene; MLR-*mef*(A)+*erm*(B), macrolide-resistant strain possessing both *mef*(A) and *erm*(B) genes.

immunity with 6B, occurring frequently among IPD cases) decreased markedly ($p<0.001$ for 4 vaccine serotypes, $p = 0.005$ for 6A). Serotype 19A, included in PCV13, increased ($p<0.001$). Other serotypes included in PCV7 or PCV13, except for 6A and 19A, changed minimally. Overall, proportions of nonvaccine serotypes not covered by PCV13 increased, particularly serotypes 15A, 15B, 15C, and 24 (each $p<0.001$), and 22F ($p = 0.004$).

Patient Age and Vaccination History in 2012

Only 23 patients had infections caused by strains of vaccine serotypes (14.7%), 21 of whom had not received PCV7 (Table 3). Of the remaining 2 patients, a 1-year-old child infected with a strain of serotype 6B had received 2 doses of vaccine. The other patient, 5 years of age, was infected with a strain of serotype 19F; vaccination history was unknown.

Discussion

Introduction of PCV7 to prevent pneumococcal infections in children was credited with dramatic declines of the incidence of IPD in the United States (*6*,*7*), European Union countries (*24*,*25*), and many other nations (*26*).

During this implementation, increases in pneumococcal infections caused by serotype 19A and other nonvaccine serotypes, including those comprising many penicillin-nonsusceptible strains, has raised problems in clinical practice (*10*,*11*,*27*). Large-scale longitudinal surveillance showed that the rate of coverage by PCV7 decreased from 70% during 1999–2000 to 4.3% during 2008–2009 (*16*). In light of these observations, PCV7 was replaced with PCV13 in the United States in 2010 (*15*). After the change to PCV13, penicillin-nonsusceptible strains and serotype 15A or 35B strains increased as they did after PCV7 was introduced (*16*).

In Japan, PCV7 vaccination of children <5 years of age began at the end of 2010 under the Provisional Special Fund for the Urgent Promotion of Vaccination. This measure led to routine vaccination with PCV7 beginning in April 2013 until the vaccine was changed to PCV13 in November 2013. Nationwide, the estimated rate of PCV7 vaccination for children <5 years of age in 2012 and 2013 was 80%–90% and >90%, respectively. We examined the influence of PCV7 against IPD infection in children in detail with active cooperation from 341 clinical laboratories at general hospitals. We therefore believe that our data are highly likely to reflect changes in trends of serotypes and strains causing IPD after PCV7 introduction in Japan. Unfortunately, we could not calculate a precise incidence of IPD per 100 000 children.

The decrease of IPD cases caused by strains of vaccine serotypes after promotion of PCV7 contributed to a reduction in overall IPD by half. Furthermore, dramatic reductions in number of cases caused by serotypes 6B, 14, 19F, and 23F—including mainly gPISP and gPRSP—have been beneficial.

However, the relative proportion of IPD caused by nonvaccine serotypes, such as 15A, 15B, 15C, 19A, 22F, 24, and 35B, increased year by year, and the PCV7 coverage rate fell drastically from 73.3% in 2010 to 14.7% in 2012. The impact of routine PCV13 vaccination implemented in

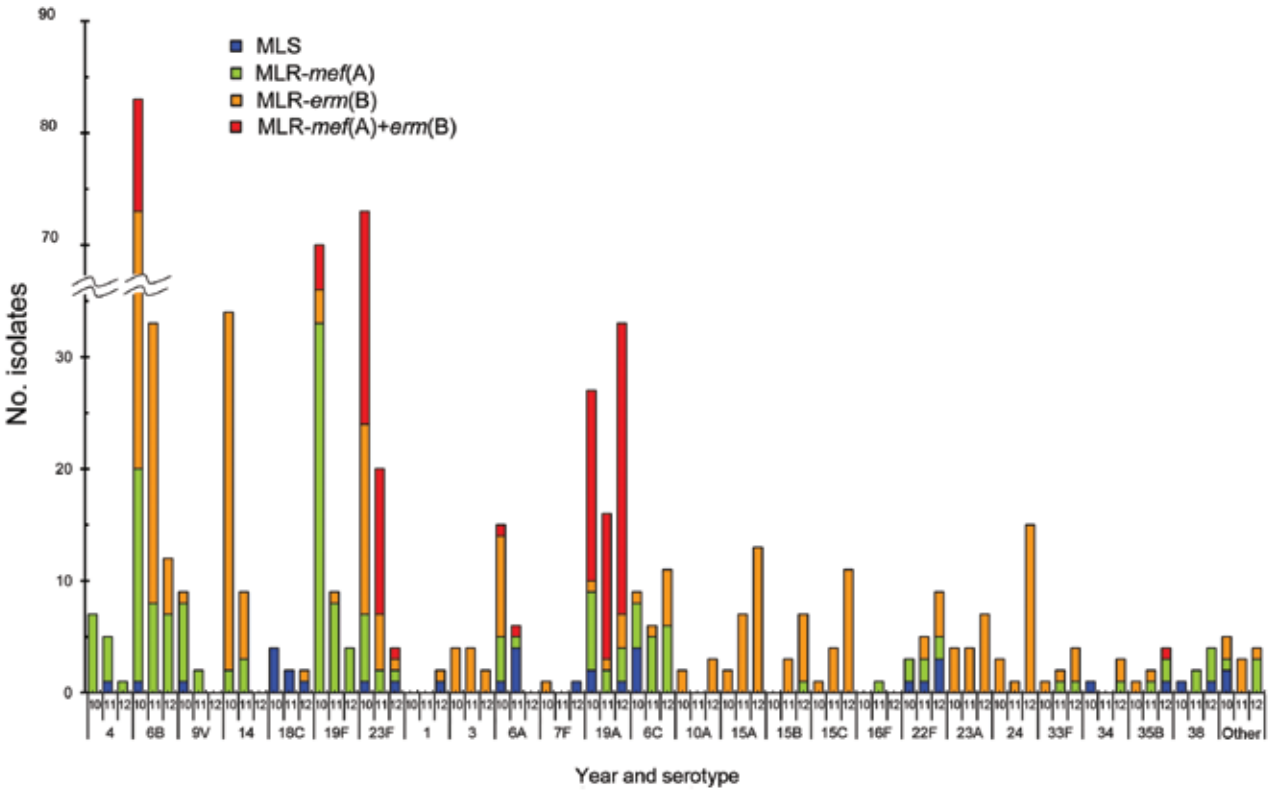

Figure 4. Changes in serotype number and macrolide resistance of *Streptococcus pneumoniae* strains according to genotype, Japan, April 2010–March 2013. MLS, macrolide-susceptible strains not possessing any resistance gene; MLR-*mef*(A), macrolide-resistant strain possessing the *mef*(A) gene; MLR-*erm*(B), macrolide-resistant strain possessing the *erm*(B) gene; MLR-*mef*(A)+*erm*(B), macrolide-resistant strain possessing both *mef*(A) and *erm*(B) genes.

December 2013 is likely to be much less than that from PCV7 because coverage already had declined to 39.1% in 2012. However, infections caused by serotype 3, included in PCV13, are expected to decrease. Although IPD caused by serotype 3 occurs infrequently among children in Japan, serotype 3 remains important as a pathogen causing acute otitis media in children and pneumonia or empyema in elderly persons.

Two findings in our study stand out. First, gPRSP strains were confirmed in serotypes 15A, 15C, 16F, and 35B. In particular, strains of serotypes 15A and 35B in 2012 all represented gPRSP. According to multilocus sequence typing analysis, serotype 15A in this study belonged to ST63 of CC63, registered as Sweden 15A-25 in the Pneumococcal Molecular Epidemiology Network clone. Serotype 35B also belonged to ST558 of CC558 from the United States. In addition, gPRSP of serotype 15C corresponded to ST83 of CC81, previously reported from Taiwan. Only 1 serotype 16F strain representing gPRSP was newly identified as ST8351 of CC3117 in the present study. Increasing migration associated with economic development is spreading pneumococcal strains worldwide.

Our finding that vaccine serotype strains are being rapidly replaced by strains of nonvaccine serotypes, such as serotypes 15C and 24, was associated with increased high

Table 3. Pneumococcal vaccination history and age for 156 children with invasive pneumococcal disease, Japan, 2012*

No. vaccine doses administered	≤6 mo	7–11 mo	1 y	2 y	3 y	4 y	5–9 y	≥10 y	Total no. (%)
0		7 [3]	15 [8]	4 [1]	5 [2]	8 [3]	4 [1]	5 [3]	47 (30.1)
1			1		5	3	1		10 (6.4)
2	3		4 [1]	1	2	1			11 (7.1)
3	2	13	24	6					45 (28.8)
Booster			16	5					21 (13.5)
Unknown	1	2	11	3	2		1 [1]	1	22 (14.1)
Total	6	22	71	19	14	12	6	6	156 (100.0)

*Numbers in brackets indicate infections with a vaccine serotype.

macrolide resistance mediated by the *erm*(B) gene. In this context, wide use of 14-membered and azalide macrolides for children, in addition to adults, will be a major factor favoring resistance. Excessive use of macrolides in Japan also has resulted in substantial increases of macrolide-resistant *Mycoplasma pneumoniae* (*28*) and macrolide-resistant *S. pyogenes* strains (*29*). This alarming problem suggests a need to strictly control macrolide use, beginning as soon as possible.

We could not analyze relationships between capsular type and death in children because deaths among children were extremely low (<2.2% every year). The low death rate reflects equal and easy access to hospitals because of Japan's universal health insurance system.

Recently, recombination of the *cps* gene encoding capsular polysaccharides among pneumococcal strains with different capsular types, called capsular switching, was observed by Brueggemann et al. (*30*). Capsular switching also is associated with recombination of PBP genes, considering that *pbp1a* and *pbp2x* genes which mediate β-lactam resistance, are positioned at both ends of the *cps* region. Dissemination of vaccine and excessive use of antimicrobial agents could favor *S. pneumoniae* with a new capsular type in the future.

In conclusion, sustained surveillance on a national and international scale is needed to control pneumococcal infections, especially considering the multifaceted consequences of vaccination programs. Also, controlling the use of antimicrobial agents is urgently needed to avoid increases in the resistant pathogens.

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Independent Lineages of Highly Sulfadoxine-Resistant *Plasmodium falciparum* Haplotypes, Eastern Africa

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Sulfadoxine-resistant *Plasmodium falciparum* undermines malaria prevention with sulfadoxine/pyrimethamine. Parasites with a highly resistant mutant dihydropteroate synthase (*dhps*) haplotype have recently emerged in eastern Africa; they negated preventive benefits of sulfadoxine/pyrimethamine, and might exacerbate placental malaria. We explored emerging lineages of *dhps* mutant haplotypes in Malawi, the Democratic Republic of the Congo, and Tanzania by using analyses of genetic microsatellites flanking the *dhps* locus. In Malawi, a triple-mutant *dhps* SGEG (mutant amino acids are underlined) haplotype emerged in 2010 that was closely related to pre-existing double-mutant SGEA haplotypes, suggesting local origination in Malawi. When we compared mutant strains with parasites from the Democratic Republic of the Congo and Tanzania by multiple independent analyses, we found that SGEG parasites were partitioned into separate lineages by country. These findings support a model of local origination of SGEG *dhps* haplotypes, rather than geographic diffusion, and have implications for investigations of emergence and effects of parasite drug resistance.

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Antimalarial drug resistance threatens to undermine efforts to control *Plasmodium falciparum* malaria. In sub-Saharan Africa, *P. falciparum* resistance to sulfadoxine/pyrimethamine (SP) is widespread, as shown by clinical treatment failures and the prevalence of molecular markers of drug resistance (*1*). Despite these findings, SP remains a major tool for malaria control when administered as a partner drug with artemisinins and as intermittent preventive therapy in infants (IPTi), children, and pregnant women (IPTp). Of these SP-based interventions, IPTi with SP is safe and effective (*2*), IPT in children receiving SP and amodiaquine has shown promise in western Africa (*3*,*4*), and IPTp-SP is used widely across sub-Saharan Africa. All 3 policies are recommended by the World Health Organization for many settings in Africa (*5*–*7*). Spread of sulfadoxine-resistant parasites will compromise the effectiveness of these programs.

IPTp-SP has been adopted most broadly; however, its efficacy appears to be decreasing in areas with increasing parasite resistance to SP (*8*,*9*). Reduced susceptibility to sulfadoxine is conferred mainly by nonsynonymous substitutions at codons 436, 437, 540, and 581 of the *P. falciparum* dihydropteroate synthase (*dhps*) gene that encodes the enzymatic target of sulfadoxine (*10*). Parasites with mutant *dhps* haplotypes are restricted to sub-Saharan Africa, and parasites with the A437G, K540E, and A581G mutations (mutant amino acids are underlined), which are known as *dhps* triple mutants (haplotype SGEG across codons 436, 437, 540, and 581), have been limited to eastern Africa. In sites in Tanzania in which the SGEG haplotype is prevalent, IPTp-SP does not appear to improve birth outcomes (*9*), and IPTi with SP is not effective (*11*).

In addition, recent evidence suggests that IPTp-SP might exacerbate placental malaria when women are

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infected with parasites that have the A581G mutation in *dhps* (*12*), which suggests that these parasites manifest increased pathogenicity under drug pressure. In contrast, there was no evidence of pathogenicity caused by A581G-bearing parasites in Malawi, and SP retained some efficacy in preventing illness caused by malaria during pregnancy (J. Gutman et al., unpub. data). These contrasting effects of this resistant parasite haplotype suggest that effects of the A581G mutation might vary among populations. However, if parasites from northern Tanzania dessiminate, parasites bearing the *dhps* A581G mutation could broadly undermine malaria control efforts in infants and pregnant women in Africa.

Because of these findings, molecular surveillance for this mutation is critical to assess the durability of SP for malaria prevention. Genetic studies have shown that mutations conferring resistance to chloroquine (*13*) and pyrimethamine (*14*) have arisen only a few times and then diffused across regions and continents. In contrast, resistance to sulfadoxine appears to have arisen independently in multiple locations (*15*,*16*), after originating only in Southeast Asia, followed by export to Africa (supported by global survey findings) (*17*). Efforts to prevent dissemination of the A581G mutation hinge on understanding whether the mutation arises de novo or is spread among locations.

To better understand the emergence of sulfadoxine-resistant *P. falciparum* in eastern Africa, we first used microsatellite genotyping to study the emergence of parasites harboring *dhps* haplotypes with the A581G mutation in a longitudinal study in Malawi during 1997–2010 (*8*). We then compared the genetic background of these triple-mutant SGEG parasites in Malawi in a cross-sectional analysis with mutant parasite haplotypes from Tanzania and the Democratic Republic of the Congo (DRC). In these 2 investigations, we hypothesized that extant SGEA haplotypes in Malawi would share a genetic lineage with recently emerged SGEG haplotypes, and that these SGEG haplotypes from Malawi would represent a distinct lineage compared with SGEG haplotypes from other settings in eastern Africa.

Methods

Ethics

All participants provided written or oral informed consent. Ethical approval for project activities was provided by the review boards of the Malawi Health Sciences Research Committee, the University of Malawi College of Medicine Research Ethics Committee, the Liverpool School of Tropical Medicine, Macro International, the School of Public Health of the University of Kinshasa, the International Clinical Studies Review Committee of the National Institutes of Health, the Seattle Biomedical Research Institute, the Tanzanian National Institute for Medical Research, and the University of North Carolina.

Sample Collection

Parasites from Malawi were obtained from peripheral blood of women who delivered children at Queen Elizabeth Central Hospital in Blantyre, Malawi, during 1997–2005 (*18*). In 2010, consecutive women who delivered children at study sites near Blantyre were offered enrollment into an observational study (F.O. ter Kuile et al., unpub. data). Dried blood spots were prepared from maternal peripheral and placental blood of enrollees.

Parasites from the DRC were obtained from adults in the 2007 Demographic and Health Survey (*19*). Parasites from Tanzania were obtained from placental blood of pregnant women delivering at Muheza Designated District Hospital during 2002–2005 (*12*).

Genotyping Procedures

For parasites from Malawi and DRC, genomic DNA was extracted from dried blood spots by using Chelex-100 or a PureLink 96 DNA Kit (Life Technologies, Grand Island, NY, USA), and *P. falciparum* was detected by using real-time PCR (*19*). These parasites were genotyped at *dhps* loci by using amplification and Sanger sequencing (*18*,*20*), and only those with pure A581G genotypes were genotyped at microsatellites. For parasites from Tanzania, the mutant alleles A437G and K540E are nearly fixed; A581G was identified by pyrosequencing (*12*). We classified parasites as having A581G if the mutant allele frequency was ≥90% within the parasitemia level of the person.

Five microsatellite loci flanking the *dhps* gene were genotyped in all isolates: –2.9 and –0.13 kB upstream, and 0.03, 0.5, and 9 kB downstream of *dhps* (*20*). PCR products of amplifications of individual loci were sized on a 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and allele lengths were scored by using GeneMapper v4.1 (Applied Biosystems). In specimens with multiple peaks, the major peak was analyzed. All specimens were amplified and sized in parallel with genomic DNA from *P. falciparum* isolate 3D7 (American Type Culture Collection, Manassas, VA, USA). These controls were used to correct allele lengths to account for batch variability in fragment sizing.

Data Analyses

We computed heterozygosity (H_e) of microsatellite loci by using GenAlEx v6.5 (*21*) to quantify the degree of selection on mutant haplotypes. To assess relatedness among *dhps* haplotypes in Malawi during 1997–2010, we used GenAlEx to compute Φ_{PT} by analysis of molecular variance (AMOVA) with 999 permutations over the whole population (*22*) and the Nei genetic distance (*23*) among

dhps haplotypes and years based on microsatellite profiles. We inputted Φ_{PT} values computed by AMOVA into a principal coordinates analysis (PCoA) in GenAlEx.

We further characterized these relationships with a network analysis. To characterize these relationships, we assigned unique haplotypes based on microsatellite profiles for the 91 isolates for which we had successfully genotyped all microsatellite loci. These unique haplotypes were inputted into NETWORK v4.6.1.1 (*24,25*), and weights were assigned to each locus in inverse proportion to the H_e, of the locus, as calculated above.

In cross-sectional analysis of parasite populations from eastern Africa defined by location and *dhps* haplotype, we first used GenAlEx to compute pairwise linear genetic distances and Φ_{PT} (by using AMOVA with 999 permutations over the full population) and then used SPAGeDi v1.4 (*26*) to compute pairwise R_{ST} (by using jackknifing with 1,000 permutations). We inputted pairwise tri-distance matrices of linear genetic distance, Φ_{PT}, and R_{ST} into separate PCoAs in GenAlEx. For testing of statistical significance, we considered a p value of 0.05 as sufficient to reject the null hypothesis and used the Bonferroni correction when computing multiple comparisons.

We constructed a neighbor-joining (NJ) network to estimate a phylogeny of *dhps* haplotypes circulating in eastern Africa. To construct this network, we first computed pairwise linear genetic distances among all 193 isolates in GenAlEx; this distance matrix was used to compute an unrooted NJ tree in PHYLIP v3.67 (*27,28*), which was computed agnostic to *dhps* haplotype and geographic location and rendered in R v3.0.1. Missing alleles precluded computation of a median-joining network with NETWORK (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/20/7/13-1720-Techapp1.pdf).

We investigated population structure of the *dhps* haplotypes from these 193 isolates by using STRUCTURE v2.3.4, a clustering algorithm designed to infer and assign individuals to subpopulations (*29*). Although it was not specifically designed to identify population structure based on linked loci, we used STRUCTURE to test our a priori hypothesis of distinct subpopulations based on *dhps* haplotype and location (*30*). We performed 3 analyses: first, of all 193 parasites of all *dhps* haplotypes; second, of 116 parasites with any mutant *dhps* haplotype; and third, of 32 parasites with only the triple-mutant SGEG *dhps* haplotype. We performed 5 simulations each at values of *K*-estimated populations from 1 to 20, and estimated the true *K* a posteriori by using estimations in STRUCTURE, as well as using the method of Evanno et al. (*31*)

Results

Longitudinal Analyses of Parasites in Malawi

We first tested 114 *P. falciparum* isolates from Malawi collected during 1997–2010. We identified 25 wild-type SAKA parasites, 1 single-mutant SGKA, 68 double-mutant SGEA, 10 triple-mutant SGEG, and 10 with other *dhps* haplotypes, including AAKA, AGEA, AGKA, and SAEA. Among major haplotypes, we observed reductions in microsatellite allele mean heterozygosity (H_e) in parasites having double-mutant SGEA (H_e 0.454, SE 0.076) and triple-mutant SGEG *dhps* haplotypes (H_e 0.485, SE 0.134) compared with those from wild-type parasites (H_e 0.798, SE 0.064). These findings are consistent with positive selection on mutant haplotypes, presumably caused by sulfadoxine pressure.

Given the recent emergence of triple-mutant SGEG haplotype in 2010, we investigated its relationship with the double-mutant SGEA haplotype that had become fixed in this population by 2005 (*32*). To quantify genetic relatedness among years and major *dhps* haplotypes, we first computed pairwise Φ_{PT} values and Nei genetic distance among major *dhps* haplotypes binned by year. In these analyses, SGEA haplotypes were closely related to each other during 1997–2005 (Φ_{PT} values 0.008–0.065, Nei value 0.016–0.073) and closely related to the SGEG haplotype that emerged by 2010 (Φ_{PT} 0.082, Nei value 0.049) (Table). We inputted Φ_{PT} estimates into a PCoA to better visualize divergence among haplotypes by year. In this analysis, coordinates 1 and 2 explained 96.3% of the variance; SGEA haplotypes from all years clustered with SGEG haplotypes from 2010, which suggested a shared lineage in Malawi of mutant *dhps* haplotypes during 1997–2010 (online Technical Appendix Figure 1).

Table. Pairwise Φ_{PT} values and Nei genetic distances among major *Plasmodium falciparum dhps* haplotypes by year, Malawi*

Haplotype, year	SAKA, 1997–1999	SAKA, 2000–2003	SGEA, 1997–1999	SGEA, 2000–2003	SGEA, 2004–2005	SGEA, 2010	SGEG, 2010
SAKA, 1997–1999		0.251	0.470	0.693	0.818	1.095	1.044
SAKA, 2000–2003	0		0.362	0.264	0.200	0.350	0.162
SGEA, 1997–1999	**0.249**	**0.331**		0.02	0.073	0.429	0.246
SGEA, 2000–2003	**0.295**	**0.347**	0.008		0.016	0.302	0.096
SGEA, 2004–2005	**0.318**	**0.404**	0.065	0.030		0.300	0.049
SGEA, 2010	**0.299**	**0.380**	**0.298**	**0.243**	**0.237**		0.031
SGEG, 2010	**0.243**	0.299	**0.202**	**0.155**	0.082	0.015	

*Values for *dhps* haplotypes are defined by amino acids at codons 436, 437, 540, and 581. Mutant amino acids are underlined and in bold. Pairwise Φ_{PT} values are shown below the diagonal; values in bold have a p value <0.05 (after Bonferroni correction for multiple comparisons) based on 999 permutations. Nei unbiased genetic distances are shown above the diagonal.

We further investigated this finding by using network analysis. To perform this analysis, we constructed a median-joining network of wild-type and mutant haplotypes by year based on microsatellite profiles (Figure 1). In this analysis, we observed clustering of triple-mutant SGEG haplotypes from 2010 in a network of double-mutant SGEA haplotypes, as well as substantial sharing of microsatellite profiles among SGEA parasites from different years and with SGEG parasites. These 2 observations suggest a shared lineage of evolved mutant *dhps* haplotypes in Malawi.

Cross-sectional Analyses of Parasite Haplotypes for Eastern Africa

Clinical consequences of infections with parasites bearing the *dhps* A581G mutation appear to vary among study sites in Africa. In Tanzania, these parasites have been associated with exacerbation of placental inflammation in women who received IPTp (*12*) and failure of IPTp-SP to prevent low birthweight of infants (*33*). In Malawi, these phenomena have not yet been observed. Because of these differing effects, we speculated that haplotypes bearing the A581G mutation may also differ among sites.

We conducted a cross-sectional analysis of parasites from 2 additional cohorts: 1) adults sampled in 2007 from the eastern DRC (*19*), and 2) pregnant women who gave birth and were enrolled during 2002–2005 in Muheza, Tanzania (*12*). In total, we compared the genetic relationships among 193 parasites grouped into 7 parasite populations: wild-type (SAKA) isolates from the DRC (n = 53) and Malawi (n = 24), those bearing double-mutant (SGEA) haplotypes from the DRC (n = 17) and Malawi (n = 67), and those bearing triple-mutant (SGEG) haplotypes from the DRC (n = 5), Malawi (n = 10), and Tanzania (n = 17). Fragment lengths are shown in online Technical Appendix Table 1.

We quantified divergence of these 7 populations based on microsatellite allele lengths by using 3 population genetic metrics: linear genetic distance, Φ_{PT}, and R_{ST}. Linear genetic distance is a simple Euclidean genetic distance metric, and Φ_{PT}, and R_{ST} are variations of Wright F-statistics that quantify divergence and estimate its variance (*22,34*). Among SGEG parasites from Malawi and Tanzania, Φ_{PT} (0.420, p = 0.001) and R_{ST} (0.436, p<0.0001) indicated significant divergence after Bonferroni correction for multiple comparisons (online Technical Appendix Table 2); Φ_{PT} and R_{ST} for other pairwise comparisons among SGEG parasites from Malawi, the DRC, and Tanzania were not significant but suggested similar divergence (values >0.420).

We visualized the output of each of these metrics with separate PCoAs (Figure 2; online Technical Appendix Figure 2). In the PCoAs of genetic distance, Φ_{PT}, and R_{ST}, the first 2 coordinates accounted for 89%, 94.3%, and 96% of variance in values, respectively. In each PCoA plot, SGEG parasites from Malawi, the DRC, and Tanzania were consistently distant from each other in the 2 plotted dimensions, and other relationships among populations were variable. These analyses suggested divergence of SGEG haplotypes in Malawi, Tanzania, and the DRC.

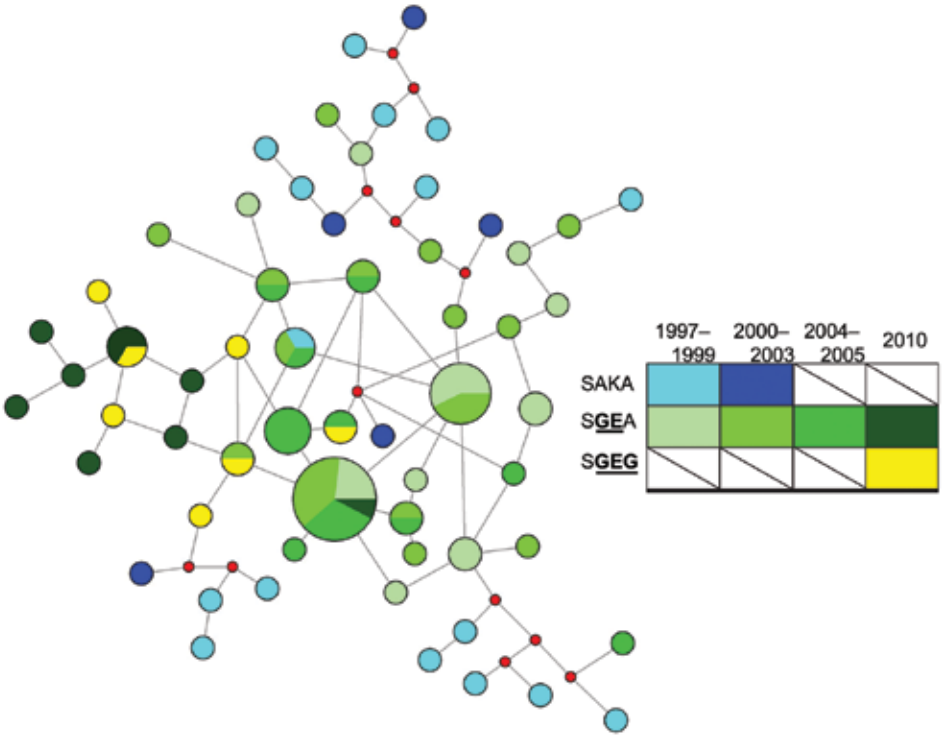

Figure 1. Genetic relatedness of *Plasmodium falciparum* dihydropteroate synthase (*dhps*) haplotypes from Malawi over time based on median-joining network of microsatellite profiles. Median-joining network was calculated based on microsatellite profiles for 91 parasites with full genotype data. Colors indicate year and *dhps* haplotype, nodes are proportional to the number of parasites with that microsatellite profile, red nodes are hypothetical profiles inserted by the program to calculate a parsimonious network, and branch lengths are arbitrary. Values were computed in NETWORK v4.6.1.1. (*24,25*). The *dhps* haplotypes are defined by amino acids at codons 436, 437, 540, and 581. Mutant amino acids are underlined and in bold. SAKA, wild-type; SGEA and SGEG, mutants.

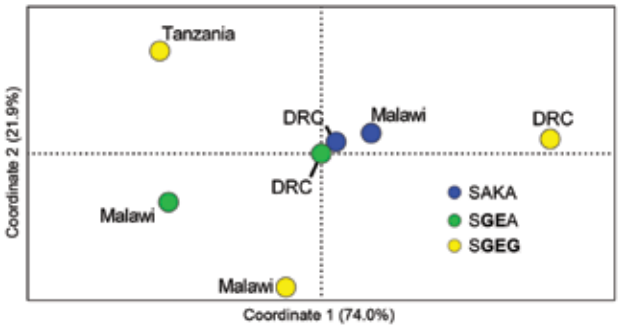

Figure 2. Principal coordinates analyses of wild-type (SAKA) and mutant (S**GE**A and S**GEG**) *Plasmodium falciparum* dihydropteroate synthase (*dhps*) halotypes from eastern Africa based on analysis of variance (R_{ST}). Pairwise R_{ST} values were computed with SPAGeDi (*26*) for microsatellite profiles of 7 populations of parasites defined by *dhps* haplotype and location: SAKA parasites from Malawi (n = 24) and the Democratic Republic of the Congo (DRC) (n = 53) (blue dots); SGEA parasites from Malawi (n = 67) and DRC (n = 17) (green dots); and SGEG parasites from DRC (n = 5), Malawi (n = 10), and Tanzania (n = 17) (yellow dots). These pairwise values were inputted into principal coordinates analyses in GenAlEx (*21*), in which coordinates 1 and 2 cumulatively accounted for 96% of the variance. The *dhps* haplotypes are defined by amino acids at codons 436, 437, 540, and 581. Mutant amino acids are underlined and in bold.

To further investigate this apparent divergence of SGEG haplotypes, we computed an NJ network based on pairwise linear genetic distances among all 193 isolates. This phylogenetic analysis, which was computed agnostic to country and *dhps* haplotype, clustered all 17 SGEG parasites from Tanzania distinctly from 8 of the 10 SGEG parasites from Malawi; haplotypes in these clusters were also distinct from 3 of the 5 SGEG parasites from the DRC (Figure 3). A large number of SGEA parasites from Malawi also clustered closely with SGEG parasites from Tanzania, which suggested some shared lineage. This inferred phylogeny further suggests that triple-mutant *dhps* haplotypes from Tanzania and Malawi bearing the A581G substitution have arisen independently.

To further investigate this partition of *dhps* lineages, we performed an analysis of population structure. The first analysis of all 193 wild-type and mutant parasites partitioned only *dhps* haplotypes into mutant and wild-type, irrespective of geographic location. When restricted to the 116 parasites with mutant *dhps* haplotypes, the program partitioned the dataset into 3 clusters with 99.9% posterior probability; these 3 clusters directly corresponded to geographic location but were irrespective of *dhps* haplotype, which suggested separate lineages of mutant *dhps* haplotypes in Malawi, the DRC, and Tanzania (Figure 4, panel A). In an analysis restricted to the 32 parasites bearing the SGEG *dhps* haplotype, 2 population clusters were identified with the highest posterior probability. All SGEG parasites from Tanzania were assigned to 1 cluster, and SGEG parasites from Malawi and the DRC were assigned to a second cluster (Figure 4, panel B). These analyses further suggest that lineages of triple-mutant *dhps* haplotypes bearing the A581G mutation from Malawi and Tanzania are divergent.

Discussion

In these longitudinal and cross-sectional analyses of *P. falciparum* haplotypes from eastern Africa that were associated with high-level parasite sulfadoxine resistance, we demonstrated emergence of a distinct lineage of triple-mutant *dhps* haplotypes in Malawi. In comparative analyses, these *dhps* triple-mutant SGEG haplotypes from Malawi were strongly divergent from haplotypes collected in northern Tanzania, which suggested independent emergence of this drug-resistant haplotype in these 2 settings in eastern Africa. This parasite haplotype appears to undermine efficacy of SP in preventing *P. falciparum* infection in infants and pregnant women (*9*,*11*); its spread could undermine current and nascent malaria control programs that are predicated on SP efficacy. To guide malaria control policies that use SP, surveillance of molecular markers of *P. falciparum* drug resistance need to be complemented by clinical studies of effects of these parasite mutations on maternal and infant birth outcomes.

Our data indicate that triple-mutant SGEG *dhps* haplotypes from Malawi, Tanzania, and the DRC represent distinct lineages. The circumscribed regional distribution is well described for single-mutant and double-mutant *dhps* haplotypes across sub-Saharan Africa (*15,20*), but our study describes this phenomenon on a subregional scale with the emerging, more highly resistant triple-mutant SGEG haplotype. In our analyses, support for this genetic divergence derives from population genetic metrics, including genetic distance and F-statistics (online Technical Appendix Table 2), PCoA (Figure 2; online Technical Appendix Figure 2), unsupervised haplotype phylogeny (Figure 3), and inferred population structure (Figure 4).

Parasites from Malawi and Tanzania were most consistently partitioned into distinct SGEG lineages and showed statistically significant divergence by Φ_{PT} (0.42, p = 0.001) and R_{ST} (0.436, p<0.0001) (online Technical Appendix Table 2), visually apparent partitioning in an NJ network (Figure 3) and in PCoA (Figure 2), and assignment to separate subpopulations in a probabilistic clustering algorithm (Figure 4). In a previous study, double-mutant SGEA haplotypes in eastern Africa (including Malawi, Tanzania, and eastern DRC) shared a common lineage (*15*). Our study supports this finding for parasites from Malawi and the DRC, with closely related SGEA parasites identified by Φ_{PT} (0.017, p = 0.228) and R_{ST} (0.0164, p = 0.4306) (online Technical Appendix Table 2). Our data suggest that despite this shared double-mutant

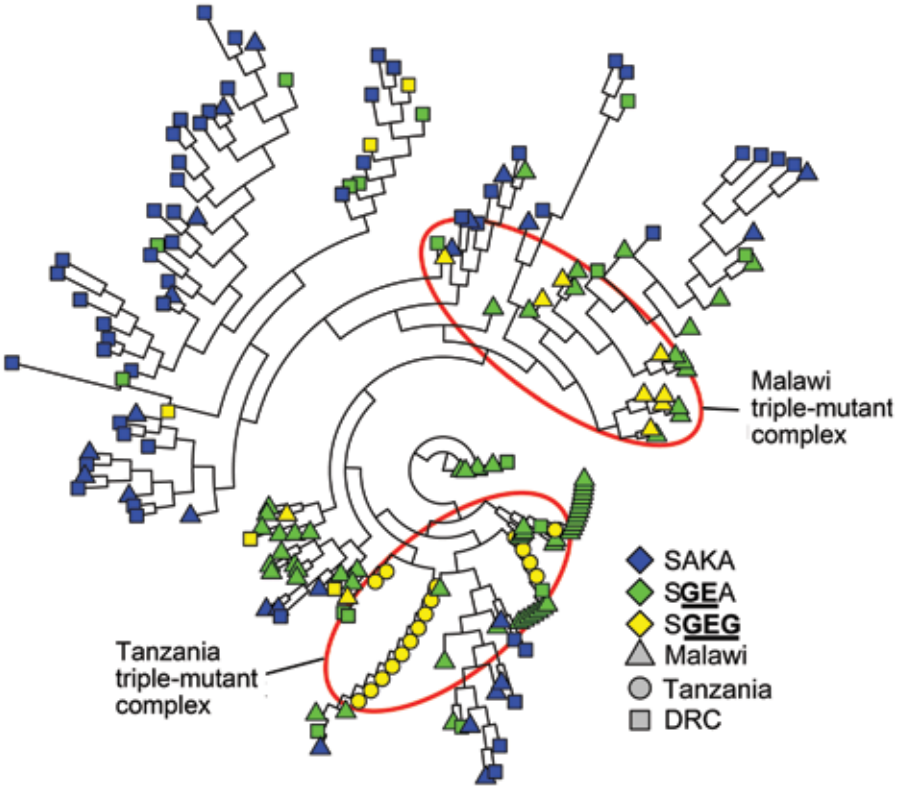

Figure 3. Neighbor-joining network of *Plasmodium falciparum* wild-type (SAKA) and mutant (SGEA and SGEG) dihydropteroate synthase (*dhps*) haplotypes, eastern Africa. Pairwise linear genetic distances among 193 parasite isolates were computed in GenAlEx (*21*) inputted into PHYLIP v3.67 (*27,28*) to calculate an unrooted neighbor-joining tree, and rendered in R v3.0.1 (http://www.r-project.org/) by using the ape package. For visualization, branch lengths were uniformly lengthened if not equal to 0. The *dhps* haplotypes are defined by amino acids at codons 436, 437, 540, and 581. Mutant amino acids are underlined and in bold. DRC, the Democratic Republic of the Congo.

dhps haplotype, the additional A581G mutation has arisen independently in different locations in, at least, Malawi and Tanzania.

Recent studies of pregnant women have implicated parasites bearing the SGEG *dhps* haplotype with undermining the efficacy of IPTp-SP (J. Gutman et al., unpub. data, *33*) and possibly exacerbating placental infection in women who received IPTp-SP (*12*). Despite these findings, results of various studies are inconsistent regarding the effect of the SGEG haplotype on IPTp-SP. On a population level, IPTp-SP appears to retain some efficacy in preventing low birthweight in infants in studies in Malawi, where the SGEG haplotype is just emerging (*35*), but not in northern Tanzania, where SGEG parasites are more prevalent (*9*). On an individual level, IPTp-SP is less effective at preventing peripheral parasitemia at delivery caused by parasites bearing the SGEG haplotype in Malawi (J. Gutman et al., unpub. data), but IPTp-SP was implicated as exacerbating placental parasite density and inflammation in the presence of SGEG parasites in Tanzania (*12*). These inconsistencies in ecologic studies may derive from different frequencies of the A581G mutation in parasite populations in the 2 settings.

A second explanation for the differences is that these effects might be derived from additional mutations that are associated with genotyped loci caused either by different genetic backgrounds or linkage disequilibrium. Such mutations may be hypothesized to exist in the *dhps* gene itself, in loci which mediate DHPS expression and thereby parasite fitness in the presence of drug, or in heretofore undescribed mediators of pathogenesis. In areas in which parasites bearing the SGEG haplotypes are emerging, studies of parasite genomics and phenotypes can further investigate these possibilities.

Our results suggest atypical spread in Africa of parasite haplotypes conferring sulfadoxine resistance: haplotypes that confer resistance to chloroquine (*13*) and pyrimethamine (*14*) appear to have limited origins and are believed to have spread across Africa largely by gene flow. Similarly, a previous global survey of *dhps* haplotypes suggested that resistant lineages originated in Southeast Asia and migrated to Africa (*17*), although this study did not include any parasites from Africa with the SGEG *dhps* haplotype. In contrast, our data support the local origination and emergence of this highly resistant haplotype, similar to rare resistant *dhfr* haplotypes (*36*). This phenomenon for *dhps* has been most clearly described on a fine scale in Southeast Asia (*16*), and our data support a similar process in eastern Africa.

This model is further supported by our longitudinal sampling and testing of parasite specimens in Malawi during 1997–2010. During this period, SGEG haplotypes arose after SGEA haplotypes had achieved near fixation.

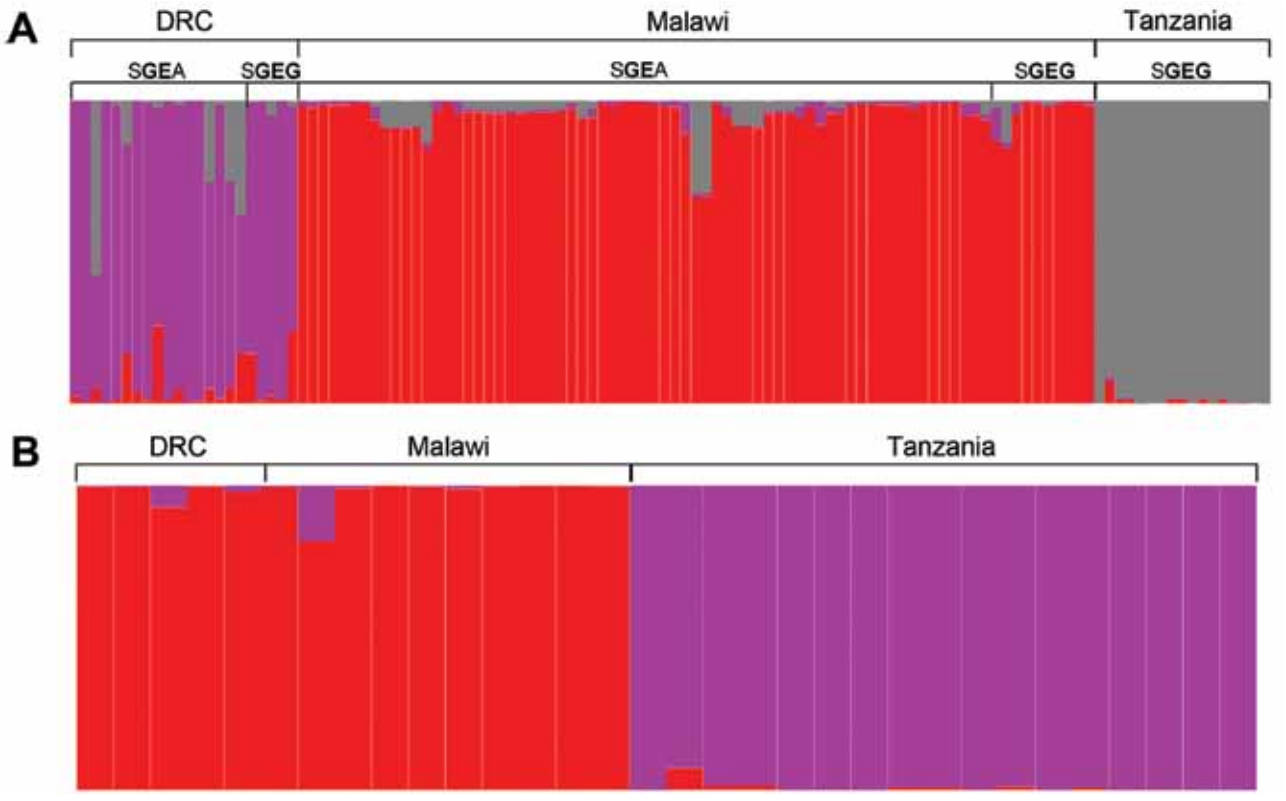

Figure 4. Predicted population structure of *Plasmodium falciparum* parasites from eastern Africa among those with A) double-mutant (S**GE**A) or triple-mutant (S**GEG**) dihydropteroate synthase (*dhps*) haplotypes and B) those with only the triple-mutant S**GEG** *dhps* haplotype. Estimations of number of populations and assignments of individual parasites to clusters were computed with STRUCTURE (*29,30*) by using a burn-in period of 10,000 and 10,000 iterations and models that assume no admixture and which use prior information related to location because of sparse data and likely subtle structure. Each vertical bar represents an individual parasite, and the color of each bar indicates the likelihood of membership in the subpopulation indicated by the color. The *dhps* haplotypes are defined by amino acids at codons 436, 437, 540, and 581. Mutant amino acids are underlined and in bold. A) Analysis of 116 parasites from Malawi, the Democratic Republic of the Congo (DRC), and Tanzania with either S**GE**A or S**GEG** *dhps* haplotypes. The posterior probability was 99.9% of the existence of 3 population clusters that are indicated by the color. B) A similar analysis restricted to 32 parasites with only the S**GEG** *dhps* haplotype from Malawi, DRC, and Tanzania. The division into 2 populations indicated by the color was supported by a posterior probability of 41%, which is higher than for those of other a priori population sizes tested.

This observation could have been caused by importation of a new parasite population bearing the SGEG *dhps* haplotype or by A581G mutation in the existing parasite population. Our data suggest that the mutation accounts for appearance of the SGEG haplotype because of the close relationship among mutant haplotypes both computationally by Φ_{PT} and visually in a median-joining network (Figure 1), which suggests that SGEA haplotypes gave rise to SGEG haplotypes in Malawi.

What are the implications of our findings for IPTp-SP? IPTp-SP is still used extensively in settings in eastern and southern Africa that have prevalent parasite resistance. Failures of IPTp-SP have prompted investigations to define the prevalence of the A581G mutation (*37*) and to more closely investigate its potential for modifying the beneficial effect of IPTp-SP. Our results have 2 practical implications for these investigations. First, these data suggest that the SGEG haplotype may arise where a SGEA haplotype is circulating without requiring the importation of a new mutant haplotype. Therefore, even in settings without major migration of parasites, continued use of sulfadoxine or other sulfonamides may promote emergence of this haplotype. Molecular surveillance is critical for detecting this step. Second, given the differing effects of parasites bearing the SGEG haplotype on IPTp-SP among settings, it would appear prudent to supplement molecular surveillance for the *dhps* A581G mutation in areas where it is present by studies of its effects on pregnant women and their infants and other clinical outcomes.

Our study has several limitations. First, the number of available parasites bearing the SGEG haplotype is limited. These parasites have emerged only recently in Africa, precluding a more widespread analysis. Second, we did not have SGEA parasites from Tanzania against

which to compare microsatellite profiles of the SGEG parasites. Third, these SGEG parasites were collected in different clinical studies in different years; the 2 principal populations of parasites were collected from women giving birth in Malawi and Tanzania during 2002–2010 and are therefore quite similar.

These analyses of malaria parasites in eastern Africa support a model of local origination and propagation of triple-mutant SGEG *P. falciparum dhps* haplotypes that confer high levels of resistance to sulfadoxine. Recent evidence indicates that these haplotypes abrogate the efficacy of SP use to prevent malaria among pregnant women and their infants in eastern Africa. Integrated clinical and molecular surveillance for these mutations in parasite populations is critical to assess the durability of prevention programs that use SP. These efforts should be complemented with ongoing investigations of more effective methods to protect vulnerable populations from malaria.

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Population-Based Analysis of Invasive Fungal Infections, France, 2001–2010

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To determine the epidemiology and trends of invasive fungal infections (IFIs) in France, we analyzed incidence, risk factors, and in-hospital death rates related to the most frequent IFIs registered in the national hospital discharge database during 2001–2010. The identified 35,876 IFI cases included candidemia (43.4%), Pneumocystis jirovecii pneumonia (26.1%), invasive aspergillosis (IA, 23.9%), cryptococcosis (5.2%), and mucormycosis (1.5%). The overall incidence was 5.9/100,000 cases/year and the mortality rate was 27.6%; both increased over the period (+1.5%, +2.9%/year, respectively). Incidences substantially increased for candidemia, IA, and mucormycosis. Pneumocystis jirovecii pneumonia incidence decreased among AIDS patients (-14.3%/year) but increased in non-HIV–infected patients (+13.3%/year). Candidemia and IA incidence was increased among patients with hematologic malignancies (>+4%/year) and those with chronic renal failure (>+10%/year). In-hospital deaths substantially increased in some groups, e.g., in those with hematologic malignancies. IFIs occur among a broad spectrum of non–HIV-infected patients and should be a major public health priority.

Invasive fungal infections (IFI) are reportedly increasing in many countries, especially candidemia and invasive aspergillosis (IA) among immunocompromised patients (*1–4*). Conversely, a decline of AIDS-associated *Pneumocystis jirovecii* pneumonia (Pjp) and cryptococcosis has been observed in Western countries since the advent of highly active antiretroviral treatments (*5*,*6*). Many publications provide insight on a given IFI and its trends in specific risk groups, but the overall burden of illness associated with IFI and its trends at a country level have not been described (*7–10*). To describe the epidemiology and trends of IFIs and to better identify public health priorities (e.g., surveillance, research, prevention strategies), we analyzed the national hospital discharge database of France, Programme de Médicalisation du Système d'Information, spanning 2001–2010.

Materials and Methods

The national hospital database covers >95% of the country's hospitals (*11*). An anonymous subset of this database can be made available for epidemiologic studies without need for ethical approval or consent of patients, according to legislation by the government of France. A unique anonymous patient identifier enables distinction among first and subsequent hospital admissions. Information filed at discharge includes the major cause of admission and associated diseases, coded according to the International Classification of Diseases, Tenth Revision, the medical and surgical procedures performed, and the outcome including transfer, discharge, or death. Details on the data source, case definitions, and methods used are available in online Technical Appendix 1 (http://wwwnc.cdc.gov/EID/article/20/7/14-0087-Techapp1.pdf).

Records of all hospital stays for which an IFI was recorded as the principal cause of admission or as a related disease were extracted from the national database for the period of January 2001 through December 2010. Records of the 5 most frequent IFIs were retained for this analysis.

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To facilitate comparisons with published studies, we restricted the study of invasive candidiasis to candidemia (i.e., excluding *Candida* endocarditis and meningitis), and invasive aspergillosis (IA) included pulmonary and disseminated cases. All cryptococcosis cases were included. Gastrointestinal mucormycoses were excluded because results of a previous study showed that cases were mostly identified on the basis of false-positive test findings (*12*). Finally, codes corresponding to "pneumocystosis" or "HIV infection resulting in pneumocystosis" were designated as Pjp only if pneumonia was associated. We excluded rare IFIs (<40 cases per year each) and endemic mycoses (histoplasmosis, blastomycosis, coccidioidomycosis, sporotrichosis). Analysis focused on metropolitan areas of France, excluding overseas territories.

After checking for multiple stays and inconsistent records within and between hospitals, we retained "incident cases," i.e., unique stays and first admissions. To reduce underreporting bias, we ensured that a risk factor that occurred during subsequent stays was integrated into the incident record (e.g., a diagnosis of diabetes recorded after a patient's transfer from a first- to a third–level hospital). Similarly, in-hospital fatality rates were estimated from the cumulative stays.

To describe risk factors associated with IFIs, we selected 9 conditions on the basis of expert opinion and published studies on the epidemiology of IFI. Considering the high diversity of conditions, and to provide a description relevant for clinical practice and health policy makers, we used hierarchical ranking to assign 1 risk factor per patient. Given that the preponderant risk factors differ among IFIs, IFIs were divided into 2 groups. In the first group, which included candidemia, IA, and mucormycosis, risk-factor ranking started with hematologic malignancies (HM, including by priority order, HM associated with hematologic stem cell transplantation [HSCT], HM not associated with HSCT but with neutropenia, and HM with none of the above factors). The following illnesses and conditions were subsequent risk factors in the first group: HIV/AIDS, solid organ transplantations, solid tumors, systemic inflammatory diseases (including inflammatory bowel diseases, sarcoidosis, rheumatoid arthritis, and systemic lupus or vasculitis of other origins), diabetes mellitus, chronic respiratory diseases (including chronic obstructive pulmonary diseases, asthma, and cystic fibrosis), chronic renal failure, and a group labeled "other diseases" that includes acute renal failure, liver cirrhosis, morbid obesity, acute or chronic pancreatitis, and severe burns. Thus, a case-patient with HM and diabetes was recorded as HM. For the second IFI group (Pjp and cryptococcosis), HIV/AIDS was the first risk factor, followed by other risk factors as described above. For all case-patients with IFI, additional risk factors were explored without hierarchical ranking: a stay in an intensive care unit; surgery; and extreme age, defined as neonates (≤28 days of age) and elderly adults (≥80 years of age). Because of lack of precise coding for several risk factors until 2003, only those documented during the 2004–2010 period were analyzed.

We expressed annual incidence rates among the general population, by gender and age groups, as cases per 100,000 population, using data from the 1999 national population census and its updates. We also analyzed trends in groups with selected risk factors, for which the respective denominators were available from routine surveillance data or from prevalence estimates, as detailed in online Technical Appendix 1: patients with HM, HIV/AIDS, solid tumors, chronic renal failure, diabetes, and HSCT recipients. In these specific populations, we estimated the annual proportion of each IFI using the given risk factor per 100,000 population (2004–2010). Finally, we used an age-polynomial fractional logistic regression (*13*) to calculate age- and sex-adjusted risk for death categorized by risk factor, and analyzed each risk factor independently from the others without hierarchical ranking. We applied Fisher or χ^2 tests to compare groups, and a Poisson regression to assess trends, considering $p \leq 0.05$ as significant, using Stata version 11.2 (StataCorp LP, College Station, TX, USA) software for all calculations.

Results

Characteristics of Case-Patients, 2001–2010

There were 35,876 cases of IFI registered in metropolitan France during 2001–2010 (Table 1). Candidemia accounted for the highest proportion of cases (43.4%); the next most frequently identified diseases were Pjp (26.1%), IA (23.9%), cryptococcosis (5.2%), and mucormycosis (1.5%). The overall incidence was 5.9/100,000 population per year. A total of 9,889 (27.6%) case-patients died while in a hospital. Candidemia and IA accounted for 87.6% of these deaths. Male patients predominated in all IFIs (64.0%), especially in Pjp and cryptococcosis (>70%). The mean age was 54.7 years (range 0–107 years). Gender and age characteristics of case-patients and of those who died differed according to the IFI. Details are provided in online Technical Appendix 2, Table 1 (http://wwwnc.cdc.gov/EID/article/20/7/14-0087-Techapp2.pdf). Incidence and fatality rates of candidemia and IA were particularly high in patients ≥60 years of age, and male patients predominated in all age groups, except in those ≥80 years of age. Case-patients in extreme age groups included 185 neonates (mainly with candidemia: 174 cases, 61.5% male patients, specific incidence 2.2/100,000 population) and 3,030 adults >80 years of age (2,283 with candidemia: 50.5% male, incidence $8.1/10^5$). Among case-patients with Pjp and cryptococcosis, the proportion of male case-patients was higher among HIV-infected persons than in non–HIV-infected persons (Pjp 74.0% vs. 62.2%; cryptococcosis 77.9% vs. 62.3%, respectively).

Table 1. Cases of invasive fungal infection and attributable deaths in metropolitan France by disease and patient sex and age, 2001–2010*

Infections	No. case-patients	Male sex, %	Age, y, median (IQR)	Illness incidence (95% CI)†	Fatality rate, % (95% CI)
Candidemia					
Cases	15,559	58.8	64 (51–75)	2.5 (2.1–2.9)	
Deaths	6,217	60.0	69 (56–77)		40.0 (38.7–42.0)
Pneumocystis pneumonia					
Cases	9,365	71.3	44 (37–55)	1.5 (1.2–1.9)	
Deaths	862	71.9	58 (43–70)		9.2 (7.6–12.4)
Invasive aspergillosis‡					
Cases	8,563	63.9	58 (45–68)	1.4 (1.2–1.6)	
Deaths	2,443	66.7	61 (49–71)		28.5 (26.9–30.5)
Cryptococcosis‡					
Cases	1,859	72.3	43 (36–55)	0.3 (0.2–0.4)	
Deaths	278	73.4	49 (39–65)		15.0 (13.2–17.9)
Mucormycosis‡					
Cases	530	57.7	58 (43–71)	0.09 (0.07–0.1)	
Deaths	89	62.9	57 (44–67)		16.8 (11.3–20.2)
Total					
Cases	35,876	64.0	56 (42–70)	5.9 (5.5–6.3)	
Deaths	9,889	63.1	65 (53–75)		27.6 (25.3–29.7)

*A total of 197 *Candida*-related endocarditis and 10 meningitis cases were excluded from analysis. IQR, interquartile range.
†Incidence expressed as number of cases per 100,000 population per year (averaged over 10 y)
‡Invasive aspergillosis includes 91.7% pulmonary and 8.3% disseminated cases. Cryptococcosis includes 63.8% cerebral or disseminated forms; 13.2% pulmonary, cutaneous, or bone localizations; and 23.0% unspecified; forms. Mucormycosis includes 50.9% pulmonary, rhinocerebral and disseminated forms; 16.9% cutaneous forms; and 32.1% unspecified forms.

The highest incidences of Pjp and cryptococcosis were observed among persons 30–59 years of age with AIDS and among those ≥60 years of age who were not infected with HIV ($p<0.001$ for each IFI). For these 2 IFIs, the fatality rate was lower in HIV-infected patients than in non-HIV-infected patients (Pjp 5.7% vs. 21.5%, $p<0.001$; cryptococcosis 13.4% vs. 17.9%, $p<0.009$).

Trends in the General Population, 2001–2010

The incidence of IFI increased by 1.5% per year and that of deaths by 2.9% per year ($p<0.001$ each) over the 10-year period of observation. Specifically, the incidence of candidemia, IA, and mucormycosis increased by 7.8%, 4.4%, and 7.3% per year, respectively ($p<0.001$ each). The fatality rate decreased by 1.6% per year ($p<0.001$) among persons with candidemia and 1.4% per year ($p = 0.04$) among those with IA, but increased by 9.3% per year ($p = 0.03$) for those with mucormycosis. Regarding Pjp and cryptococcosis, incidence decreased by 8.6% and 9.8% per year ($p<0.001$ each), and the fatality rate increased by 11.7% ($p<0.001$) and 4.7% ($p = 0.03$) per year, respectively (Figure 1, panels A, B; Tables 2, 3). However, trends differed according to HIV status (online Technical Appendix 2, online Figure 1); incidence of both IFIs decreased among HIV-infected patients (Pjp -14.3%; cryptococcosis -14.9% per year, $p<0.001$ each), and Pjp increased in non–HIV-infected patients (+13.3% per year, $p<0.001$); there was no significant trend for cryptococcosis in non–HIV-infected patients. The fatality rate trend was only significant for HIV-associated Pjp (+5.6% per year, $p = 0.001$).

Risk Factor Distribution and Trends in the General Population, 2004–2010

We studied risk factors among 25,933 IFI case-patients identified during the 2004–2010 period. Candidemia remained the most frequent IFI (46.4%) followed by IA (24.8%) and Pjp (22.9%). The distribution of risk factors differed for each IFI (online Technical Appendix 2, Table 2). Solid tumors were mainly found in patients with candidemia (30.6%), HM in those with IA and mucormycosis (54.3% and 34.8%, respectively), and HIV/AIDS in those with Pjp and cryptococcosis (>55% each). The incidence of candidemia, IA, and mucormycosis in patients with HM (especially with neutropenia) increased significantly, as did the incidence of candidemia and IA in solid organ transplant recipients, and patients with solid tumors or chronic renal failure. The incidence of Pjp decreased in patients with HM and increased in patients with solid organ transplants, solid tumors, and chronic renal failure.

IFI Trends in Specific Risk Groups, 2004–2010

We estimated trends from the annual proportion of risk factor–associated IFIs in the corresponding risk population. Only statistically significant trends are shown in Figure 2. In the general population, the number of patients with HM, solid organ transplantations, chronic renal failure, HIV/AIDS, and diabetes substantially increased over time, and the population of HSCT recipients remained unchanged. In patients with HM, there was a statistically significant increase of candidemia, IA, and mucormycosis, and a decrease of Pjp (Figure 2, panel A). In HSCT recipients, candidemia and IA increased (Figure 2, panel B).

During the study period, candidemia increased among patients who had solid tumors (Figure 2, panel C). Among patients with chronic renal failure, the incidence of candidemia, IA, and Pjp increased (Figure 2, panel D). Among patients with HIV/AIDS, the incidence of Pjp and cryptococcosis decreased (Figure 2, panel E). There was no substantial trend among patients with diabetes (data not shown).

Odds Ratio of Death by Risk Factors, 2004–2010

We assessed the risk for death associated with each risk factor by logistic regression, considering each factor independently and expressed as an odds ratio for death; except for age, significant results are shown in online Technical Appendix 2, Table 3. The risk for death was lower in female patients with IA, but did not differ by sex for other infections. The role of age varied according to the IFI type; for instance, in-hospital fatality rates increased in persons >20 years of age who had candidemia and Pjp, and in those >70 years who had IA. HM represented a substantial risk factor for death in patients with candidemia, IA, mucormycosis, and in non-HIV cryptococcosis. Solid tumors were a substantial risk factor for death in patients with candidemia, IA, and Pjp, regardless of HIV status. Cirrhosis and acute renal failure were also substantial risk factors for death in patients with candidemia, IA, and non-HIV Pjp and cryptococcosis. Hospitalization in an intensive care unit was associated with a higher risk for death among patients with all IFIs except candidemia. Inversely, chronic renal failure decreased the risk for death among those with IA or Pjp, respiratory diseases decreased the risk in patients with IA, and surgical procedures decreased the risk for those with candidemia.

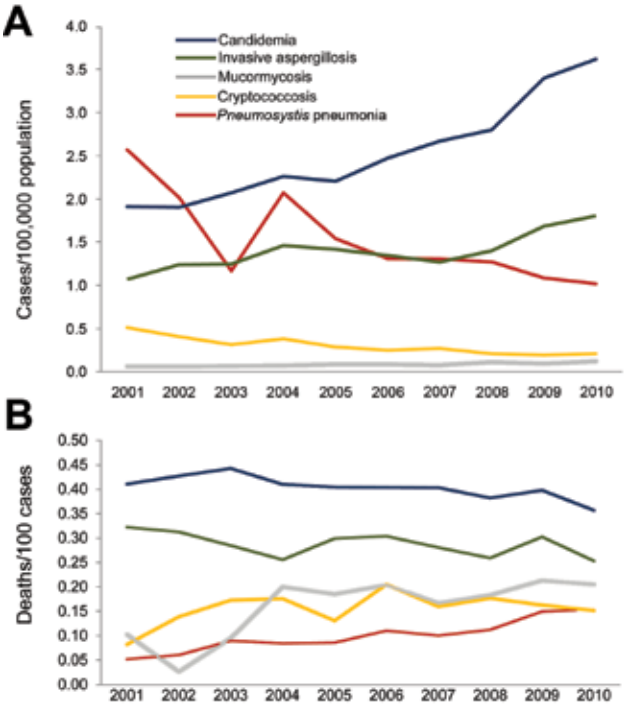

Figure 1. A) Trends in the incidence of invasive fungal infections in France, 2001–2010. The incidence increased (p<0.001) for candidemia, invasive aspergillosis, and mucormycosis, but decreased for cryptococcosis and pneumocystosis (Poisson's regression). B) Trends in the fatality rate by invasive fungal infections during 2001–2010. Fatality rates decreased for candidemia (p<0.001) and invasive aspergillosis (p = 0.04), but increased for mucormycosis (p = 0.03), pneumocystosis (p<0.001), and cryptococcosis (p = 0.03).

Discussion

This nationwide study provides evidence that ≈3,600 patients have IFI each year in France, of whom 28% die. The incidence of candidemia, IA, mucormycosis, and non-HIV Pjp has increased over the last decade, predicting a protracted trend over the coming years.

Studies on the epidemiology of the 5 predominant IFIs have reached conflicting results, depending on the IFI studied (most studies focused on a single IFI), the study design, and source of data (active surveillance system, cohorts, multicentric or monocentric, laboratory-based diagnosis, hospital discharge data), the population of interest (neutropenic patients, HM, HSCT and solid organ transplant recipients), and the practices regarding antifungal agents use (prophylactic, empiric, preemptive, or curative therapy). Here, we analyzed the hospital dataset at a country level, covering all persons who were admitted to hospitals over a period of 10 years, regardless of age or underlying conditions. We included those with illness caused by IFIs that have straightforward diagnostic criteria (candidemia, cryptococcosis) or well-characterized clinical entities (pulmonary or disseminated IA, pulmonary Pjp), as well as mucormycosis, for which we previously validated the accuracy of diagnostic coding in the hospital national database (*14*,*15*). Despite potential bias in the precise classification of cases, particularly for mold infections, and other limitations of administrative datasets that have been previously discussed (*12*,*14*,*16*), several points validate the findings obtained through this large database. The predominance of candidemia and IA has been described in other studies of a variety of IFIs in the general population or in other groups (*7*,*9*,*17*). For candidemia, the incidence and trends we estimated are comparable to many other, although smaller scale, population-based studies from Europe and North America (*18*–*22*). For IA in France, we observed a lower incidence and higher mortality rate than were found by Dasbach et al. in their analysis of US hospital discharge data (*23*). The differences may be explained by the researchers' use of the International Classification of Diseases, Ninth Revision case definitions in that study, which would impair the comparison of invasive and noninvasive forms.

The decreasing incidence of Pjp and cryptococcosis was expected after the advent of active antiretroviral

Table 2. Cases of invasive fungal infections per 100,000 population, metropolitan France, 2001–2010

Disease	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Pneumocystis pneumonia	2.6	2.0	1.2	2.1	1.5	1.3	1.3	1.3	1.1	1.0
Candidemia	1.9	1.9	2.1	2.3	2.2	2.5	2.7	2.8	3.4	3.6
Invasive aspergillosis	1.1	1.2	1.3	1.5	1.4	1.4	1.3	1.4	1.7	1.8
Cryptococcosis	0.5	0.4	0.3	0.4	0.3	0.3	0.3	0.2	0.2	0.2
Mucormycosis	0.07	0.06	0.07	0.07	0.09	0.09	0.08	0.11	0.10	0.12

therapy (*5*,*6*,*24*,*25*). However, we observed some noteworthy changes: Pjp incidence in non-HIV–infected patients has currently reached the levels observed in HIV-infected patients, as observed in the United Kingdom during the same period (*26*); incidence of cryptococcosis is also increasing in the seronegative population, and the mortality rate of both IFIs among non-HIV–infected patients is higher than among HIV-infected patients.

Most risk factors described in this study are well known in clinical practice. The major risk factors for candidemia, IA, and mucormycosis, i.e., HM, HSCT, and solid tumors, are described in many studies, such as those by the Transplant Associated Infections Surveillance Network, known as TRANSNET, and Prospective Antifungal Therapy Alliance, known as PATH (*3*,*27*–*29*), albeit sometimes reported as differently distributed. The hierarchical ranking process used here may have influenced the risk factor distribution, underestimating some conditions. Most studies of risk factors are performed on the basis of cohorts of cases in referral centers where a large number of high-risk patients are recruited, whereas in our population-based approach, we used a national dataset covering all levels of care, thus selecting a wider range of underlying conditions, including those less commonly recognized as risk factors. As a result, we documented substantial increases of candidemia, IA, and Pjp in patients with chronic renal failure, suggesting that the increase is not uniquely caused by the growing number of persons at risk (Figure 2). The growing number and longer survival of patients with protracted immunosuppression beyond traditional hematology patients, transplant patients, and HIV/AIDS populations are major challenges. The fact that 2 IFIs that are frequently associated with health care settings (candidemia and IA) are still on the rise despite existing infection control recommendations is of specific concern (*30*).

Hospital data are not collected for clinical research purposes. Thus, it is very hazardous to explain the trends on the basis of our limited observations. Specific analyses should be encouraged, aiming at better understanding the role of comorbid conditions in the occurrence of IFI (e.g., chronic renal failure) or the effect of the improved overall survival of patients, even those who are immunocompromised.

Another noteworthy finding of this study is that the risk for death was altered by factors that were not frequently documented before. For instance, cirrhosis was found in 1.3% of all patients with IFIs but was an independent risk factor for death among all except those with mucormycosis, suggesting underrecognition of IFIs in such populations, possibly leading to delayed prevention or treatment. Similarly, patients with HM showed an increased risk for death when cryptococcosis was also diagnosed, as did those with cirrhosis and acute renal failure, which suggest that specific attention should be paid to patients with these conditions; this could modify their clinical management.

This population-based study has limitations. The increase in IFIs observed parallels a better awareness of clinicians and microbiologists of the threat of IFIs in at-risk populations, improving the sensitivity of the hospital-based dataset. The availability of a broader antifungal drug armamentarium and efficient treatment could have the paradoxical effect of improving the prevention of IFI for selected groups of at-risk patients, thus lowering the population of infected patients. We report trends and risk factors for invasive mycosis in France. Hence, our findings may not apply to other countries with different endemic mycoses, population structures, and health care systems. Our observations are based on hospital discharge coding, which is subject to many biases, including misdiagnosis and incorrect coding. More notably, the advent of new diagnostic tools for the detection of many invasive mycoses may have affected our ability to diagnose these diseases over the study period, which may have had a substantial impact on the temporal trends observed.

Nevertheless, this large-scale study provides benchmarking data on the current burden of illness of major IFIs and shows the effects of disease trends and death rates spanning a decade in a Western European country. The need for baseline data was recently highlighted (*10*). Our data provide complementary information to specific studies

Table 3. Deaths attributed to invasive fungal infections per 100,000 cases, metropolitan France, 2001–2010

Disease	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Candidemia	0.41	0.43	0.44	0.41	0.40	0.40	0.40	0.38	0.40	0.36
Invasive aspergillosis	0.32	0.31	0.28	0.26	0.30	0.30	0.28	0.26	0.30	0.25
Mucormycosis	0.10	0.03	0.10	0.20	0.19	0.20	0.17	0.18	0.21	0.21
Cryptococcosis	0.08	0.14	0.17	0.18	0.13	0.21	0.16	0.18	0.16	0.15
Pneumocystis pneumonia	0.05	0.06	0.09	0.08	0.09	0.11	0.10	0.11	0.15	0.15

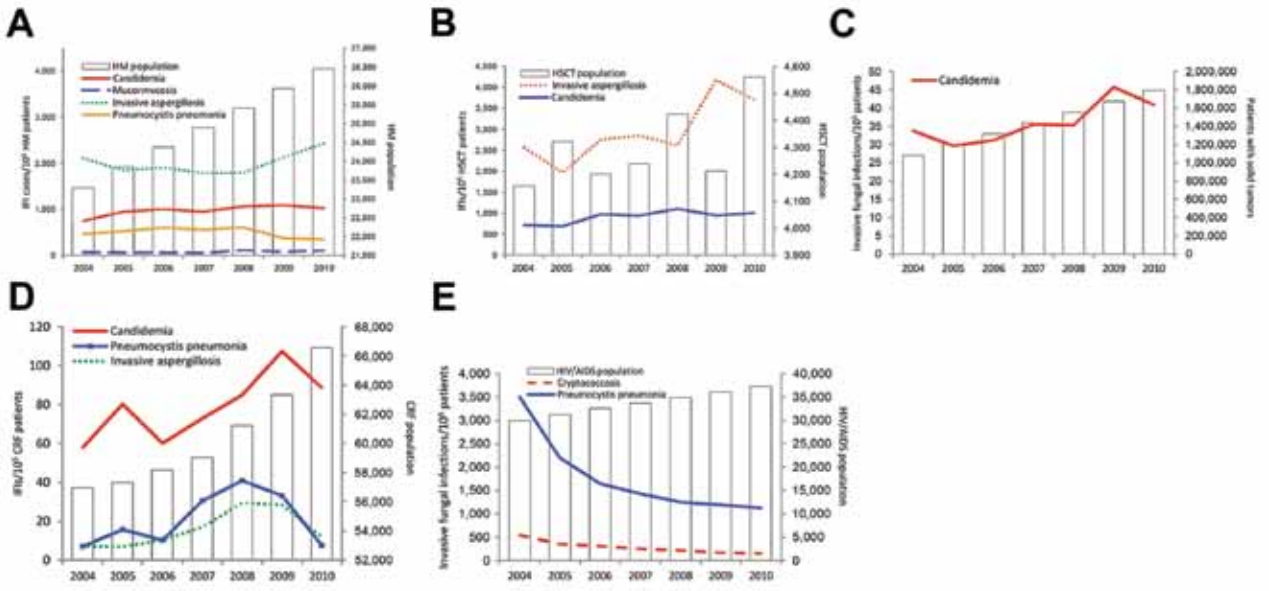

Figure 2. A) Invasive fungal infections in patients with hematologic malignancies (HM) in France, 2004–2010. The case count continuously increased (p<0.001) over the period. Candidemia increased from 751.4 to 1,028.2 cases (+4.3%, p = 0.001), invasive aspergillosis (IA) from 2,112.4 to 2,434.2 cases (+2.7%, p = 0.002), and mucormycosis from 73.0 to 105.8 cases (+8.7%, p = 0.05) per 100,000 patients per year. Inversely, the incidence of *Pneumocystis jirovecii* pneumonia (Pjp) decreased from 468.0 to 351.5 cases/100,000 patients/year (–4.4%, p = 0.006). B) In HSCT recipients (average 4,300 cases per year, no significant trend), candidemia increased from 721.5 to 1008.6 cases (+6.0%, p = 0.05) and invasive aspergillosis from 2,573.4 to 3,705.3 cases (+9.8%, p<0.001) per 100,000 HM patients per year. C) The number of patients with solid tumors continuously increased (p<0.001), and candidemia increased among those patients from 33.7 to 40.9 cases/100,000 patients/year (+6.2%, p<0.001). D) The number of patients with chronic renal failure continuously increased (p<0.001). Candidemia increased from 57.9 to 88.6 cases/100,000 patients/year (+8.1%), IA from 7.0 to 12.0 cases/100,000 patients/year (+18.4%, p = 0.007), and Pjp increased with a peak during 2007–2008 (+11.1%, p = 0.052). E) In the HIV/AIDS population (increase p<0.001), incidence of Pjp and cryptococcosis decreased by –17.9% and –19.0%, respectively (p<0.001). HSCT, hematologic stem cell transplant.

or investigations linked to outbreaks (*31*,*32*). IFIs in this study occurred among a broad spectrum of patients and the fatality rate was high; clinicians should be made aware of risk factors, signs, and symptoms. Beyond the specific issues addressed by our study, such as the identification and management of patients in potentially under-recognized risk groups, the expected consequences of the increasing incidence of IFIs should be anticipated in terms of hospital and laboratory workload, antifungal use, and need for new systemic antifungal drugs and strategies (*33*). The development of epidemiologic studies is also of specific concern to clarify the determinants of the trends and identify effective interventions that can reduce deaths and the general public health burden of illness. These questions should be addressed jointly by clinicians and public health authorities at national and international levels.

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Staphylococcus aureus Infections in New Zealand, 2000–2011

Deborah A. Williamson, Jane Zhang, Stephen R. Ritchie, Sally A. Roberts, John D. Fraser, and Michael G. Baker

The incidence rate for invasive and noninvasive *Staphylococcus aureus* infections in New Zealand is among the highest reported in the developed world. Using nationally collated hospital discharge data, we analyzed the epidemiology of serious *S. aureus* infections in New Zealand during 2000–2011. During this period, incidence of *S. aureus* skin and soft tissue infections increased significantly while incidence of staphylococcal sepsis and pneumonia remained stable. We observed marked ethnic and sociodemographic inequality across all *S. aureus* infections; incidence rates for all forms of *S. aureus* infections were highest among Māori and Pacific Peoples and among patients residing in areas of high socioeconomic deprivation. The increased incidence of *S. aureus* skin and soft tissue infections, coupled with the demographic disparities, is of considerable concern. Future work should aim to reduce this disturbing national trend.

Despite advances in diagnostics and therapeutics, the clinical and economic burdens of *Staphylococcus aureus* infections remain a substantial public health problem (*1*). During the past decade in several parts of the world, most notably in North America, the epidemiology of *S. aureus* infections has changed dramatically, predominantly because of the epidemic spread of a strain of community-associated methicillin-resistant *S. aureus* (MRSA) (*2*,*3*). Infections caused by community-associated MRSA are most commonly skin and soft tissue infections (SSTIs) and typically occur in patients with no history of exposure to health care facilities (*1*). In addition, specific sociodemographic associations for community-associated MRSA infection have been described and include younger patient age, specific ethnic groups, and economic deprivation (*1*,*4*,*5*). Although the epidemiology of *S. aureus* infections has been well studied in North America, comparatively little is known about the trends and patient demographics for *S. aureus* infections in other geographic settings, particularly in the Southern Hemisphere. Knowledge of the overall prevalence and distribution of *S. aureus* infections, regardless of methicillin resistance, at a population level is crucial for informing prevention and control strategies.

The incidence of invasive and noninvasive *S. aureus* infections is reportedly higher In New Zealand than in other developed countries; rates are highest among Māori (indigenous New Zealanders) and Pacific Peoples (*6*–*9*). For example, in 1 study, *S. aureus* bacteremia was 2 times more likely to develop among Māori patients and 4 times more likely to develop among Pacific Peoples than among European patients (*7*). To date, however, studies describing *S. aureus* infections in New Zealand have generally been confined to 1 geographic region, to children, or to 1 specific aspect of *S. aureus* disease such as bloodstream or MRSA infection (*4*,*6*–*8*). Accordingly, we sought to describe the longitudinal trends for *S. aureus* infection and demographic characteristics of patients across the entire New Zealand population for the 12-year period 2000–2011.

Methods

Study Setting

New Zealand is an island nation in the southwestern Pacific Ocean and has ≈4.4 million residents. The population is ethnically diverse, consisting of the following ethnicities: 67% European, 15% Māori, 10% Asian, 7% Pacific Peoples, and 1% other (*10*). New Zealand has a public health care system; data on all publicly funded hospital admissions are recorded by the New Zealand Ministry of Health in the National Minimum Dataset (NMDS). In addition to basic patient information such as age, sex, and ethnicity, these data include principal and additional hospital discharge diagnoses, which since July 1999 have been coded according to the International Classification of

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Diseases, Tenth Revision (ICD-10). Our study population included all patients discharged from New Zealand hospitals from January 2000 through December 2011.

Data Collection and Definitions

In New Zealand, a unique identifier (the National Health Index number) is assigned to each person who accesses public health services; this number can be used to extract information from the NMDS about patient hospitalizations. Patients were identified from the NMDS on the basis of *S. aureus*–associated ICD-10 discharge codes. These ICD-10 codes were A410 (sepsis due to *S. aureus*), J152 (pneumonia due to staphylococci), and B956 (*S. aureus* as the cause of diseases classified elsewhere). A case of *S. aureus* SSTI was defined as infection in a patient who had 1) a principal discharge diagnosis of SSTI (according to an epidemiologic case definition validated in a previous study [*11*]), 2) an additional discharge diagnosis of B956, and 3) no additional discharge diagnoses containing either A410 or J152. The National Health Index number can also be used to filter out unrelated hospital admissions. We filtered our data to exclude the following groups: overseas visitors, patients on waiting lists, hospital attendees who did not stay overnight, hospital transferees, and patients readmitted to the hospital within 30 days of first admission.

The following information about each patient who was discharged from the hospital for an *S. aureus*–associated cause was extracted from the NMDS: age, sex, ethnicity, and socioeconomic status (derived from the New Zealand deprivation index [NZDep] (*12*). The NZDep score is an area-based measure of socioeconomic deprivation derived from New Zealand census data; the score is based on various measures of deprivation, including household income, household ownership, household occupancy, employment and education levels, and access to telecommunications. It is expressed as a score between 1 and 10; a score of 10 represents the most deprived neighborhoods. To determine whether any increasing trends in *S. aureus* infection were associated with a general increase in all hospital admissions, we obtained information from the NMDS on all patients acutely hospitalized overnight in New Zealand over the study period, applying the same exclusion filters described above.

Statistical Analyses

Age-adjusted incidence rates were calculated per 100,000 population and standardized to the age distribution of the 2006 New Zealand census (*10*). These incidence rates were stratified according to sex, ethnicity, NZDep score, and geographic region. For analysis, we used 4 major ethnic groups: European, Māori, Pacific Peoples, and Asian/other. To determine possible geographic differences in incidence of *S. aureus* infection across New Zealand, we analyzed 4 broad geographic regions: northern, midland, central, and southern (Figure 1). Population denominator data were obtained from Statistics New Zealand (http://www.stats.govt.nz). A Poisson regression model, with log population data as an offset variable, was used to assess trends over time. The Kruskal-Wallis analysis of variance

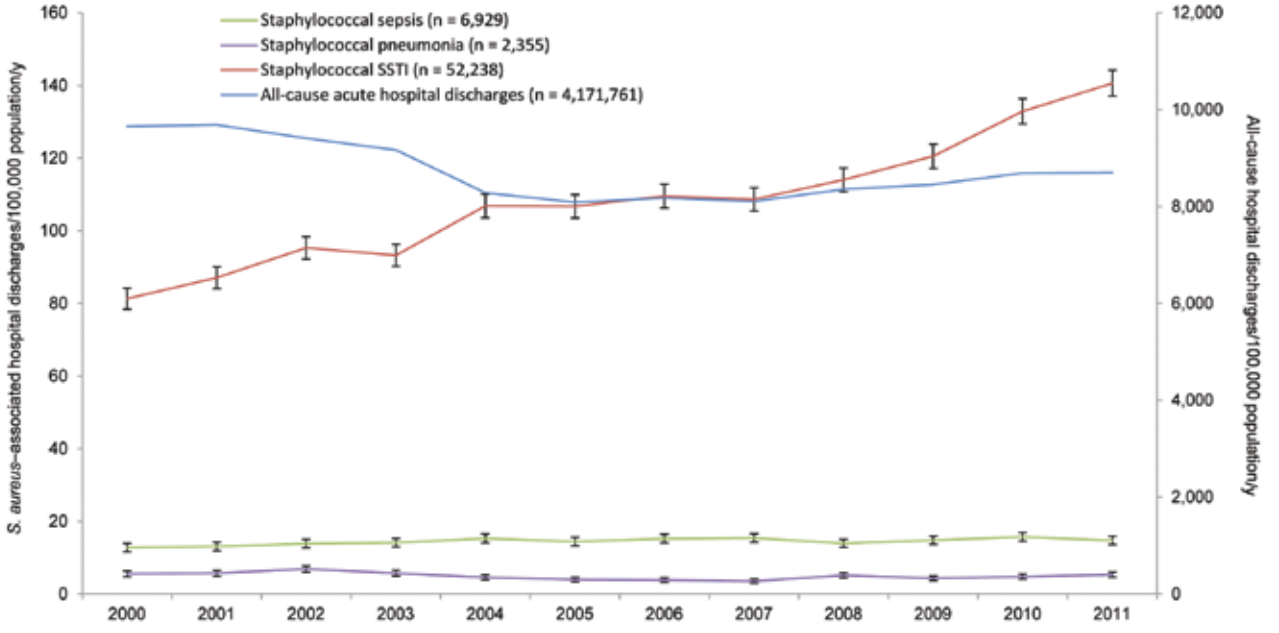

Figure 1. Annual rates of *Staphylococcus aureus*–associated hospital discharge (no. cases/100,000 population) and all-cause acute hospital discharge rates (no. cases/100,000 population), New Zealand, 2000–2011. Error bars indicate 95% CIs; for all-cause hospital discharges, error bars are too small to be visible on this chart. SSTI, skin and soft tissue infection.

test was used to determine differences in the geographic incidence of *S. aureus* infections. Relative risks were calculated with 95% CIs, and all statistical analyses were performed by using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) or STATA version 11.1 (StataCorp, College Station, TX, USA). We considered $p<0.05$ to be statistically significant.

Results

For the study period, 61,522 *S. aureus*–associated hospital discharges were identified. The overall averaged 12-year incidence rate for all *S. aureus* infections was 127 (95% CI 122–133) per 100,000 population per year. The overall incidence rate for *S. aureus* SSTIs was 108 (95% CI 105–111) per 100,000 population, *S. aureus* sepsis 14 (95% CI 13–16) cases per 100,000, and staphylococcal pneumonia 5 (95% CI 4–6) cases per 100,000. The incidence rate for sepsis caused by *S. aureus* and pneumonia caused by staphylococci did not change significantly over the study period; however, the incidence rate for *S. aureus* SSTIs increased significantly, from 81 (95% CI 78– 84) cases per 100,000 population in 2000 to 140 (95% CI 137–144) cases per 100,000 in 2011 ($p<0.001$) (Figure 1), which represents an increase of ≈5% each year. In contrast, the rate of acute all-cause hospital discharges in New Zealand fell significantly, from 9,657 (95% CI 9,625–9,689) per 100,000 population in 2000 to 8,701 (95% CI 8,673–8,729) per 100,000 population in 2011 ($p<0.001$). Consequently, the relative proportion of *S. aureus* SSTIs to all hospital discharges doubled, from 0.8% in 2000 to 1.6% in 2011.

Incidence of staphylococcal pneumonia did not vary significantly by geographic location ($p = 0.8$); however, incidence of staphylococcal sepsis ($p = 0.02$) and SSTIs ($p = 0.01$) did (Figure 2). In particular, there was a distinct north–south gradient for staphylococcal SSTIs; rates in the northern and central regions were ≈3 times rates in the southern region.

Incidence of *S. aureus* infections also varied markedly by sociodemographic characteristics (Table, http://wwwnc.cdc.gov/EID/article/20/7/13-1923-T1.htm). Staphylococcal infections of all forms were significantly more likely to occur among male than female patients; this difference was most marked for *S. aureus* sepsis (relative risk [RR] 1.9; 95% CI 1.8–2.0). The incidence rates for sepsis and pneumonia were significantly higher among patients >70 years of age (62 and 24 cases/100,000 population/year, respectively) than among patients of other age groups (Appendix Table). In contrast, the incidence rate for *S. aureus* SSTIs was highest among those <5 years of age (242 cases/100,000 population/year). The incidence of all disease types was highest among Māori and Pacific Peoples (Appendix Table). In particular, Māori were 3 times more likely and Pacific Peoples almost 5 times more likely than Europeans to have an *S. aureus* SSTI.

The incidence of *S. aureus* disease also varied significantly according to socioeconomic deprivation; the

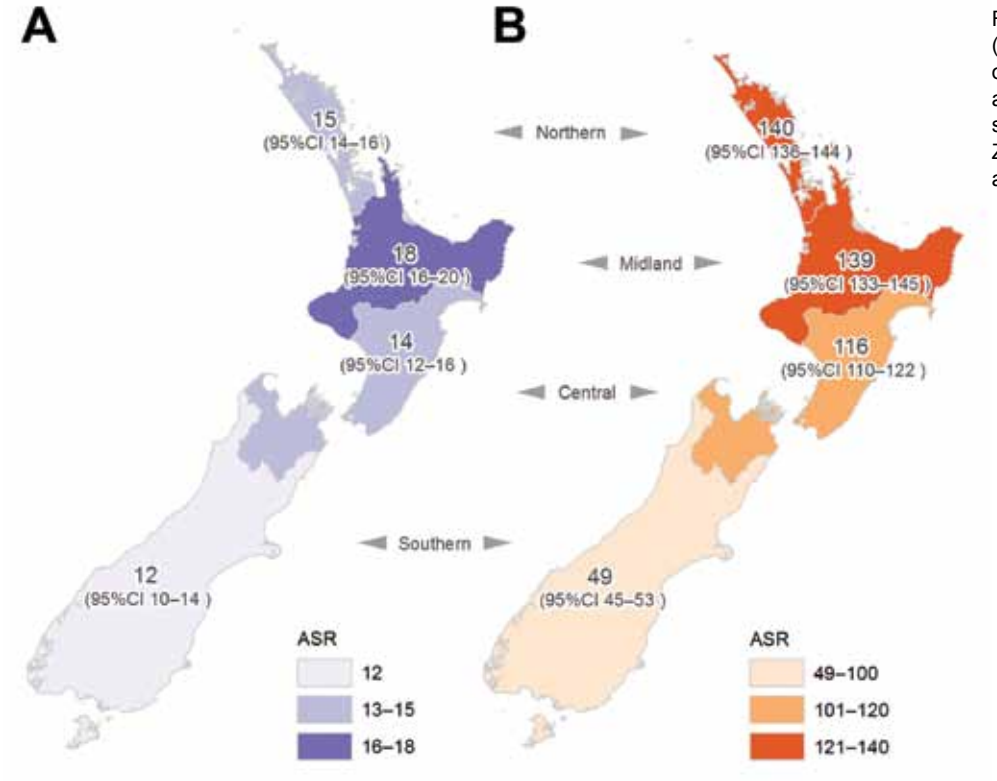

Figure 2. Average annual ASR (no. cases/100,000 population) of staphylococcal sepsis (A) and staphylococcal skin and soft tissue infections (B), New Zealand, 2000–2011. ASR, age-standardized rate.

incidence rates for sepsis, pneumonia, and SSTI were significantly higher among patients residing in areas of high socioeconomic deprivation. This disparity was most marked for SSTIs; patients residing in areas of high deprivation were almost 4 times more likely to have *S. aureus* SSTIs than were those residing in areas of low deprivation (RR 3.7, 95% CI 3.6–3.8). An independent association seemed to exist between *S. aureus* disease and ethnicity after socioeconomic status was adjusted for, such that for each tier of socioeconomic deprivation, all 3 types of *S. aureus* disease were more common among Māori and Pacific Peoples than among those of European or other ethnicity (Figure 3).

Discussion

In this study, we analyzed the longitudinal incidence and epidemiology of serious *S. aureus* disease across the entire New Zealand population during 2000–2011. Incidence of *S. aureus* SSTI increased dramatically while incidence of *S. aureus* sepsis and pneumonia remained relatively stable. Our finding of a persistent increase in serious *S. aureus* SSTIs over the past decade is a substantial public

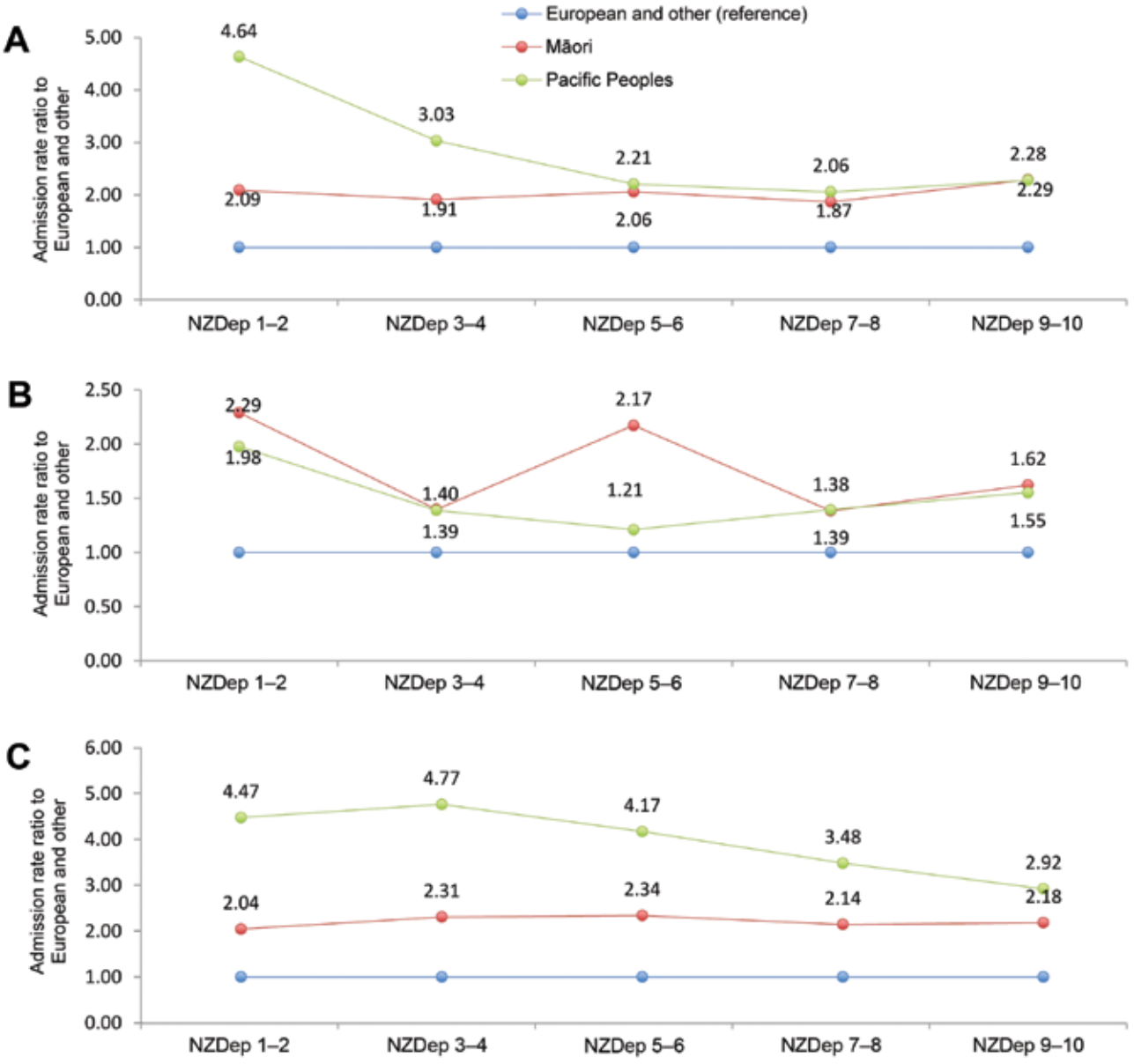

Figure 3. Admission rate ratios for *Staphylococcus aureus*–associated hospital discharges by ethnicity according to level of deprivation, New Zealand, 2000–2011. A) Staphylococcal sepsis; B) staphylococcal pneumonia; C) staphylococcal skin and soft tissue infections. NZDep, New Zealand Deprivation Index.

health concern, particularly given the overall decrease in acute overnight hospital admissions in New Zealand.

The factors underlying the increase in such infections are unknown, but risk factors for the development of *S. aureus* SSTI are multifactorial and probably include household crowding, delayed or inadequate access to health care, and issues associated with household hygiene (*13*,*14*). The reasons for the relatively unchanged rate of *S. aureus* sepsis and staphylococcal pneumonia in New Zealand are unclear; however, recent studies highlight the decreasing incidence of invasive *S. aureus* infections in other geographic settings, particularly among those patients recently exposed to health care facilities or receiving health care (*15*,*16*). Although improvements in infection prevention practices probably contribute to the decrease of invasive infections (*17*), other possible unexplored factors include changes in host susceptibility to *S. aureus* infection (e.g., improved management of concurrent conditions such as cardiovascular disease and diabetes) or temporal changes in the virulence profiles or transmissibility of circulating *S. aureus* strains.

Consistent with the findings of other studies of infectious diseases in New Zealand (*13*,*14*), we found notable sociodemographic disparity in the incidence of *S. aureus* infections; incidence of all *S. aureus* infections was highest among Māori or Pacific Peoples and among those residing in areas of high socioeconomic deprivation. Even after adjusting for socioeconomic deprivation, we found that the incidence of all *S. aureus* disease was significantly higher among Māori and Pacific Peoples than among patients of European and other ethnicities; this pattern is seen for infectious diseases generally in New Zealand (*14*). The underlying reasons for this apparent ethnic disparity in staphylococcal disease are uncertain. Unexplored possibilities include a higher prevalence of *S. aureus* colonization among Māori or Pacific Peoples or differences in the circulating *S. aureus* strain types among distinct ethnic groups, as previously described for our setting (*4*). However, an alternative possibility is that the area-based NZDep score used to record socioeconomic deprivation does not fully represent those facets of poverty that contribute to the development and prevention of serious *S. aureus* disease. These unmeasured risk factors include aspects of health literacy relating to early management of insect bites and skin infections, availability of household amenities such as hot water, and affordable and timely access to health care. Specific individual-level and household-level studies are required for determination of the relative contribution of such potentially modifiable risk factors.

We also observed significant geographic variation in the incidence of *S. aureus* SSTI, with a distinct north–south gradient. This finding can probably be explained by the distribution of population groups in New Zealand; the groups most affected by *S. aureus* SSTI reside predominantly in the North Island (*13*). However, other possible contributory factors include geographic differences in access to and provision of health care and climate differences; the climate in the upper North Island is relatively warmer and more humid than that in the southern regions.

A limitation of our study was our use of hospital discharge data for case ascertainment. Use of these data meant that we were unable to determine the proportion of cases occurring in the community versus in the hospital setting, although previous studies have demonstrated that most *S. aureus* infections in New Zealand originate in the community (*4*,*7*,*8*). However, our aim was not to provide detailed information on individual *S. aureus* infections but rather to provide a broad overview of the trends and demographics of serious *S. aureus* infections across the entire New Zealand population. In addition, our data represent only those patients whose hospital discharge was associated with *S. aureus* disease; they do not represent those patients who sought care from a primary care physician or who sought care at a hospital but were not admitted. For example, a recent study of children with SSTIs in 1 New Zealand region found an estimated 14 primary care cases for every 1 hospital admission (*18*). Furthermore, these data represent only those instances in which an etiologic agent was described and recorded in the discharge diagnoses. It is therefore highly likely that the overall prevalence of staphylococcal disease in our setting is substantially higher than that estimated here.

In summary, our study provides valuable longitudinal data on the prevalence of serious *S. aureus* disease in the New Zealand population and represents one of the few studies that systematically assessed the epidemiology and demographics of staphylococcal infections across an entire nation. The steady and significant increase in serious *S. aureus* SSTI coupled with notable sociodemographic disparity in disease incidence is a disturbing national trend. A concerted multimodal public health intervention is urgently required to tackle this problem.

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Dr Williamson is a clinical microbiologist and a clinical research training fellow of the Health Research Council of New Zealand. Her research interests are the clinical and molecular epidemiology of *S. aureus* infections and infections caused by antimicrobial drug–resistant pathogens.

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Epidemiology of Influenza Virus Types and Subtypes in South Africa, 2009–2012[1]

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Release date: June 16, 2014; Expiration date: June 16, 2015

Learning Objectives

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

1. Describe differences in characteristics of patients hospitalized with severe acute respiratory illness (SARI) in South Africa by infection with different influenza types and subtypes, based on a surveillance study
2. Compare characteristics of patients hospitalized with SARI in South Africa by infection with different influenza types and subtypes during the first and second influenza A(H1N1)pdm09 waves
3. Compare case fatality and severity rates among infections with different types and subtypes and between the first and second influenza A(H1N1)pdm09 waves.

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[1]This information has been presented at Options for the Control of Influenza VIII, Cape Town, South Africa, September 5–9, 2013.

To determine clinical and epidemiologic differences between influenza caused by different virus types and subtypes, we identified patients and tested specimens. Patients were children and adults hospitalized with confirmed influenza and severe acute respiratory illness (SARI) identified through active, prospective, hospital-based surveillance from 2009–2012 in South Africa. Respiratory specimens were tested, typed, and subtyped for influenza virus by PCR. Of 16,005 SARI patients tested, 1,239 (8%) were positive for influenza virus. Patient age and co-infections varied according to virus type and subtype, but disease severity did not. Case-patients with influenza B were more likely than patients with influenza A to be HIV infected. A higher proportion of case-patients infected during the first wave of the 2009 influenza pandemic were 5–24 years of age (19%) than were patients infected during the second wave (9%). Although clinical differences exist, treatment recommendations do not differ according to subtype; prevention through vaccination is recommended.

Most influenza in humans is caused by 2 types of influenza virus: A and B. On the basis of the hemagglutinin and neuraminidase proteins on the surface of the virus, influenza A viruses are further subdivided into subtypes, 2 of which have commonly caused disease in humans over the past century: H3N2 and H1N1. The proportion of these 3 types and subtypes of influenza virus—A(H3N2), A(H1N1), and B—that circulate among humans varies each year. In 2009, a novel pandemic strain of influenza A(H1N1) virus, now called influenza A(H1N1) pdm09 virus, became the dominant H1N1 virus strain circulating worldwide (*1*).

It is generally not possible to distinguish infection caused by different influenza types and subtypes by clinical features (*2*,*3*), although differences in severity have been observed (*4*–*6*). Analyses of vital statistics data from the United States and South Africa have suggested that the numbers of excess deaths associated with influenza are higher in years when influenza A(H3N2) virus is circulating than when influenza B or prepandemic influenza A(H1N1) virus is circulating (*4*,*7*). Some studies have suggested that influenza A(H1N1)pdm09 virus infection led to more severe outcomes than did other types and subtypes (*8*,*9*). In the first 3 months after influenza A(H1N1)pdm09 virus was identified in South Africa, 91 deaths among 12,331 patients with laboratory-confirmed cases were identified; rates of HIV infection and pregnancy among those who died were high (*10*). After the influenza pandemic, studies showed that A(H1N1) pdm09 virus was more likely than previously circulating virus types and subtypes to affect children and young adults and that severe disease was associated with clinical characteristics such as obesity (*11*,*12*). The data conflict with regard to whether severity of disease increases with subsequent waves of A(H1N1)pdm09 virus infection (*13*–*17*).

Little data have been reported from Africa on clinical and epidemiologic differences caused by different influenza virus types and subtypes. The objective of our study was 2-fold. First, we sought to compare the demographic and clinical characteristics, factors associated with infection, and disease severity among case-patients hospitalized with severe acute respiratory illness (SARI) associated with influenza A(H1N1)pdm09, A(H3N2), and B viruses in South Africa during 2009–2012. Second, we sought to compare the characteristics of case-patients infected during the first wave of influenza A(H1N1)pdm09 infection in 2009 with those of case-patients infected during the subsequent wave in 2011. Because this surveillance was started in 2009, we did not include prepandemic A(H1N1) virus strains in this study.

Materials and Methods

Setting and Time

The SARI program is an active, prospective, sentinel, hospital-based surveillance system that monitors children and adults hospitalized with pneumonia in 4 provinces in South Africa (*18*). In February 2009, SARI surveillance was implemented in 3 of the 9 provinces of South Africa (Chris Hani-Baragwanath Academic Hospital, an urban site in Gauteng Province; Edendale Hospital, a periurban site in KwaZulu-Natal Province; and Matikwana and Mapulaneng Hospitals, rural sites in Mpumalanga Province). In June 2010, an additional surveillance site was introduced at Klerksdorp and Tshepong Hospitals, periurban sites in Northwest Province. This surveillance, which includes testing for influenza virus and HIV, has received human subjects review and approval by the University of Witswatersrand, South Africa. The US Centers for Disease Control and Prevention deemed this a nonresearch surveillance activity. The study was conducted during 2009–2012.

Case Definitions and Patient Enrollment

A case of SARI was defined as acute lower respiratory tract infection (or pneumonia) in a patient hospitalized within 7 days of illness onset. Children 2 days through <3 months of age with physician-diagnosed sepsis or acute lower respiratory tract infection (including, for example bronchitis, bronchiolitis, pneumonia, and pleural effusion) and children 3 months through <5 years of age with physician-diagnosed acute lower respiratory tract infection were enrolled. Among patients ≥5 years of age, we enrolled those who met the World Health Organization case definition of SARI: sudden onset of reported or measured fever (>38°C), cough or sore throat, and shortness of breath or difficulty breathing (*19*).

All patients admitted to a hospital during Monday–Friday were eligible for enrollment in the study; adult patients at Chris Hani-Baragwanath Academic Hospital

were systematically sampled 2 of every 5 working days per week. Patients were enrolled within the first 24 hours of admission. We determined the number of patients who were admitted, met study case definitions, and were enrolled. Study staff were centrally trained and completed case report forms until discharge for all enrolled patients; staff collected respiratory (nasopharyngeal) aspirates from patients <5 years of age and nasopharyngeal and throat swab specimens from patients ≥5 years of age and blood specimens from consenting patients. Patients were admitted to an intensive care unit, and specimens for bacterial culture and tuberculosis testing were collected at the discretion of the attending physician. For children <5 years of age, we gathered data on additional clinical signs and symptoms; for adolescents and adults ≥12 years of age, we gathered information on smoking and alcohol use. Informed consent was obtained for all enrollment, laboratory testing, and anonymized, linked HIV testing.

Laboratory Methods

Respiratory specimens were placed in viral transport media, kept at 4–8°C, and sent to the National Institute for Communicable Diseases in Johannesburg within 72 hours of collection. Respiratory specimens were tested by multiplex real-time reverse transcription PCR for 10 respiratory viruses (influenza A and B viruses; parainfluenza viruses 1, 2, and 3; respiratory syncytial virus; enterovirus; human metapneumovirus; adenovirus; and rhinovirus) (*20*). Influenza-positive specimens were subtyped by using the Centers for Disease Control and Prevention real-time reverse transcription PCR protocol for detection and characterization of influenza virus (*21*). *Streptococcus pneumoniae* was identified by quantitative real-time PCR that detected the *lytA* gene from whole-blood specimens (*22*). When available, data on HIV infection status were obtained through routine standard-of-care testing at the treating hospital. When those data were not available, HIV testing was implemented at the National Institute for Communicable Diseases through anonymized, linked, dried blood-spot specimen testing by HIV PCR for children <18 months of age and by ELISA for patients ≥18 months of age.

Statistical Analyses

We excluded from the analysis influenza virus–positive case-patients for whom subtyping could not be performed because of low concentration of virus. Univariate comparisons were performed by using multinomial or logistic regression. We conducted multinomial regression to compare demographic and clinical characteristics, associated factors, and disease severity among patients infected with the 3 influenza types and subtypes. Multinomial regression enables modeling of outcome variables with >2 categories and relates the probability of being in a category (in this instance either influenza A[H3N2] or B virus) to the probability of being in a baseline category (in this instance influenza [H3N2] virus). A complete set of coefficients are estimated for each of the categories being compared with the baseline, and the effect of each predictor in the model is measured as relative risk ratio (RRR). For this analysis, we used the influenza virus A(H3N2)–infected group as the baseline category because influenza A(H3N2) virus is considered to induce more severe illness (*4*,*7*). We conducted 2 logistic regression models to compare patients infected with influenza A with those infected with influenza B and to compare patients infected during the first wave of influenza A(H1N1)pdm09 with patients infected during subsequent waves of influenza A(H1N1)pdm09. All models were built by using stepwise forward selection. Covariates for which p value was <0.2 at the univariate analysis were assessed for significance with multivariable analysis, and statistical significance was assessed at $p<0.05$ for all multivariable models. We assessed 2-way interactions by inclusion of product terms for all variables remaining in the final models. Additional modeling is shown in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/20/7/13-1869-Techapp1.pdf).

Results

From February 2009 through December 2012, a total of 21,792 patients hospitalized with lower respiratory tract infection were approached for enrollment in SARI surveillance. Of those, 16,005 (73%) were enrolled and 1,239 (8%) had positive influenza virus test results. Of the 5,876 patients who were approached but not enrolled, the most common reasons for not enrolling were unavailability of a legal guardian (among children <5 years of age; 1,452 [25%]), refusal (1,296 [22%]), and being confused or too ill (431 [7%]). Of the influenza-positive SARI cases, 463 (37%) were caused by influenza A(H3N2), 338 (27%) by influenza A(H1N1)pdm09, and 418 (34%) by influenza B viruses; 20 (2%) influenza A viruses could not be further subtyped because of low viral yield in the samples. Influenza epidemics occur annually during the colder months in South Africa (May–September), and little activity occurs during the rest of the year (Figure). The circulating types and subtypes varied between study years and within annual epidemics. During 2009, influenza virus activity occurred in 2 peaks; the first was caused by subtype A(H3N2) (194/379, 51%), which occurred earlier than in the other years, and the second was caused by subtype A(H1N1) pdm09 (160/379 42%) (Table 1 [an expanded version of this table is available in the online Technical Appendix]; Figure). The predominant influenza virus types or subtypes in the other years were as follows: B (164/273, 60%) in 2010, A(H1N1)pdm09 (140/362, 39%) in 2011, and A(H3N2) (99/205, 48%) and B (105/205, 51%) in 2012. Most (71%) case-patients were at Chris Hani-Baragwanath

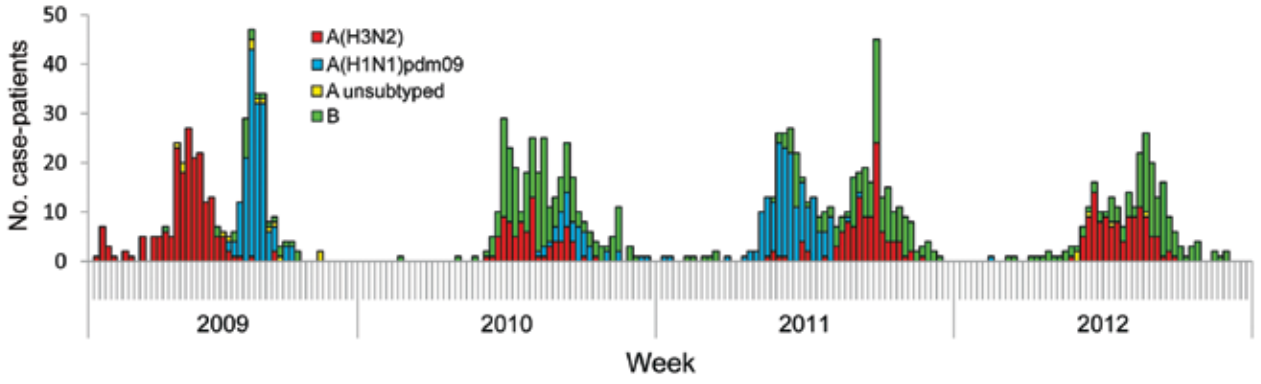

Figure. Number of case-patients hospitalized with influenza-associated severe acute respiratory illness, by week and virus strain at 4 sites, South Africa, 2009–2012.

Academic Hospital, which reflects the higher number of SARI case-patients enrolled there. Of 12,494 SARI case-patients for whom treatment data were available, 7 (0.1%) received oseltamivir, 1 of whom had laboratory-confirmed influenza. Of 12,173 SARI case-patients for whom influenza vaccine histories were available, 19 (0.2%) reported having been vaccinated. HIV test results were available for 947 (76%) of influenza case-patients. Of those, 399 (42%) were positive for HIV: 377 (94%) from anonymized testing at the National Institute for Communicable Diseases and 22 (6%) from standard-of-care testing at the treating hospitals.

The age distribution of SARI case-patients with influenza was bimodal: most of the 1,239 influenza case-patients were <5 years of age (613 [49.5%]), followed by those 25–44 years of age (306 [24.7%]); few patients were ³65 years of age (53 [4.3%]). This bimodal age distribution is repeated for each of the types and subtypes (Table 1) except that the first wave of A(H1N1)pdm09 infection disproportionately affected those 5–24 years of age (Table 2). According to univariate analysis, case-patients infected with influenza A(H1N1)pdm09 virus were less likely than case-patients infected with influenza A(H3N2) virus to be co-infected with another virus (crude RRR [cRRR] 0.6, 95% CI 0.4–0.8), and case-patients infected with influenza B virus were more likely to be infected with HIV (cRRR 1.7, 95% CI 1.2–2.3), have stridor (cRRR 2.1, 95% CI 1.2–3.6), have symptoms ≥3 days before admission (cRRR 1.6, 955 CI 1.2–2.1), and to have been hospitalized for ≥2 days (cRRR 1.6, 95% CI 1.2–2.2), and were less likely to have a measured fever of ≥38°C (cRRR 0.5, 95% CI 0.4–0.7) (Table 1). In the multivariate analysis model, only age and year remained statistically significant (Table 1). We found no statistical difference in case-fatality rates between virus types and subtypes (2.8% for A[H3N2], 1.5% for A[H1N1] pdm09, and 3.9% for B) and no difference in other markers of severity, such as admission to an intensive care unit, need for mechanical ventilation, need for supplemental oxygen, or prolonged hospitalization (Table 1).

To further explore the association between influenza types and characteristics such as HIV status, we conducted a univariate analysis and constructed a multivariable logistic regression model comparing influenza B virus with influenza A (both A[H3N2] and A[H1N1]pdm09) viruses. Except for co-infection with any virus other than influenza, the same variables were significant on this univariate analysis as were significant on the previous analysis. According to multivariate analysis, only year and HIV status remained statistically significant and were retained in the final model. Because age group was not significantly associated with virus type and did not have an interaction with HIV infection in the multivariate model, we did not include age in the final model. When we controlled for year, this model showed that case-patients with influenza B virus infection were more likely than patients with influenza A virus infection to also be infected with HIV (adjusted odds ratio 1.4, 95% CI 1.02–1.80).

According to univariate analysis, case-patients in the second wave of the A(H1N1)pdm09 pandemic were less likely than case-patients in the first wave to have had a measured fever of ≥38°C (crude odds ratio [cOR] 0.2, 95% CI 0.1–0.4) and more likely to have been co-infected with respiratory syncytial virus (cOR 6.4, 95% CI 1.4–29.6), have had symptoms for ≥3 days at admission (cOR 2.0, 95% CI 1.2–3.1), and to have needed supplemental oxygen (cOR 2.6, 95% CI 1.6–4.2; Table 2). According to multivariable logistic regression, only age group and surveillance site remained statistically significant (Table 2). Severity of hospitalization, as measured by admission to an intensive care unit, need for mechanical ventilation, need for supplemental oxygen, or prolonged hospitalization, did not differ between waves (Table 2). In addition, case-fatality rates did not differ between the first (1.3%) and second (1.5%) waves.

Discussion

The influenza virus types and subtypes that circulated during the annual winter influenza seasons in South Africa

Table 1. Characteristics of patients hospitalized with influenza-associated severe acute respiratory illness, by virus type and subtype, 4 sites, South Africa, 2009–2012*

	Influenza type and subtype				
	A(H3N2) (reference)	A(H1N1)pdm09		B	
Characteristic	No. pos/no. tested (% pos)	No. pos/no. tested (% pos)	Adjusted RRR (95% CI)	No. pos/no. tested (% pos)	Adjusted RRR (95% CI)
Age group, y					
<5	265/463 (57.2)	167/338 (49.4)	Reference	171/418 (40.9)	Reference
5–24	35/463 (7.6)	49/338 (14.5)	2.3 (1.4–3.8)	43/418 (10.3)	2.0 (1.2–3.4)
25–44	96/463 (20.7)	78/338 (23.1)	1.3 (0.9–2.0)	128/418 (30.6)	1.5 (1.0–2.1)†
45–64	44/463 (9.5)	35/338 (10.4)	1.4 (0.9–2.4)	55/418 (13.2)	1.4 (0.9–2.2)
≥65	23/463 (5.0)	9/338 (2.7)	0.6 (0.2–1.3)	21/418 (5.0)	1.1 (0.6–2.2)
Male	207/461 (44.9)	149/336 (44.4)		177/417 (42.5)	
Black African	452/460 (98.3)	327/336 (97.3)		407/416 (97.8)	
Year					
2009	194/463 (41.9)	160/338 (47.3)	Reference	25/418 (6.0)	Reference
2010	72/463 (15.6)	37/338 (11.0)	0.6 (0.4–1.0)†	164/418 (39.2)	16.8 (10.1–27.9)
2011	98/463 (21.2)	140/338 (41.4)	1.7 (1.2–2.5)	124/418 (29.7)	9.5 (5.7–15.6)
2012	99/463 (21.4)	1/338 (0.3)	0.0 (0.0–0.1)	105/418 (25.1)	7.8 (4.7–13.0)
Co-infections and underlying medical conditions					
HIV infection	112/311 (36.0)	110/271 (40.6)		170/352 (48.3)	
Tuberculosis	42/458 (9.2)	34/335 (10.2)		38/411 (9.3)	
Underlying medical condition excluding tuberculosis, HIV‡	34/460 (7.4)	31/336 (9.2)		38/417 (9.1)	
Pregnancy	3/251 (1.2)	2/187 (1.1)		3/24 (1.3)	
Pneumococcal co-infection detected by PCR	23/310 (7.4)	25/286 (8.7)		32/325 (9.9)	
Clinical presentation and course					
Temperature ≥38°C	181/364 (49.7)	141/287 (49.1)		138/407 (33.9)	
Cough§	255/264 (96.6)	162/167 (97.0)		163/170 (95.9)	
Tachypnea§	99/250 (39.6)	73/161 (45.3)		62/159 (39.0)	
Difficulty breathing§	188/264 (71.2)	125/167 (74.9)		111/170 (65.3)	
Chest wall indrawing§	96/264 (36.4)	77/167 (46.1)		56/170 (32.9)	
Stridor§	30/264 (11.4)	20/167 (12.0)		36/170 (21.2)	
Symptoms ≥3 d before admission	206/452 (45.6)	153/335 (45.7)		239/415 (57.6)	
Admitted to ICU	4/457 (0.9)	3/336 (0.9)		4/411 (1.0)	
Mechanical ventilation needed	3/457 (0.7)	1/336 (0.3)		4/411 (1.0)	
Supplemental oxygen needed	138/457 (30.2)	117/336 (34.8)		144/411 (35.0)	
Antimicrobial drugs prescribed on admission	402/421 (95.7)	321/335 (95.8)		384/395 (97.2)	
Hospitalized for ≥2 d	319/451 (70.7)	255/332 (76.8)		323/407 (79.4)	
No. deaths/no. patients (case-fatality ratio)	13/459 (2.8)	5/334 (1.5)		16/412 (3.9)	

*Pos, positive; RRR, relative risk ratio; ICU, intensive care unit. An expanded version of this table is available in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/20/7/13-1869-Techapp1.pdf).
†p<0.05.
‡Asthma, other chronic lung disease, chronic heart disease (valvular heart disease, coronary artery disease, or heart failure excluding hypertension), liver disease (cirrhosis or liver failure), renal disease (nephrotic syndrome, chronic renal failure), diabetes mellitus, immunocompromising conditions excluding HIV infection (organ transplant, immunosuppressive therapy, immunoglobulin deficiency, malignancy), neurologic disease (cerebrovascular accident, spinal cord injury, seizures, neuromuscular conditions), or pregnancy. Concurrent conditions were considered absent for patients for whom the medical records stated that the patient had no underlying medical condition or when there was no direct reference to that condition.
§Patients <5 y of age.

varied from 2009 (the year of the A(H1N1)pdm09 pandemic) to 2012. Characteristics of patients hospitalized with SARI differed by infection with different influenza types and subtypes, particularly with regard to age and co-infection with HIV. In South Africa, the age distribution of those hospitalized with influenza during the second wave of the A(H1N1)pdm09 pandemic was more similar to the age distribution of those infected by seasonal influenza types and subtypes (a bimodal distribution with a peak in young adults 25–44 years of age) than to that of those who experienced severe disease during the first wave of the A(H1N1)pdm09 pandemic (*18*). This age distribution of respiratory influenza infection in South Africa is driven by the high prevalence of HIV infection among young adults in South Africa because HIV-infected adults are at increased risk for severe disease from influenza virus infection (*18*). In South Africa in 2009, the prevalence of HIV infection among the total population was 11% (*23*) and the prevalence among women attending antenatal care was 29% (*24*). In other settings, infection with influenza B virus is associated with less severe disease than is infection with influenza

Table 2. Characteristics of patients hospitalized with influenza A(H1N1)pdm09–associated severe acute respiratory illness, by wave, 4 sites, South Africa, 2009–2012*

Characteristic	A(H1N1)pdm09		Crude OR (95% CI)	Adjusted OR (95% CI)
	First wave (2009), no. pos/no. tested (% pos)	Second wave (2011), no. pos/no. tested (% pos)		
Age group, y				
<5	87/160 (54.4)	67/140 (47.9)	Reference	Reference
5–24	31/160 (19.4)	12/140 (8.6)	0.5 (0.2–1.1)	0.6 (0.3–1.4)
25–44	24/160 (15.0)	42/140 (30.0)	2.3 (1.3–4.1)	2.8 (1.5–5.1)
45–64	13/160 (8.1)	16/140 (11.1)	1.6 (0.7–3.6)	2.0 (0.9–4.6)
≥65	5/160 (3.1)	3/139 (2.1)	0.8 (0.2–3.4)	1.1 (0.3–5.1)
Male	76/159 (47.8)	57/139 (41.1)	0.8 (0.5–1.2)	
Black African	156/159 (98.1)	135/139 (97.1)	1.5 (0.3–7.0)	
Site				
Soweto	104/160 (65.0)	98/140 (70.0)	Reference	Reference
Klerksdorp	Not applicable	12/140 (8.6)	Not calculated	Not calculated
Pietermaritzburg	6/160 (3.8)	12/140 (8.6)	0.4 (0.2–0.7)	0.4 (0.2–0.8)
Agincourt	50/160 (31.3)	18/140 (12.9)	2.1 (0.8–5.9)	2.7 (0.96–7.8)
Co-infections and underlying medical conditions				
HIV-infected	47/119 (39.5)	49/117 (41.9)	1.1 (0.7–1.9)	
Tuberculosis	18/158 (11.4)	12/139 (8.6)	0.7 (0.3–1.6)	
Underlying medical condition excluding tuberculosis, HIV	11/159 (6.9)	15/139 (10.8)	1.6 (0.7–3.7)	
Pregnancy	2/83 (2.4)	0/82 (0)	Not calculated	
Bacterial/viral respiratory co-infection				
Pneumococcal co-infection detected by PCR	15/129 (11.6)	7/127 (5.5)	0.4 (0.2–1.1)	
Respiratory syncytial virus	2/153 (1.3)	11/140 (7.9)	6.4 (1.4–29.6)	
Adenovirus	0/153 (0)	18/140 (12.9)	Not calculated	
Parainfluenzavirus 1, 2, or 3	10/160 (6.3)	3/140 (2.1)	0.3 (0.1–1.2)	
Human metapneumovirus	6/153 (3.9)	1/140 (0.7)	0.2 (0.0–1.5)	
Rhinovirus	16/153 (10.5)	11/140 (7.9)	0.7 (0.3–1.6)	
Enterovirus	2/153 (1.3)	2/140 (1.4)	1.1 (0.2–7.9)	
Clinical presentation and course				
Temperature ≥38°C	76/110 (69.1)	46/139 (33.1)	0.2 (0.1–0.4)	
Cough†	83/87 (95.4)	66/67 (98.5)	3.2 (0.3–29.1)	
Tachypnea†	32/84 (38.1)	34/65 (52.3)	1.8 (0.9–3.4)	
Difficulty breathing†	69/87 (79.3)	45/67 (67.2)	0.5 (0.3–1.1)	
Chest wall indrawing†	44/87 (50.6)	25/67 (47.3)	0.6 (0.3–1.1)	
Stridor†	4/87 (4.6)	11/67 (16.4)	4.1 (1.2–13.4)	
Tachycardia†	44/87 (50.6)	43/67 (64.2)	1.8 (0.9–3.4)	
Diarrhea†	16/87 (18.4)	9/67 (13.4)	0.7 (0.3–1.7)	
Unable to eat†	29/87 (33.3)	11/67 (6.4)	0.4 (0.2–0.9)	
Vomiting †	26/87 (29.9)	22/67 (32.8)	1.1 (0.6–2.3)	
Lethargy†	19/87 (21.8)	10/67 (14.9)	0.6 (0.3–1.5)	
Symptoms ≥3 d before admission	58/158 (36.7)	74/139 (53.2)	2.0 (1.2–3.1)	
Admission to intensive care unit	1/159 (1.0)	2/139 (1.4)	2.3 (0.2–25.7)	
Mechanical ventilation needed	0/159 (0)	1/139 (1.0)	Not calculated	
Supplemental oxygen needed	37/159 (23.3)	61/139 (43.9)	2.6 (1.6–4.2)	
Antimicrobial drugs prescribed on admission	151/158 (95.6)	134/139 (96.4)	1.2 (0.4–4.0)	
Duration of hospitalization ≥2 d	117/157 (74.5)	107/138 (77.5)	1.2 (0.7–2.0)	
No. deaths/no. patients (case-fatality ratio)	2/158 (1.3)	2/138 (1.5)	1.1 (0.2–8.3)	

*Pos, positive; OR, odds ratio.
†Patients <5 y of age.

A(H3N2) virus (*4–6*). We found that hospitalization with influenza B virus infection was associated with HIV infection. This finding suggests that underlying immunosuppression can trigger severe influenza illness requiring hospitalization for infection caused by virus types, such as influenza B, that can cause milder illness in immunocompetent persons.

Unlike case-fatality rates and disease severity previously reported from South Africa and other countries, we found no differences in case-fatality rates or severity in South Africa during the years studied among the virus types and subtypes or between the first and second waves of the A(H1N1)pdm09 pandemic. Previous excess death models have suggested increased deaths in years when influenza A(H3N2) virus circulated in South Africa (*7*). The contrast between case-fatality and severity found in this analysis and that observed in previous studies in South Africa might be the result of different methods

or different study periods. Although our study was conducted over fewer years and might have had less power to detect differences at a population level, we were able to look at markers of severity in individual cases and to compare different waves of A(H1N1)pdm09 virus infection.

This study has several limitations. We compared influenza types and subtypes across 4 years, so some associations might have resulted from changes in prevalence of other diseases such as HIV over the same period. We do not have data on nonrespiratory influenza disease, which might have different associations with influenza virus types and subtypes than respiratory influenza disease. Although obesity and pregnancy have been associated with infection with influenza A(H1N1)pdm09 virus, we identified few case-patients who were pregnant, and obesity was not included in our analysis because so few obese case-patients were identified by surveillance. Other factors and conditions, such as neuromuscular disorders that are associated with severe influenza disease, might be associated with specific types and subtypes, but we were unable to evaluate this association because of the small number of patients with these conditions. Patients were not enrolled on weekends, which could introduce bias if patients had more or less severe disease on weekends than patients enrolled during the week. Last, most patients were identified at a single surveillance site, so the results might more strongly reflect differences observed at that site.

Vaccination remains the best way to prevent influenza infection. Influenza vaccination coverage is very low in South Africa (*25*). In that country, influenza vaccination is recommended for HIV-infected persons (*26*), and efforts should be made to encourage higher vaccine coverage. Although differences exist between infection with different influenza types and subtypes, particularly with regard to age distribution and co-infections, it can be difficult for the clinician to differentiate infection by different types and subtypes for individual patients. Current treatment recommendations do not differ according to the subtype with which a patient is infected, in part because it is not common to type and subtype the virus in individual patients in time for clinical decision-making.

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Dr Cohen is a pediatrician, medical epidemiologist, and director of the Influenza Program at the Centers for Disease Control and Prevention–South Africa, Pretoria, South Africa. His research interests involve public health in the fields of pneumonia and global child health.

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Deaths Attributable to Carbapenem-Resistant *Enterobacteriaceae* Infections

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We evaluated the number of deaths attributable to carbapenem-resistant *Enterobacteriaceae* by using studies from around the world published before April 9, 2012. Attributable death was defined as the difference in all-cause deaths between patients with carbapenem-resistant infections and those with carbapenem-susceptible infections. Online databases were searched, and data were qualitatively synthesized and pooled in a metaanalysis. Nine studies met inclusion criteria: 6 retrospective case–control studies, 2 retrospective cohort studies, and 1 prospective cohort study. *Klebsiella pneumoniae* was the causative pathogen in 8 studies; bacteremia was the only infection in 5 studies. We calculated that 26%–44% of deaths in 7 studies were attributable to carbapenem resistance, and in 2 studies, which included bacteremia and other infections, –3% and –4% of deaths were attributable to carbapenem resistance. Pooled outcomes showed that the number of deaths was significantly higher in patients with carbapenem-resistant infections and that the number of deaths attributable to carbapenem resistance is considerable.

Carbapenem-resistant strains have emerged among species belonging to the *Enterobacteriaceae* family (*1*,*2*). Carbapenemases are a class of enzymes that can confer resistance to carbapenems and other β-lactam antibiotic drugs, but not all carbapenemase-producing isolates are carbapenem-resistant (*3*,*4*). Among the known carbapenemases are *Klebsiella pneumoniae* carbapenemase (KPC) and Verona integrin–encoded metallo-β-lactamase (VIM) (*5*). Several outbreaks caused by carbapenem-resistant *Enterobacteriaceae* (CRE) have been recorded in health care facilities around the world (*6*–*13*), and in some places, CRE have become endemic (*14*–*18*). Serious concurrent conditions (*3*,*4*,*19*–*22*) and prior use of fluoroquinolones (*20*,*23*,*24*), carbapenems (*22*,*25*), or broad-spectrum cephalosporins (*20*,*22*) have been independently associated with acquisition of infections caused by CRE.

Several studies have provided data regarding clinical outcomes for CRE infections. However, controversy remains concerning the number of deaths among persons infected with CRE compared with the number among persons infected with carbapenem-susceptible *Enterobacteriaceae* (CSE) (*23*,*26*). In this context, the goal of our study was to evaluate the number of deaths attributable to CRE infections by conducting a systematic review and metaanalysis of the available data.

Methods

Literature Search

We performed a systematic search in the PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and Scopus (http://www.scopus.com/home.url?zone=header&origin=searchbasic) databases on April 9, 2012, by using the following search terms: carbapenem-resistant or carbapenemase-producing or KPC and outcome or mortality. We also conducted a hands-on search of the reference lists of relevant studies to identify additional studies. Articles published in languages other than English, French, German, Italian, Spanish, or Greek were not evaluated.

Study Selection Criteria

Any article that compared death rates between CRE-infected patients and CSE-infected patients was considered eligible for inclusion in the review. Studies that reported only on carbapenem-resistant isolates (without comparison

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with susceptible isolates) were excluded, as were studies that compared patients who had carbapenem-resistant infections with patients who were not infected. Other excluded studies were those that did not distinguish the outcomes for infected patients from those for colonized patients and studies that reported on isolates resistant to a carbapenem other than imipenem, meropenem, or doripenem. Studies that compared infection-related but not all-cause deaths among CRE-infected patients with those among CSE-infected patients were excluded because of homogeneity of the outcome analysis. Unpublished studies presented as abstracts at scientific conferences were not eligible for inclusion because of the low quantity of information provided in these types of articles.

Data Extraction

Literature search, study selection, and data extraction were performed independently by 2 of the authors (G.S.T. and K.Z.V.). Any disagreement was resolved by consensus in meetings with all investigators and by reviewing the original articles to assess validity of the abstracted data. Extracted data included study characteristics (author, design, country, period, number of patients) and patient characteristics (type of infection, causative pathogen, and concurrent condition or severity of illness score at admission). We also recorded the all-cause deaths in each group of patients (CRE and CSE), deaths attributable to carbapenem resistance, and the independent predictors of all-cause deaths evaluated in the total population of each study.

For studies in which analyses were performed for the individual patient groups (CRE and CSE) rather than the study population as a whole, we could not conclude whether carbapenem resistance predicted death. Thus, we did not extract results from such studies.

Definitions and Outcomes

We compared 2 groups of patients: CRE-infected and CSE-infected patients. The primary outcome of our analysis was the comparison of all-cause deaths between CRE and CSE groups with the same type of infection (i.e., bacteremia or pneumonia) caused by the same species (i.e., *K. pneumoniae*). The secondary outcome was deaths attributable to carbapenem resistance in *Enterobacteriaceae* infections. Attributable death was defined as the difference in all-cause deaths between the 2 compared groups.

Carbapenem resistance was defined as the resistance of a pathogen to imipenem, meropenem, or doripenem, according to the susceptibility breakpoints that had been applied by the investigators of each study. Carbapenemase production was not considered as carbapenem resistance if the MIC of an antibiotic was within the susceptible range according to those breakpoints.

Statistical Analysis

We calculated pooled risk ratios (RRs) and 95% CIs regarding deaths. The statistical heterogeneity between studies was assessed by using the χ^2 test ($p<0.10$ was defined to indicate the presence of heterogeneity) and the I^2 index (for assessing the degree of heterogeneity) (*27*). The random effects model was applied because we considered the nonrandomized, comparative studies that we analyzed to be heterogeneous by definition. We used RevMan 5.1 software (Nordic Cochrane Centre of the Cochrane Collaboration, Copenhagen, Denmark) to perform the metaanalysis.

Results

A total of 364 articles were retrieved during the search process: 152 in PubMed, 207 in Scopus, and 5 from hands-on searches of the reference lists of relevant studies. Of the 364 articles, 9 were considered eligible for inclusion in the analysis (*3*,*4*,*19*,*21–23*,*26*,*28*,*29*). The study selection process is depicted in the Figure. A total of 985 patients were included in the 9 eligible studies.

The characteristics and outcomes of the included studies are presented in the online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/20/7/12-1004-Techapp1.pdf. Of the 9 studies, 8 were retrospective: 6 case-control studies with a total of 527 patients (*3*,*19*,*21–23*,*28*) and 2 cohort studies with a total of 296 patients (*26*,*29*). The remaining study was a prospective cohort study with 162 patients (*4*). The causative pathogen was *K. pneumoniae* in 8 studies (*3*,*4*,*21–23*,*26*,*28*,*29*) and *Escherichia coli* in 1 study (*19*). Among studies that provided relevant data, metallo-β-lactamases were the carbapenemases produced by *Enterobacteriaceae* in 2 studies (*3*,*4*), and KPC and VIM were the carbapenemases produced by *Enterobacteriaceae* in another study (*21*). In 6 studies, bacteremia represented the only infection or the majority of infections (*3*,*4*,*19*,*21*,*22*,*26*). In another study, cases of bacteremia constituted 26% of all infections (*23*). The remaining 2 studies included patients with undetermined infections (*28*) or infections other than bacteremia (*29*). An MIC of ≤4 μg/mL was considered the susceptibility breakpoint for imipenem, meropenem, and doripenem in 8 of the 9 studies (*3*,*4*,*19*,*21–23*,*26*,*29*); relevant data were not provided by 1 study (*28*).

In 3 studies, CRE-infected and CSE-infected patients had similar underlying diseases (*21*,*23*,*28*). However, in 3studies that provided specific relevant data, CRE-infected patients were more likely than CSE-infected patients to experience heart or liver failure or malignancy or to be transplant recipients (*19*,*22*,*26*). In 5 of the 9 studies, concurrent condition scores or severity of illness scores for the 2 groups of patients were compared by using the Acute Physiology and Chronic Health Evaluation II severity of disease classification system, Sequential Organ

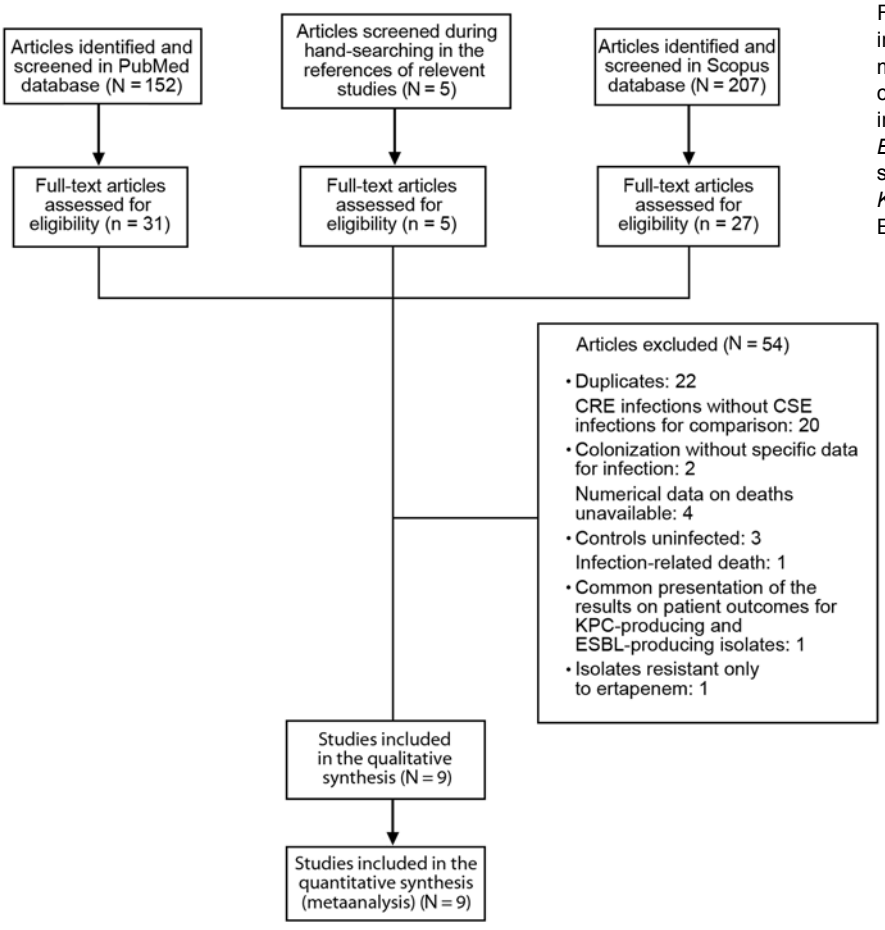

Figure. Selection process for studies included in a systematic review and metaanalysis of deaths attributable to carbapenem-resistant *Enterobacteriaceae* infections. CRE, carbapenem-resistant *Enterobacteriaceae*; CSE, carbapenem-susceptible *Enterobacteriaceae*; KPC, *Klebsiella pneumoniae* carbapenemase; ESBL, extended-spectrum β-lactamase.

Failure Assessment scoring system, Pitt bacteremia score, or Charlson comorbidity index (*3*,*21*,*23*,*26*,*28*). Of the 5 studies, 3 showed significantly higher scores for CRE-infected than CSE-infected patients (*19*,*21*,*26*). Comparative data on the appropriateness of empirical antibiotic treatment were provided by only 2 studies (*4*,*26*). Patients with infections caused by CRE were more likely than those with infections caused by CSE to receive inappropriate empirical treatment (88% vs. 39%, odds ratio 4.1, 95% CI 1.3–12.9).

Deaths

Reported all-cause deaths differed significantly between the 2 groups of patients in 5 of 9 studies (*3*,*4*,*19*,*22*,*26*). The CRE-attributable deaths that we calculated varied from 26% to 44% in 7 studies (*3*,*4*,*19*,*21*,*22*,*26*,*29*) and were -3% and -4%, respectively, in 2 studies (*23*,*28*).

Our pooled analysis of the 9 studies (985 patients) showed that the death rate was higher among CRE-infected than CSE-infected patients (RR 2.05, 95% CI 1.56–2.69) (online Technical Appendix Figure). Moderate heterogeneity was detected between all studies (I^2 = 51%). Subgroup analysis was performed for studies that included only or mostly patients with bacteremia. In 6 studies (718 patients), the patients who had bacteremia caused by CRE had higher death rates than those who had bacteremia caused by CSE (RR 2.19, 95% CI 1.82–2.63) (online Technical Appendix Figure) (*3*,*4*,*19*,*21*,*22*,*26*). No heterogeneity was detected among these studies. Three studies (267 patients) provided data regarding patients with bacteremia or other infections (*23*,*28*,*29*). The death rate did not differ between CRE-infected patients and CSE-infected patients in those studies (RR 1.46, 95% CI 0.47–4.49) (online Technical Appendix Figure). Considerable heterogeneity was detected among studies (I^2 = 77%).

Predictors of Death

We performed a multivariable analysis of deaths for the total study population in 7 of the 9 studies (*3*,*4*,*21–23*,*26*,*29*); in 6 of the 7 studies, adjustment was made for concurrent condition score or severity of illness score (*3*,*4*,*21*,*23*,*26*,*29*). In 7 studies, higher concurrent condition score at hospital admission or more severe patient condition independently predicted death (*3*,*4*,*21–23*,*26*,*29*). Five studies showed that carbapenem resistance was independently associated with death (*3*,*4*,*22*,*26*,*29*), and another

study, which did not specifically analyze carbapenem resistance, showed that KPC production was an independent predictor of death (*21*).

Discussion

The main finding of this metaanalysis is that the rate of CRE-attributable deaths ranged from 26% to 44% in 7 studies (*3*,*4*,*19*,*21*,*22*,*26*,*29*) and was –3% and –4%, respectively, in 2 studies (*23*,*28*). Furthermore, CRE-infected patients had an unadjusted number of deaths 2-fold higher than that for CSE-infected patients.

Six of the included studies showed significantly more deaths among CRE-infected than CSE-infected patients (*3*,*4*,*19*,*22*,*26*,*28*). In the 3 remaining studies, the lack of a significant difference in death rates for the CRE-infected and CSE-infected patients could be explained by the similarity of underlying disease characteristics for the 2 groups of patients (*21*,*23*,*28*). On the contrary, in the 3 studies that provided relevant data, concurrent condition scores or severity of illness scores were higher in CRE-infected than CSE-infected patients (*19*,*22*,*26*). In 2 studies, the Acute Physiology and Chronic Health Evaluation II score was independently associated with death (*3*,*23*).

A critical finding of our metaanalysis is that the number of deaths was 2-fold higher among patients with bacteremia caused by CRE than among patients with bacteremia caused by CSE (*3*,*4*,*19*,*21*,*22*,*26*). However, a significant difference in death rates was not detected between the 2 compared groups in studies reporting on patients with undetermined infections, patients with infections other than bacteremia, or patients among whom the percentage of bacteremia cases was low (*23*,*28*,*29*). Therefore, it could be suggested that the higher rate of death among patients with CRE infections, compared with CSE infections, is due to the higher rate of death among patients with bacteremia caused by CRE. The smaller number of patients included in this subgroup analysis (267 patients) compared with the number in the group who had bacteremia as the only infection (718 patients), along with the considerable heterogeneity among the included studies, but not among the type of infection, may justify the absence of statistical significance. Apart from the sample size, other variables that have not been analyzed might have affected the strength of the death (or outcomes) analysis. Additional and larger studies reporting on infections other than bacteremia could elucidate this issue.

Many factors other than underlying concurrent condition or severity of illness at the initial medical visit could be responsible for the higher rate of death among patients with infections caused by CRE. A key relevant factor could be the higher frequency of inappropriate empirical treatments among the CRE patients. Only 2 of the included studies provided comparative data for patients who received appropriate empirical antibiotic treatment (*4*,*26*). Those studies showed that patients with infections caused by CRE were significantly more likely than those infected by CSE to receive inappropriate antibiotic treatment. In addition, another study showed that inappropriate empirical antibiotic treatment was independently associated with death in patients infected with KPC-producing *K. pneumoniae* (*30*). Apart from empirical treatment, the antibiotics used for treatment might be less effective against carbapenem-resistant infections as well. There are few published clinical data available on the effectiveness of colistin, tigecycline, fosfomycin, and gentamicin (which are likely to be active in vitro against CRE) for the treatment of CSE infections. From a pharmacokinetic–pharmacodynamic perspective, these agents might be suboptimal for the treatment of serious CRE infections, particularly bloodstream infections (*31*).

Five studies showed that carbapenem resistance (*3*,*4*,*26*,*29*) or KPC production (*21*) were independent predictors of death after adjustment for concurrent condition or severity of illness. KPC ST258, a widely distributed clone of KPC-producing *K. pneumoniae*, is considered a successful pathogen because of its ability to persist and spread, causing nosocomial outbreaks (*32*).

Data regarding the association between carbapenem resistance and virulence are scarce. In vivo and in vitro findings from1 study argued that carbapenem-resistant *K. pneumoniae* isolates are less virulent and fit than carbapenem-susceptible isolates in an antibiotic-free environment (*33*).This reduction in virulence and fitness was due to the loss of the major porins OmpK35/36 (through which β-lactams penetrate into *K. pneumoniae* isolates) and the presence and expression of OmpK26 in the resistant isolates.

In addition, the number of deaths attributable to CRE infections varied between studies; the susceptibility profile of the microbes in the control groups could have an influence on this outcome. Metaanalyses have shown that death rates are higher among patients with infections caused by extended-spectrum β-lactamase–producing or multidrug-resistant *Enterobacteriaceae* isolates than among patients with infections caused by non–extended-spectrum β-lactamase or non–multidrug-resistant isolates (*34*–*36*). However, the type of infections, concurrent conditions, prior antibiotic use, and length of preinfection hospital stay could also have played a role in the observed differences in attributable death in our metaanalysis. Also, the virulence characteristics of the carbapenem-resistant isolates may differ among isolates with different types of carbapenemases or among strains that belong to different clones. This is important because some of the studies might have only included clonal isolates (e.g., KPC isolates in an endemic setting), and others might have included isolates from different clones (e.g., VIM producers that are typically polyclonal).

It should be emphasized that the findings of this systematic review and metaanalysis may not apply to the current Clinical and Laboratory Standards Institute breakpoints for carbapenem susceptibility; in 2010, the susceptibility breakpoint for imipenem, meropenem, and doripenem was lowered from 4 μg/mL to 1 μg/mL (*37*). There were no available data among the included studies that we could use to classify deaths according to the new breakpoints.

Our study findings should be interpreted in light of certain other limitations. The effect of the possible confounding factors (i.e., concurrent condition, severity of illness) on death could not be detected in the pooled analysis because only unadjusted data were entered. Furthermore, 8 of the 9 included studies had a retrospective study design. Data from such studies may be suboptimal compared with data from prospective studies, but this could not be tested due to the lack of prospective studies.

In conclusion, our findings suggest that the number of deaths attributable to carbapenem resistance is considerably high among persons with *Enterobacteriaceae* infections. Further original studies are needed to determine the reason(s) for the increased risk for death from carbapenem-resistant isolates versus carbapenem-susceptible isolates. Our findings imply a need for strict infection control measures and a need for new antibiotics to protect against CRE infections.

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Undiagnosed Acute Viral Febrile Illnesses, Sierra Leone

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Sierra Leone in West Africa is in a Lassa fever–hyperendemic region that also includes Guinea and Liberia. Each year, suspected Lassa fever cases result in submission of ≈500–700 samples to the Kenema Government Hospital Lassa Diagnostic Laboratory in eastern Sierra Leone. Generally only 30%–40% of samples tested are positive for Lassa virus (LASV) antigen and/or LASV-specific IgM; thus, 60%–70% of these patients have acute diseases of unknown origin. To investigate what other arthropod-borne and hemorrhagic fever viral diseases might cause serious illness in this region and mimic Lassa fever, we tested patient serum samples that were negative for malaria parasites and LASV. Using IgM-capture ELISAs, we evaluated samples for antibodies to arthropod-borne and other hemorrhagic fever viruses. Approximately 25% of LASV-negative patients had IgM to dengue, West Nile, yellow fever, Rift Valley fever, chikungunya, Ebola, and Marburg viruses but not to Crimean-Congo hemorrhagic fever virus.

The West African country of Sierra Leone is located in a Lassa fever–hyperendemic region that also includes Guinea and Liberia. The causative agent of Lassa fever is Lassa virus (LASV), a member of the *Arenaviridae* family. Lassa fever is a severe, often fatal, hemorrhagic illness; the virus causes 100,000–300,000 infections and 5,000 deaths each year in the region (*1*).

In 2002, Sierra Leone emerged from a brutal 11-year civil war that left the country with little infrastructure and much of its formal economy destroyed. Today Sierra Leone is undergoing substantial economic growth; however, poverty, unemployment, and inadequate health care remain major challenges. Through the Mano River Union–Lassa Fever Network, a variety of organizations are building diagnostic capacity for Lassa fever that will lead to better understanding of the disease and its treatment (*2–5*). These scientific efforts are centered in eastern Sierra Leone at the Kenema Government Hospital (Kenema, Sierra Leone) within the Lassa fever–hyperendemic region (Figure). The Lassa Fever Ward, a 16-bed facility on the hospital grounds, is dedicated to treating patients suspected of having Lassa fever and is supported by the Lassa Diagnostic Laboratory.

Each year, suspected Lassa fever infections result in submission of ≈500–700 samples to the Kenema Government Hospital Lassa Diagnostic Laboratory (J. Bangura, unpub. data). Samples come from throughout the Lassa fever–hyperendemic region and initially are screened for malaria by thick blood smear and, if negative, are tested for LASV. LASV infection is determined by the presence of virus detected by an antigen-detection ELISA and by the presence of IgM determined by using an IgM-capture ELISA. Generally only 30%–40% of samples tested are positive for LASV antigen and/or LASV-specific IgM; therefore, 60%–70% of patients have acute diseases of unknown origin. We investigated what other arthropod-borne and hemorrhagic fever viral diseases might be causing serious illness in the region and confounding the diagnosis of Lassa fever. We tested samples from these patients using IgM-capture ELISAs to virus pathogens that could occur in the region and mimic Lassa fever. We tested for IgM to dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV), Rift Valley fever virus (RVFV), chikungunya virus (CHIKV), Ebola virus (EBOV), Marburg virus (MBGV), and Crimean-Congo hemorrhagic fever virus (CCHFV). Follow up analyses included IgG ELISAs and/or confirmatory plaque-reduction neutralization tests (PRNTs). This study provides a better understanding of the differential diagnoses for Lassa fever in the region, which can lead to improved diagnostic capability and disease treatment.

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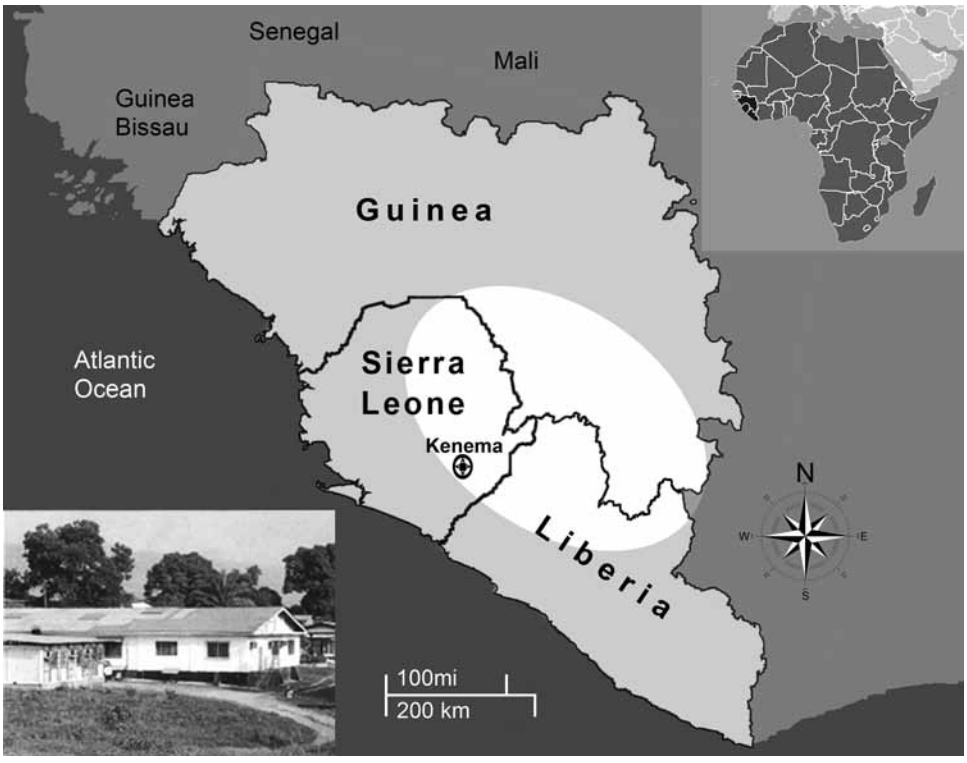

Figure. Lassa fever–hyperendemic region (white area) comprising parts of Guinea, Sierra Leone, and Liberia in West Africa. Inset image: Lassa Diagnostic Laboratory, Kenema Government Hospital, Kenema, Sierra Leone.

Materials and Methods

Patient Samples

Blood samples collected during October 2006–October 2008 from patients with suspected Lassa fever were submitted to the Lassa Diagnostic Laboratory. Most samples were from acutely ill patients from eastern Sierra Leone; some were submitted from Liberia and a few from Guinea. Samples were from patients whose illness met a surveillance case definition for Lassa fever (Table 1) and were processed as described elsewhere (*5*). Samples that were malaria negative by thick blood smear and were from patients whose illness failed to respond to antimicrobial drugs were initially tested in the Lassa Diagnostic Laboratory for LASV antigen by antigen detection ELISA and for LASV-specific IgM by IgM-capture ELISA. LASV-negative samples were then tested for IgM to DENV, WNV, YFV, RVFV, and CHIKV before being transferred to the US Army Medical Research Institute of Infectious Diseases for additional testing for IgM to EBOV, MBGV, and CCHFV. IgM-positive samples were tested for IgG by ELISA and/or confirmatory PRNT, depending on the volume of sample available.

Research on human subjects was conducted in compliance with US Department of Defense, federal, and state statutes and regulations relating to the protection of human subjects, and adhered to principles identified in the Belmont Report (1979) (www.hhs.gov/ohrp/humansubjects/guidance/belmont.html). All data were gathered and human subjects research was conducted under an institutional review board–approved protocol (no. HP-09-32).

Antigens and Antiserum

Viruses used in production of ELISA antigenic materials were the 4 DENVs (DENV-1 Hawaii, DENV-2 New Guinea C strain [*6*,*7*], DENV-3 H87 strain, and DENV-4 H241 strain [*8*]); WNV EG101 strain (*9*); YFV Asibi strain (IgM ELISA) or YFV 17D strain (Connaught) (IgG ELISA) (*10*); CHIKV Indo23574 strain; RVFV ZH 501 strain (*11*); EBOV Zaire-76 strain (*12*); MBGV Musoke strain (*13*); CCHFV IbAr10200 strain (*14*); and LASV Josiah strain (*15*). The antigens were prepared and optimized as previously described (*16*). All viruses were propagated at Biosafety Level 3 or 4, as appropriate. Briefly, the viruses were grown in appropriate continuous cell lines until cytopathic effects were observed in 50%–75% of the cells. Cell culture supernatants, used in the IgM- and IgG-capture ELISAs, were clarified by centrifugation, inactivated by treatment with 0.3% β-propiolactone, aliquoted, and stored at –70°C. Cell lysates for the IgG sandwich ELISAs were produced from virus-infected cell pellets that were resuspended, sonicated, clarified, aliquoted, and stored at –70°C. Virus-infected cell culture supernatants and cell lysates were inactivated by γ-irradiation (3×10^6 rad) and safety tested to ensure inactivation. Optimal dilutions of antigens were determined by checkerboard

Table 1. Case definition used to detect suspected Lassa fever at Kenema Government Hospital, Kenema, Sierra Leone, October 2006–October 2008*

Major criteria	Minor criteria
Known exposure to person with suspected Lassa fever	General myalgia or arthralgia
Abnormal bleeding (from mouth, nose, rectum, or vagina)	Headache
Edema of the neck and/or face	Sore throat
Conjunctivitis or subconjunctival hemorrhage	Vomiting
Spontaneous abortion	Abdominal pain/tenderness
Petechial or hemorrhagic rash	Retrosternal pain
Onset of tinnitus or altered hearing	Cough
Persistent hypotension	Diarrhea
Elevated liver transaminases (aspartate aminotransferase higher than alanine aminotransferase)	Profuse weakness
	Proteinuria
	Leukopenia (leukocytes <4,000 μL)

*To be tested for suspected Lassa fever, a patient had to have a fever >38°C and not respond to appropriate antimalarial and antimicrobial drug treatment within 72 h.

titrations against virus-specific antibodies. Negative or mock antigens, both supernatant and cell lysate, used as negative controls, were prepared from uninfected cell monolayers as described above.

IgM-Capture ELISA

The IgM-capture ELISAs were performed as described elsewhere (*17*,*18*). Briefly, 96-well round bottom polyvinyl chloride microtiter plates were coated with diluted goat anti–human IgM heavy chain capture antibody overnight at 4°C. The capture plates were washed, and then patient samples, diluted 1:100, were added and incubated for 1 h at 37°C. With every assay, we included at least 1 known positive control serum sample to ensure assay was working. and 4 known negative control serum samples were used to determine assay cutoff. After washing the plates, the inactivated cell culture supernatant antigen or mock antigen was added, and the plates were incubated for 1 h at 37°C. Unlike the other assays that used a single virus strain, the DENV IgM ELISA antigen was a mixture of all 4 viruses and was recognized by antibodies against each (*19*). Samples were tested in duplicate against each virus and mock antigen. After the plates were washed, a secondary detector antibody (mouse or rabbit polyclonal serum antibody titers raised against the target virus) was added, and the plates were incubated for 1 h at 37°C. After additional washing, horseradish peroxidase (HRP)–labeled antidetector species antibody (goat anti–mouse IgG, heavy and light chain, or HRP-labeled goat anti–rabbit IgG, heavy and light chain), was added to the mixture, and the plates were incubated for 1 h at 37°C. The plates were washed again, ABTS (2, 2′ -azino-di[3-ethylbenzthiazoline-6-sulfonate]) substrate was added, and the plates were incubated for 30 min at 37°C. The optical densities (OD) were determined at 410 nm in an automated ELISA reader. We determined an adjusted OD for each sample by subtracting the average mock antigen OD from the average positive antigen OD. For each assay, the mean deviation and SD of the adjusted ODs were determined for all 4 negative control samples. The cutoff of each assay was the mean OD plus 3 SDs rounded up to the nearest tenth. This OD was typically 0.2. A sample was considered positive if the OD was greater than or equal to this OD cutoff.

IgG ELISA

We conducted the IgG ELISAs on selected serum samples using a modification of the IgM-capture ELISAs described above. Briefly, polyvinyl chloride microtiter plates were coated with inactivated cell lysate or mock antigen overnight at 4°C (*19*). To detect RVFV IgG, we used a sandwich IgG ELISA; plates were coated with an RVFV-specific nucleocapsid monoclonal antibody overnight at 4°C to capture inactivated cell culture supernatant onto the plate surface (1 h at 37°C) (*18*). Antigen-coated plates were washed; diluted patient samples (1:100) were added, and plates were incubated for 1 h at 37°C. Samples were tested in duplicate against each virus and mock antigen, and we included at least 1 known positive control sample and 4 known negative control samples with every assay. After the plates were washed, diluted HRP-labeled mouse anti–human IgG (Fc-specific) conjugate was added, and the plates were incubated for 1 h at 37°C. After again washing the plates, ABTS substrate was added, and the plates were incubated for 30 min at 37°C, and the absorbance at 410 nm was determined. Mathematical calculations and assay cutoffs were determined as described for the IgM-capture ELISA.

PRNT

PRNTs were conducted on selected serum samples as described elsewhere (*20*). Briefly, heat-inactivated serum samples were diluted 4-fold from 1:10 to 1:10,240 and were tested for their ability to neutralize ≈100 PFU of the challenge virus. Each sample dilution was tested in duplicate. Both known positive and negative control serum samples were included with every assay. Serum–virus mixtures were incubated overnight at 4°C and then inoculated onto 85%–100% confluent monolayers of the appropriate cell lines grown in 6-well tissue culture plates. After incubation for 1 h at 37°C, a nutrient 0.5%–1% agarose overlay was

added, and plates were incubated at 37°C for the appropriate number of days for the virus, then stained with a second overlay containing 4%–5% neutral red; plaques were counted 24–48 h later. To detect a wider range of viruses neutralized by the serum, we recorded the reciprocal of the highest serum dilution reducing 50% of the plaque assay dose. A titer ≥10 was considered positive. The virus strains used in the PRNT were the same as the strains used for ELISA antigen, with the exception of WNV(NY99 strain) (*21*). In addition, the PRNT used the alphaviruses, o'nyong-nyong (ONNV), Semliki Forest (SFV), and Sindbis (SINV); and the ebolaviruses, Sudan Gulu strain (SUDV) and Taï Forest (Côte d'Ivoire) virus (TAFV).

Results

We tested serum samples from 253 patients submitted to the Lassa Diagnostic Laboratory during 2006–2008 for IgM to the arthropod-borne and hemorrhagic fever viruses of interest (Table 2). Because of limited amounts of serum, not all samples were tested for antibodies to all viruses. Of the arthropod-borne viruses, the prevalence of DENV antibodies (4.3%) was highest, followed by CHIKV (4.0%). The prevalences of other viruses were <3.0%; WNV, 2.8%; YFV, 2.5%; and RVFV, 2.0% of patients tested. No antibodies to the tick-borne virus, CCHFV, were found in any samples tested. Antibody prevalence to the hemorrhagic fever viruses, EBOV and MBGV, were 8.6% and 3.6%, respectively.

Samples positive for IgM and with sufficient volumes were tested for IgG. Of the 11 DENV IgM–positive patients, 6 (2.4% of total) were IgM positive only (Table 2). Of the 7 WNV IgM–positive patients, 3 (1.2%) were IgM positive only. Of the 10 CHIKV IgM–positive patients, 5 (2.0%) were IgM positive only. Of the 8 MBGV IgM–positive patients, 7 (3.2%) were IgM positive only. No IgG was detected in the patients identified as IgM positive for YFV, RVF, or EBOV. Two patients were IgM positive for both EBOV and MBGV; 1 sample neutralized EBOV in PRNT and 1 did not. MBGV PRNTs were not possible because of a lack of positive control serum samples. No IgM-only samples reacted in >1 flavivirus assay. Testing of patients with undiagnosed acute febrile illness for antibodies against the 8 viruses suggested a possible cause for illness in 25.7% of the patients originally suspected of having Lassa fever; of these, 19.4% demonstrated only an IgM response, suggesting an acute infection.

Malaria parasites and LASV are known to occur in the region, and we excluded samples with evidence of either because of our interest in undiagnosed acute febrile illnesses. However, the sample group was tested for LASV IgG to better understand the prevalence of Lassa fever. Of 237 patients, 25.5% were positive for LASV-specific IgG (data not shown). Because of the high number of IgG-positive patients, we retested the samples for LASV IgM and demonstrated that 7 (3.0%) of the patients tested had LASV-specific IgM.

Table 2. Patients' antibody reactions to arthropod-borne and hemorrhagic fever virus antigens, Lassa Diagnostic Laboratory, Kenema, Sierra Leone, October 2006–October 2008*

Virus	No. positive /total (%)	No. IgM only positive/total (%)
Dengue	11/253 (4.3)	6/250 (2.4)
West Nile	7/253 (2.8)	3/250 (1.2)
Yellow fever	5/201 (2.5)	5/201 (2.5)
Rift Valley fever	5/253 (2.0)	5/253 (2.0)
Chikungunya	10/253 (4.0)	5/253 (2.0)
Ebola	19/220 (8.6)	18/219 (8.2)
Marburg	8/220 (3.6)	7/219 (3.2)
Crimean-Congo hemorrhagic fever	0/220	Not tested
Total	65/253 (25.7)	49/253 (19.4)

*Detected by IgM-capture ELISA in serum samples submitted to Lassa Diagnostic Laboratory (Kenema, Sierra Leon) for suspected Lassa fever. All samples tested were malaria negative by thick blood smear and Lassa virus negative by antigen detection and IgM-capture ELISA when initially tested. Samples with sufficient volumes were tested for the presence of IgG to determine samples that were IgM positive only.

Antibodies detected by ELISA cross-react, especially within a genus and particularly for antibodies elicited by alphaviruses and flaviviruses. Immunodiagnosis conventionally is confirmed by virus isolation or a rise in PRNT titer (*22*). We did not attempt to isolate viruses because the samples were heat inactivated to protect the laboratory personnel. Confirmation by PRNT ideally uses paired serum samples (acute- and convalescent- phase), demonstrating a 4-fold rise in titer. In our retrospective study, we had only acute-phase serum samples, but we performed PRNTs in an attempt to clarify the specific viruses causing severe disease in this region. Flavivirus-reactive patient serum, positive for IgM only, was tested for its ability to neutralize DENV-3, WNV, and YFV. Comparison of neutralizing titers could not attribute a specific virus as the cause of disease, with the exception of YFV (data not shown). Three of the 5 YFV IgM–positive serum samples demonstrated neutralizing titers to YFV and not to the other flaviviruses tested. These serum samples also were not reactive by IgM ELISA to any of the other 8 viruses tested. CHIKV-reactive patient serum, positive for IgM only, was tested for its ability to neutralize CHIKV, ONNV, SFV, and SINV (Table 3). Four of the 5 samples tested neutralized ONNV to a greater degree than CHIKV. We found no correlation between OD and neutralization titer. Five samples had evidence of IgM against RVFV, none of which had evidence of IgG. Of the 3 samples tested for neutralizing antibodies, only 1 neutralized RVFV (data not shown). No other bunyaviruses were available for comparison.

Comparative PRNTs for the ebolaviruses used EBOV, SUDV, and TAFV, all of which are known to have circulated in Africa. Eighteen samples were IgM positive only by ELISA. Of these, 14 had sufficient volume for PRNT

Table 3. Results of Immunologic assays for serum samples that tested IgM positive only for alphaviruses, Lassa Diagnostic Laboratory, Kenema, Sierra Leone, October 2006–October 2008*

	CHIKV ELISA		Alphavirus PRNT			
Sample no.	IgM	IgG	CHIKV	ONNV	SFV	SINV
051–5	0.34	0.07	160	2,560	10	10
055–1	0.34	0.00	160	2,560	<10	10
132–1	1.32	0.00	640	2,560	40	<10
168–1	0.76	0.03	10	640	10	10

*Patient samples were tested for IgM and IgG reactivity in a CHIKV ELISA. Samples with only IgM were tested for their ability to neutralize specific alphaviruses, CHIKV, chikungunya virus; PRNT, plaque-reduction neutralization test; ONNV, o'nyong-nyong virus; SFV, Semliki Forest virus; SINV, Sindbis virus.

against all 3 ebolaviruses (Table 4). Eight of these neutralized EBOV, 7 of which were 4 times more reactive to EBOV than to the other ebolaviruses tested. One sample was 4 times more reactive to SUDV, and 1 neutralized TAFV but only at a 1:10 dilution. Four patient samples did not neutralize any of the ebolaviruses tested. We found no correlation between ELISA OD and neutralization titer. We did not test samples with evidence of MBGV-specific IgM by PRNT because no known neutralizing antibody was available to use as a control.

Discussion

In West Africa, as in many regions of Africa, infectious disease is part of everyday life. The cause of disease is often unknown or incompletely understood because of nonspecific clinical features, lack of diagnostic laboratory support, or little or no knowledge about disease prevalence in a region (*23*). Within the LASV-hyperendemic region, Lassa fever is always possible, but early signs and symptoms are similar to those of other viral, bacterial, and rickettsial diseases, which can confound a clinical diagnosis (*24*). Our aim was to investigate other viral diseases that cause acute febrile illnesses originally thought be Lassa fever. We investigated arthropod-borne and hemorrhagic fever viruses that were likely to occur in the region. More than 25% of the LASV-negative patients had evidence of infection with other arthropod-borne or hemorrhagic fever viruses.

Using only retrospective field-collected samples limited the analysis and thus our conclusions. In a prospective study, patients would be sampled during the acute phase and again during the convalescent phase of illness. Virus isolations, antigen-detection ELISAs, and/or reverse transcription PCR would be attempted on all acute-phase samples. Testing acute- and convalescent-phase serum would enable both IgM and IgG testing and confirm positive results by a ≥4-fold increase in neutralizing titer. In this retrospective study we had only acute-phase samples; therefore, our results can be considered presumptive only.

Because the samples submitted to the Lassa Diagnostic Laboratory were from patients with acute illness, IgM-capture ELISAs were used to detect the earliest antibody elicited in response to viral infection. We found evidence of IgM for flaviviruses (DENV, WNV, and YFV); the bunyavirus RVFV; the alphavirus CHIKV; and the filoviruses EBOV and MBGV (Table 2). We tested IgM-positive samples for IgG when possible. Most samples exhibited only IgM or very low IgG levels, suggesting acute-phase disease or the beginning of class switching (data not shown). Exceptions were the samples that had CHIKV antibodies; 6 of the 10 patients had higher IgG than IgM against CHIKV, suggesting late acute-phase or early convalescent-phase infection.

PRNT is the laboratory standard for immunologic assays. It measures in vitro virus neutralization and is the most virus-specific serologic test to confirm immunologic test results. Testing CHIKV-positive serum demonstrated that the patients were more likely to have been infected by ONNV, a related but separate virus species. The viruses can be distinguished genetically by sequence analysis but with greater difficulty by serologic testing (*25*). Antibodies to the 2 viruses are generally distinguishable only by PRNT. We demonstrated that the CHIKV ELISA we used can detect antibodies to both viruses and confirmed the results by PRNT (Table 3). Most patient samples that reacted in the CHIKV IgM ELISA were ONNV upon confirmatory testing in the PRNT (Table 3). PRNT results for other viruses provided some additional information but at times were incomplete because of limitations of available virus strains or appropriate positive controls. We found clear evidence for YFV infections in the samples tested, but data were incomplete for other flaviviruses. PRNT results for RVFV and MBGV infections were similarly inconclusive.

Table 4. Results of immunologic assays for serum samples testing IgM positive only for ebolaviruses, Lassa Diagnostic Laboratory, Kenema, Sierra Leone, October 2006–October 2008*

	ELISA		PRNT		
Sample no.	IgM	IgG	EBOV	SUDV	TAFV
060–1	0.35	0.00	40	<10	<10
076–1	0.45	0.00	40	<10	10
085–1	0.20	0.00	40	<10	<10
090–1	0.26	0.06	40	<10	<10
118–2	0.23	0.00	40	<10	<10
119–1	0.24	0.06	<10	<10	<10
120–1	0.38	0.00	<10	<10	10
121–1	0.58	0.00	<10	<10	<10
122–1	0.66	0.00	40	<10	<10
125–1	0.24	0.00	ND	ND	ND
129–2	0.37	0.00	<10	<10	<10
130–1	0.40	0.03	<10	40	<10
131–1	0.25	0.00	<10	<10	<10
132–1	0.21	0.09	ND	ND	ND
143–1	0.30	0.00	ND	ND	ND
144–1	0.35	0.06	40	<10	<10
182–1	0.38	0.00	ND	ND	ND
261–1	0.29	0.00	10	<10	<10

*Patient samples were tested for IgM and IgG reactivity in an EBOV ELISA. Samples with IgM only were tested for their ability to neutralize specific ebolaviruses, PRNT, plaque-reduction neutralization test; EBOV, Ebola virus; SUDV, Sudan Gulu strain; TAFV, Taï Forest (Côte d'Ivoire) viruses.

PRNT results for the ebolaviruses clearly indicated that most resulted from EBOV infections (Table 4). We found evidence that SUDV was responsible for 1 infection but no evidence for TAFV infection, the only ebolavirus isolated in West Africa. In the ebolavirus PRNTs, we did not include the newest discovered ebolavirus, Bundibugyo virus, which cross-reacts with EBOV in immunoassays (*26*). Ebolavirus infections in Sierra Leone might be the result of Bundibugyo virus or an ebolavirus genetic variant and not EBOV.

Several arthropod-borne viruses are known to circulate in West Africa (*23*). Using ELISA to look for IgM and IgG, we found indication of infections with the flaviviruses DENV, WNV, and YFV; the bunyavirus RVFV; the alphavirus CHIKV (shown to be ONNV by PRNT); and the filoviruses EBOV and MBGV. Evidence of flavivirus infections was not unexpected. DENV, WNV, and YFV infections have been reported in Sierra Leone and the surrounding region (*23*,*27*–*30*). CHIKV is thought to be enzootic in West Africa, maintained in a sylvatic cycle involving nonhuman primates and *Aedes* species mosquitoes (*25*). ONNV is a distinct virus species but closely related to CHIKV. CHIKV fever is described throughout the region, but ONNV disease has not been described in this immediate region. In 2003, an outbreak of ONNV was reported in Côte d'Ivoire (*31*).

RVFV, a bunyavirus in the *Phlebovirus* genus, is endemic to East and South Africa, but may not be established in West Africa (*32*–*34*). In this study, we found evidence of RVFV IgM and confirmation of at least 1 of them as neutralizing the virus. CCHFV, another bunyavirus in the *Nairovirus* genus, circulates in West Africa, but we found no evidence of CCHFV infections in any patient samples tested (*35*). The filoviruses represented the largest group of patient samples that reacted in our study. This finding was surprising because no filovirus has been reported in the region or in West Africa other than the initial isolation of TAFV in Côte d'Ivoire (*36*). These serologic results provide evidence that ebolaviruses are circulating and infecting humans in West Africa. All of the ebolavirus-reactive samples demonstrated only IgM and no evidence of IgG, suggesting acute infection. PRNT results indicated that the infecting virus was most closely related to EBOV, except for 1 SUDV-reactive patient sample. This finding was unexpected because our assumption was that any ebolavirus would more likely be TAFV, the only species described in West Africa. Although the serum samples were able to neutralize EBOV only at a low level (1:40 dilution), it is possible that the virus is an EBOV genetic variant. This presumptive diagnosis of EBOV infection extends the ebolavirus geographic region to Sierra Leone and the surrounding region. The MBGV-reactive samples, similar to the ebolavirus samples, had evidence only of IgM, suggesting acute infection. Unfortunately, we were unable to determine whether the samples could neutralize any MBGV because we were unable to acquire a known neutralizing serum to use as a positive control.

Our presumptive results provide some insight into the other viruses causing acute disease in the patients whose samples were submitted to the Lassa Diagnostic Laboratory. Although our results are not definitive, they demonstrate arthropod-borne and hemorrhagic fever viruses that should be considered when Lassa fever is suspected. These continued studies will add to the body of knowledge for Lassa fever and other arthropod-borne diseases and hemorrhagic fevers that occur naturally within Sierra Leone and West Africa.

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Borrelia miyamotoi sensu lato Seroreactivity and Seroprevalence in the Northeastern United States

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Borrelia miyamotoi sensu lato, a relapsing fever *Borrelia* sp., is transmitted by the same ticks that transmit *B. burgdorferi* (the Lyme disease pathogen) and occurs in all Lyme disease–endemic areas of the United States. To determine the seroprevalence of IgG against *B. miyamotoi* sensu lato in the northeastern United States and assess whether serum from *B. miyamotoi* sensu lato–infected persons is reactive to *B. burgdorferi* antigens, we tested archived serum samples from area residents during 1991–2012. Of 639 samples from healthy persons, 25 were positive for *B. miyamotoi* sensu lato and 60 for *B. burgdorferi*. Samples from ≈10% of *B. miyamotoi* sensu lato–seropositive persons without a recent history of Lyme disease were seropositive for *B. burgdorferi.* Our results suggest that human *B. miyamotoi* sensu lato infection may be common in southern New England and that *B. burgdorferi* antibody testing is not an effective surrogate for detecting *B. miyamotoi* sensu lato infection.

Relapsing fever, an arthropod-borne infection caused by several *Borrelia* spp. spirochetes, is transmitted by ticks and lice (*1*,*2*). In 1995, Fukunaga et al. (*3*) discovered a novel relapsing fever spirochete in the hard-bodied (ixodid) tick *Ixodes persulcatus* and named it *Borrelia miyamotoi*. This discovery greatly expanded the potential geographic range of relapsing fever borreliae for humans. Before this finding, only soft-bodied ticks were known to transmit tick-borne relapsing fever spirochetes to humans. In 2001, a related spirochete was detected in *I. scapularis* ticks in the northeastern United States (*4*); this and similar organisms have been designated *B. miyamotoi* sensu lato to distinguish them from the *B. miyamotoi* sensu stricto isolates from Japan (*5*). A subsequent study showed that ticks in 15 states in the northeastern and northern midwestern regions of the United States are infected with *B. miyamotoi* sensu lato and have an average prevalence of infection of 1.9% (range 0–10.5%) (*6*). *B. miyamotoi* sensu lato has now been found in all tick species known to be vectors of Lyme disease, including *I. pacificus* in the western United States, *I. ricinus* in Europe, and *I. persulcatus* and *I. ricinus* in Russia (*7*–*9*). The first human cases of *B. miyamotoi* sensu lato infection were reported from central Russia in 2011 (*9*). Several reports of *B. miyamotoi* sensu lato infection in humans have subsequently been published, including 3 in the United States, 1 in Europe, and 1 in Russia (*10*–*14*). Some of these reports suggest that *B. miyamotoi* sensu lato infection causes a nonspecific, virus-like illness. *B. miyamotoi* sensu lato and *B. burgdorferi*, the agent of Lyme disease, share several antigens that might cause cross-reactivity during serologic testing, which could lead to a misdiagnosis.

There are few data on the seroprevalence of *B. miyamotoi* sensu lato infection. To increase knowledge of the seroprevalence of this infection, we used assays for antibodies against *B. miyamotoi* sensu lato glycerophosphodiester phosphodiesterase (GlpQ), a protein that is absent

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from all Lyme disease *Borrelia* species (*15*), for evaluation of >1,000 archived serum samples from persons living in a Lyme disease–endemic region of the United States. We also performed standard 2-tiered testing for *B. burgdorferi* antibodies (*16*). Our aim was to compare the seroprevalence of *B. miyamotoi* sensu lato with that of *B. burgdorferi*. We also sought to determine whether persons seropositive for *B. miyamotoi* sensu lato would also have positive results for standard *B. burgdorferi* antibody testing.

Materials and Methods

Study Population

The serum samples evaluated in our study were obtained during 1991–2012 from 3 groups of persons living in areas of the northeastern United States where Lyme disease is endemic. Group 1 consisted of 639 persons from Block Island and Prudence Island, Rhode Island, and from Brimfield, Massachusetts, who participated in serosurveys for tick-borne infections. Persons participating in the serosurvey were healthy at the time of blood sampling and were enrolled during the spring and autumn of each year (*16*). All participants were asked to respond to a questionnaire and to provide a blood sample for serologic analyses of tick-borne infections.

Group 2 consisted of 194 patients from Block Island; Nantucket, Massachusetts; Mansfield, Connecticut; and the Lower Hudson Valley, New York, who were enrolled in studies of tick-borne diseases. At or near the time of sample collection, persons in this group were treated with doxycycline, amoxicillin, or amoxicillin/clavulanic acid for acute Lyme disease.

Group 3 consisted of 221 adult patients who experienced a febrile illness in the late spring or summer without features suggestive of an upper respiratory tract infection or gastroenteritis. A subgroup of group 3 consisted of 17 patients from the Lower Hudson Valley who were enrolled in a study during 1992–2009 to better characterize the clinical and laboratory features of human granulocytic anaplasmosis as a single infection or as a co-infection with early Lyme disease. Results for serologic testing, culture, buffy coat examination for morulae, and/or PCR showed that none of the patients was infected with *Anaplasma phagocytophilum* (*17*). All these patients resided in an area where *I. scapularis*–transmitted infection is highly endemic and, thus, had possible exposure to *I. scapularis* ticks. A second subgroup of group 3 consisted of 204 adult patients from Block Island, Mansfield, or Nantucket who had suspected Lyme disease or babesiosis. Testing showed that 25 of these patients had babesiosis but none had Lyme disease or anaplasmosis.

Serum samples were also obtained from 2 patients from the Udmurtia Republic, Russia, who had PCR-confirmed acute *B. miyamotoi* sensu lato infection. In addition, Creative Testing Solutions (Tempe, AZ, USA) provided an aliquot of residual serum used for blood screening from 300 blood donors who lived in Tempe or in Miami, Florida.

De-identified serum samples were used in this study. The study was approved by the Yale School of Public Health Human Investigation Committee, the New York Medical College Institutional Review Board, and the University of Connecticut Institutional Review Board.

Laboratory Procedures

Production of *B. miyamotoi* sensu lato GlpQ Antigen

B. miyamotoi sensu lato *glpQ* from strain LB-2001 cloned into the prokaryotic expression vector pXT7 (*18*), a derivative of pGEM4Z and pSP64T (Promega, Madison, WI, USA), was transformed into BL21 Star (DE3)/pLysS cells (Invitrogen, Carlsbad, CA, USA), and transformants were used for protein production (*6*). The chromosome sequence for the protein is in GenBank (accession no. CP006647) (*19*). The 39.1-kDa recombinant GlpQ (rGlpQ) containing an N-terminal His tag was purified over an Ni-NTA Superflow affinity column (QIAGEN, Valencia, CA, USA) as described by the manufacturer. Purity was assessed by sodium dodecyl sulfate electrophoresis of ≈1 μg of rGlpQ on a 4%–20% polyacrylamide gel and by Coomassie blue staining (Figure 1).

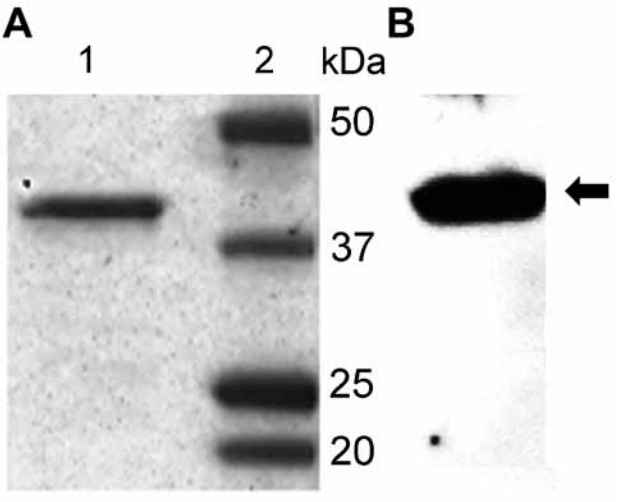

Figure 1. Polyacrylamide gel electrophoresis purification (A) and Western blot analysis (B) of recombinant glycerophosphodiester phosphodiesterase (rGlpQ). A) Coomassie blue staining of purified *Borrelia miyamotoi* sensu lato rGlpQ (lane 1) and of Precision Plus Protein Prestained Standards (Bio-Rad, Laboratories, Hercules, CA, USA) (lane 2). B) Western blot analysis of *B. miyamotoi* sensu lato–positive control mouse serum shows 39-kDa rGlpQ-specific band (arrow).

GlpQ Antibody ELISA

We developed a *B. miyamotoi* sensu lato IgG ELISA by using 20 C3H/HeJ mice (Jackson Laboratory, Bar Harbor, ME, USA). Ten of the mice were not infected. The other 10 age-matched mice were infected by using *B. miyamotoi* sensu lato–infected *I. scapularis* nymphal ticks. A month after the mice were infected, blood was obtained from all 20 mice for testing. Titrating concentrations of GlpQ protein and secondary antibody were tested in a checkerboard assay to determine the optimal concentrations for detecting *B. miyamotoi* sensu lato antibody. Results for the *B. miyamotoi* sensu lato ELISA were positive for all 10 *B. miyamotoi* sensu lato–infected mice and negative for all 10 uninfected mice.

To test the human serum samples, we coated ELISA plates with 100 μL of 1 μg/mL GlpQ protein in phosphate-buffered saline (PBS) and incubated the plates at 4°C for 18 h. We then added 300 μL of 1% bovine serum albumin in PBS buffer to the plates and incubated them for 2 h at room temperature. The plates were then emptied, and serum was added at a 1:320 dilution and incubated for 1 h. If acute- and convalescent-phase serum samples were available for a study participant, the initial dilution of the acute-phase sample was 1:80, and convalescent-phase samples were diluted to endpoint. The plates were then washed 3 times with wash buffer, and 100 μL of goat antihuman IgG secondary antibody was added at 0.002 mg/mL, incubated for 1 h, and then washed 3 times. BluPhos substrate (Kirkegaard & Perry, Gaithersburg, MD, USA) was added and allowed to react for 20 min before absorbance at 630 nm was determined. *B. miyamotoi* sensu lato–infected mouse serum was used as a positive control. As a negative control for each plate, we used serum samples that were negative for *B. miyamotoi* sensu lato antibody, as determined by ELISA and Western blot. The serum was obtained from 3 healthy participants who had no history of tick bite or tick-borne disease and who lived in an area where Lyme disease is endemic. The serum samples were tested by PCR for amplifiable *B. miyamotoi* sensu lato DNA and were negative. For mouse and human serum samples, a signal ≥3 SD above the mean of 3 noninfected serum controls was considered positive for *B. miyamotoi* sensu lato infection.

GlpQ Western Blot Antibody Assay

Purified GlpQ (500 ng) was electrophoresed on each replicate lane of a precast mini 4%–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to a nitrocellulose membrane using the Bio-Rad MiniTrans Blot Cell (Bio-Rad Laboratories). Replicate strips containing rGlpQ were blocked overnight at 4°C in PBS (pH 7.2)/5% dried milk/0.05% Tween 20. The blocked strips were then individually incubated with human serum at a 1:250 dilution at room temperature in PBS (pH 7.2)/2.5% dried milk/0.05% Tween 20 for 1 h. The strips were then washed 3 times and incubated for 1 h with horseradish peroxidase–conjugated rabbit anti-human IgG (Sigma-Aldrich, St. Louis, MO, USA) or with horseradish peroxidase–conjugated goat anti-human IgM (Invitrogen) at a 1:5,000 dilution in PBS (pH 7.2)/2.5% dried milk/0.05% Tween 20. Bound antibodies were detected by using Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Serum from ≈10% of the study participants reacted to a ≈55-kDa band, presumably a trace contaminant copurified with the rGlpQ generated in a bacterial expression system. Samples with a 39-kDa band corresponding to GlpQ on positive control mouse serum samples were considered GlpQ antibody–positive (Figure 1).

PCR DNA Amplification

We used a *B. miyamotoi* sensu lato PCR as described (*4*) to amplify *B. miyamotoi* sensu lato DNA in serum samples. *B. burgdorferi* DNA was amplified by using a standard PCR assay (*16*).

B. burgdorferi Antibody Detection

We detected serologic evidence of exposure to *B. burgdorferi* by using a whole-cell sonicate ELISA, C6 ELISA, or Western blot assay as described (*16*,*20*–*22*). Specimens were considered positive according to the criteria of the US Centers for Disease Control and Prevention (http://www.cdc.gov/lyme/diagnosistesting/LabTest/TwoStep/index.html).

Case Definitions

B. miyamotoi sensu lato–seropositive serum samples were defined by the presence of *B. miyamotoi* sensu lato antibody as determined by using ELISA and confirmatory Western blot assays for IgG alone or IgG plus IgM antibody. *B. burgdorferi* seropositive serum samples were defined by the presence of *B. burgdorferi* antibody as determined by ELISA and supplemental Western blot IgM or IgG assays.

Study participants were considered to have *B. miyamotoi* sensu lato infection if they had exhibited a fever >37.5°C and a ≥4-fold rise in antibody to *B. miyamotoi* sensu lato GlpQ protein between acute- and convalescent-phase serum samples, as determined by ELISA and confirmatory Western blot assays for IgG alone or IgG plus IgM. The time between acute- and convalescent-phase samples ranged from 2 wk to 2 mo. Study participants were considered to have Lyme disease if they had a physician-diagnosed erythema migrans skin lesion or a virus-like illness plus a test result that showed either PCR amplification

of *B. burgdorferi* DNA in blood or *B. burgdorferi* seroconversion from negative to positive between acute- and convalescent-phase serum samples.

Statistical Analysis

A 2-tailed Fisher exact test was used to compare the frequency of *B. miyamotoi* sensu lato–seropositive and –seronegative study participants in groups 1, 2, and 3. The McNemar χ^2 test was used to compare the seroprevalence of *B. miyamotoi* sensu lato and *B. burgdorferi* among group 1 participants.

Results

Seroprevalence of *B. miyamotoi* sensu lato Infection

Serum samples from 52 of the 1,054 study participants were seroreactive to *B. miyamotoi* sensu lato antigen by rGlpQ ELISA and Western blot assay (Table 1). The percentage of *B. miyamotoi* sensu lato–seropositive persons was greater among participants with Lyme disease (group 2; 19/194 [9.8%]) than among those who were healthy (group 1; 25/639 [3.9%], p<0.01 by Fisher exact test, odds ratio [OR] 2.66 [range 1.35–5.16]) or those who had a febrile illness in the late spring or summer (group 3; 8/221 [3.6%], p<0.05 by Fisher exact test, OR 2.89 [range 1.17–7.81]). *B. miyamotoi* sensu lato DNA could not be amplified from any serum samples (including 27 acute-phase serum samples) from the 52 participants who had test results positive for *B. miyamotoi* sensu lato antibody.

Of the 639 serum samples from group 1 participants, 25 (3.9%) were seroreactive to *B. miyamotoi* sensu lato antigen and 60 (9.4%) were seroreactive to *B. burgdorferi* antigen, as determined by using the standard 2-step ELISA and Western blot procedure (McNemar χ^2 test, p<0.0001, OR 10.23 [range 7.84–13.57]). About half (51%) of group 1 participants were male, and the mean age of group 1 participants was 55 years (range 2–102). There was no significant difference in the sex of the group 1 study participants who were seropositive for *B. miyamotoi* sensu lato (40% male) and those who were seropositive for *B. burgdorferi* (53% male; p = 0.34). The mean age also did not differ significantly between participants who were seropositive for *B. miyamotoi* sensu lato (59 years [±15]) and those who were seropositive for *B. burgdorferi* (61 years [±15]; p = 0.62).

Of the participants from Brimfield, Massachusetts, 9.3% (10/107) were seropositive for *B. miyamotoi* sensu lato and 7.5% (8/107) were seropositive for *B. burgdorferi*, compared with 3.2% (15/474) and 11% (52/474), respectively, of the participants from Block Island, Rhode Island. None of the 58 participants from Prudence Island, Rhode Island, were seropositive for *B. miyamotoi* sensu lato or *B. burgdorferi*.

Serodiagnosis of *B. miyamotoi* sensu lato Infection

To assess *B. miyamotoi* sensu lato ELISA and Western blot assay accuracy in patients with confirmed *B. miyamotoi* infection, we tested acute- and convalescent-phase serum samples from 2 patients in Russia with *B. miyamotoi* sensu lato infection confirmed by real-time PCR–(*9*). Both patients had a ≥4-fold rise in *B. miyamotoi* sensu lato GlpQ antibody between acute- and convalescent-phase serum samples (1:80 and 1:2,560, respectively, for 1 patient and 1:640 and 1:2,560, respectively, for the other), as determined by ELISA and confirmed by Western blot.

To assess *B. miyamotoi* sensu lato ELISA and Western blot assay performance in persons at low risk for Lyme disease or *B. miyamotoi* sensu lato infection, we performed the GlpQ ELISA on 300 serum samples from healthy blood donors living in Tempe or Miami. For the 9 microtiter plates used for this serosurvey, the mean and standard deviation of the ELISA optical density values for 3 negative control serum samples ranged from 0.108 to 0.136 and from 0.03 to 0.07, respectively. Of the 300 samples, 19 (6.3%) exceeded the mean of the negative control serum by ≥3 SDs, but none was reactive by Western blot.

We determined whether *B. miyamotoi* sensu lato infection might be misdiagnosed as Lyme disease in persons whose serum was reactive by *B. burgdorferi* antibody testing. Of the 36 *B. miyamotoi* sensu lato–seropositive study participants without a clinical history of Lyme disease within the previous 2 years, 7 (19.4%) had test results positive for *B. burgdorferi* by IgG and/or IgM ELISA, 6 (16.7%) had test results positive for C6 ELISA, and 4 (11.1%) had test results positive for standard 2-tier ELISA plus confirmatory Western blot (Table 2). The 2-tier *B. burgdorferi* ELISA and Western blot assay combination used in our laboratory has a 2% false-positive rate.

Clinical Manifestations among Patients with *B. miyamotoi* sensu lato Seroconversion

A clinical description of illness was available for 5 symptomatic patients who experienced a ≥4 fold rise in *B. miyamotoi* sensu lato IgG and/or IgM antibody between acute- and convalescent-phase serum samples, as determined by ELISA and confirmatory Western blot assays (Western blot data shown in Figure 2). Of the 5 patients, 4 were co-infected with Lyme disease, 1 of whom was also co-infected with babesiosis (determined by blood smear). The 4 patients all had an erythema migrans skin lesion, and 2 had culture results positive for *B. burgdorferi*. The fifth patient had no evidence of co-infection and was the only 1 of 17 (5.9%) participants with a febrile summertime illness who had acute- and convalescent-phase serum tested for *B. miyamotoi* sensu lato antibody and who seroconverted. Three of these 5 patients have been reported previously (*11*). All 5 patients had fever, but a relapsing fever pattern was not reported. Symptoms resolved

Table 1. Assay results for patient samples seroreactive to *Borrelia miyamotoi* sensu lato antigen, northeastern United States, 1991–2012

Group no, description, participant no.	Year sample obtained	IgG ELISA	Western blot IgM	Western blot IgG
Group 1, healthy participants, n = 639				
1	1995	1:320	Positive	Positive
2	2000	≥1:1280	Negative	Positive
3	1991	≥1:1280	Positive	Positive
4	1993	≥1:1280	Negative	Positive
5	2000	≥1:1280	Negative	Positive
6	2000	≥1:1280	Negative	Positive
7	2012	≥1:1280	Negative	Positive
8	2012	≥1:1280	Negative	Positive
9	2012	≥1:1280	Negative	Positive
10	2012	≥1:1280	Negative	Positive
11	1993	≥1:1280	Negative	Positive
12	1993	1:320	Negative	Positive
13	2012	1:320	Negative	Positive
14	2012	≥1:1280	Positive	Positive
15	2012	1:640	Negative	Positive
16	2002	≥1:1280	Negative	Positive
17	2002	≥1:1280	Negative	Positive
18	2002	≥1:1280	Negative	Positive
19	2002	≥1:1280	Negative	Positive
20	2000	≥1:1280	Negative	Positive
21	2000	≥1:1280	Negative	Positive
22	2002	≥1:1280	Negative	Positive
23	2002	≥1:1280	Negative	Positive
24	2002	≥1:1280	Positive	Positive
25	2002	≥1:1280	Positive	Positive
Group 2, adults with Lyme disease, n = 194				
26				
Acute-phase serum	1992 Jul 17	1:80 (negative)	Negative	Negative
Convalescent-phase serum	1992 Jul 27	1:1280	Negative	Positive
27				
Acute-phase serum	1997 Jul 27	1:160 (negative)	Negative	Negative
Convalescent-phase serum	1997 Aug 26	1:1280	Positive	Positive
28				
Acute-phase serum	1996 Jun 30	1:80 (negative)	Positive	Positive
Convalescent-phase serum	1996 Jul 10	1:320	Negative	Positive
29				
Acute-phase serum	1997 Aug 7	1:80 (negative)	Negative	Negative
Convalescent-phase serum	1997 Aug 17	≥1:1280	Negative	Positive
30	1995	≥1:1280	Negative	Positive
31	1991	≥1:1280	Positive	Positive
32	2004	1:640	Negative	Positive
33	2004	1:320	Positive	Positive
34	2000	≥1:1280	Negative	Positive
35	2011	≥1:1280	Negative	Positive
36	1995	≥1:1280	Negative	Positive
37	1994	≥1:1280	Negative	Positive
38	1998	1:320	Positive	Positive
39	2000	≥1:1280	Negative	Positive
40	1998	≥1:1280	Positive	Positive
41	2006	≥1:1280	Negative	Positive
42	2002	≥1:1280	Negative	Positive
43	2002	1:320	Negative	Positive
44	1995	1:320	Positive	Positive
Group 3, adults with virus-like illness, n = 221				
45				
Acute-phase serum	1996 Jul 8	1:80 (negative)	Positive	Negative
Convalescent-phase serum	1996 Jul 19	1:320	Positive	Positive
46	2011	≥1:1280	Positive	Positive
47	1997	≥1:1280	Negative	Positive
48	1991	≥1:1280	Negative	Positive
49	1991	≥1:1280	Negative	Positive
50	1993	≥1:1280	Negative	Positive
51	1997	1:320	Negative	Positive
52	1992	1:320	Negative	Positive

Table 2. Number of false-positive *Borrelia burgdorferi* assay results for participants in various relapsing fever studies*

Borrelia burgdorferi assay	No. participants seroreactive to *B. burgdorferi*/no. total (%)				
	B. miyamotoi (current study)†	*B. hermsii* (*23*)	*B. hermsii* (*24*)	*B. recurrentis* (*24*)	*B. recurrentis* (*25*)
Whole-cell sonicate ELISA	7/36 (19)	ND	7/11 (64)	3/11 (27)	5–7/11 (45–64)
C6 ELISA	6/35 (17)‡	1/14 (7)	ND	ND	ND
Whole-cell sonicate ELISA and Western blot	4/36 (11)	2/14 (14)	ND	ND	ND

*ND, not determined.
†Study participants had no history of Lyme disease within the 2 years before serum was obtained for testing.
‡The quantity of 1 serum sample was insufficient to test.

in 4 of the patients after treatment with doxycycline for 7–14 days, and symptoms resolved in the fifth patient after treatment with amoxicillin/clavulanic acid for 14 days.

Discussion

We found evidence of human infection with the spirochete *B. miyamotoi* sensu lato in 52 residents residing in southern New England or New York State during 1991–2012. Among healthy study participants from southern New England, the seroprevalence of *B. miyamotoi* sensu lato infection was about one third that of *B. burgdorferi* infection (3.9% vs. 9.4%, respectively). This finding is consistent with the higher rate of *B. burgdorferi* infection in *I. scapularis* ticks in the region (range 2:1–20:1) (*4*–*6*). As expected, the seroprevalence of *B. miyamotoi* sensu lato infection was higher in serum samples from patients with acute Lyme disease and recent *I. scapularis* tick bites than in serum samples from patients whose tick-bite status was unclear. *B. miyamotoi* sensu lato seroprevalence rates were similar among study participants with a febrile late spring or summertime illness and healthy participants, probably because *B. miyamotoi* sensu lato infection is unlikely to be a common cause of nonspecific febrile illness in the late spring or summer. The seroprevalence of *B. miyamotoi* sensu lato was less than that of *B. burgdorferi* but similar to that of *Babesia microti* among residents of the same southern New England and New York region (*16*,*26*–*28*).

Approximately 10 percent of the *B. miyamotoi* sensu lato–seropositive patients without a recent history of Lyme disease reacted to *B. burgdorferi* antigen by 2-tier testing. The reactivity could have represented a prior *B. burgdorferi* infection, a false-positive test reaction, and/or cross-reactivity of *B. miyamotoi* sensu lato antibody against ≥1 *B. burgdorferi* antigens. The frequency of antibody reactivity to *B. burgdorferi* in patients with relapsing fever is shown in Table 2 (*23*–*25*,*29*). Several proteins are found in common between *B. burgdorferi* and *B. miyamotoi* sensu lato, including the flagellin FlaB protein, the GroEL heat shock proteins, and the BmpA (P39) protein (*19*,*25*). Misdiagnosis of *B. miyamotoi* sensu lato infection as Lyme disease is therefore possible. Results of *B. burgdorferi* testing may be positive for *B. miyamotoi* sensu lato–infected patients who are co-infected with *B. burgdorferi* (as was the case for some persons in this study). Our findings suggest, however, that testing for antibodies against *B. burgdorferi* is not an appropriate surrogate for testing for antibodies against

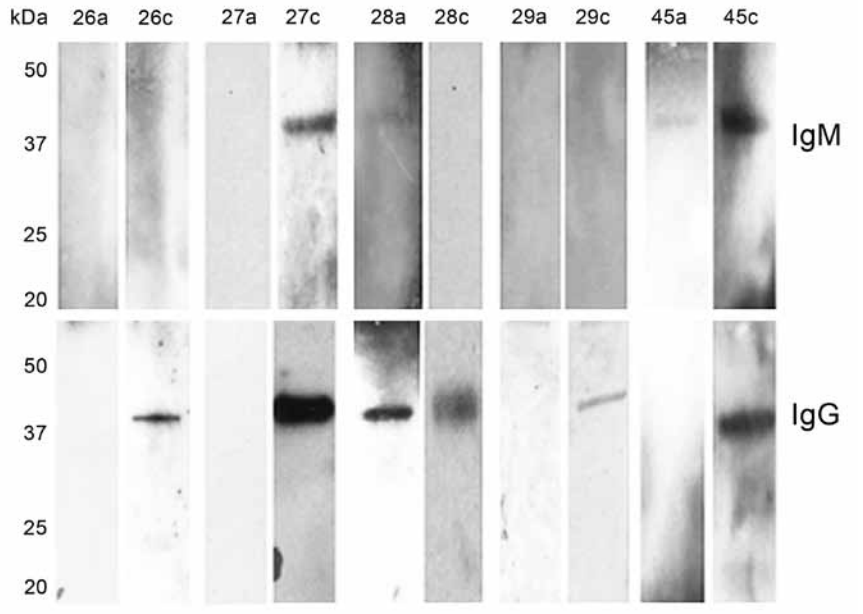

Figure 2. Western blot reactivity to recombinant *Borrelia miyamotoi* glycerophosphodiester phosphodiesterase in serum samples from 5 *Borrelia miyamotoi* sensu lato–seropositive patients in the northeastern United States, 1991–2012. Numbers at the top of rows are patient numbers and correspond to patients 26–29 and 45 in Table 1. The letters a and c that follow patient numbers indicate acute- and convalescent-phase serum samples, respectively. Western blot results that show no seroreactive IgG and/or IgM band in acute-phase serum samples and a reactive IgG and/or IgM band in convalescent-phase serum samples are consistent with ELISA results showing a 4-fold rise in *B. miyamotoi* sensu lato antibody titer from acute-phase (negative) and convalescent-phase (positive) serum samples. The acute-phase serum of patient 28 was nonreactive for IgG in the ELISA assay (Table 1), but the sample was reactive for IgM and IgG on Western blot.

B. miyamotoi sensu lato; *B. burgdorferi* antibody testing should not be used in place of an assay for antibody against *B. miyamotoi* sensu lato GlpQ or another *B. miyamotoi* sensu lato–specific antigen.

Our study had several limitations. First, laboratory evidence for acute *B. miyamotoi* sensu lato infection was based on ELISA and Western blot antibody assay rather than on culture, blood smear, or *B. miyamotoi* sensu lato PCR. However, in agreement with the case definition commonly used for many infectious diseases by the US Centers for Disease Control and Prevention (*30*), we considered results positive if a ≥4-fold rise in antibody occurred between acute- and convalescent-phase serum samples.

Second, *B. miyamotoi* sensu lato from North America has not been cultured, and blood smears were not available from the patients in our study. We were unable to detect *B. miyamotoi* sensu lato DNA in frozen, archived serum samples; however, the process of preparing serum from whole blood likely removed some spirochetes from the samples, and freeze–thaw cycles may have destroyed bacterial DNA. Furthermore, almost half of the serum samples that we tested were obtained after the period of acute illness, when the bacteremia may have cleared.

Third, our seroprevalence rates presumably would have been higher if we had tested for both IgM and IgG antibody by ELISA and included patients with IgM antibody alone as seropositive patients; however, we chose a more stringent definition of seropositivity by requiring the presence of IgG antibody. On the other hand, our seroprevalence data may have been inflated as a result of cross-reactivity of antibodies from other infections reacting against *B. miyamotoi* sensu lato GlpQ antigen. Although all other relapsing fever species have the *glpQ* gene, no other relapsing fever *Borrelia* sp. has been identified in *I. scapularis* ticks or humans in the northeastern United States (*2*,*4*–*6*,*15*,*17*,*31*).

Last, we do not have travel histories for the *B. miyamotoi* sensu lato–seroreactive patients included in the study, but the probability that many of our patients would have had exposure to other relapsing fever *Borrelia* spp. in the United States seems highly unlikely because these infections are infrequent and occur in the western states (*2*). Cross-reactivity against other tick-borne infections in the Northeast also appears unlikely because the agents of Lyme disease, human granulocytic anaplasmosis, and Powassan virus disease lack a *glpQ* gene (*15*). Proteins homologous to the GlpQ protein of relapsing fever borreliae are found in some gram-negative bacteria, including *Escherichia coli*, but they are so distant in sequence that antibody cross-reactivity is not expected (*15*).

The determination of *B. miyamotoi* sensu lato seroprevalence in our population is important because it indicates that this pathogen may infect persons at a rate that is similar to that of *B. microti* in the northeastern United States (*16*,*26*,*27*). Our data suggest that acute *B. miyamotoi* sensu lato infection in some persons may be misdiagnosed as Lyme disease because of the presence of antibody to *B. burgdorferi* from a previous *B. burgdorferi* infection, a false-positive test reaction, and/or cross-reactivity. Antibody testing for *B. burgdorferi*, however, is not adequate to detect infection with *B. miyamotoi* sensu lato in the United States. The potential for misdiagnosis may be greater in locations like northern California, were the prevalence of *B. miyamotoi* sensu lato in ticks equals or exceeds the prevalence of *B. burgdorferi* in ticks (*32*). Further studies are needed to better characterize the epidemiology and improve the serodiagnosis of human *B. miyamotoi* sensu lato infection.

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Diversity of *Francisella tularensis* Subsp. *holarctica* Lineages, China

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We analyzed 10 isolates of *Francisella tularensis* subspecies *holarctica* from China and assigned them to known clades by using canonical single-nucleotide polymorphisms. We found 4 diverse subtypes, including 3 from the most basal lineage, biovar *japonica*. This result indicates unprecedented levels of diversity from a single region and suggests new models for emergence.

Tularemia is a disease caused by distinct subspecies and phylogenetic groups within the bacterial species *Francisella tularensis* (*1*,*2*). These groups exhibit distinct phylogeographic patterns; *F. tularensis* subsp. *tularensis* (type A) is restricted to North America, whereas *F. tularensis* subsp. *holarctica* (type B) is found throughout many parts of the Northern Hemisphere (*3*) and has been reported recently in Tasmania (*4*). Both subspecies exhibit highly clonal population structures, as determined by phylogenetic analysis using data from multilocus variable number tandem repeat analysis, single-nucleotide polymorphisms (SNPs), and indels (*5*–*7*). The wide geographic distribution and low diversity of *F. tularensis* subsp. *holarctica* isolates have been used to argue that this clade is recently emerged and highly fit (*3*), but the geographic origin of its emergence has not been determined.

F. tularensis subsp. *holarctica* has been further subdivided by whole-genome sequencing and canonical SNP (canSNP) genotyping into multiple clades (*7*) (Figure 1). The most basal clade consists of strains assigned to the biovar *japonica*; this biovar had previously only been reported from Japan (*8*), but a recent report suggests that it may be found in Turkey (*9*). The next derived clade (B.2/3) has been described only from 2 isolates from California, USA (*7*). Isolates from these 2 most basal clades are rare, and apparently geographically restricted, but still provide insights into the origin of *F. tularensis* subsp. *holarctica*. The global expansion of the more derived clades is extensive, and closely related isolates are common and widely distributed. The source for emergence of the main type B has been proposed for either North America or Scandinavia, on the basis of the presence of the OSU18 clade isolates in both locations (*6*,*7*). However, a sampling bias toward both of these geographic regions has left *F. tularensis* subsp. *holarctica* diversity in much of the rest of the world poorly understood. We analyzed 10 isolates of *F. tularensis* subsp. *holarctica* from China (*10*) to determine their placement within the current global phylogeographic framework of this pathogen.

The Study

The *F. tularensis* subsp. *holarctica* isolates we analyzed were collected over a long period but have been preserved by lyophilization and have been verified every 5 years since they were isolated (Table 1, http://wwwnc.cdc.gov/EID/article/20/7/13-0931-T1.htm). We assigned these isolates into previously defined (*6*,*7*) phylogenetic clades and conducted a phylogeographic analysis by using a panel of 12 canSNPs specific for *F. tularensis* subspecies or clades within *F. tularensis* subsp. *holarctica* (Table 2); these canSNPs were obtained from previous reports (*6*,*7*). The canSNP analysis was PCR based and performed as described (*7*). Table 1, lists the derived or ancestral allele status for these isolates and for 13 control isolates. These data facilitated the assignment of the 10 *F. tularensis* subsp. *holarctica* isolates to major phylogenetic subgroups previously identified within this subspecies (*6*,*7*).

The isolates were of wide phylogenetic diversity for isolates from a single country. The 10 isolates we analyzed were assigned to 4 distinct phylogenetic clades: 3 were assigned to the basal *japonica* clade (B.16), 3 to the OSU18 clade (B.4), 3 to the FSC200 clade (B.20), and 1 to clade B.6 (Figure 2; Table 1,). Two of these clades are very basal (B.16 and B.4; Figure 1), whereas the other 2 are relatively derived (B.6 and B.20). Regardless, these results demonstrate the presence of multiple distinct *F. tularensis* subsp. *holarctica* lineages in China. Within China, isolates from the Tibetan plateau in the areas bordering Nepal, Bhutan, India, and central Asia were particularly diverse; all 7 strains assigned to clades B.4, B.6, and B.16 were from this region.

The substantial diversity of *F. tularensis* subsp. *holarctica* from the Tibetan region provides evidence for an Asian

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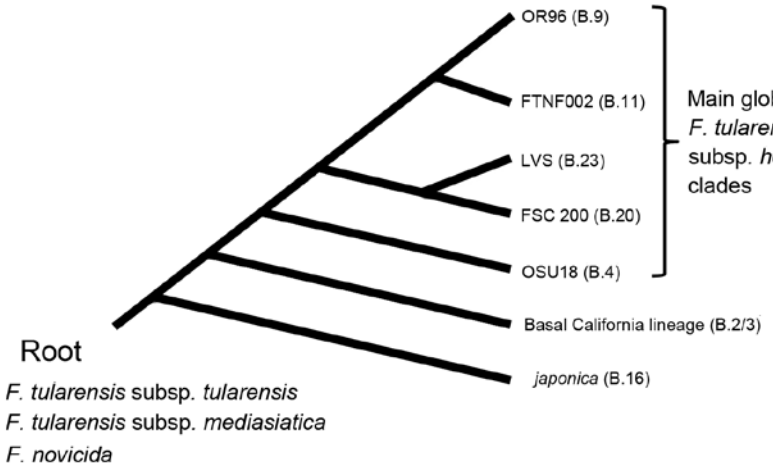

Figure 1. Evolutionary development of *Francisella tularensis* subsp. *holarctica*. Previous studies (*6,7,11*) defined the major lineages of this subspecies on the bases of whole-genome sequences and single-nucleotide polymorphism analysis. The other *F. tularensis* subspecies and closely-related *Francisella* species are shown at the root.

ancestral focus of this subspecies. With the exception of the rare B.2/3 California group, all major basal lineages were represented in this small sample from this region. The center of diversity rationale would suggest that *F. tularensis* subsp. *holarctica* diversified in Asia and then spread outward to the rest of the world. The presence of representatives of the basal *japonica* and OSU18 lineages further argues for ancestral populations in this region. In contrast, the derived and terminal position of the B.20 (FSC200) isolates in northern China suggests that this lineage was introduced to this region from other regions—perhaps Europe, in which B.20 is found (*6*)—after ancestral strains dispersed to other regions from Asia and diversified in these new locations. The analyses used in this current study show that the B.4 (OSU18) isolates from China are indistinguishable from B.4 isolates from North America or Europe and could represent an ancestral population or a reintroduction after global dissemination.

Although strong evidence shows that *F. tularensis* subsp. *holarctica* is a highly fit and recently emerged clone (*3*), we know little about the basis for its great fitness. It is possible that certain, as yet unidentified, adaptive features developed that led to an increase in its fitness. Alternatively, a stochastic event may have led to the emergence and subsequently circumpolar expansion of this subspecies. However, our

Table 2. Primers used in sequencing to obtain canonical SNP loci for *Francisella tularensis* subsp. *holarctica* isolates*

SNP	SCHU S4 SNP position†	SNP state (D/A)‡	Primer sequence, 5′ → 3′	Annealing temperature, °C§
F.3	910179	G/A	F: GCTGTATCATCATTTAATAAACTGCTG R: TTGGGAAGCTTGTATCATGGCACT	55
B.2	5162	A/C	F: TTAGTCTATGAGCAGCCAG R: TAATATCACCAAGGTAGCC	50
B.3	470841	A/G	F: ACGCTAGGTGTCTTGGT R: CTATATCCGCTCAACAT	50
B.4	823672	T/A	F: TAGACGCACTGGATTTAGGT R: AACCATCACGCCACCATAAG	53.5
B.5	1853655	T/C	F: TGGATCAAACAACCGT R: TCTCAAGAGCTGGTGC	50
B.6	713647	A/G	F: AGTAGTGGTAGCGAGGC R: TACCGTTAGCCCAACAG	53.5
B.12	109781	T/A	F: TACTGCCCAACATAGAG R: ATCGTGATAAGGCTGGA	55
B.16	608245	T/G	F: ATGCTAGCAAATTACCATCAAAAG R: AACTCTTCTCGCCATCAACTTCTAT	57
B.17	1743207	A/C	F: CCAAGAGCTAAATTAGCTTCAA R: TGACCAAGAAGGTAGAGGTATTGGTT	53.5
B.19	1373999	A/C	F: TTGCTACTGATGGTTTAACT R: CAATACGTCACTTATGCAGTGAT	57
B.20	1396082, 1789417	C/T	F: ATGGGTCGGACTATCACATC R: ATTATTGTTAAACGGCATCG	56
B.23	253120	A/C	F: GGCAACAGCAGATTCGTGAG R: TGAAAGCAGGTTTAGAAGGACAG	56

*SNP, single-nucleotide polymorphism; D, derived; A, ancestral; F, forward; R, reverse.
†SNP position based on the reference isolate Schu S4 (NC_006570).
‡Top strand orientation of SCHU S4.
§Sequencing conditions as described in (10).

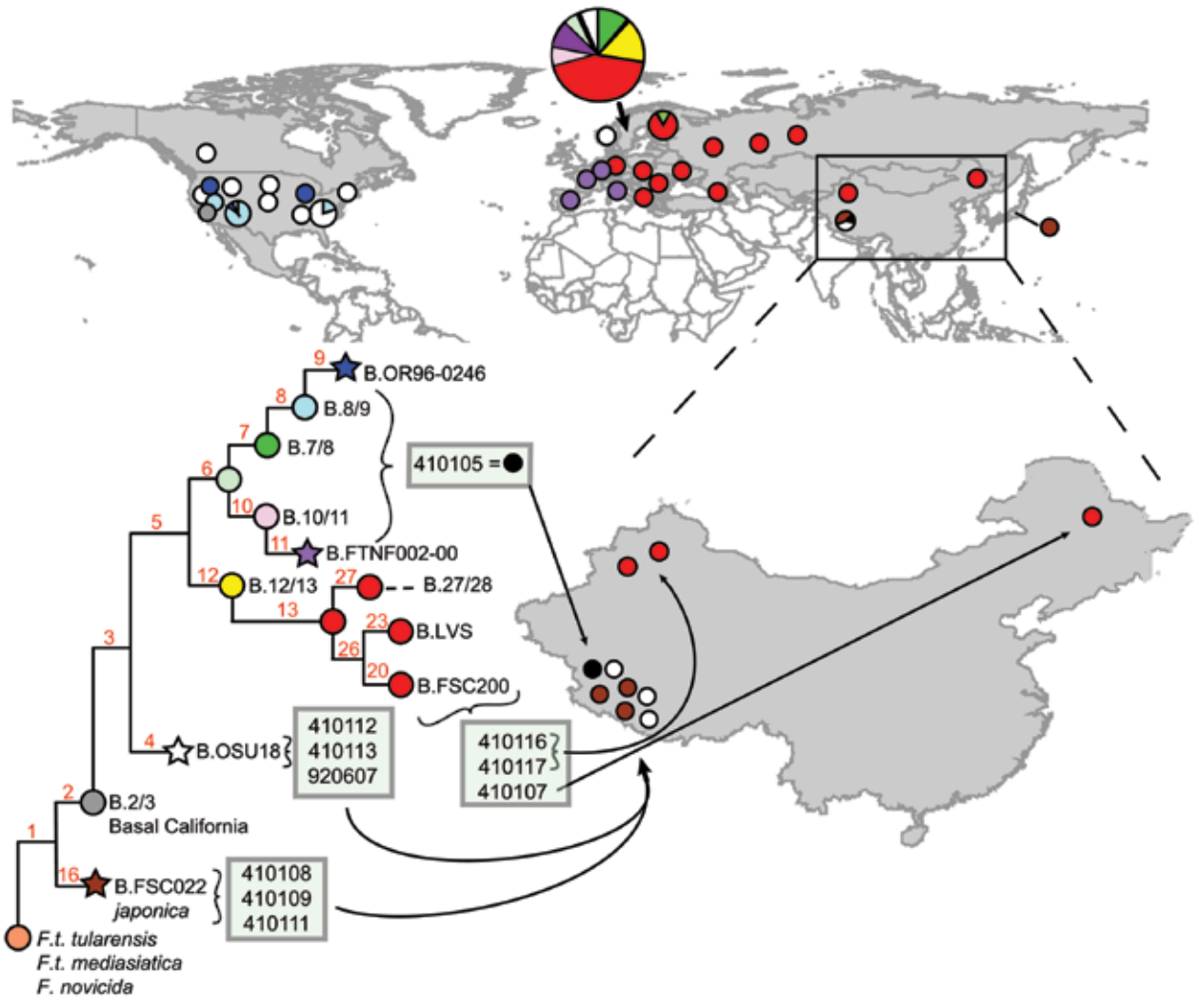

Figure 2. Phylogeography of *Francisella tularensis* (*F.t.*) subsp. *holarctica*. The global distribution of different clades (indicated by colored stars, circles, and circle sections) and their phylogenetic relationships (tree) are shown as described (*6,7,11*). Stars indicate sequenced reference strains. The phylogenetic positions of the 10 isolates from China (boxes on tree) and their sites of isolation (circles within China) are indicated. The exact lineage of strain 410105 (black circle) was not determined.

understanding of the ecology of *F. tularensis* subsp. *holarctica* is severely limited, so the dispersal mechanisms that led to its wide geographic distribution have yet to be identified.

Conclusions

Wide diversity in *F. tularensis* subsp. *holarctica* strains, including basal lineages, has been observed in China and underscores a lack of phylogeographic knowledge of this subspecies. Previous arguments (*1*) about the emergence of this highly fit subspecies have been based on highly biased sampling of strains in North America, Europe, and Japan. Our data suggest a broader distribution in Asia of the *japonica* clade (B.16) in particular. The OSU18 clade (B.4) also appears to have a broader distribution in Asia than has been observed from both North America and Europe. These clades are thought to be basal to the highly fit clonal expansion on these continents. Sampling of additional regions in Asia and characterization of those isolates would greatly advance the literature on the phylogeography of *F. tularensis* subsp. *holarctica*.

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Widespread Rotavirus H in Domesticated Pigs, United States

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We investigated the presence in US pigs of rotavirus H (RVH), identified in pigs in Japan and Brazil. From 204 samples collected during 2006–2009, we identified RVH in 15% of fecal samples from 10 US states, suggesting that RVH has circulated in the United States since 2002, but probably longer.

Rotaviruses (RVs) belong to the *Reoviridae* family and are a major cause of severe diarrhea in humans and animals worldwide (*1*). According to the International Committee on Taxonomy of Viruses, the *Rotavirus* genus is divided into 5 antigenically distinct groups or species (RVA, RVB, RVC, RVD, RVE), 2 tentative species (RVF, RVG), and an unassigned species (ADRV-N), recently confirmed to be distinct from the other RV species, and now referred to as RVH (*2*,*3*).

Three human RVH strains from Asia (ADRV-N, J19, B219) (*4*–*8*) and a porcine RVH strain (SKA-1) (*9*) were identified during 1997–2002. In 2012, three Brazil porcine RVH strains BR63, BR60, and BR59 (GenBank accession nos. KF021621, KF021620, and KF021619) were identified, bringing to only 7 the total number of known RVH strains. To investigate the presence of RVH in US swine, we screened 204 porcine samples collected during 2006–2009.

The Study

We identified RVH in a porcine intestinal sample (RVH/Pig-wt/USA/AR7.10-1/2012/GXP[X]) submitted from a farm in Arkansas in 2012. Subsequently, we rescreened 204 available RVA-, RVB-, and/or RVC-positive porcine samples collected during 2006–2009 from 16 US states for RVH. The samples were from 5 different age groups of pigs: 1–3 days (21 samples), 4–7 days (23), 8–20 days (19), 21–55 days (110), and >55 days (9); 22 samples were from pigs of unknown age. Sample selection, histologic examination, extraction of genomic material, reverse transcription PCR (RT-PCR) amplification, sequencing of viral protein (VP) 6 gene, and statistical and sequence analysis are described in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/20/7/14-0034-Techapp1.pdf).

We identified RVH in 30 (15%) of the 204 samples, including sample AR7.10-1 (online Technical Appendix Table). RVH strains were identified in samples from 10 US states (Figure 1, panel A). The first US sample was identified on November 7, 2006. Of samples from age groups in which we detected positive results, most (20/111, 18%) were from 21–55-day-old pigs; RVH was not detected in 1–3-day-old piglets. We also detected RVH-positive samples in 4–20-day-old (5/42, 12%) and >55-day-old (5/9, 56%) pigs. The number of positive and negative samples differed significantly between age groups ($p = 0.036$, Fisher exact test). The odds of 21–55 day-old pigs being RVH positive was not significant (odds ratio [OR] 1.63, $p = 0.36$); however, in the >55-day group, the odds of being RVH positive was significant (OR 5.92, $p = 0.031$), compared with odds for the 4–20-day group. The trend for increased RVH positivity by age group was not significant ($p = 0.94$, Wald χ^2 test).

Although we identified only 5 samples with RVH in pigs co-infected with RVA and RVB, co-infections with RVH and RVA, RVB, both RVA and RVC, or both RVB and RVC (1 sample each) also were identified but did not differ significantly ($p>0.05$, Fisher exact test) (Figure 1, panel B). We did not identify RVH co-infected with only RVC. Most RVH samples (21 [70%]) were identified from pigs co-infected with RVA, RVB, and RVC, which was significantly higher from any other RVH co-infections with RVA, RVB, RVC, RVAB, RVAC, or RVBC ($p<0.001$, Fisher exact test). Of these 21 RVA, RVB, RVC, and RVH co-infected samples, 15 were from 21–55-day-old pigs (Figure 1, panel B).

The US porcine RVH VP6 sequences (GenBank accession nos. KF757260–KF757289) exhibited 91%–100% nt identity with each other and shared 89%–92% nt identity with Japan porcine strain SKA-1 and 85%–87% nt identity with Brazil porcine strains BR63, BR60, and BR59 (Table 1). The US porcine and human RVH VP6 sequences shared 70%–73% nt identity. The US porcine RVH VP6 sequences were 97%–100% aa identical with each other and 97%–98% and 96%–98% aa identical with the Japan and the Brazil porcine strains, respectively. The US porcine and human RVH VP6 sequences were 75.3%–76.8% aa identical (Table 1). The nucleotide and amino acid pairwise

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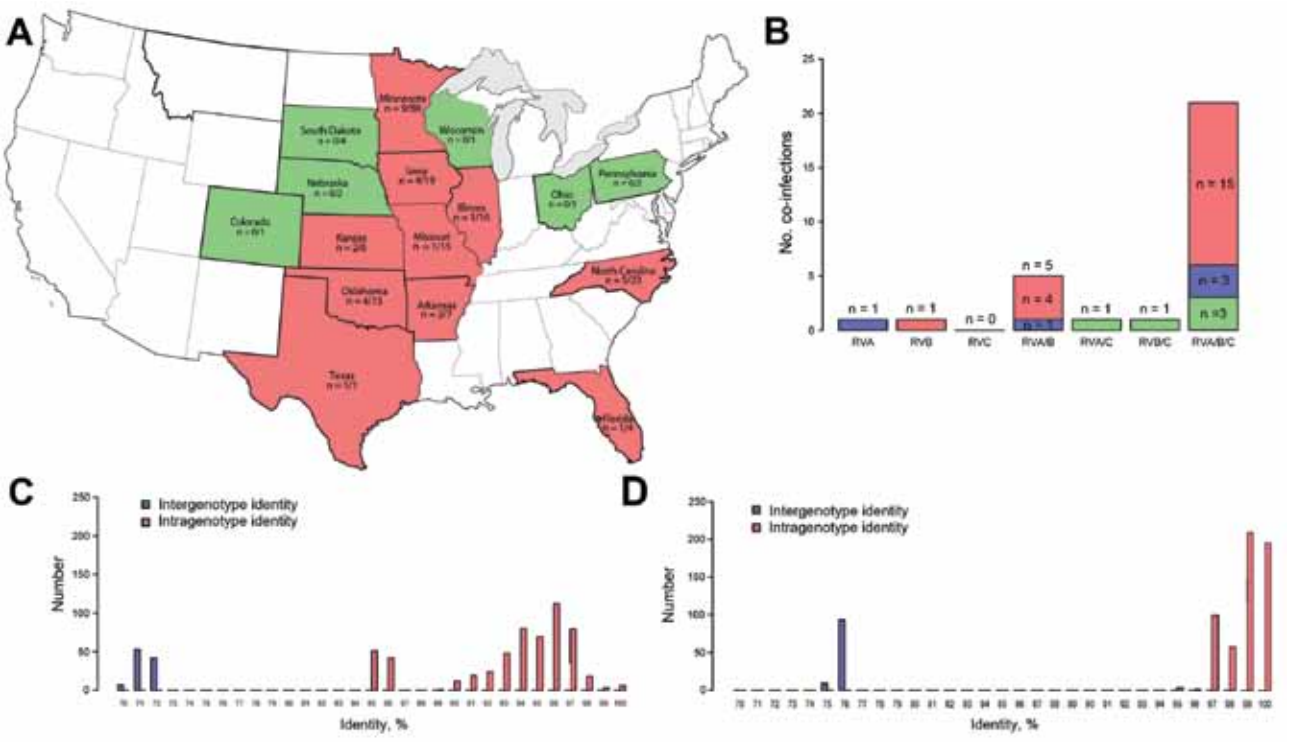

Figure 1. Epidemiologic and molecular distribution of porcine rotavirus H (RVH) strains, United States, 2006–2009. A) Geographic distribution of RVH-positive porcine samples/total number of samples tested. Pink indicates states containing positive samples; green indicates states negative samples; white indicates states from which samples were not submitted. B) Distribution of RVH-positive samples and age group in pigs co-infected with RVA, RVB, and/or RVC. Blue indicates samples from the 4–20-day age group; pink indicates samples from the 21–55-day age group; green indicates samples from the >55-day age group. C) RVH viral protein 6 nt pairwise identity. D) RVH amino acid pairwise identity.

identity charts (Figure 1, panels C and D) and phylogenetic trees (Figure 2, panel A) suggest the existence of at least 2 distinct RVH VP6 (I) clusters/genotypes containing human and porcine strains, respectively.

Compared with other RV species, the US RVH VP6 sequences shared the highest nucleotide and amino acid identities with RVG (51%–53% and 39%–41%, respectively) and RVB (47%–52% and 34%–39%, respectively) (Table 2). In the RV VP6 phylogenetic tree, The RVH, RVG, and RVB VP6 sequences clustered in 1 large branch, whereas the RVA, RVC, RVF, and RVD sequences clustered separately in another large branch (Figure 2, panel A). The RVH evolutionary rate (substitution/site/year) from BEAST (http://tree.bio.ed.ac.uk/) was estimated at 2.6×10^{-3} (95% CI 5.83×10^{-4} to 4.46×10^{-3}). On the basis of the estimate of the time from the most recent common ancestor for the VP6 gene segment, we believe that US RVH strains circulated in US swine for at least a decade and possibly much longer (the time from the most recent common ancestor 1963–2002, 95% highest posterior

Table 1. Nucleotide and amino acid percentage identities of RVH*

RVH type	US porcine RVH, %	Japan porcine RVH, %	Brazil porcine RVH, %	Human RVH, %
US porcine RVH				
Nucleotide	91–100	89.2–91.9	85.2–86.8	70.4–72.8
Amino acid	97–100	96.5–98.2	95.7–97.7	75.3–76.8
Japan porcine RVH				
Nucleotide	89.2–91.9	NA	85.5	71.7–72.3
Amino acid	96.5–98.2	NA	97	76.5-76.8
Brazil porcine RVH				
Nucleotide	85.2–86.8	85.5	100	71.1–71.2
Amino acid	95.7–97.7	97	100	75.8-76
Human RVH				
Nucleotide	70.4–72.8	71.7-72.3	71.1-71.2	94–100
Amino acid	75.3–76.8	76.5–76.8	75.8–76	98.7–100

*RVH, rotavirus H; NA, not applicable

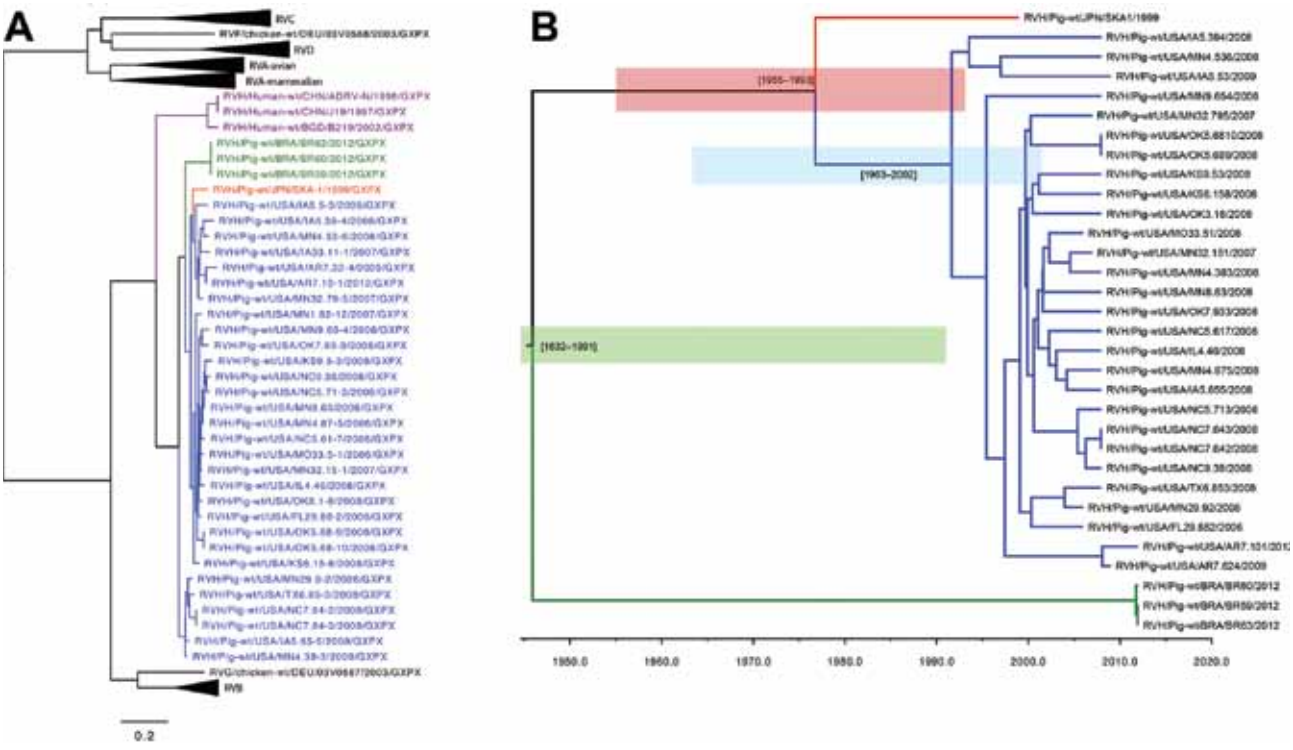

Figure 2. A) Nucleotide neighbor-joining phylogenetic tree of rotavirus (RV) A–D and F–H viral protein (VP) 6 sequences. Blue strains are from the United States; green strains are from Brazil; and the red strain is from Japan. Purple strains are from humans. Scale bar indicates percentage of dissimilarity between sequences. B) Time-scaled phylogeny of swine RVH VP6 sequences using a Bayesian Markov chain Monte Carlo approach. Blue shaded region indicates the time from the most recent common ancestor range (tMRCA) of the US strain; red shaded region indicates the US and Japan RVH tMCRA range; green shaded region indicates the tMRCA range for all swine RVH VP6 sequences.

density [HPD]) (Figure 2, panel B). The US and Japan RVH VP6 sequences diverged during 1955–1993, 95% HPD, and the estimated divergence of the Brazil RVH VP6 sequences from the US and Japan RVH VP6 sequences was 1832–1991, 95% HPD.

Conclusions

Our data indicate that RVH is widespread in US swine herds. Although the samples analyzed already were known to be positive for RV species A, B, and/or C, our identification of RVH in 15% of samples is remarkable. In the United States, piglets are weaned at 21 days of age and then mixed with other piglets from different production sites, which may explain the higher rate of RV co-infections in 21–55-day-old pigs (*10*,*11*). These findings suggest that RVH is underdiagnosed in US swine herds and requires further surveillance.

Our phylogenetic analysis indicates that the RVH strains circulating in US swine is evolutionarily distinct from that found in humans, as well as from swine in Brazil and Japan. Although our low sample number and sequencing of a single gene (VP6) makes the genetic diversity of RVH in US swine herds difficult to fully assess, the lack of spatial structure in the tree indicates extensive gene flow of RVH between swine herds in different US regions. Inferring the circulation of RVH in US swine herds is difficult because of the small sample size, although our time-structured phylogenetic analysis indicates at least 1 decade of circulation. Although US swine are routinely transported to South America, the phylogeny indicates that the VP6 gene of US swine RVH viruses is more closely related to that of Japan strain SKA-1 than to those of the 3 Brazil strains included in this analysis.

In conclusion, we identified RVH in 30 samples from pigs co-infected with RVA, RVB, and/or RVC in the United States, which indicates that RVH has been circulating in US swine for at least 1 decade and perhaps for longer. The human and porcine RVH VP6 sequences clustered into separate branches in the phylogenetic tree, but the presence of RVH in swine clearly raises the possibility of interspecies transmission. Because the swine samples were co-infected with RVA, RVB, and/or RVC, the role of RVH in pathogenesis remains unknown but this circumstance illustrates the need for molecular epidemiologic studies.

Table 2. Nucleotide and amino acid percentage identities of RVs*

RV type	RVA	RVB	RVC	RVD	RVF	RVG	RVH
RVA							
Nucleotide	65.2–100	29.7–36.2	48.5–55.7	46.4–52.1	46.3–50.8	32.9–36.7	31.7–36.2
Amino acid	65–100	7.5–11.3	36.3–42.9	33.3–39.9	31.8–37.2	11.1–13.5	9.9–13.1
RVB							
Nucleotide	29.7–36.2	64.8–100	30.5–34.4	29.2–32.9	30.1–32.9	50.7–57.1	47.4–51.7
Amino acid	7.5–11.3	66.2–100	10.6–13.9	10.4–12.7	11.3–13.4	46.1–49.4	34.4–39.4
RVC							
Nucleotide	48.5–55.7	30.5–34.4	81.4–100	47.2–49.8	47.4–48.3	33.8–34.2	31.5–34.6
Amino acid	36.3–42.9	10.6–13.9	87.1–100	34.7–35.4	32.7–33.9	14.4–14.6	13.4–14.7
RVD							
Nucleotide	46.4–52.1	29.2–32.9	47.2–49.8	90.1–99.6	49.8–50.7	33–34	31.9–34.4
Amino acid	33.3–39.9	10.4–12.7	34.7–35.4	98.2–99.7	36.6–37.6	12–12.5	14.5–16.8
RVF							
Nucleotide	46.3–50.8	30.1–32.9	47.4–48.3	49.8–50.7	NA	32.3	31–32.2
Amino acid	31.8–37.2	11.3–13.4	32.7–33.9	36.6–37.6	NA	11.1	12.6–14
RVG							
Nucleotide	32.9–36.7	50.7–57.1	33.8–34.2	33–34	32.3	NA	50.7–52.2
Amino acid	11.1–13.5	46.1–49.4	14.4–14.6	12–12.5	11.1	NA	39.1–41.4
RVH							
Nucleotide	31.7–36.2	47.4–51.7	31.5–34.6	31.9–34.4	31–32.2	50.7–52.2	70.4–100
Amino acid	9.9–13.1	34.4–39.4	13.4–14.7	14.5–16.8	12.6–14	39.1–41.4	75.3–100

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Severe Malaria Not Responsive to Artemisinin Derivatives in Man Returning from Angola to Vietnam

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Resistance to artemisinin derivatives, the most potent antimalarial drugs currently used, has emerged in Southeast Asia and threatens to spread to Africa. We report a case of malaria in a man who returned to Vietnam after 3 years in Angola that did not respond to intravenous artesunate and clindamycin or an oral artemisinin-based combination.

Artemisinin derivatives are used in combination with other drugs for treatment of *Plasmodium falciparum* malaria. Nevertheless, *P. falciparum* resistance to artemisinins has been recently detected in 4 countries (Cambodia, Thailand, Myanmar, and Vietnam) in the Greater Mekong subregion in Southeast Asia (*1*). Artemisinin resistance could spread from these countries to other regions, including sub-Saharan Africa, where the incidence of malaria is highest, and where artemisinin resistance would have devastating consequences. Increased and uncontrolled travel between Asia and Africa might contribute to the spread of artemisinin-resistant malaria parasites.

An estimated 40,000 Vietnamese workers travel annually to Angola (*2*), where *P. falciparum* malaria is the leading cause of illness and death (*3*,*4*). Over the past year, an increasing number of severe malaria cases have been identified among Vietnamese migrants returning from Angola (*5*). During March–mid-April 2013, five deaths from malaria among Vietnamese workers in Angola were reported (*6*–*8*). We report a case of malaria in a Vietnamese man who returned from Angola and did not respond to intravenous artesunate or oral artemisinin-based combination therapy.

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The Study

In April 2013, a 58-year-old Vietnamese man was admitted to Bach Mai Hospital in Hanoi, Vietnam, because of high fever, jaundice, and lack of consciousness. Eleven days before hospitalization, he had returned from Saurimo City in Angola, where he had been working in the construction industry for the past 3 years, to his home village in malaria-free Nam Dinh Province, 80 km from Hanoi. Four days after returning from Angola, he reported fatigue, fever with chills, and a cough. Two days later, he went to the local district hospital where he was given a diagnosis of bronchitis and received a third-generation cephalosporin (cefixime) and antipyretics for 3 days. Ten days after his arrival in Vietnam, the patient came to the emergency department of Bach Mai Hospital because of continuous high fever (body temperature 39°C–40°C), dyspnea, and urinary incontinence. He was given fosfomycin for pyelonephritis.

Because his clinical condition worsened rapidly, the patient was transferred the next day to the Infectious Diseases Department in the same hospital because cerebral malaria was diagnosed. At admission to the department, the patient (weight 58 kg) was in a confused state and had a Glasgow coma score of 13/15, generalized convulsions, jaundice, and tachypnea (respiration rate 25 breaths/min), a body temperature of 38.5°C, a blood pressure of 130/80 mm Hg, and pulse rate of 121 beats/min. Clinical examination detected hepatomegaly and cracklings in both lungs. Blood tests showed the following results: leukocyte count 13.3×10^9 cells/L, hemoglobin 15.3 g/L, platelet count 20.9×10^9/L, blood urea nitrogen 19.8 mmol/L, serum creatinine 135 mmol/L, aspartate aminotransferase 125 IU/L, alanine aminotransferase 45 IU/L, C-reactive protein 16 mg/L, procalcitonin >120 ng/mL; total bilirubin 116 μmol/L; direct bilirubin 11.5 μmol/L; and standard levels of electrolytes.

Parasite density/microliter of blood was calculated after counting the total number of *P. falciparum* trophozoites/200 leukocytes and assuming a leukocyte concentration of 8,000 cells/μL. Microscopy identified *P. falciparum* trophozoites at a concentration of 378,470/μL and some schizonts. A chest radiograph showed bilateral pneumonia. Analysis of cerebrospinal fluid and a computed tomographic scan of the brain showed standard results. Tests results for HIV and hepatitis B surface antigen were negative.

The patient was given a diagnosis of *P. falciparum* cerebral malaria and treated with intravenous (IV) artesunate (batch no. 511004; Pharbaco, Hanoi, Vietnam) (60 mg every 12 h, loading dose 120 mg at admission) and IV

clindamycin (600 mg every 12 h). After 24 h of hospitalization, the patient had a Glasgow coma score of 11, a body temperature of 39.7°C, and a parasite density of 329,411 parasites/μL (Figure 1), a blood pressure of 70/40 mm Hg, and a respiration rate of 30 breaths/min. An ultrasound examination showed hepatomegaly and bilateral pleural effusion. At day 2 of hospitalization, the patient was treated with intubation and mechanical ventilation. At day 4, the hemoglobin level had decreased to 9.3 g/dL and the patient was given a blood transfusion. After 5 consecutive days of treatment with IV artesunate and clindamycin, the patient remained comatose and had continuous fever (39°C) and high parasite density (148,000 parasites/μL).

At day 6 of hospitalization, artesunate and clindamycin were discontinued, and oral treatment with dihydroartemisinin (40 mg/tablet)/piperaquine (320 mg/tablet) was administered through a nasogastric tube for 3 days (4 tablets/day the first day, then 2 tablets/day). After 3 days of treatment, the patient was still febrile (38.5°C) and had a Glasgow coma score of 12 and a parasite density of 113,409 parasites/μL (Figure 1). Quinine (1,750 mg/day) and doxycycline (200 mg/day) were then administered at day 9 through a nasogastric tube (injectable quinine was not available). Twelve hours after the first dose of quinine, parasite density was 55,558 parasites/μL. It decreased to 9,668 parasites/μL 72 h at day 11 after starting quinine treatment, when fever eventually subsided. Two days later, the patient regained consciousness, and at day 15 of hospitalization, blood slides were negative for parasites (Figure 1). The patient eventually recovered and was discharged 35 days after admission.

Species-specific PCR (*9*) of a blood sample obtained on day 3 of hospitalization confirmed the diagnosis of *P. falciparum* monoinfection. Further genotyping by using nested PCR (merozoite surface proteins 1 and 2 repeat markers) and capillary electrophoresis (*10*) of several filter paper blood spots obtained during the 23 days of hospitalization confirmed that the patient had a polyclonal *P. falciparum* infection with ≥2 clones. These clones might have persisted from admission through day 10 of hospitalization (Figure 2).

The quality of IV artesunate used was determined by using high-performance liquid chromatography according to USP34 NF29 specifications at the National Institute of Drug Quality Control (Hanoi, Vietnam) (http://nidqc.org.vn/). The drug was found to be acceptable.

Conclusions

This clinical case of suspected artemisinin resistance originating from sub-Saharan Africa is of concern because the patient had probably been infected in Angola

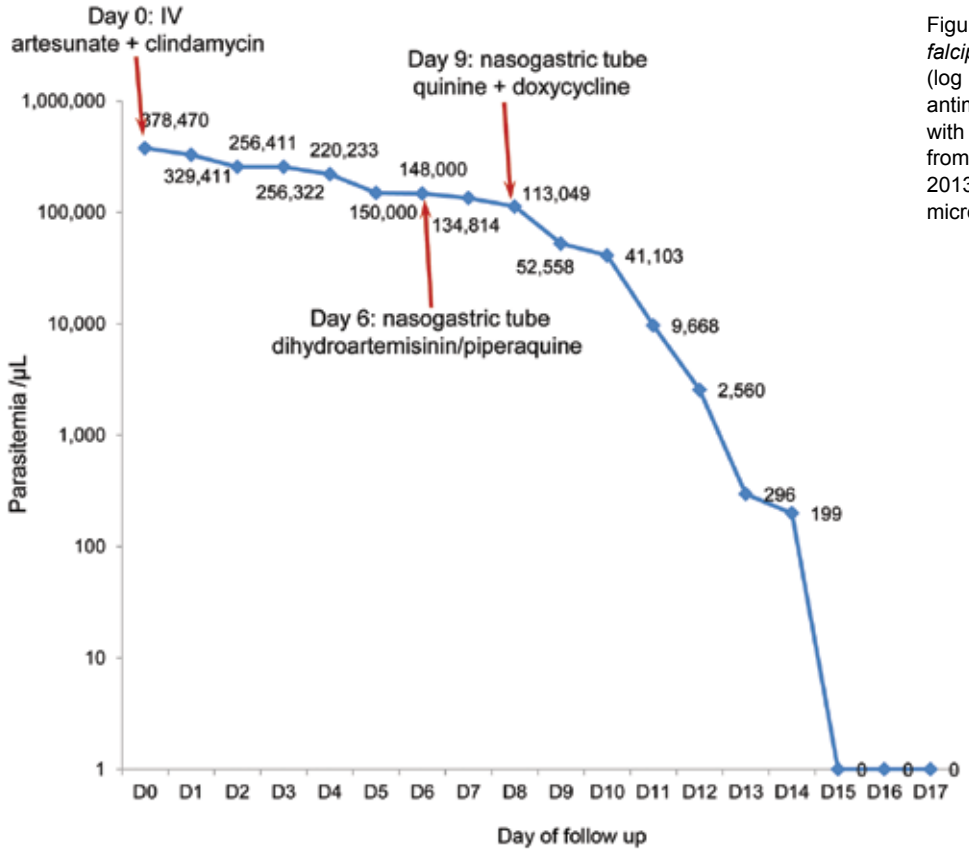

Figure 1. Evolution of *Plasmodium falciparum* parasite density (log scale) by day after start of antimalaria treatments for man with severe malaria who returned from Angola to Vietnam in April 2013. Values are parasites/microliter of blood. IV, intravenous.

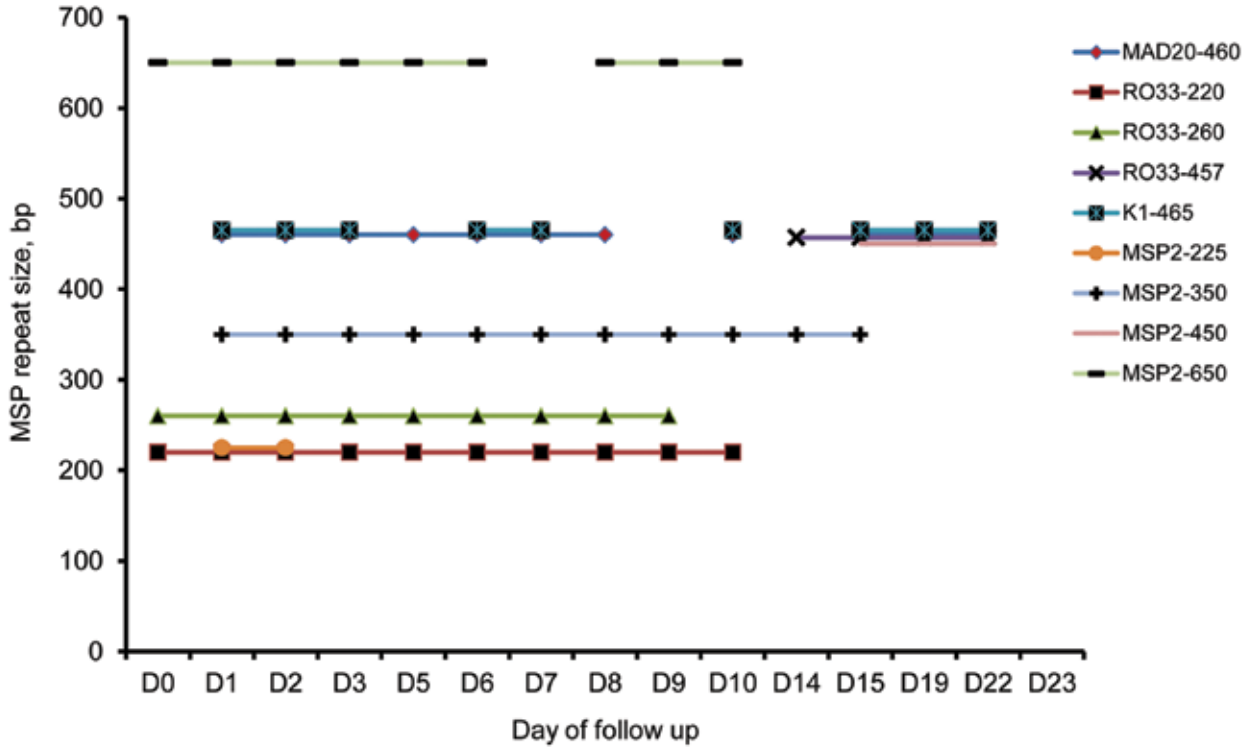

Figure 2. Clonal complexity of *Plasmodium falciparum* strain that caused severe malaria in man who returned from Angola to Vietnam after treatment, as determined by merozoite surface protein 1 (MSP1) and MSP2 repeat length molecular typing. Allele sizes were detected by capillary electrophoresis of amplified MSP repeat regions and shown for each follow-up sample.

and because the clinical presentation differed from delayed parasite clearance reported in Southeast Asia (*1*). The patient had never had malaria and had worked continuously in Angola for the past 3 years before his return (direct flight to Hanoi and then directly by car to his home village) to a malaria-free area in Vietnam. Given the onset of symptoms only 4 days after his return, the patient was most likely infected in Luanda Sul Province, Angola, to which malaria is hyperendemic and where Vietnamese workers have reportedly died of malaria (*6*–*8*).

Five days of treatment with intravenous artesunate and clindamycin and a 3-day regimen of dihydroartemisinin/piperaquine did not clear the infection because parasite density remained high (>100,000 parasites/μL) until the eighth day of hospitalization. Parasite density showed a logarithmic decrease only after treatment was changed to quinine and doxycycline.

An external quality control of all blood slides was performed by an expert microscopist at the National Institute of Malariology, Parasitology and Entomology in Hanoi; the parasite densities were confirmed. Therefore, this case is suggestive of *P. falciparum* tolerance to artemisinin derivatives. However, because no blood samples for pharmacokinetic or in vitro studies were obtained, drug resistance could not be confirmed.

Identification of *P. falciparum* malaria parasites tolerant to arteminins raises serious concerns that artemisin-resistant strains have emerged or spread in Africa. This finding would be a major public health disaster and needs to be urgently confirmed by larger treatment efficacy studies in Angola.

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Dr Van Hong is a physician, junior scientist, and doctoral student at the National Institute of Malariology, Parasitology and Entomology, Hanoi, Vietnam. Her research interests are genetic diversity and population structure of *P. vivax* and antimalarial drug resistance in *P. falciparum* and *P. vivax*.

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Rates of Influenza-like Illness and Winter School Breaks, Chile, 2004–2010

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To determine effects of school breaks on influenza virus transmission in the Southern Hemisphere, we analyzed 2004–2010 influenza-like–illness surveillance data from Chile. Winter breaks were significantly associated with a two-thirds temporary incidence reduction among schoolchildren, which supports use of school closure to temporarily reduce illness, especially among schoolchildren, in the Southern Hemisphere.

Influenza pandemic preparedness plans to mitigate effects of a severe pandemic recommend layered medical and social distancing interventions, including school closings, cancellation of large public gatherings, and face mask use (*1*). Because schoolchildren are considered to be high transmitters of influenza virus (higher contact rates, enhanced susceptibility to infection, and increased virus shedding relative to that among persons in other age groups), prompt school closure is expected to reduce transmission during a pandemic (*2*).

Although several empirical studies have linked school activities with influenza virus transmission (*2–10*), few studies have considered data from multiple epidemic periods, and little information is available from the Southern Hemisphere. School breaks and school teacher strikes provide natural experiments in which the effect of school terms on influenza transmission dynamics can be explored. On the basis of 21 years of surveillance data, Cauchemez et al. (*5*) found a 16%–18% reduction in incidence of influenza-like illness (ILI) associated with the 2-week school winter break periods in France. A study of variation in contact rate patterns in Europe suggested a 13%–40% reduction in the basic reproduction number associated with school breaks in Belgium, Great Britain, and the Netherlands (*11*). A 12-day teacher strike in Israel in the winter of 1999 was also associated with a reduction (43%) in weekly rates of respiratory disease (*12*). A single study is available from the Southern Hemisphere and indicates a 14% reduction in ILI incidence during winter break in Argentina during 2005–2008; the largest decrease was observed among children 5–14 years of age (*6*). In our study, we quantified the effect of school break cycles on the age distribution of ILI patients in Chile during 2004–2010.

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The Study

We obtained weekly age-specific ILI incidence rates during 2004–2010 from a systematic national surveillance system in Chile (*13*). ILI surveillance relies on 42 sentinel outpatient sites located throughout the country; these sites are representative of the general population and systematically report weekly age-specific physician visits for ILI (*13*) (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/7/13-0967-Techapp1.pdf). We characterized the effect of the 2-week winter break period on influenza transmission during 2004–2010 by comparing trends in weekly ILI incidence rates among schoolchildren (5–14 and 15–19 years of age) and adults (20–64 and ≥65 years of age). To estimate changes in the age distribution of ILI patients, on the basis of methods used in previous work (*8*,*14*), we compared the weekly ratios of ILI incidence rates for schoolchildren and adults during the 2-week period before, during, and after the winter break by using a 1-sided Z test. We also considered a 6-week window (*8*,*14*) before and after the winter break as a sensitivity analysis. A decline in the schoolchildren-to-adult incidence rate ratio indicates a shift in the age distribution of patients toward adults, suggestive of decreased influenza transmission among schoolchildren (*8*).

In Chile, wintertime influenza activity peaks during May–September, which is typical of temperate regions in the Southern Hemisphere (*15*). The 2-week winter school break typically coincides with the influenza season and is synchronous throughout the country; ≈95% of educational institutions follow the break periods set by the Ministry of Education.

Figure 1 illustrates trends in ILI incidence rates among schoolchildren 5–19 years of age and adults ≥20 years of age throughout the year and the associated schoolchildren-to-adults incidence rate ratio. In Chile, ILI incidence displays bimodal patterns of activity; activity increases before and after the winter break, and transmission is reduced during the break. The schoolchildren-to-adults ratios decreased substantially (40%–68%) during the 2-week winter break period relative to the 2-week period immediately preceding the winter break (Table 1). Also, the reduction in ratios coinciding with the first week of the winter break occurred every year of our study,

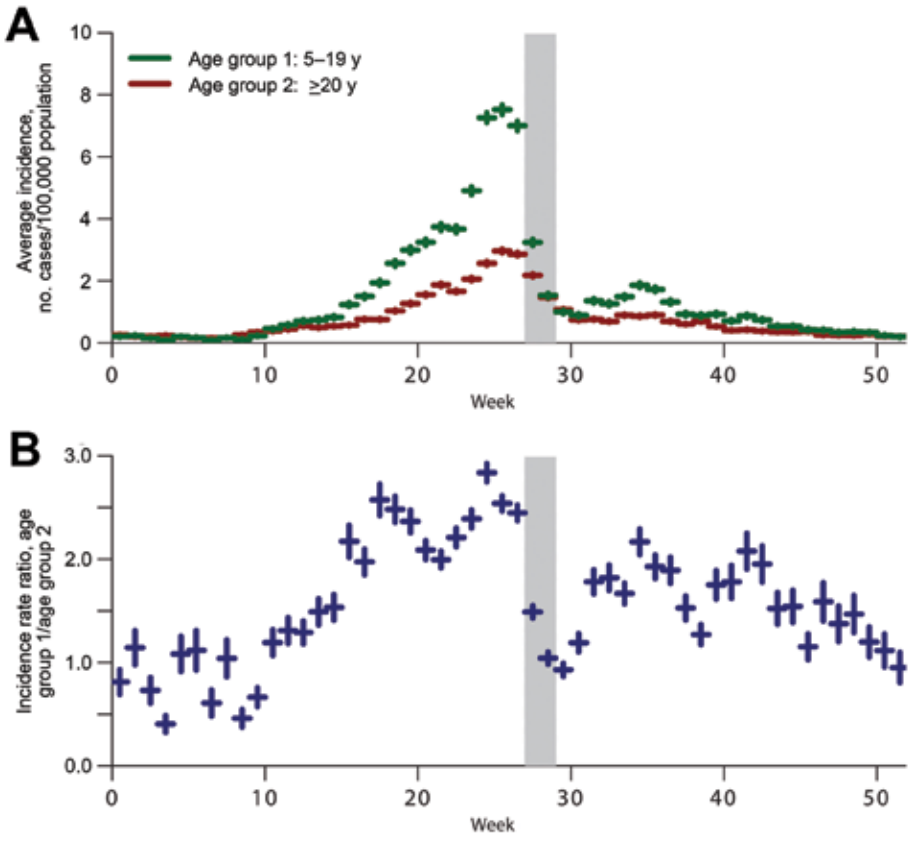

Figure 1. Average weekly incidence rates for influenza-like illness (ILI) among schoolchildren 5–19 years of age and adults ≥20 years of age, Chile, 2004–2010. Error bars represent the standard errors of the mean within each week. The shaded area represents the period of the 2-week winter break. A) Average ILI incidence per 100,000 population, by week. B) Average ILI incidence rate ratio of schoolchildren-to-adult incidence by week. Examination of a 2-week period and comparison of the averaged within–week-of-year ILI incidence rate ratio for children (5–19 years of age) to adults (≥20 years of age) to the average of the ratio in the 2-week period immediately before provided 50 such comparisons. The Bonferroni corrected $\alpha = 0.05$; significance level is thus $\alpha = 0.05/50 = 0.001$. The only 2-week periods in which the ratio comparison p value was less than $\alpha = 0.001$ were the periods beginning week 28 and 29 (which corresponds to the winter break), week 44 (which corresponds to the Reformation/All Saints Day 4-day weekend), week 21 (the week of the Naval Glories Day break), and week 38 (the Independence Day break).

including during the pandemic (2009) and postpandemic (2010) seasons.

The reduction in the schoolchildren-to-adults incidence rate ratios was maintained for an average of 2 weeks after the end of the winter break. The decline in ratios was primarily caused by a decrease in ILI rates among schoolchildren; the average (± standard error of the estimate) reduction in ILI incidence among schoolchildren (5–19 years of age) in the 2 weeks during the winter break compared with the 2 weeks before was 67.2% ± 2.1% ($p<0.001$). This reduction occurred systematically in each winter of the study period. In contrast, the average reduction in adult ILI incidence (≥20 years of age) was more modest but remained significant at 37.4% ± 0.9% ($p<0.001$).

Furthermore, the incidence rate ratios for school-age children to middle-age adults significantly increased after the winter break, signaling a return toward a higher proportion of ILI cases among children, although the ratio did not return to prebreak levels (Table 1). In contrast, the ratio comparing rates for children with rates for adults did not change. Our results did not change when we used a 6-week period before and after the winter break period instead of a 2-week period (Table 2) or when we excluded the 2009 pandemic year from our analysis (online Technical Appendix Figure 1).

Conclusions

We have shown that a two-thirds decline in ILI incidence among schoolchildren coincided with the onset of the school winter break in Chile; this pattern was consistent across the 7 years of the study. In line with a prior study in Argentina (*6*), the average reduction in schoolchildren-to-adults incidence rate ratio was sustained for up to 2 weeks after school sessions resumed. This time scale is consistent with the natural history of influenza virus infection, which has a serial interval (interval between cases) of 2–3 days, so that it takes a few successive chains of transmission to reach full-scale transmission.

Similar to findings from prior studies (*5*,*6*), our findings are based on analysis of ILI incidence, which is a broad indicator of respiratory disease activity in a community and is not entirely specific for influenza. Our results could be affected by changes in health-seeking behavior during the winter break. However, our ILI data are well correlated with influenza virus activity data (*15*) (Figure 2), and large increases in incidence among schoolchildren during winter 2009 coincide with the influenza A(H1N1)

Table 1. ILI incidence rate ratios for schoolchildren compared with adults during 2-week periods surrounding school winter breaks, by age group, Chile, 2004–2010*

Age group, y	ILI incidence rate ratio			p value	
	Before school break	During school break	After school break	Before break vs. during break†	During break vs. after break‡
Adults ≥20					
Schoolchildren 5–14	2.68 (0.06)	1.28 (0.16)	1.88 (0.117)	<0.001	0.002
Schoolchildren 15–19	2.11 (0.12)	1.22 (0.04)	1.63 (0.14)	<0.001	0.002
Schoolchildren 5–19	2.49 (0.06)	1.26 (0.11)	1.80 (0.08	<0.001	<0.001
Adults 20–64					
Schoolchildren 5–14	2.52 (0.06)	1.25 (0.16)	1.92 (0.13)	<0.001	<0.001
Schoolchildren 15–19	1.98 (0.10)	1.18 (0.04)	1.66 (0.13)	<0.001	<0.001
Schoolchildren 5–19	2.34 (0.05)	1.23 (0.11)	1.83 (0.09)	<0.001	<0.001
Adults ≥65					
Schoolchildren 5–14	5.07 (0.48)	1.61 (0.23)	1.65 (0.16)	<0.001	0.451
Schoolchildren 15–19	4.01 (0.56)	1.52 (0.08)	1.44 (0.22)	<0.001	0.626
Schoolchildren 5–19	4.71 (0.50)	1.58 (0.16)	1.58 (0.17)	<0.001	0.505

*The "after break" period begins 2 weeks after the winter break ends because the reduction in the incidence rate ratio during the winter break was maintained on average for 2 weeks after the end of the winter break. ILI, influenza-like illness.
†p value of a 1-sided Z test comparing the average incidence rate ratio (ratio of incidence rate for schoolchildren to incidence rate for adults) during the 2-week period before the school break to that during the winter break. Small p values indicate that the incidence rate ratio for the period before the break is significantly higher than that for the period during the break;. p values near 1.00 indicate that the incidence rate ratio for the period before the break is significantly lower than that for during the break.
‡p value of a 1-sided Z test comparing the average incidence rate ratio during the 2-week period after the school break to that during the winter break period. Small p values indicate that the incidence rate ratio for the period after the winter break period is significantly higher than that for the period during the winter break; p values near 1.00 indicate that the incidence ratio for the period after the break is significantly lower than that for the period during the break.

pdm09 virus pandemic period, suggesting that fluctuations in ILI incidence in Chile are primarily attributable to influenza. Our data also support the conclusion that school closure during pandemic situations is effective. Although the winter break took place near the peak of the 2009 influenza A(H1N1) pandemic in Chile, it was correlated with changes in the age distribution of patients hospitalized for influenza A(H1N1)pdm09 virus infection (*7*).

Overall, our study findings add to the body of information provided by empirical studies, supporting the implementation of school closure to achieve temporary reductions in ILI incidence rates, especially among school-age children, including in the Southern Hemisphere temperate setting (*2*–*7*). Our finding that ILI incidence was more modestly reduced among adults during winter breaks is consistent with past work on the age-specific transmission dynamics of influenza (*2*–*7*). School closure may be particularly useful in pandemic situations to gain time until pharmaceutical measures (vaccines, antiviral medications) become available and to mitigate the burden on health care institutions by reducing the surge of influenza patients. There is still,

Table 2. ILI incidence rate ratios for schoolchildren compared with adults during 6-week periods surrounding school winter breaks, by age group, Chile, 2004–2010*

Age group, y	ILI incidence rate ratio			p value	
	Before school break	During school break	After school break	Before break vs. during break†	During break vs. after break‡
Adults ≥20					
Schoolchildren 5–14	2.59 (0.14)	1.28 (0.16)	1.98 (0.10)	<0.001	<0.001
Schoolchildren 15–19	2.02 (0.07)	1.22 (0.04)	1.66 (0.07)	<0.001	<0.001
Schoolchildren 5–19	2.40 (0.11)	1.26 (0.11)	1.87 (0.07)	<0.001	<0.001
Adults 20–64					
Schoolchildren 5–14	2.52 (0.11)	1.25 (0.16)	2.00 (0.10)	<0.001	<0.001
Schoolchildren 15–19	1.97 (0.05)	1.18 (0.04)	1.68 (0.07)	<0.001	<0.001
Schoolchildren 5–19	2.33 (0.08)	1.23 (0.11)	1.89 (0.08)	<0.001	<0.001
Adults ≥65					
Schoolchildren 5–14	3.82 (0.62)	1.61 (0.23)	1.85 (0.14)	<0.001	0.187
Schoolchildren 15–19	2.94 (0.46)	1.52 (0.08)	1.56 (0.11)	0.001	0.401
Schoolchildren 5–19	3.52 (0.56)	1.58 (0.16)	1.75 (0.12)	<0.001	0.206

*The "after break" period begins 2 weeks after the winter break ends because the reduction in the incidence rate ratio during the winter break was maintained on average for 2 weeks after the end of the winter break. ILI, influenza-like illness.
†p value of a 1-sided Z test comparing the average incidence rate ratio (ratio of incidence rate for schoolchildren to incidence rate for adults) during the 2-week period before the school break to that during the winter break. Small p values indicate that the incidence rate ratio for the period before the break is significantly higher than that for the period during the break; p values near 1.00 indicate that the incidence rate ratio for the period before the break is significantly lower than that for during the break.
‡p value of a 1-sided Z test comparing the average incidence rate ratio during the 2-week period after the school break to that during the winter break period. Small p values indicate that the incidence rate ratio for the period after the winter break period is significantly higher than that for the period during the winter break; p values near 1.00 indicate that the incidence rate ratio for the period after the break is significantly lower than that for the period during the break.

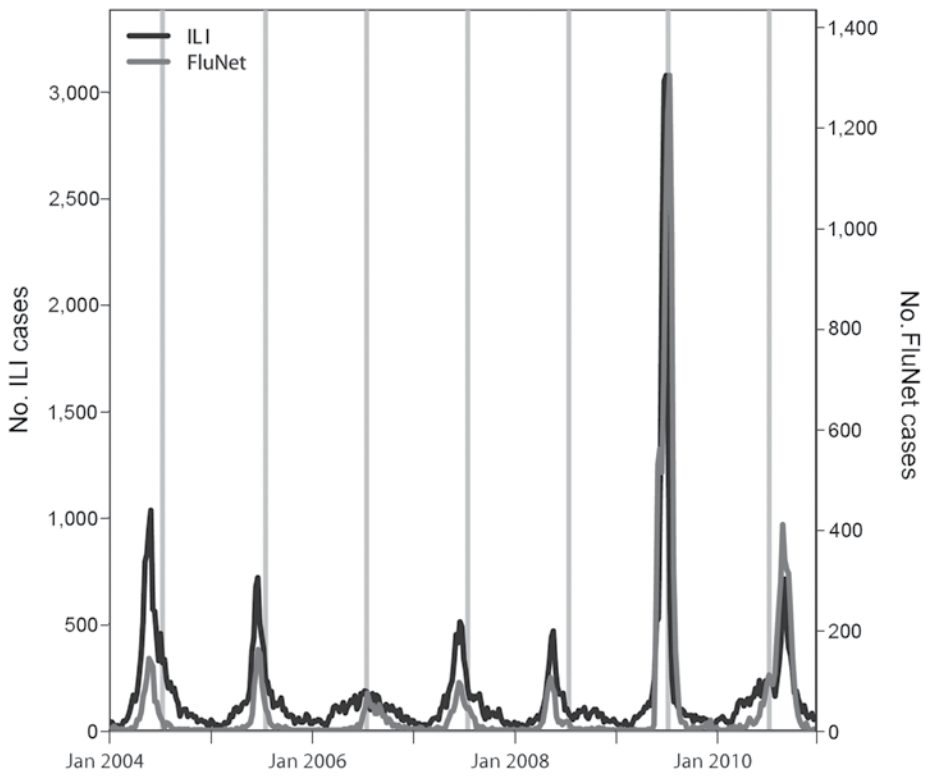

Figure 2. Weekly time series of influenza-like illness (ILI) cases and laboratory-confirmed influenza notifications (FluNet [*15*]), in Chile, 2004–2010. The shaded areas represent the 2-week winter break periods.

however, little information available from tropical and Southern Hemisphere settings, which are characterized by complex influenza seasonality patterns and/or low connectivity with the rest of the world and particular demographic and health conditions. Systematic multicountry and multiyear comparison of the effects of school closures could shed light on the effectiveness of school-based intervention policies under different epidemiologic, behavioral, and demographic situations.

This research was conducted in the context of the Multinational Influenza Seasonal Mortality Study, an ongoing international collaborative effort to understand influenza epidemiological and evolutionary patterns led by the Fogarty International Center, National Institutes of Health (http://www.origem.info/misms/index.php). Funding for this project came in part from the Office of Global Affairs, International Influenza Unit, in the Office of the Secretary of the Department of Health and Human Services.

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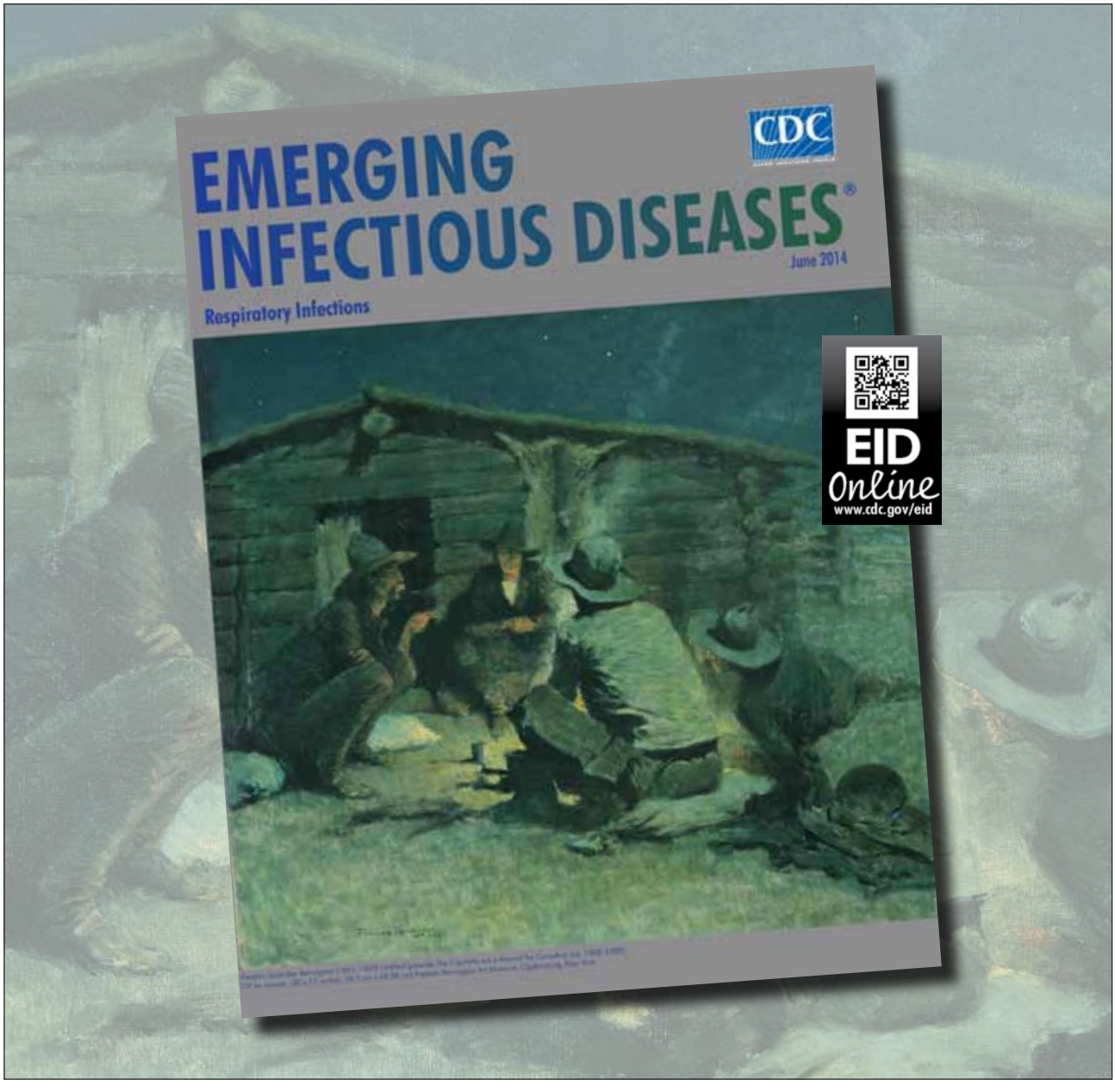

Highly Pathogenic Fowlpox Virus in Cutaneously Infected Chickens, China

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We investigated an acute outbreak of the cutaneous form of fowlpox among chickens in China in November 2009. Using pathologic and virologic methods, we identified a novel type of fowlpox virus that carried an integrated genomic sequence of reticuloendotheliosis virus. This highly pathogenic virus could lead to severe ecologic effects and economic losses.

Fowlpox has been reported worldwide as a mild to severe poultry disease (*1*). Caused by fowlpox virus (FWPV), the disease is primarily found in 2 forms, cutaneous and diphtheritic (*2*). The cutaneous form is usually mild and characterized by multifocal cutaneous lesions on unfeathered areas of the skin. The more severe diphtheritic form is characterized by fibrous necrotic proliferative lesions on the mucous membranes of the respiratory and gastrointestinal tracts and causes more deaths than the cutaneous form, usually resulting from asphyxiation.

In recent years, fowlpox outbreaks in poultry flocks have been gradually increasing because of an emerging novel type of FWPV (*3*–*5*). The pathogenic traits of this virus type are likely enhanced by integrated reticuloendotheliosis virus (REV) sequences of various lengths in the FWPV genome (*6*–*8*). Although this variant FWPV has been found widely (*7*,*9*–*14*), the reported illness and death rates from the cutaneous form of fowlpox in chickens have not reached 100%. We investigated a severe outbreak of cutaneous fowlpox in a poultry flock in northeastern China in which all infected chickens died. The flock had not been vaccinated with an FWPV vaccine.

The Study

In November 2009, a natural outbreak of the cutaneous form of fowlpox occurred in a poultry flock in Jilin Province in northeastern China (125°35′ E, 43°88′ N). A total of 10,000 brown breeding, 46-day-old chickens (Jilin Zhengda Co., Ltd, Changchun, China) used for egg production were affected. The flock had not received vaccination against FWPV.

Clinical signs, including listlessness, anorexia, and typical skin pock lesions, were observed in all infected chickens. Lesions types varied in size and type: ulcerated, multifocal, or coalescing proliferative cutaneous exanthema variolosum. The lesions appeared on the skin in unfeathered areas of the backs, the eyelids, and the wings (Figure 1). All of the birds died within 10 days after clinical signs first appeared.

Postmortem examinations were performed for pathol ogic evaluation. Samples submitted for histopathologic examination included skin from the varioliform exanthema areas, trachea, thymus gland, bursa of fabricius, and internal organs. Microscopic examination of skin lesions showed swelling, vacuolation, and characteristic eosinophilic cytoplasmic inclusion bodies in the stratified squamous epithelial cells of the folliculus pili (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/20/7/13-1118-Techapp1.pdf). No obvious lesions were observed in other organs.

Electron microscopy of the clarified supernatant of the scab specimens collected from the skin of infected chickens showed characteristic FWPV virions, which have an ovoid shape (online Technical Appendix Figure 1). We attempted to isolate the virus by injecting the chorioallantoic membranes (CAM) and allantoic cavities of 9-day-old specific pathogen free (SPF) chicken embryos with the scab specimens that were positive for FWPV. White, raised varioles were observed on the CAMs of the embryos 4 days after injection (online Technical Appendix Figure 1). Electron microscopy also showed FWPV-shaped virions in the supernatant of the CAMs. After 5 blind passages at 4-day intervals, no other viruses were isolated from the allantoic cavities of the SPF chicken embryos.

We used indirect immunofluorescence and a DF-1 chicken embryo fibroblast cell line to test the ability of the FWPV isolate from the CAMs to invade cells in vitro. Chicken anti-FWPV polyclonal antibody was used as the primary antibody; the secondary antibody was fluorescein isothiocyanate–conjugated goat anti-chicken IgG. Cellular nuclei were stained by using 4′,6-diamidino-2-phenylindole. In some cells, typical bright, DNA-containing poxvirus factories were evident, often coincident with virus antigen–specific green fluorescence, at 3 days postinfection (dpi) (online Technical Appendix Figure 1).

Viral genomic DNA was extracted from scab specimens, and PCR amplification was performed immediately by using the specific primers for FWPV P4b gene (P4b

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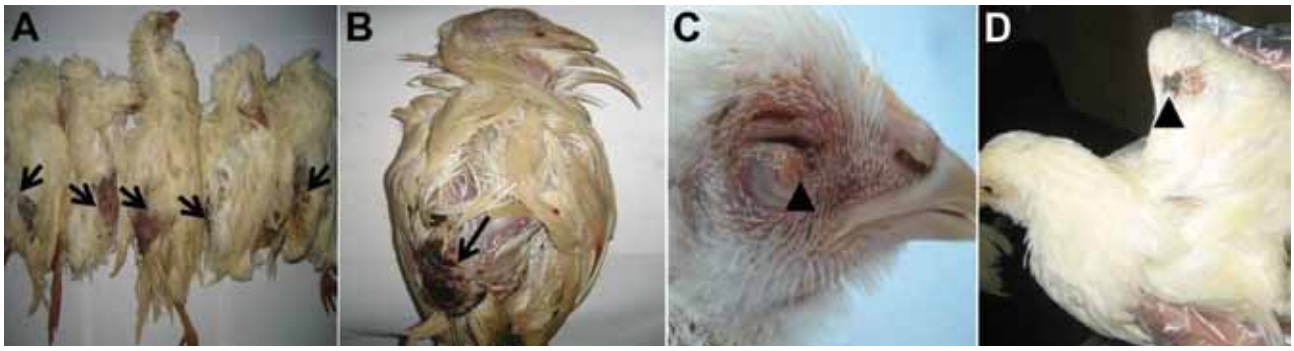

Figure 1. Macropathologic images of fowlpox virus infection in chickens from a commercial flock in northeastern China. A, B) Severe lesions on the skin in unfeathered areas of the backs (arrows). C) Cutaneous exanthema variolosum of the eyelids (arrowhead). D) Skin pock lesions in the wings (arrowhead).

Fw1: 5′-GATAGAGGATCGTACATCCA-3′; and P4b Rv1: 5′-CATCTACTCATGACTGGCAA-3′). The size of the product was 1,381 bp (online Technical Appendix Figure 2). The amplicons were sequenced, and the obtained P4b gene sequence was submitted to GenBank (accession no. KF875986). We then used the neighbor-joining method in MEGA4 (*15*) to construct a phylogenetic tree on the basis of the nucleotide sequences of P4b gene with corresponding reference sequences (online Technical Appendix Figure 3). The resulting tree showed that the FWPV isolate clustered in the same branch with other FWPVs and that the P4b gene shared a close relationship with other FWPVs (99.9%–100%). This result indicates that the P4b genes were highly conserved among FWPV isolates. No nucleic acid sequences of other potentially pathogenic viruses (i.e., avian influenza virus, Newcastle disease virus, Marek's disease virus, chicken anemia virus, avian leukosis virus J subgroup, infectious bursal disease virus) were detected by using PCR or reverse transcription PCR. These findings indicate that FWPV may have been the causative pathogen in the infected chickens.

To investigate the possibility of an integration of an REV gene sequence into the FWPV genome, we designed another 2 sets of primers for the amplification of a partial REV *env* gene and the REV *env*–FWPV open reading frame 203, which contains the entire REV 3′ long terminal repeat. The primer sequences were as follows: REV-env Fw1, 5′-ACCACTCTCGACTCAAGAAA-3′; REV-env Rv1, 5′-CCACACACAAATACATGACCC-3′; REV env-FWPV 203 Fw1, 5′-GAAATCTTACGAGGCTATGTC-3′; and REV env-FWPV 203 Rv1, 5′-TTCAACCACCAGGCTACATAAAGG-3′. Specific products of the expected sizes, 1,089 bp and 1,437 bp, were amplified from the skin lesions (online Technical Appendix Figure 2). The results indicated that the FWPV isolate had integrated partial REV sequences.

We further determined the pathogenesis of the FWPV isolate by experimentally infecting 18-day-old, 53-day-old, and 145-day-old SPF chickens. The experimental groups (10 chickens per group) were inoculated by scarification of the wing and skin scarification into the feather folliculus pili by using purified virus containing 200 50% egg infectious doses of the virus. A control group (10 chickens) was injected with 0.2 mL of phosphate-buffered saline. All inoculated chickens had characteristic skin pock lesions develop at 7–14 dpi (Figure 2) and died at 18–25 dpi; illness and death rates were 100%. Scab specimens were collected at 7, 9, 14, and 20 dpi for histologic

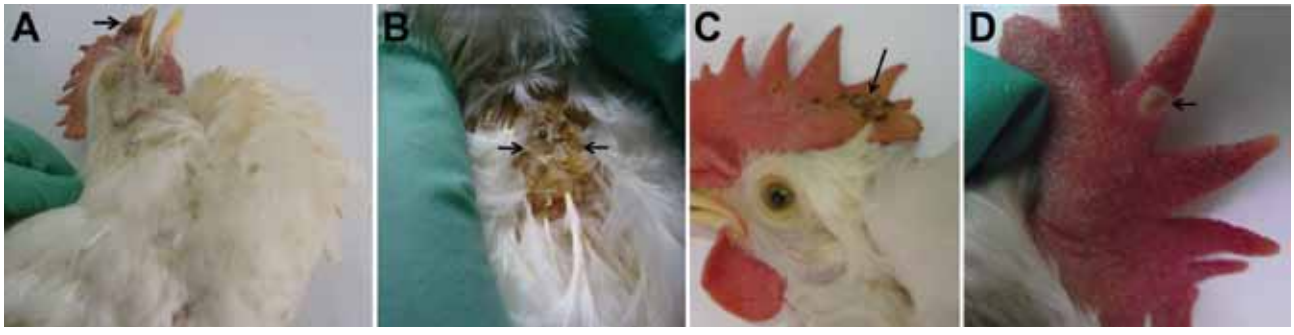

Figure 2. Macropathologic images of fowlpox virus infection in experimentally infected specific pathogen free (SPF) chickens. China. A) Brown variolar crusts on the combs of 18-day-old SPF chickens at 14 days postinfection (dpi). B) Large areas of brown scabs on the backs of 18-day-old SPF chickens at 14 dpi. C) Multifocal to coalescing pock lesions on the combs of 53-day-old SPF chickens at 14 dpi. D) Cutaneous exanthema variolosum on the combs of 145-day-old SPF chickens at 14 dpi.

examination. The chickens in the control group did not show any clinical signs.

The paraffin sections of scab samples from the SPF chickens inoculated with FWPV were positive not only for FWPV, tested by using a chicken anti-FWPV polyclonal antibody, but also for REV, tested by using a monoclonal antibody that specifically recognized the envelope protein of REV in the cytoplasm of stratified squamous epithelial cells of the folliculus pili by immunohistochemical assay (online Technical Appendix Figure 1).

Conclusions

Our investigation of an acute outbreak of the cutaneous form of fowlpox determined that the outbreak was caused by a novel type of FWPV that carried integrated REV genomic sequences. Illness and death rates of up to 100% occurred in this commercial poultry flock in northeast China. Our results show that the novel FWPV we isolated was much more pathogenic than common FWPV strains obtained from other chickens infected with the cutaneous form of fowlpox. This highly pathogenic FWPV variant is a potential threat to chickens and could lead to severe ecologic effects and economic losses. The virulence of this FWPV is probably dependent on the presence of the REV sequences in the FWPV genome, although this conclusion needs experimental confirmation. Because these sequences are also found in less virulent isolates, other determinants may account for this unusual phenotype. Identifying the genomic changes responsible for the increased pathogenicity of this FWPV variant will require considerable effort in sequencing and molecular virology.

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Neisseria gonorrhoeae Strain with Reduced Susceptibilities to Extended-Spectrum Cephalosporins

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The spread of *Neisseria gonorrhoeae* strains with reduced susceptibility to extended-spectrum cephalosporins is an increasing public health threat. Using Etest and multiantigen sequence typing, we detected sequence type 1407, which is associated with reduced susceptibilities to extended-spectrum cephalosporins, in 4 major populated regions in California, USA, in 2012.

Neisseria gonorrhoeae infections are the second most common sexually transmitted infection in the United States (*1*). Gonorrhea typically presents as urethritis in men and cervicitis in women but when left untreated can result in severe sequelae, such as infertility (*2*,*3*). Gonorrhea infections continue to be a public health problem worldwide, and control efforts have been complicated because of the ability of the organism to develop resistance to all first-line antimicrobial drugs used in treatment, including penicillins, tetracyclines, and fluoroquinolones.

Treatment failures and isolates with reduced susceptibilities to extended-spectrum cephalosporins (ESCs) have been detected in Asia (*4*,*5*), Canada (*6*), Europe (*7*,*8*), and South Africa (*9*). In the United States, ESCs are currently the foundation of treatment recommendations. However, the increasing prevalence of isolates with reduced susceptibility to ESCs has led to dual treatment with ceftriaxone plus azithromycin or doxycycline. This combination is the only recommended treatment regimen of the US Centers for Disease Control and Prevention (CDC) (*10*).

N. gonorrhoeae isolates with reduced susceptibility to ESCs have been linked to altered penicillin-binding protein, which is encoded by the *penA* gene. Molecular and genomic epidemiologic studies have been used to describe *N. gonorrhoeae* antimicrobial drug resistance to ESCs in much of the world. In the United States, isolates with reduced susceptibility to cefixime have been associated with a specific strain of *N. gonorrhoeae* (*11*). However, little is known about the molecular epidemiology of strains with reduced susceptibilities to ceftriaxone, the current recommended ESC. In this study, we conducted surveillance for genotypic and phenotypic diversity of *N. gonorrhoeae* strain diversity in California during January 2012–September 2013.

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The Study

Urethral gonococcal isolates were obtained from men with urethral infections during the CDC-sponsored Gonococcal Isolate Surveillance Project (GISP) at sites in Los Angeles, Orange, San Diego and San Francisco Counties in California. Duplicates of all GISP isolates from these sites were analyzed at the San Francisco Department of Public Health Laboratory by using Etest and at the GISP regional laboratory by using agar dilution. Antimicrobial drug susceptibility testing was performed by using Etest (bioMérieux, Marcy l'Etoile, France) as part of the California rapid response project to enhance surveillance efforts aimed at detecting treatment failures and containing the spread of isolates that require increased MICs of third-generation cephalosporins.

A total of 718 isolates were tested for susceptibility to ceftriaxone, cefixime, and azithromycin by Etest. In brief, frozen isolates were thawed in a biosafety cabinet at room temperature, inoculated onto a chocolate II agar plate containing 1% Iso VitaleX (Becton Dickinson, Franklin Lakes, NJ, USA), and incubated at 37°C with in an atmosphere of 5% CO_2 for 20–25 h. A sterile swab was then used to collect colonies on the plate and suspend them in typticase soy broth containing 15% glycerol to give a turbidity of 0.5–1 MacFarland units. Using another sterile swab, we plated out the liquid culture onto 3 chocolate II agar plates in three 90° turnings. We then placed an Etest strip in the center of each plate by using a manual applicator. After incubation for 20–25 h at 37°C in an atmosphere of 5% CO_2, the plates were examined, and MICs of each drug were recorded according to the manufacturer's instructions.

Molecular epidemiologic typing was performed by using *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) on all isolates. NG-MAST was performed as described (*12*) and allele numbers of *porB* and *tbpB* genes and sequence types (STs) were assigned by using the NG-MAST website (http://www.ng-mast.net).

Of 718 urethral isolates that were sequence typed, 258 STs were identified that had 192 *porB* alleles and 60 *tbpB* alleles. Eighty-three STs were present among ≥2 isolates in the collection, and the remaining 175 STs were unique.

Table 2. MICs required for *Neisseria gonorrhoeae* isolates of the 5 most common genogroups in 4 counties in California, USA, 2012*

Genogroup	Cefixime, no. (%)			Ceftriaxone, no. (%)			Azithromycin, no. (%)		
	Low	Intermediate	High	Low	Intermediate	High	Low	Intermediate	High
2992	69 (98.5)	1 (0.015)	0	70 (100)	0	0	52 (74.3)	18 (25.7)	0
2400	19 (32)	40 (68)	0	8 (13.6)	51 (86.4)	0	55 (93)	4 (7)	0
3307	51 (98)	1 (2)	0	51 (98)	1 (2)	0	50 (96)	2 (4)	0
1407	4 (9)	27 (60)	14 (31)	7 (15.5)	28 (62.2)	10 (22.3)	26 (58)	19 (42)	0
3935	30 (97)	1 (3)	0	30 (97)	1 (3)	0	14 (45)	17 (55)	0
Other	436 (94.5)	24 (5)	1 (0.5)	384 (83.3)	76 (16.5)	1 (0.02)	432 (94)	27 (5.8)	2 (0.2)

*Cefixime: low ≤0.023 μg/mL, intermediate 0.032–0.094 μg/mL, high ≥0.125 μg/mL; ceftriaxone: low ≤0.023 μg/mL, intermediate 0.032–0.094 μg/mL, high ≥0.125 μg/mL; azithromycin: low ≤0.380 μg/mL, intermediate 0.5–1.5 μg/mL, high ≥2.0 μg/mL.

The 5 most prevalent STs identified were ST2400 (55, 7.7%), ST2992 (46, 6.4%), ST3307 (45, 6.3%), ST1407 (38, 5.3%), and ST7268 25, 3.5%). These STs constituted 29% of all isolates obtained. ST1407, the fourth most prevalent ST in this study, has been associated with ESC treatment failures in Europe and Canada. In California, this ST and closely related STs were associated with reduced susceptibility to ESCs, but these STs remained susceptible to azithromycin.

Because of the high discriminatory power of NG-MAST, which assigns STs on the basis of single basepair differences, we sought to assess diversity on the basis of genogroups. Genogroups were defined as having identical *tbpB* alleles and ≥99% DNA sequence similarity within *porB* alleles as defined by NG-MAST (*13*). The 718 urethral isolates sequence typed for this study formed 56 genogroups (≥2 isolates) and 92 ungrouped isolates with unique STs. The 5 most common genogroups were G2992 (n = 70, 9.7%), G2400 (n = 59, 8.2%), G3307 (n = 52, 7.2%), G1407 (n = 45, 6.3%) and G3935 (n = 31, 4.3%) (Table 1, http://wwwnc.cdc.gov/EID/article/20/7/13-1396-T1.htm).

Most isolates in our study required low MICs (≤0.023 μg/mL) for ceftriaxone and cefixime. Proportions of low, intermediate, and high MICs required by isolates in the 5 most prevalent genogroups are shown in Table 2. The G2400 and G1407 isolates were the only genogroups to require predominantly intermediate MICs of ceftriaxone and cefixime (0.032–0.094 μg/mL). G1407 comprised 91% (10/11) of isolates that required a high MIC of ceftriaxone and 93% (14/15) of isolates that required a high MIC of cefixime. Overall, 99.7% (716/718) of isolates required an MIC of azithromycin ≤1.5 μg/mL, of which 88% (629/716) required an MIC of azithromycin ≤0.380 μg/mL. Only 2 isolates required an MIC of azithromycin ≥2.0 μg/mL (2.0 μg/mL and 3.0 μg/mL).

Seventeen isolates that had reduced susceptibility phenotypes for ESCs (ceftriaxone MIC ≥0.125 μg/mL and cefixime MIC ≥0.125 μg/mL) were subjected to *penA* sequencing to determine the presence an altered *penA* gene. Sequencing was performed as described (*14*). Sequencing of *penA* genes showed that 100% (17/17) had the mosaic *penA* XXXIV allele. Genetic characteristics of isolates that required high MICs of ESCs are shown in Table 3. The *tbpB* 110 allele was found in 100% (17/17) of the isolates that required high MICs of ESCs Cs, and 94% (16/17) were G1407.

Conclusions

Genotypic surveillance of *N. gonorrhoeae* has been performed in the United States and elsewhere, but little is known about the genetic diversity of strains circulating

Table 3. Genetic characteristics of, and MICs required for, 17 *Neisseria gonorrhoeae* isolates of the 5 most common genogroups in 4 counties, California, USA, 2012*

Isolate	Genogroup	ST	*porB* allele	Ceftriaxone MIC	Cefixime MIC	Azithromycin MIC
1	1407	1407	908	0.25	0.25	0.75
2	1407	1407	908	0.25	0.19	0.75
3	1407	1407	908	0.25	0.19	0.5
4	1407	1407	908	0.19	0.125	1.0
5	1407	1407	908	0.125	0.125	0.38
6	1407	1407	908	0.125	0.125	0.25
7	1407	1407	908	0.125	0.125	0.75
8	1407	1407	908	0.125	0.125	0.5
9	1407	1407	908	0.094	0.125	0.38
10	1407	1407	908	0.094	0.125	0.5
11	1407	1407	908	0.064	0.125	0.38
12	1407	1407	908	0.047	0.125	0.38
13	1407	1407	908	0.125	0.094	0.75
14	1407	1407	908	0.125	0.094	0.75
15	1407	3158	1914	0.094	0.125	0.19
16	1407	8417	1903	0.094	0.125	0.38
17	Unique	8476	4878	0.125	0.125	0.5

*All 17 isolates had the 110 allele for *tbpB* and the XXXIV allele for *penA*. MICs are shown in micrograms per milliliter. ST, sequence type.

in the United States. We sought to perform phenotypic and genotypic surveillance of isolates from major population centers in California in 2012. ST1407, a widespread strain linked to ESC treatment failures, was found in all 4 counties in California and was the fourth most prevalent ST overall.

In this study, ST1407 was associated with reduced susceptibility to ceftriaxone and cefixime. These data show that ST1407 is well established in California and are consistent with results from Asia (*4*,*5*), Canada (*15*), Europe (*8*), and the United States (*11*). All G1407 isolates that required increased MICs of ceftriaxone or cefixime had *penA* allele XXXIV. Among the 718 isolates in this study, none had reduced susceptibility to azithromycin and an ESC. The relatively high prevalence of this strain type, combined with the evolutionary capacity of *N. gonorrhoeae* to evade antimicrobial drug pressure, may eventually lead to circumstances where even dual therapy is not sufficient.

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Cefotaxime-Resistant *Salmonella enterica* in Travelers Returning from Thailand to Finland

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During 1993–2011, cefotaxime resistance among *Salmonella enterica* isolates from patients in Finland increased substantially. Most of these infections originated in Thailand; many were *qnr* positive and belonged to *S. enterica* serovar Typhimurium and *S. enterica* monophasic serovar 4,[5],12:i:-. Although cefotaxime-resistant salmonellae mainly originate in discrete geographic areas, they represent a global threat.

Salmonella spp. are a common cause of foodborne illnesses globally, but illnesses caused by *Salmonella* infections vary from mild diarrhea (travelers' diarrhea) to severe generalized infections (*1*). Certain *Salmonella* serotypes are more commonly linked to human infections and for example, the monophasic 4,[5],12:i:- variant of *S. enterica* serovar Typhimurium has caused an increasing number of *Salmonella* infections in humans during the last decade (*2*). Antimicrobial agents, usually fluoroquinolones and extended-spectrum cephalosporins, are needed for the treatment of patients with invasive *Salmonella* infections (*3*).

The abundant use of antibiotics in human and veterinary medicine and in food production has led to antimicrobial drug resistance (*4*), and the numbers and proportions of extended-spectrum β-lactamase (ESBL)– and AmpC β-lactamase–producing strains of *Enterobacteriaceae* have increased worldwide (*3*,*5*–*7*). Although reduced fluoroquinolone susceptibility among *S. enterica* isolates has increased since the late 1990s (*8*,*9*), *Salmonella* spp. have remained cephalosporin-susceptible. Coexistence of ESBL and plasmid-mediated quinolone resistance genes in *Salmonella* and in other *Enterobacteriaceae* genera have been reported and there are existing reports on extended-spectrum cephalosporin-resistant and ESBL-producing *Salmonella* isolates (*3*,*4*,*10*).

To date, *Salmonella* isolates that have acquired resistance determinants against fluoroquinolones and extended-spectrum cephalosporins have been reported only anecdotally in Finland. This study describes a systematic analysis of extended-spectrum cephalosporin–resistant *Salmonella* isolates in Finland during a 19-year period.

The Study

During 1993–2011, 43,171 *S. enterica* isolates were sent to the National Salmonella Reference Centre of the National Institute for Health and Welfare (THL) in Finland. This *Salmonella* collection contains ≈85% (range 75.9%–91.1%) of all *Salmonella* isolates collected annually in Finland during the study period. All of these isolates were screened for cefotaxime susceptibility (*11*). A total of 225 cefotaxime-nonsusceptible *S. enterica* isolates were identified; 183 of these, collected during 2000–2011, were genotyped. The isolates were screened and serotyped in the Bacteriology Unit at THL.

We confirmed phenotypic ESBL using disk diffusion tests (*11*). Cefotaxime-nonsusceptible isolates were screened for the ESBL genes TEM, SHV, and CTX-M by PCR (*7*). CTX-M–positive *Escherichia coli*, SHV-positive *Klebsiella pneumoniae*, and TEM-positive *E. coli* were used as positive ESBL controls. Isolates having only a TEM determinant were further classified by pyrosequencing (*12*).

We also screened the cefotaxime-nonsusceptible isolates for AmpC production. PCR was used to amplify the AmpC b-lactamase genes CMY, FOX, DHA, ACC, MOX, and EBC by using previously described primers (*13*). The AmpC multiplex-PCR reaction (50 μL) consisted of 0.2 pmol/μL of each primer, 0.06 U/μL AmpliTaq Gold DNA polymerase, 5 μL AmpliTaq Gold buffer, 2 mM $MgCl_2$, and 0.2 mM dNTP mix (Life Technologies Europe, Espoo, Finland). The PCR program consisted of an initial denaturation at 94°C for 10 minutes, then 38 cycles of DNA denaturation at 94°C for 30 seconds, primer annealing at 64°C for 30 seconds, and extension at 72°C for 1 minute.

We determined susceptibility to the antimicrobial drugs ciprofloxacin, nalidixic acid, and meropenem using the standard agar dilution method according to the Clinical Laboratory and Standards Institute guidelines (*11*). We screened isolates showing reduced fluoroquinolone susceptibility; specifically, to ciprofloxacin (MIC ≥0.125 μg/mL), that were susceptible or resistant on a low level to nalidixic acid (MIC ≤32 μg/mL) (*9*) for transferable plasmid-mediated quinolone resistance determinants. We screened the *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib-cr* genes with a previously described method (*14*).

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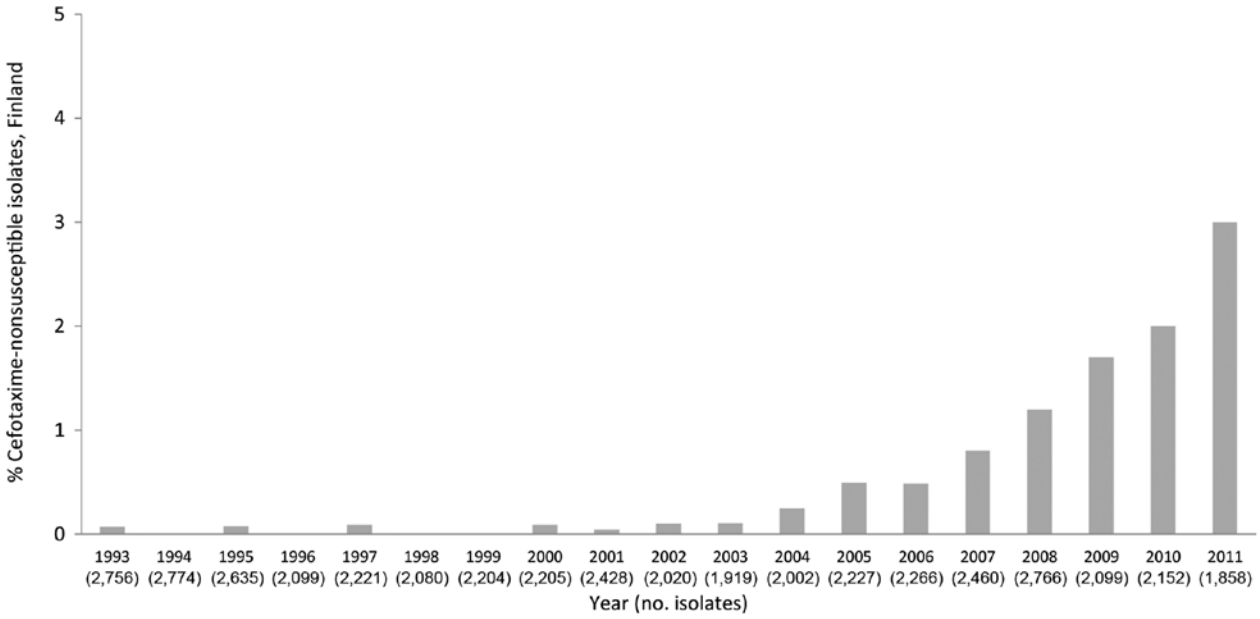

Figure 1. The increasing trend (p<0.001) in the proportion (%) of cefotaxime-nonsusceptible (30-μg disk diameter ≤22 mm) S*almonella enterica* isolates in Finland during 1993–2011.

We performed the statistical analysis using a log-binomial model and year as an explanatory variable to assess the log-linear trend in time in the percentage/proportion of cefotaxime-nonsusceptible *S. enterica* isolates. A p value <0.05 was considered significant. Statistical analyses were performed by using IBM SPSS Statistics Version 21 (IBM Corporation, Armonk, NY, USA).

During 1993–2011, we found 225 cefotaxime-nonsusceptible *S. enterica* isolates and observed a significantly increasing trend (p<0.001) of cefotaxime-nonsusceptible *S. enterica* isolates (Figure 1). During 1993–1999, 6 *S. enterica* isolates showed nonsusceptibility to cefotaxime. From the year 2000 onwards, cefotaxime-nonsusceptible isolates were detected more frequently, and in the mid-2000s, the absolute number as well as the proportion of cefotaxime-nonsusceptible *Salmonella* isolates started to increase rapidly: 55 (2.96%) of 1,858 isolates were positive for this resistance phenotype in 2011 (Figure 1).

During 2000–2011, of the 183 cefotaxime-nonsusceptible isolates, 95 produced ESBL and 88 produced AmpC.

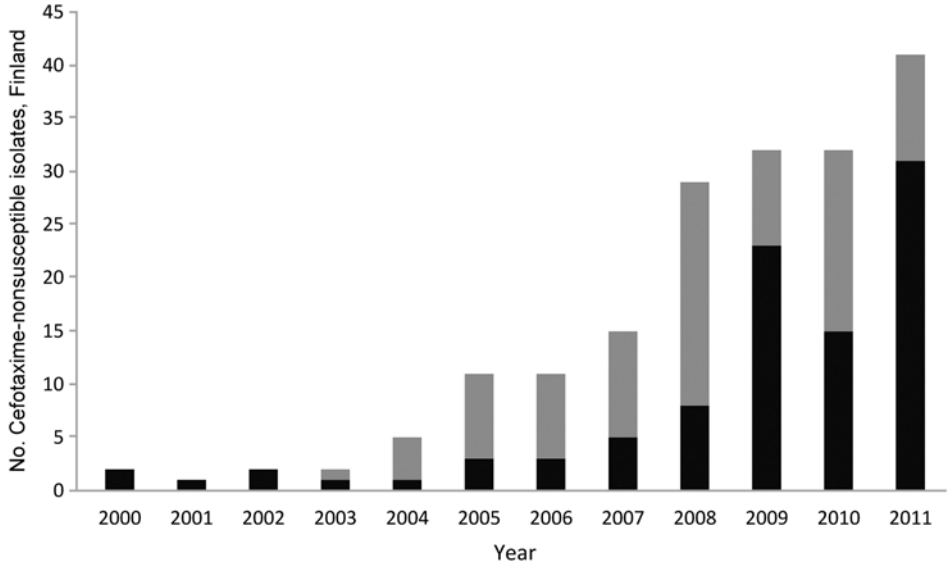

Figure 2. Number of cefotaxime-nonsusceptible *S. enterica* isolates carrying extended-spectrum β-lactamase (black bars) and AmpC genes (gray bars) in Finland, 1993–2011.

Table. β-lactamase and plasmid-mediated quinolone resistance genes linked to origin and serovar in cefotaxime-nonsusceptible *Salmonella enterica* isolates, 2006–2011

Gene profile (no. isolates)	Primary origin (no. isolates)	Serovar(s) (no. isolates)
CTX-M + *qnrS* (32)	Thailand (30)	Typhimurium (5); *S. enterica* B 4,[5],12:i:- (18)
CTX-M + *qnrB* (2)	Spain (1)/India (1)	Grumpensis (1)/ Minnesota (1)
CTX-M + *qnrA* (1)	Ethiopia (1)	Concord (1)
SHV + *qnrS* (1)	Egypt (1)	Heidelberg (1)
SHV + *qnrB* (1)	Germany (1)	Senftenberg (1)
CMY + *qnrS* (8)	Thailand (6)	Rissen (4)
CMY/DHA + *qnrS* (1)	Thailand (1)	*S. enterica* B 4,[5],12:i:- (1)
DHA+ *qnrS* (1)	China (1)	Typhimurium (1)

The number and proportion of ESBL- and AmpC-positive isolates varied and the number of cefotaxime-nonsusceptible isolates increased (Figure 2). The number of AmpC-positive *S. enterica* isolates was highest in 2008, and the number of ESBL-positive isolates was highest in 2011. During 2000–2005, 10 ESBL-positive isolates were found; these isolates had been identified in samples collected from travelers from Finland returning from the Mediterranean area, Egypt, and European countries. Isolates positive for the SHV gene mainly originated from Egypt. From 2006 onwards, the main geographic origin of ESBL-positive isolates was Southeast Asia; 61% (52/85) of the ESBL isolates originated from Thailand. During the same time, the CTX-M determinant (72/85 isolates) became more common than SHV. Of the ESBL positive isolates, 44 of 95 belonged to *S. enterica* ser. Typhimurium or the monophasic 4,[5],12:i:- variant of this serovar; 38 of these originated from Thailand.

AmpC-positive isolates were found from 2003 onwards. During 2003–2004, the AmpC-positive isolates were found in travelers from Finland returning from Spain, India, Mexico, and Africa. From 2005 onwards, the AmpC-positive isolates also commonly originated from Thailand (61/83 isolates). The most common AmpC gene was CMY. Of the AmpC positive isolates, 21 of 88 belonged to *S. enterica* ser. Typhimurium or a monophasic 4,[5],12:i:- variant of *S. enterica* ser. Typhimurium serotypes; 8 of these originated from Thailand.

Of the 183 cefotaxime-nonsusceptible *Salmonella* isolates, 47 had the *qnr* phenotype; i.e., they showed reduced susceptibility to ciprofloxacin (MIC ≥0.125 μg/mL) but were susceptible or only resistant on a low level to nalidixic acid (MIC ≤32 μg/mL). These isolates were collected from travelers during 2006–2011. Co-resistance to ESBL determinants were detected in 37 isolates: 35 isolates were CTX-M+*qnr*–positive, including 1 CTX-M+SHV+*qnr*–positive isolate. Two *Salmonella* isolates were SHV+*qnr* positive. Of the 35 CTX-M+*qnr*–positive isolates, 30 isolates originated from Thailand and 23 of them belonged to the serovar *S. enterica ser. Typhimurium* or a monophasic 4,[5],12:i:- variant of *S. enterica* ser. Typhimurium serovars. Ten isolates with an AmpC phenotype were also *qnr*-positive. Nine of these originated from Southeast Asia and 3 of them were *S. enterica* ser.Typhimurium or *S. enterica* ser. 4,[5],12:i:- (Table).

Conclusions

In this study, we described a significant increase ($p<0.001$) in cefotaxime nonsusceptibility among *Salmonella* isolates, collected from patients in Finland during 1993–2011. In *Salmonella* spp., cefotaxime nonsusceptibility is thought to be linked to AmpC-type β-lactamases, and production of ESBLs to be more rare (*3*). According to our results, ESBL and AmpC production (51.9% vs. 48.1%) were equally common among the cefotaxime-nonsusceptible *Salmonella* serovars.

During the study period, a change in the geographic origin of cefotaxime-nonsusceptible *Salmonella* isolates was observed: its predominance in Egypt and the Mediterranean area shifted to Thailand and other Southeast Asian countries. We previously reported that *Salmonella* isolates with the *qnr* phenotype are concentrated in Southeast Asia, mainly Thailand (*9*). In this study, 37 ESBL-positive and 10 AmpC-positive *S. enterica* isolates were also *qnr* positive and 40/47 isolates were from Southeast Asia. These results were in concordance with previous reports: ESBL-producing *Enterobacteriaceae* are commonly isolated from patients returning from Southeast Asia (*15*) and ESBL and plasmid-mediated quinolone-resistance mechanisms are commonly found in the same plasmids in *Enterobacteriaceae* and *Salmonella* (*4*,*6*).

We conclude that cefotaxime-nonsusceptible *Salmonella* isolates are already a threat for travelers to Southeast Asia. Because of the mobile nature of the ESBL and AmpC genes, *qnr* resistance determinants, and increased travel, this is a worldwide threat, and makes the treatment for invasive *Salmonella* infections even more challenging.

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etymologia

Artemisinin [ahr″tə-mis′ĭ-nin]

Artemisinin is an antimalarial lactone derived from *qing hao* (*Artemisia annua* or sweet wormwood). The medicinal value of this plant has been known to the Chinese for at least 2,000 years. In 1596, Li Shizhen recommended tea made from *qing hao* specifically to treat malaria symptoms. The genus name is derived from the Greek goddess Artemis and, more specifically, may have been named after Queen Artemisia II of Caria, a botanist and medical researcher in the fourth century BCE.

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New Viruses in Idiopathic Human Diarrhea Cases, the Netherlands

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Emerging viral infections can be identified by using a viral metagenomics approach for clinical human material. Diarrhea samples of patients with unexplained gastroenteritis from the Netherlands were analyzed by using viral metagenomics. Novel circular DNA viruses, bufaviruses, and genogroup III picobirnaviruses were identified. These data expand our knowledge of the human virome.

The list of emerging viral pathogens is ever-changing. The recognition that an increasing number of diseases that were once unexplained are caused by infectious agents has increased substantially in recent years because of breakthroughs in the metagenomics field (*1*). The human gut is a reservoir of a wide variety of microorganisms. In industrialized countries, diarrheal diseases are a major cause of illness among persons of all age groups, and most gastroenteritis cases are caused by viruses (*2*). However, despite extensive diagnostic analysis, the cause of many diarrhea cases remains unresolved.

We analyzed stool samples from 27 patients in the Netherlands who had acute gastroenteritis of unknown etiology for (un)known viruses by using a metagenomics approach. Samples were obtained from patients with sporadic cases and from patients involved in outbreaks of diarrhea and vomiting, for which most common causes of gastroenteritis had been ruled out.

The Study

Thirteen diarrhea stool samples were obtained from patients with gastroenteritis during 2005–2009 whose infection was not identified despite extensive testing at the reference laboratory for viral gastroenteritis at the National Institute for Public Health and the Environment, Bilthoven, the Netherlands (*3*). In addition, we obtained 14 stool samples from patients hospitalized during 7 gastroenteritis outbreaks in 2008 and 2009 (Table) (*4*). All procedures were performed in compliance with relevant laws (Medical Ethical Committee, University Medical Center Utrecht approval no. 07–310). Samples were analyzed by using a viral metagenomics approach and 169,305 trimmed reads were characterized according to BLAST searches as described (*5*).

Mammalian viral sequences were detected in stool samples from 13 of 27 patients (Table). Anelloviruses that displayed ≈60%–91% nt identities with known anelloviruses were obtained from patients VS6600014 and VS6600015. Because anelloviruses are endemic worldwide, present in many different tissues, and were found in ≈0.05% of the total number of reads, we did not consider it likely that they played a causative role in the gastroenteritis of the patients. Patient VS6600014 was infected with human herpesvirus 4 and an aichivirus; the aichivirus is associated with diarrhea (*6*) and constituted ≈1.7% of the total number of reads. A partial viral protein 2 nucleotide sequence (336 bp covered by 7 reads; KJ206565) of a bufavirus was detected in patient VS6600009. This sequence, which aligned with corresponding sequences of a recently described bufavirus in children with diarrhea in Burkina Faso (*7*), was phylogenetically analyzed and showed 67%–73% nt identity (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/20/7/14-0190-Techapp1.pdf). Attempts to obtain more sequences from this virus were unsuccessful, and results for real-time PCRs specific for a nonstructural protein 1 gene remained negative, probably because of low virus titers in the sample. New picobirnaviruses and circular DNA viruses were identified and further characterized.

Picobirnaviruses are highly variable, double-stranded RNA viruses with a bisegmented genome. Segment 1 (2.2–2.7 kb) encodes the capsid (Cap) protein and potential hypothetical protein(s), and segment 2 (1.2–1.9 kb) encodes the RNA-dependent RNA polymerase (RdRP). On the basis of sequence diversity in RdRP, picobirnaviruses are classified into 2 genogroups (*8*). They have been detected in humans and a wide range of animals (*8*) and might be opportunistic enteric pathogens (*8*,*9*). Stool samples from 7 patients had virus sequences with relatively high homology with known group I picobirnaviruses (Table; online Technical Appendix Figure 2). A near-complete highly divergent picobirnavirus genome was obtained by 454-sequencing (Roche, Basel, Switzerland) of samples from patient VS6600008 (GenBank accession nos. KJ206568 and KJ206569). The genome organization is highly similar to that of picobirnaviruses (Figure 1, panel A).

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Table. Detection of mammalian viral sequences in 27 patients with diarrhea by using viral metagenomics, the Netherlands, 2005–2009*

Patient no.	Age, y	Diarrhea type or source	Year of sampling	No. trimmed reads	No. trimmed viral reads	Virus species (no. reads; % nucleotide identity)
VS6600008	7	Sporadic	2008	7,851	271	Human picobirnavirus (221; NA)
VS6600009	25	Sporadic	2008	8,079	10	Bufavirus 1 (7; 67–94)
VS6600010	87	Sporadic	2008	3,237	1	NA
VS6600011	66	Sporadic	2008	3,866	6	NA
VS6600013	48	Sporadic	2008	2,849	19	NA
VS6600014	40	Sporadic	2008	8,079	143	Aichivirus (139; 98) Human herpesvirus 4 (1; 91) Anellovirus (3; 61–85)
VS6600015	84	Sporadic	2008	11,197	9	Anellovirus (6; 81–97)
VS6600016	37	Sporadic	2008	7,333	1	NA
VS6600017	62	Sporadic	2008	546	0	NA
VS6600018	<1	Sporadic	2008	3,936	4	NA
VS6600019	30	Sporadic	2008	9,590	18	NA
VS6600020	57	Sporadic	2009	10,389	37	Porcine picobirnavirus (6; 57–80)
VS6600021	52	Sporadic	2009	4,587	0	NA
VS6600022	27	OB2005111	2005	4,877	113	Fur seal–associated circular DNA virus (98; NA);
VS6600023	47	OB2005111	2005	7,423	117	Human picobirnavirus (91; 82–87)
VS6600024	47	OB2005115	2005	6,852	338	NA
VS6600025	6	OB2005115	2005	8,949	34	Human picobirnavirus (23; 75–84)
VS6600026	12	OB2006097	2006	6,879	52	Otarine picobirnavirus (42; 70–88)
VS6600027	13	OB2006097	2006	9,481	32	NA
VS6600028	52	OB2008169	2008	5,222	74	NA
VS6600029	32	OB2008169	2008	1,568	4	Human picobirnavirus (3; 86)
VS6600030	26	OB2008190	2008	7,377	57	NA
VS6600031	10	OB2008190	2008	4,541	14	NA
VS6600032	90	OB2008217	2008	4,185	13	Fur seal–associated circular DNA virus (4; NA)
VS6600033	97	OB2008217	2008	3,299	11	NA
VS6600034	89	OB2009024	2009	14,797	168	Human picobirnavirus (115;68%–77%)
VS6600035	91	OB2009024	2009	2,256	8	Human picobirnavirus (3; 70%)

*Classification of sequences was based on taxonomic origin of the best-hit sequence and was performed by using MEGAN version 4.40.4 (http://ab.inf.uni-tuebingen.de/software/megan/) with E cutoff values of 0.001 and 10^{-10} for BLASTn and BLASTx searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi), respectively (*5*,*6*). NA, not applicable; OB, outbreak

The pairwise amino acid identity of the partial RdRP of the human picobirnavirus PBVIII/*Homo sapiens*/VS6600008/2008/NL/KJ206569 and that of other representative picobirnaviruses was determined (Figure 1, panels B–D). The intragenogroup amino acid identity between picobirnavirus species ranged from 34.5% to 99.7% in RdRP (Figure 1, panel D). The intergenogroup amino acid identity between genogroup I and II picobirnaviruses ranged from 17.5% to 24.1% (Figure 1, panel D). PBVIII/*Homo sapiens*/VS6600008/2008/NL/KJ206569 showed low amino acid identity (19.4%–26.1%) in the intergenogroup range with genogroup I and II picobirnaviruses (Figure 1, panels B–D), which justifies the placement of this virus in a new genogroup III. Only a few Cap sequences of picobirnaviruses are available; these sequences show <25% amino acid identity to each other, and a clear genogroup division cannot be distinguished (Figure 1, panel C). A picobirnavirus VS6600008-specific real time PCR was performed on the total sample set with primers VS791 (5′-CGATGGATCTTTATGTTCCCG-3′), VS792 (5′-GTAGTTGAAATGTTGATCCATTT-3′), and VS793 (5′-CAAACTTTCCAGCAACCGCTT-3′) labeled with 6-carboxy-fluorescein and 6-carboxy-tetramethyl-rhodamine as described (*10*). Only the sample from patient VS6600008 had a positive result (cycle threshold 25.1).

Novel circular small DNA viruses containing a rolling circle replication initiator protein gene (Rep) have been discovered at increasing rates from animals and humans (*11*). These viruses are extremely diverse and encode at least Cap protein and Rep protein located in opposite genomic orientations and separated by 2 intergenic regions. On the basis of genome organization and amino acid sequence identity of Rep proteins, novel circular DNA viruses seem most closely related to others viruses of the family *Circoviridae* (*11*). A complete circular virus genome (2,836 nt) was obtained from patient VS6600022 by rolling circle amplification and 454-sequencing (KJ206566) (Table). The genome showed an ambisense organization and 2 major inversely arranged open reading frames encoding the Rep and Cap proteins (Figure 2, panel A). A stem-loop structure with the conserved circovirus nonanucleotide motif (5′-TAGTATTAC-3′) was found in the 5′-intergenic region. However, genome size, presence of 2 putative other open reading frames with no sequence homology to any sequence in GenBank, and deviations in WWDGY, DDFYGW, DRYP, FTLNN, TPHLQG, and

CSK motifs in the Rep protein, which are ordinarily conserved, indicate that this virus is different from characteristic circoviruses.

Pairwise amino acid identity between the Rep protein of virus VS6600022 and other representative circovirus-like viruses was determined, and a phylogenetic tree was generated (Figure 2, panel B and C). The Rep protein of VS6600022 showed <20% aa identity with all circoviruses and was most closely related to a circular DNA virus from feces of a New Zealand fur seal (33% identity) (*12*). A similar phylogenetic relationship was observed in the Cap protein.

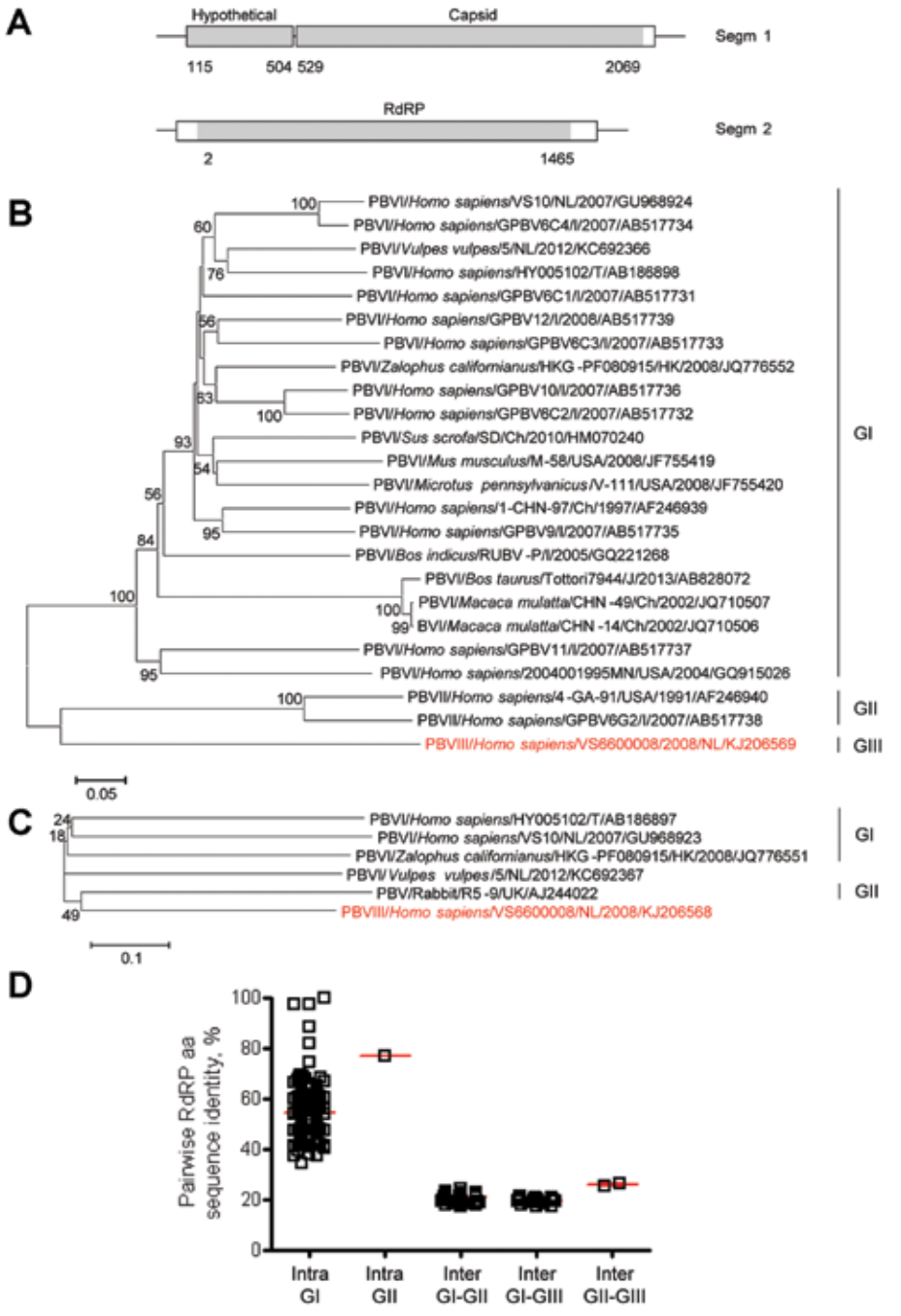

Figure 1. Genome organization and phylogenetic analysis of human picobirnavirus (PBV) VS6600008 isolated in the Netherlands, 2005–2009. A) Putative schematic genome organization of human PBV VS6600008. Locations of major open reading frames are indicated in white and sequences obtained by next-generation sequencing are indicated in gray. Segm, segment; RdRP, RNA-dependent RNA polymerase. B) Phylogenetic neighbor-joining tree with p-distances and 1,000 bootstrap replicates of amino acid sequences of partial RdRP genes corresponding to aa 80–377 of reference PBV strain HY005102; AB186898, PBV VS6600008, and representative PBVs. Alignments were created by using ClustalX 2.0 (http://www.clustal.org/). Viruses are shown as virus/host species/strain/country/year/GenBank accession no. (if available). Virus isolated in this study is indicated in red. Genogroups are indicated on the right. Scale bar indicates amino acid substitutions per site. NL, the Netherlands; I, India; T, Thailand; Ch, China; USA, United States; UK, United Kingdom; J, Japan; HK, Hong Kong. C) Phylogenetic neighbor-joining tree with p-distances and 1,000 bootstrap replicates of the amino acid sequences of the partial capsid genes corresponding to aa 1–220 of reference PBV strain HY005102; AB186897, PBV VS6600008, and representative PBVs. Alignments were created by using ClustalX 2.0. Virus isolated in this study is indicated by underline. Genogroups are indicated on the right. Scale bar indicates amino acid substitutions per site. D) Pairwise intragenogroup (Intra) and intergenogroup (Inter) amino acid sequence identities determined by using Bioedit 7.0.9.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) between the partial RdRP sequences (corresponding to amino acids 80–377 of reference PBV strain HY005102; AB186898). Each square represents pairwise RdRP amino acid sequence identity between viruses in panel B. Red bars indicate mean and SEM.

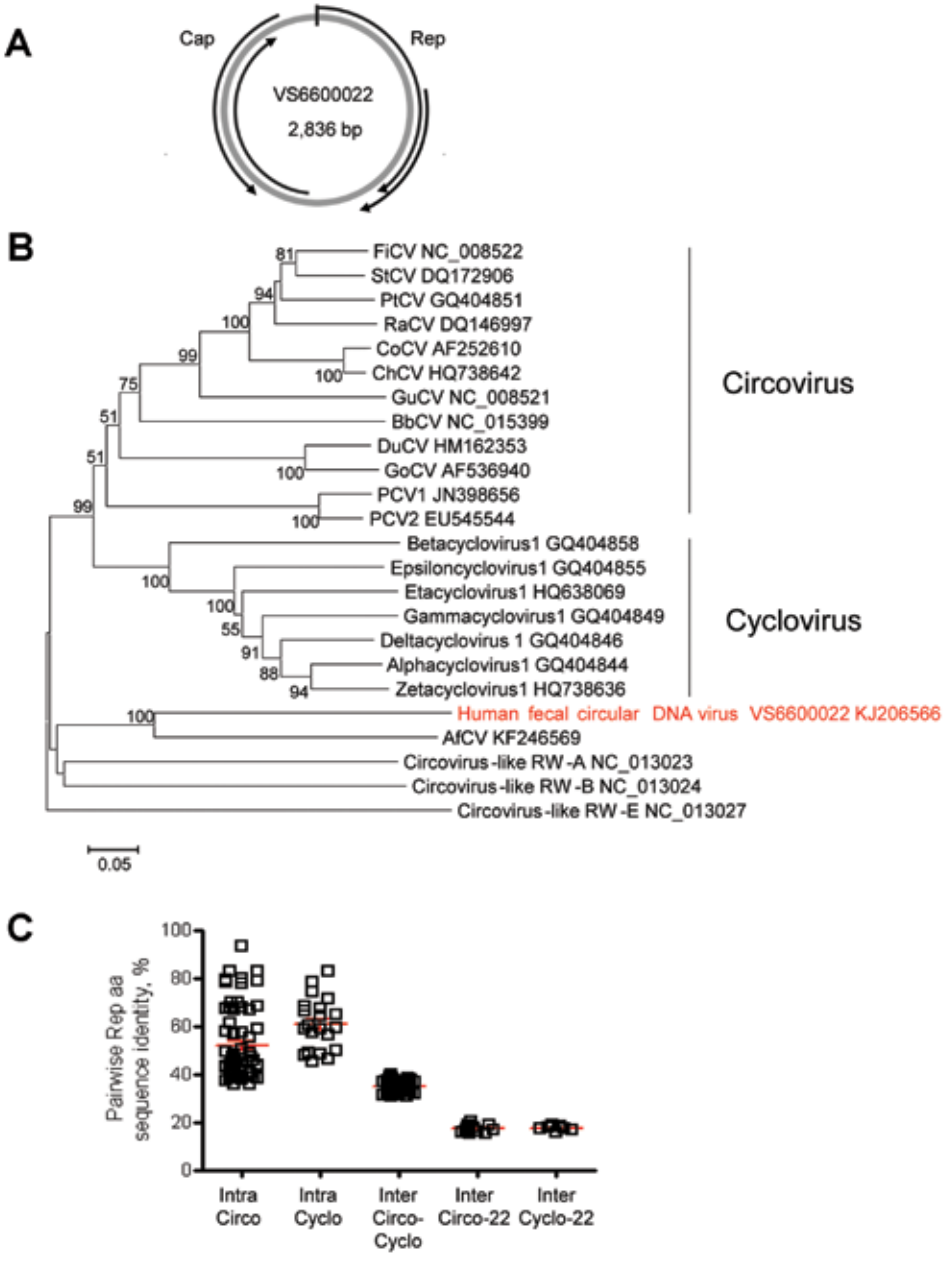

Figure 2. Genome organization and phylogenetic analysis of human fecal circular DNA virus VS6600022, the Netherlands, 2005–2009. A) Putative schematic genome organization. Arrows indicate major open reading frames. Cap, capsid; Rep, rolling circle replication initiator protein. B) Phylogenetic neighbor-joining tree with p-distances and 1,000 bootstrap replicates created with MEGA5 of amino acid sequences of the Rep genes of human fecal circular DNA virus VS6600022 and representative circoviruses that were aligned by using ClustalX2.0 (http://www.clustal.org/). Virus isolated in this study is indicated by underline. Scale bar indicates amino acid substitutions per site. FiCV, finch circovirus; StCV, starling circovirus; PtCV, *Pan troglodytes* circovirus; RaCV, raven circovirus; CoCV, columbid circovirus; ChCV, chicken circovirus; GuCV, gull circovirus; PCV, porcine circovirus; BbCV; *Barbus barbus* circovirus; DuCV, duck circovirus; GoCV, goose circovirus; AfCV, *Arctocephalus forsteri* circovirus. C) Pairwise intraspecies (Intra) and interspecies (Inter) amino acid sequence identities determined by using Bioedit 7.0.9.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) between the Rep protein sequences of VS6600022 and representative species in the genera *Circovirus* (Circo) and *Cyclovirus* (Cyclo). Each square represents the pairwise RdRP amino acid sequence identity between the viruses in panel B. Red bars indicate mean and SEM.

A partial viral genome was identified in patient VS6600032 (KJ206567), and the partial Rep protein of this virus was most closely related to that of VS6600022 (45% aa identity). The cellular host for the novel circular DNA viruses from patients with diarrhea cannot be deduced, and although replication in human cells is conceivable, these viruses might also originate from the diet of the patient. A VS6600022-specific real-time PCR was performed on the total sample set with primers VS794 (5′-ATCGAAGRWCAYCCTGGAAC-3′), VS795 (5′-TKRCACAGGGTACTTGTATC-3′), and VS796 (5′-ACTGTCCTCGTGTACATTGGCAA-3′) labeled with 6-carboxy-fluorescein and 6-carboxy-tetramethyl-rhodamine as described (*13*). Only the sample from patient VS6600022 had a positive result (cycle threshold 34.8).

Conclusions

Viral metagenomics of patients samples from unexplained diarrhea cases in Netherlands identified viruses of the families *Anelloviridae*, *Picobirnaviridae*, *Herpesviridae*, and *Picornaviridae*, some of which might be associated with development of gastroenteritis (*6–8*,*14*,*15*). The discoveries of a new genogroup III picobirnavirus and circular DNA virus from human diarrhea samples expands

our knowledge of virus diversity in the human gut. We also showed that recently identified bufaviruses are present beyond the boundaries of Africa (*7*). Mammalian viral sequences were detected in patients with sporadic gastroenteritis and in persons during outbreaks in relatively equal proportions. In addition, specific viral infections were not identified in samples from the same gastroenteritis outbreaks. On the basis of these findings, we cannot conclude or rule out that these viruses cause disease. Further studies are needed to clarify the epidemiology and possible pathogenicity of these viruses in humans.

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Outbreak-Related Porcine Epidemic Diarrhea Virus Strains Similar to US Strains, South Korea, 2013

Sunhee Lee and Changhee Lee

In late 2013, outbreaks of porcine epidemic diarrhea virus (PEDV) infection recurred in South Korea. Genetic and phylogenetic analyses showed that isolates from the outbreaks were most closely related to emergent US strains of PEDV. These US strain–like PEDV variants are prevalent in South Korea and responsible for recent outbreaks in the country.

Porcine epidemic diarrhea (PED), a devastating swine disease, is characterized by watery diarrhea, followed by dehydration, and a high death rate among suckling pigs (*1*,*2*). The disease was first recognized in England in 1971 (*3*), and since then, outbreaks have been reported in Europe, Asia, and recently, the United States (*4*–*7*). The causative agent of this disease, PED virus (PEDV), is a member of the order *Nidovirales*, family *Coronaviridae*, genus *Alphacoronavirus* (*2*,*8*). The virus first emerged in South Korea in 1992 (*9*), and PED outbreaks subsequently occurred every year until early 2010, causing economic losses to the pork industry. However, after South Korea experienced severe outbreaks of foot-and-mouth disease in 2010–2011, the prevalence of PEDV infections was low and only sporadic outbreaks occurred. This decline in PED epidemics likely resulted from the culling of >3 million pigs in South Korea during the 2010–2011 foot-and-mouth disease outbreaks. However, starting in late 2013, outbreaks of PED increased remarkably and swept rapidly across the country. To determine the origin and diversity of the PEDVs responsible for the ongoing outbreaks in South Korea, we sought to determine the full-length sequences of the spike proteins of field isolates and the complete genome sequence of a representative strain.

The Study

During December 2013–January 2014, specimens of small intestine or feces were collected from 10 pigs that had watery diarrhea; each of the pigs lived at a different swine farm in South Korea. All samples were prepared as 10% suspensions as described elsewhere (*10*) and subjected to reverse transcription PCR using a Transmissible Gastroenteritis and Porcine Epidemic Diarrhea Detection Kit (iNtRON Biotechnology, Seongnam, South Korea) according to the manufacturer's protocol. The full-length spike glycoprotein sequences of 10 PEDVs identified from the pigs were subsequently determined as previously described (*10*) and deposited in GenBank under the accession numbers shown in Figure 1. In addition, the complete genome of a PEDV strain, KNU-1305, was sequenced and analyzed. The 5′ and 3′ ends of the KNU-1305 genome were determined by rapid amplification of cDNA ends as described elsewhere (*11*). Ten overlapping cDNA fragments were generated to encompass the entire genome, pooled in equimolar amounts, and subjected to next-generation sequencing as previously described (*12*); the sequencing reads were assembled by using complete PEDV reference genomes from GenBank (*13*,*14*). The KOR/KNU-1305/2013 PEDV sequence data were deposited in GenBank under accession no. KJ662670. The sequences of 46 fully sequenced spike genes and the 21 complete genomes of PEDV strains were independently used in sequence alignments and phylogenetic analyses as described elsewhere (*10*).

We determined that the full-length spike genes of the PEDV strains were 9 nt longer than that of the prototype PEDV strain, CV777; this difference was caused by the presence of genetic signatures for recent PEDV field isolates as described elsewhere (*10*). The similarity between the spike genes was determined; sequence homology results are described in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/20/7/14-0294-Techapp1.pdf). Nucleotide sequence analysis showed high homology (98.8%–99.9%) among the 10 tested isolates. In contrast, the isolates all shared only 94.3%–94.7% nt sequence identity with a previously sequenced field isolate from South Korea, KNU-0801. However, the sequences of the 10 isolates were compared with those of other published PEDV strains and found to consistently share 99.2%–99.9% nt identity with recently emergent US strains.

The complete genomic sequence of KNU-1305 was determined to be 28,038 nt in length, excluding the 3′ ploy(A) tail. The complete PEDV genome of KNU-1305 shared 96.3%–99.9% nt identity with other complete PEDV genomes available in GenBank; the highest nucleotide identity (99.9%) was with US strains CO/13, IA1, IN17846, and MN. Compared with the complete genome of US strain IA1, KNU-1305 showed 49 different nucleotides: 1 each was in the 5′ untranslated region and the membrane gene, 7 were in the spike gene, and 35 and 5 were in open reading frames 1 and 3, respectively. Together, our results indicate that the PEDV isolates from South Korea

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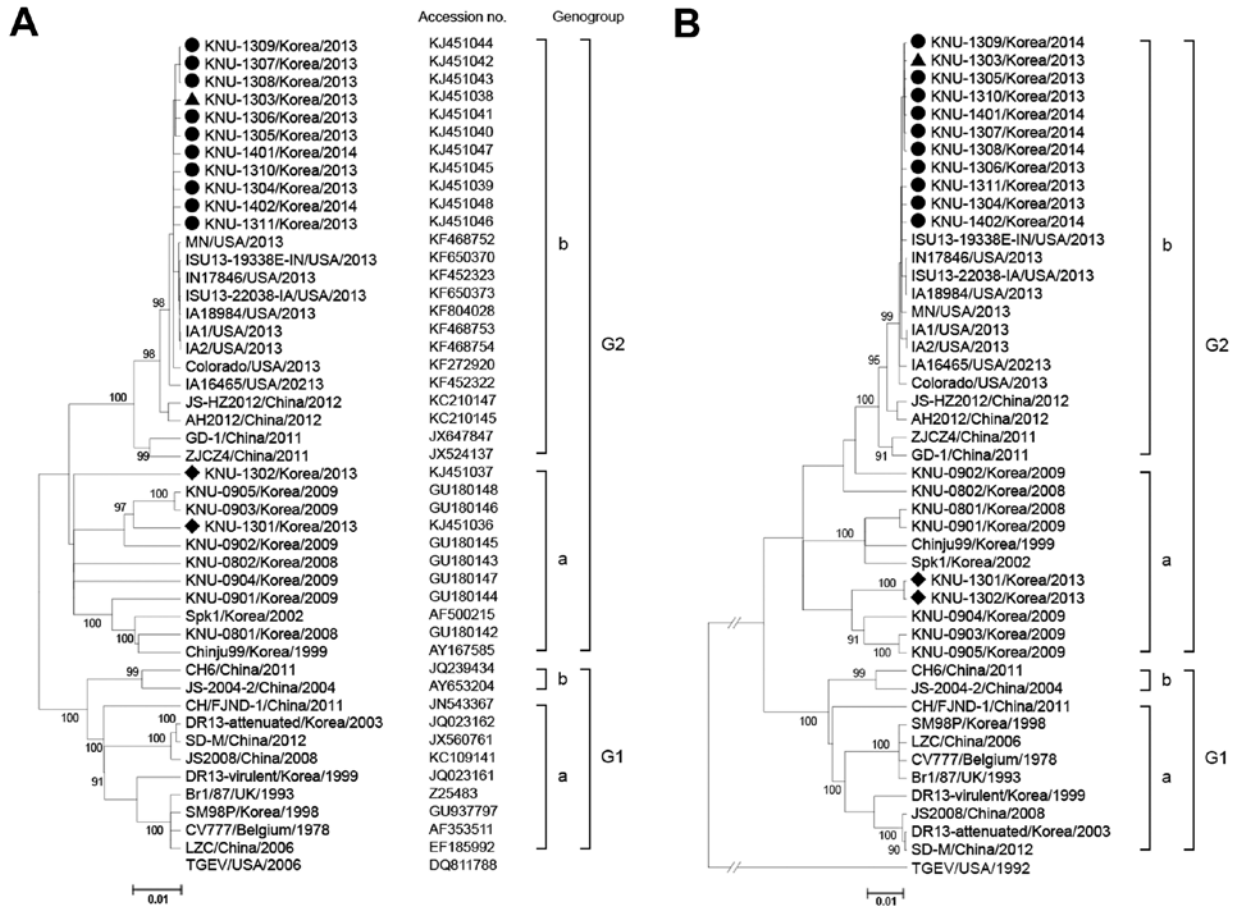

Figure 1. Phylogenetic analyses based on the nucleotide sequences of the spike gene (A) and S1 portion (B) of porcine epidemic diarrhea virus (PEDV) strains. A putative similar region of the spike protein of transmissible gastroenteritis virus (TGEV) was included as an outgroup in this study. Multiple-sequencing alignments were performed by using ClustalX (http://www.clustal.org/), and the phylogenetic tree was constructed from the aligned nucleotide sequences by using the neighbor-joining method. Numbers at each branch represent bootstrap values >50% of 1,000 replicates. Names of the strains, countries and years of isolation, GenBank accession numbers, and genogroups and subgroups proposed in this study are shown. Solid circles indicate the strains from South Korea from this study that are similar to US PEDV strains; solid triangle indicates the early 2013 strain that is similar to the US PEDV strains; solid diamonds indicate the early 2013 strains that are similar to the previous PEDV strains from South Korea. Scale bars indicate nucleotide substitutions per site.

were highly homologous with strains responsible for recent outbreaks in the United States.

The full-length spike gene–based phylogenetic analysis revealed that the PEDV strains were clearly defined into 2 separate clusters, designated genogroup 1 (G1) and genogroup 2 (G2); each of the groups can be further divided into subgroups 1a, 1b, 2a, and 2b (Figure 1, panel A). All 10 PEDV strains from South Korea were classified into subgroup 2b and most closely clustered together with the recent US strains in an adjacent clade with the same subgroup, suggesting that the US strains may be the origin of the recurrence of PEDV infections in South Korea. Subsequent phylogenetic analysis of the S1 protein showed the grouping structure was the same as that in the spike gene–based tree (Figure 1, panel B). In addition, phylogenetic analysis based on the entire genome sequences demonstrated that strain KNU-1305 is grouped within the same cluster with the US stains (Figure 2).

Conclusions

Sequence comparison and phylogenetic analyses indicated that the South Korean PEDV isolates in this study differed genetically from previous isolates from South Korea and were most genetically similar to PEDV strains emerging in the United States during 2013. Therefore, our data suggest that the recent strains from South Korea might have originated from the United States, likely by the importation of pig breeding stock during or after the sudden emergence of PEDV in the United States. However, it remains unclear whether the US strain–like

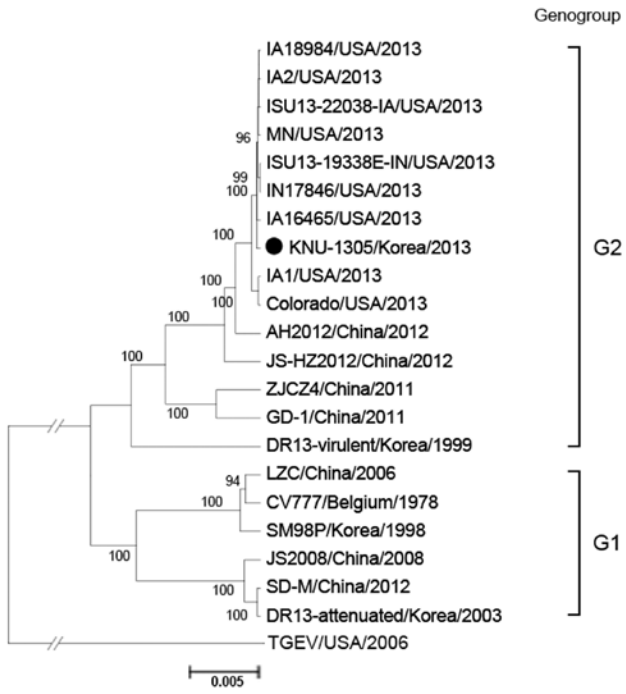

Figure 2. Phylogenetic analysis based on the nucleotide sequences of the full-length genomes of porcine epidemic diarrhea virus (PEDV) strains (GenBank numbers are shown in Figure 1, panel A). The complete genome sequence of transmissible gastroenteritis virus (TGEV) was included as an outgroup in this study. Numbers at each branch represent bootstrap values >50% of 1,000 replicates. Names of the strains, countries and years of isolation, and genogroups and subgroups proposed in this study are shown. Solid circle indicates the strain from South Korea, KNU-1305, that is similar to the US PEDV strains. The scale bar indicates nucleotide substitutions per site.

PEDVs had existed in South Korea before the emergence of PEDV in the United States. Our retrospective study, using PEDV-positive fecal samples obtained during early 2013 (KNU-1301–03), verified the presence of a PEDV isolate (KNU-1303) in South Korea in May 2013 that was placed in the same clade as the US strains (Figure 1). Thus, it is also conceivable that the strains may have already been present in South Korea as a minor lineage before the recent emergence of PEDV in the United States. Given that situation, the virus could have evolved independently by recombination, or the virus could have originated directly from China and have subsequently become dominant, leading, under suitable circumstances, to the current acute outbreak in South Korea.

Further molecular epidemiologic study is needed to find temporal and geographic evidence for the exact origin and evolution of the recent US strain–like PEDVs in South Korea. In addition, the existence of distinct PEDV lineages in South Korea suggests the potential for recombination events between different PEDV lineages or sublineages and possible cocirculation of different PEDV subgroups. During the 2010–2011 foot-and-mouth disease outbreaks, more than one third of the total pig population in South Korea was slaughtered, and since then, the importation of breeding pigs has greatly increased. However, the vaccination program for PED prevention in South Korea was not fully implemented before this importation began. Thus, it is not unexpected that the pig population appears to have a low level of immunity against PEDV and, as a result, large-scale outbreaks of PED could occur. To prevent the periodic recurrence of acute PEDV outbreaks in South Korea, a proper vaccination program should be implemented to enhance overall immunity to the virus in all stock, and strict biosecurity measures should be established. In addition, current quarantine procedures should be adequately reinforced with respect to breeding stock imported from the United States. Our findings will provide insights into a better understanding of the genetic diversity of PEDV strains and contribute to the development of more effective preventive measures against PED.

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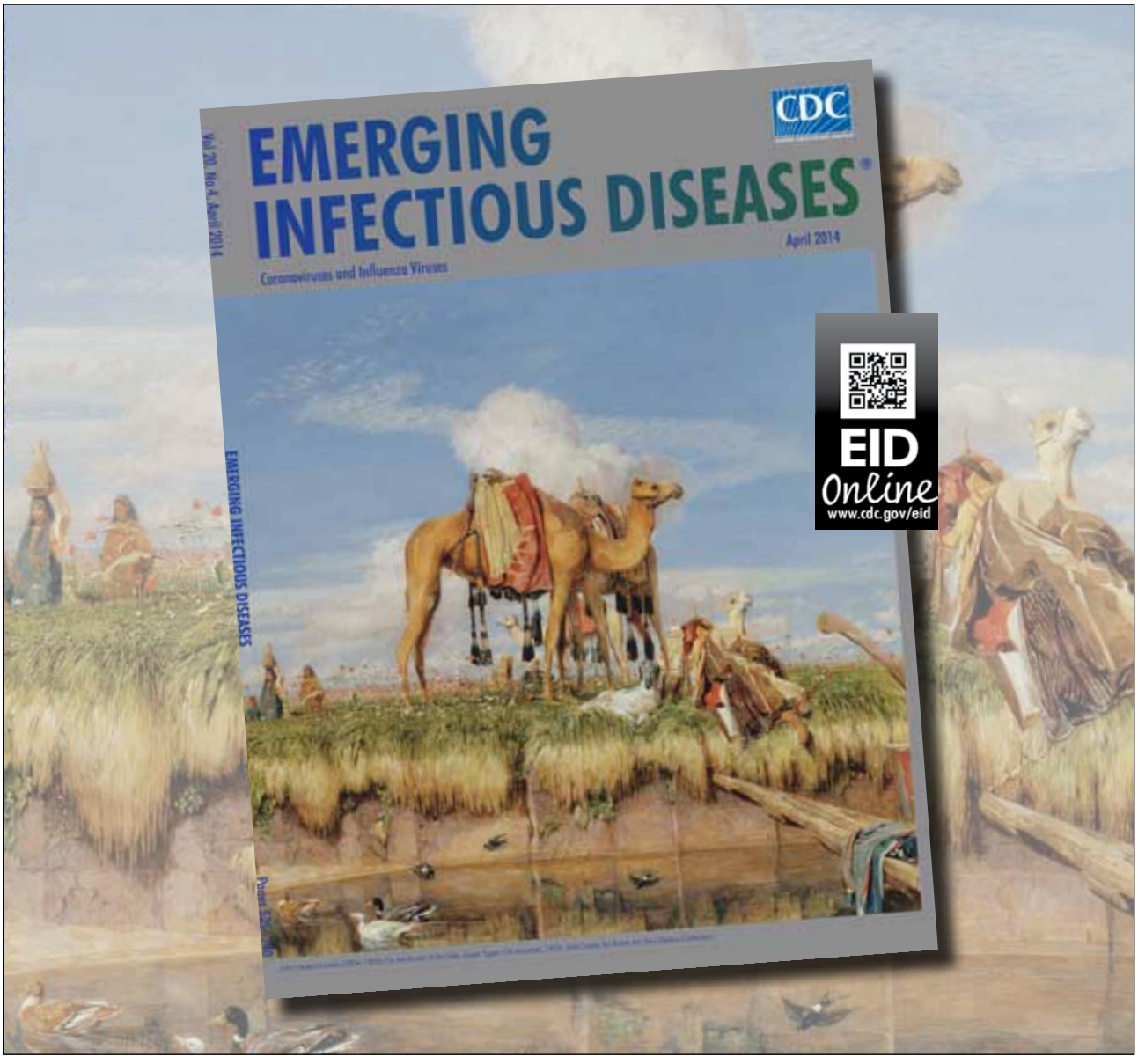

Detection and Genetic Characterization of Deltacoronavirus in Pigs, Ohio, USA, 2014

Leyi Wang,[1] Beverly Byrum, and Yan Zhang[1]

In Ohio, United States, in early 2014, a deltacoronavirus was detected in feces and intestine samples from pigs with diarrheal disease. The complete genome sequence and phylogenetic analysis of the virus confirmed that the virus is closely related to a porcine deltacoronavirus (porcine coronavirus HKU15) reported in Hong Kong in 2012.

Coronaviruses (order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*) are single-stranded, positive-sense, enveloped RNA viruses with a genome size ranging from 25.4 to 31.7 kb (*1*). Coronaviruses traditionally were classified into groups 1, 2, and 3 on the basis of their antigenic relationships (*2*). The traditional classification recently was replaced by 4 genera (*Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*), as described by the International Committee for Taxonomy of Viruses (http://www.ictvonline.org/virusTaxonomy.asp?msl_id=27). Virus from each coronavirus genus has been found in diverse host species, including mammals and birds (*3*). Viruses of the *Alphacoronavirus*, *Betacoronavirus*, and *Deltacoronavirus* genera have been detected in swine (*3*). Transmissible gastroenteritis virus and porcine epidemic diarrhea virus (PEDV) are members of the genus *Alphacoronavirus*; these viruses cause severe diarrhea in swine herds, leading to significant economic loss in many countries, including the United State (*4*–*6*). There is little information about deltacoronavirus infections in pigs, and only 1 surveillance study from Hong Kong reported its detection in pigs (*1*). The virus has not been reported to be associated with clinical disease in these animals. We report the detection and genetic characterization of a deltacoronavirus in pigs from farms in Ohio, United States; the pigs all had clinical diarrheal disease.

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The Study

During the end of January and the beginning of February in 2014, feces and intestine samples from pigs on 5 Ohio farms were submitted to the Animal Disease Diagnostic Laboratory at the Ohio Department of Agriculture. The farm managers reported outbreaks of diarrheal disease in sows and piglets. The clinical signs were similar to those associated with PEDV infection, including watery diarrhea in sows and death in piglets. However, the death rate in piglets (30%–40%) was lower than that typically observed with PEDV infection. Test results for samples from Farm 1 were negative for PEDV, transmissible gastroenteritis virus, rotavirus, and *Salmonella* spp. Examination of the samples by electron microscopy showed that most contained coronavirus-like virus particles. This finding prompted the laboratory to look further for the presence of viruses other than alphacoronaviruses.

Because deltacoronavirus has been reported in pigs (*1*), we designed a deltacoronavirus-specific reverse transcription PCR (RT-PCR). RNA was extracted from feces and intestine samples by using the TRIzol (Invitrogen, Carlsbad, CA, USA) method, and RT-PCR was performed by using the QIAGEN OneStep RT-PCR kit (QIAGEN, Hilden, Germany) with porcine deltacoronavirus–specific primers for membrane (M) gene 67F(5′-ATCCTCCAAGGAGGCTATGC-3′), 560R(5′-GCGAATTCTGGATCGTTGTT-3′), and nucleocapsid (N) gene 41F(5′-TTTCAGGTGCTCAAAGCTCA-3′), 735R(5′-GCGAAAAGCATTTCCTGAAC-3′). These primers were designed by using the conserved regions of 2 available porcine deltacoronavirus sequences (*1*). RT-PCR was performed under the following cycling conditions: 50°C for 30 min and 95°C for 15 min for the RT reaction, followed by 40 cycles of amplification at 95°C for 15 s, 55°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. All samples (3 piglet small intestines and 9 feces samples) from Farm 1 were positive for deltacoronavirus by the 2 RT-PCR assays for M and N genes (Table 1). Nucleotide sequences were determined for both amplified M and N fragments. A BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search of the sequences of both M and N fragments showed 99% nt identity with the porcine coronavirus HKU15 (PorCoV HKU15)-155. Therefore, the virus detected in this study was PorCoV HKU15, which belongs to the *Deltacoronavirus* genus.

RT-PCR was then run on samples from Farms 2–5. The results from the 5 farms are summarized in Table 1. Of the total 42 samples from the 5 farms, 39 (92.9%) were positive for PorCoV HKU15 by RT-PCR (Table 1). In addition, 5 (11.9%) of the 42 samples were positive for classical US PEDV instead of a recently reported variant PEDV (*7*) (Table 1). None of the samples tested were positive for transmissible gastroenteritis virus. Four (9.5%) of the 42 samples were positive for PorCoV HKU15 and PEDV,

[1]These authors were co–principal investigators.

Table 1. Detection of porcine coronavirus HKU15 and porcine epidemic diarrhea virus in samples from pigs on 5 farms in Ohio, USA, 2014*

Farm no	No. samples positive/total samples tested for: Porcine coronavirus HKU15	Porcine epidemic diarrhea virus
1	12/12†	0/12
2	8/11‡	2/11
3	8/8	1/8
4	4/4	1/4
5	7/7	1/7

*Porcine coronavirus HKU15 was detected by reverse transcription PCR. Porcine epidemic diarrhea virus was detected by real-time reverse transcription PCR. The samples consisted of 42 feces and intestine samples from pigs with diarrheal disease.
†Of the positive samples, 3 were from piglets.
‡Of the positive samples, 2 were from piglets.

indicating that mixed infection with PorCoV HKU15 and PEDV occurred in some pigs.

On the basis of 2 complete genome sequences from GenBank, of PorCoV HKU15-44 and HKU15-155, we designed 16 pairs of primers to determine the whole genome of extracted RNA samples (OH1987) from Farm 1 (Table 2). The genome of PorCoV HKU15 OH1987 comprised 25,422 nt (GenBank accession no. KJ462462). The genome organization and the transcription regulatory sequence motif 5′-ACACCA-3′ of PorCoV HKU15 OH1987 are the same as those reported for PorCoV HKU15-155 (*1*). Similar to the BLAST search results of partial N and M fragments, the BLAST search of the whole genome of the PorCoV HKU15 OH1987 showed 99% nt identity to PorCoV HKU15-155. BLAST search of the spike gene of PorCoV HKU15 OH1987 showed 99% nt identity to PorCoV HKU15-44. These results confirmed that the coronavirus detected was a deltacoronavirus.

Phylogenetic analysis of the complete genome of PorCoV HKU15 OH1987 showed that the OH1987 strain clustered with the other 2 PorCoVs, HKU15-155 and HKU15-44, and was distinct from the bird deltacoronaviruses (Figure 1). In addition, the phylogenetic trees constructed by using the amino acid sequences of the spike glycoprotein and nucleocapsid protein showed that the OH1987 virus clustered with HKU15-155 and HKU15-44 (Figure 2); this finding is in agreement with that in a previous study (*1*). The OH1987 virus differs from HKU15-155 in the spike gene at nt 19469 and in the noncoding region at nt 25044; a 3-nt insertion is present at each location, making the whole-genome sequence of the OH1987 virus 6 nt longer than that of HKU15-155. The 2 insertion sites of the virus are identical to those of the HKU15-44 strain of porcine deltacoronavirus. However, the genome of OH1987 virus is 1 nt longer than

Table 2. Oligonucleotide primers used for amplification of the porcine coronavirus HKU15 genomic fragments by reverse transcription PCR, Ohio, USA, 2014

Primer identification	Sequence (5′→3′)	Nucleotide position*	Fragment
Dcor-1-F	ACATGGGGACTAAAGATAAAAATTATAGC	1–29	1
DCor-1610-R	AGACGGGCCAATTTTGACCG	1591–1610	
DCor-1481-F	TGATGATGTT CTGCTAGCCT	1481–1500	2
DCor-3300-R	GCTCATCGCCTACATCAGTA	3281–3300	
DCor-3091-F	CGGATTTAAAACCACAGACT	3091–3110	3
Dcor-4860-R	ACGACTTTACGAGGATGAAT	4841–4860	
DCor-4741-F	CTCCTGTACAGGCCTTACAA	4741–4760	4
DCor-6420-R	TCACACGTATAGCCTGCTGA	6401–6420	
DCor-6291-F	CTCAATGCAGAAGACCAGTC	6291–6310	5
DCor-8041-R	CAGCTTGGTCTTAAGACTCT	8041–8060	
DCor-7920-F	GGTACTGCTTCTGATAAGGAT	7920–7940	6
DCor-9660-R	TAGGTACAGTTGTGAACCGA	9641–9660	
DCor-9541-F	CTCTGCCCATTATCATGCCT	9541–9560	7
DCor-11040-R	AAAGAGAGGCATTTTGCTGG	11021–11040	
DCor-10861-F	ACTTGGACCCTCCTATGCGC	10861–10880	8
DCor-12840-R	GGCTCAAGATACTTATCTGC	12821–12840	
DCor-12721-F	TATGCAGGATGGTGAAGCGG	12721–12740	9
DCor-14400-R	TCACAATAAATCGCAGTGCC	14381–14000	
DCor-14281-F	TGTTACGCAGACTACACATA	14281–14300	10
DCor-16020-R	TCATAGCCGCAGCGCTTAAA	16001–16020	
DCor-15901-F	TGTGGTGTTTAGGCAGGCAA	15901–15920	11
DCor-17760-R	GTGGCGGTTACGCCTAAACC	17741–17760	
DCor-17641-F	CAAACTCTTTGACAATCGCA	17641–17660	12
DCor-19200-R	GCTAAAGGAGAATAGGTTGGTG	19179–19200	
DCor-18981-F	CTGAACATTCATTCTCACCC	18981–19000	13
DCor-20910-R	GAAGGTGGTGGCATTTGTGG	20891–20910	
DCor-20761-F	GTCTTACCGTGTGAAACCCC	20761–20780	14
DCor-22440-R	AACATCCCACTGAGGAGGTG	22421–22440	
DCor-22321-F	TTTTATAACACCACCGCTGC	22321–22340	15
DCor-24004-R	GGCCATGATAGATTGGTGTC	23985–24004	
DCor-23881-F	ATGGTGAGCCTTTACTGCTT	23881–23900	16
DCor-25417-R	TGCTCCATCCCCCCTATAAG	25398–25417	

*Positions correspond to porcine coronavirus HKU15-155 strain (GenBank accession no. JQ065043).

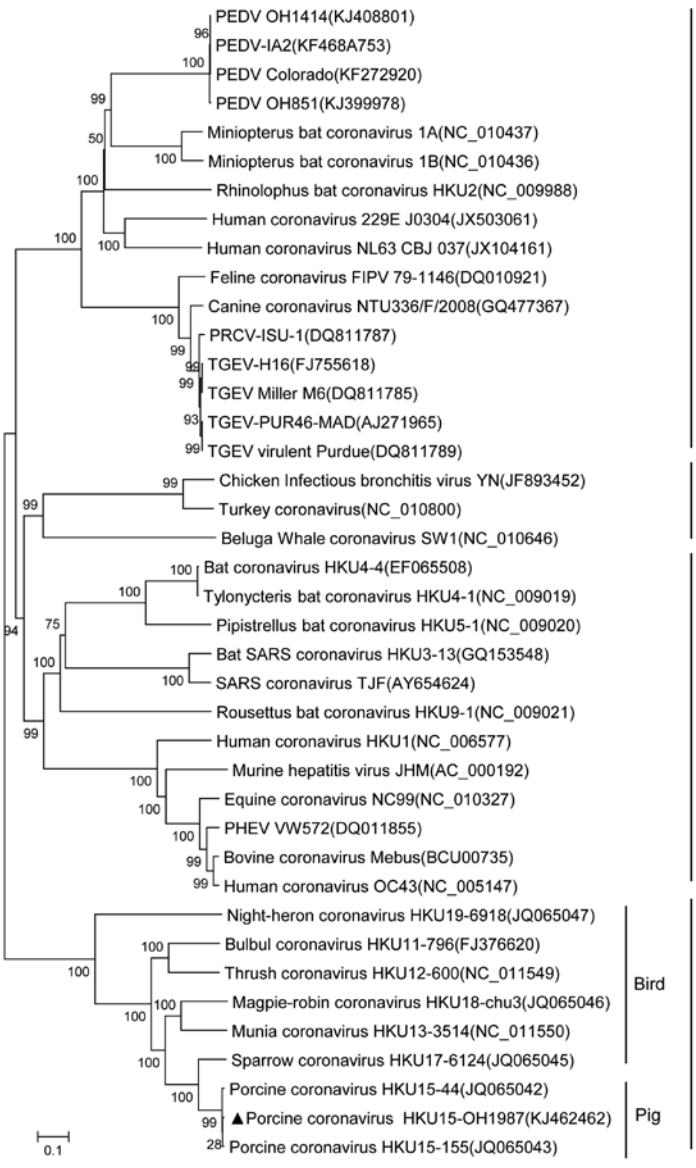

Figure 1. Phylogenetic tree constructed on the basis of the whole-genome sequences of virus strains from 4 coronavirus genera (*Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*), including the porcine coronavirus HKU15 OH1987 strain (indicated with triangle). The dendrogram was constructed by using the neighbor-joining method in the MEGA software package, version 6.05 (http://www.megasoftware.net/). Bootstrap resampling (1,000 replications) was performed, and bootstrap values are indicated for each node. Reference sequences obtained from GenBank are indicated by strain name and accession number. Scale bar represents 0.1 nt substitutions per site. PEDV, virus and porcine epidemic diarrhea virus; PRCV, porcine respiratory coronavirus; TGEV, transmissible gastroenteritis virus; SARS, severe acute respiratory syndrome. PHEV, porcine hemagglutinating encephalomyelitis virus.

that of HKU15-44 because of the insertion site at nt 25263, located in 3′ untranslated region. Of interest, strain OH1987 was most closely related to HKU15-155 when whole-genome sequence was used for phylogenetic analysis (Figure 1), but strain OH1987 was more closely related to HKU15-44 when phylogenetic analysis of spike protein and nucleocapsid protein was performed (Figure 2). On basis of the partial genome sequence, strain OH1987 was also closely related to a deltacoronavirus found in the Asian leopard cat (the whole-genome sequence is not available in GenBank).

Conclusions

We detected a porcine deltacoronavirus in pigs in the United States. Although the genetic and phylogenetic analyses showed that the newly emergent strain, PorCoV HKU15 OH1987, was closely related to 2 strains from China, HKU15-155 and HKU15-44, in the genus *Deltacoronavirus*, when and how this virus was introduced into United States remain unknown. Further investigation is needed to determine whether infection with PorCoV HKU15 results in disease in pigs and if the virus was responsible for the clinical disease observed in outbreaks on the 5 Ohio farms in this study. In addition, surveillance should be conducted in other US states to define the distribution of the virus among the US pig population. Moreover, whole-genome sequence analysis should be performed for other strains from different locations to determine whether the virus was introduced into the United States by a single entry or by multiple entries.

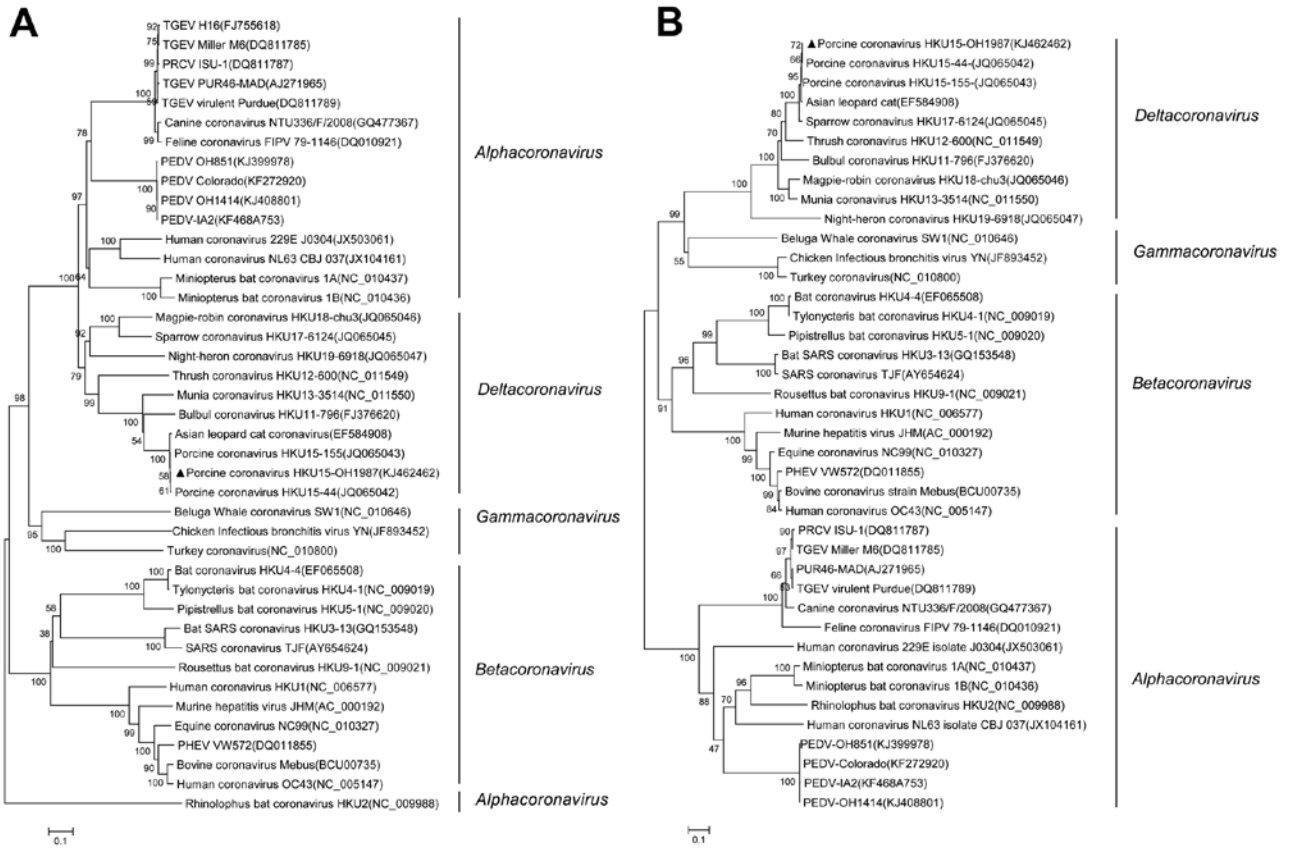

Figure 2. Phylogenetic analyses of spike protein (A) and nucleocapsid protein (B) of virus strains of 4 coronavirus genera (*Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*), including the porcine coronavirus HKU15 OH1987 strain (indicated with triangle). The dendrogram was constructed by using the neighbor-joining method in the MEGA software package, version 6.05 (http://www.megasoftware.net/). Bootstrap resampling (1,000 replications) was performed, and bootstrap values are indicated for each node. Reference sequences obtained from GenBank are indicated by strain name and accession number. Scale bars represent 0.1 aa substitutions per site. PEDV, virus and porcine epidemic diarrhea virus; PRCV, porcine respiratory coronavirus; TGEV, transmissible gastroenteritis virus; PHEV, porcine hemagglutinating encephalomyelitis virus; SARS, severe acute respiratory syndrome.

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MERS Coronavirus in Dromedary Camel Herd, Saudi Arabia

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A prospective study of a dromedary camel herd during the 2013–14 calving season showed Middle East respiratory syndrome coronavirus infection of calves and adults. Virus was isolated from the nose and feces but more frequently from the nose. Preexisting neutralizing antibody did not appear to protect against infection.

Ongoing transmission of Middle East respiratory syndrome coronavirus (MERS-CoV) to humans underscores the need to understand the animal sources of zoonotic infection (*1*,*2*). MERS-CoV RNA has been detected in dromedary camels (*3*,*4*), and dromedary infection precedes human infection (*5*). We conducted a prospective study in dromedary herds in Al-Hasa, Saudi Arabia, through the peak calving season (December 2013–February 2014) to document virologic features of MERS-CoV infection in these animals.

The Study

We studied dromedaries at 2 farms in Al-Hasa, 4–5 km apart. Farm A had 70 animals; 4 were 1 month of age, 8 were ≈1 year of age, and the rest were adults (≥2 years of age). The herd did not go to pasture in the desert ("zero-grazing"; type of grazing may influence types of potential exposures). The animals were sampled on 5 occasions during November 2013–February 2014. Farm B ("semi–zero-grazing") had 17 adults and 3 calves; its herd was sampled in February 2014. Nasal, oral, or rectal swab samples and blood samples were collected (Table 1; online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/20/7/14-0571-Techapp1.pdf). Swab and serum samples were stored frozen at −80°C until testing.

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Hydrolysis probe–based real-time reverse transcription PCR (RT-PCR) targeting MERS-CoV upstream of E (UpE) and open reading frame (ORF) 1a genes and a broad-range RT-PCR reacting across the CoV family to detect other CoVs were used as described (*4*). Specimens initially positive for MERS-CoV were re-extracted and retested to confirm the positive results.

The full genome of MERS-CoV was obtained directly from the clinical specimens with 3–4 times coverage by sequencing PCR amplicons with overlapping sequence reads and sequence assembly (*4*). Dromedary MERS-CoV full genomes obtained in this study (GenBank accession nos. KJ650295–KJ650297) were aligned with human MERS-CoV genomes retrieved from GenBank. We constructed full-genome phylogenies using MEGA5 with neighbor-joining and bootstrap resampling of 500 replicates (*6*). Virus isolation was attempted in Vero E6 cells. We tested serum samples for neutralizing antibody titers using a validated MERS-CoV spike pseudoparticle neutralization test (*7*) (online Technical Appendix).

At farm A, we detected MERS-CoV in 1 of 4 dromedaries sampled on November 30, none of 11 sampled on December 4, nine of 11 sampled on December 30, and none of 9 sampled on February 14 (Table 1). Of the 10 dromedaries that tested positive for MERS-CoV, 9 had parallel nasal and fecal specimens tested, with virus detected in the nasal swab specimens from 8 and the fecal specimen from 1. At the December 30 sampling, 7 of 8 calves and 2 of 3 adults tested positive for MERS-CoV, indicating that when MERS-CoV circulates on a farm, both calves and adults can be infected (online Technical Appendix Table). Because all 12 adults with serum collected before December 30 were seropositive (titers ≥320), it is likely, though not certain, that the MERS-CoV infections in the 2 adults (nos. 21, 19Dam) sampled on December 30 were reinfections, as has been reported for other CoVs (*8*). The seronegative 1-year-old calves, nos. 13 and 14, had the highest nasal viral loads (UpE assay 1.3×10^8 to 1.78×10^8/mL specimen), and a 2-week-old calf, no. 22, with (presumably passively acquired) titers of 1,280 became infected but had a much lower viral load. Overall, these data suggest that prior infection or passively acquired maternal antibody might not provide complete protection from infection (online Technical Appendix Table).

Four MERS-CoV–positive calves had mild respiratory signs (cough, sneezing, respiratory discharge), abnormally elevated body temperature, and loss of appetite at the December 30 sampling, which resolved over a few days. Three calves from which paired serum samples were available (Table 2; nos. 13, 15, 17) demonstrated ≥4-fold rising antibody titers to MERS-CoV. Calf no. 13 (1 year of age) had

[1]These authors contributed equally to this article.

Table 1. RT-PCR of dromedary camel samples for MERS-CoV, Al-Hasa, Saudi Arabia*

Farm, sampling date	Age†/no. sampled	No. specimens positive/no. tested		
		Nasal	Oral	Fecal
Farm A				
2013 Nov 30	Calf, 0	ND	ND	ND
	Adult, 4	1/1	0/2	0/4
2013 Dec 4	Calf, 9	ND	0/9	0/7
	Adult, 2	ND	0/2	0/2
2013 Dec 30	Calf, 8	7/8	0/1	0/6
	Adult, 3	1/3‡	0	1/3‡
2014 Feb 14	Calf, 7	0/7	ND	0/7
	Adult, 2	0/2	ND	0/2
Farm B: 2014 Feb 11	Calf, 3	0/3	ND	0/3
	Adult, 3	0/3	ND	0/3

*Data on individual dromedaries are provided in online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/20/7/14-0571-Techapp1.pdf. RT-PCR, reverse transcription PCR; MERS-CoV, Middle East respiratory syndrome coronavirus; ND, not done.
†Adults are 6–14 y of age; calves are 40 d to 2 y of age.
‡Two different dromedaries were positive in nasal and fecal swabs.

a high viral load and was seronegative at the first MERS-CoV–positive result (indicating that it had been recently infected) but was MERS-CoV RNA negative 6 weeks later, suggesting that virus shedding is not prolonged. We did not detect virus RNA by RT-PCR in the 3 acute-phase serum samples from infected dromedaries (nos. 1, 16, 17), suggesting that acute infection is not associated with prolonged viremia. Dromedaries from farm B were sampled once on February 11; all results were negative.

The full genomes of MERS-CoV sequenced directly from a nasal swab specimen collected on November 30 were identical to those from a nasal swab specimen and a fecal specimen collected on December 30. In addition, the complete spike gene was sequenced from 4 other MERS-CoV–positive nasal swab specimens, and these spike genes were genetically identical.

Virus isolation in Vero E6 cells was attempted from 7 positive nasal swab and fecal specimens that had $>10^6$ copies/mL in the original sample in the UpE RT-PCR. Viruses were isolated from 2 nasal swab (nos. 13, 14) and 1 fecal swab (no. 19Dam) specimens collected on December 30; these were the specimens with high numbers of MERS-CoV copies (9.27×10^7 to 1.78×10^8 copies/mL). The full-genome sequence of 1 virus culture isolate was obtained in parallel with that of the original virus in the original clinical specimen. We observed 3 nucleotide changes in ORF1b, spike, and membrane protein genes in the isolates after 2 passages in Vero E6 cells, of which 2 were nonsynonymous, leading to changes in spike (S1251F) and membrane proteins (T8I). This finding highlights the importance of sequencing the viral genome directly from clinical specimens.

MERS-CoVs circulating in dromedaries on farm A during a 1-month period were genetically identical over the full 30,100-nt genome in 3 viruses and the spike protein of 4 more viruses, giving a mutation rate of 0 nt substitutions per site per day (95% credible interval 0 to 2.7×10^{-6}). The estimated mutation rate for epidemiologically unlinked human MERS-CoV was 3.1×10^{-6} (95% CI 2.4×10^{-6} to 3.8×10^{-6}) (*9*).

Conclusions

The unusual genetic stability of MERS-CoV in dromedaries, taken together with its high seroprevalence (*7*,*10*–*13*), raises the hypothesis that dromedaries might be the natural host for this virus. Further longitudinal studies of MERS-CoVs in dromedaries are needed to confirm this hypothesis.

Genome organization of the dromedary MERS-CoV detected in this study was identical to that of the virus in humans. The virus strains clustered phylogenetically within clade B (*9*) and were most closely related to the strain MERS-CoV_FRA/UAE and to MERS-CoV detected in Buraidah (Saudi Arabia) and Al-Hasa (Figure). The farm is ≈300 km from United Arab Emirates and 600 km from Buraidah. Dromedaries move between Al-Hasa and Buraidah and, more limitedly, between Al-Hasa and United Arab Emirates.

The full-genome sequence of MERS-CoV from dromedaries in this study is 99.9% similar to genomes of human clade B MERS-CoV. The spike gene is the major determinant for virus host specificity. In comparison with other publically available human MERS-CoV sequences, we found 6-nt mutations in the spike gene unique to these dromedary viruses. Of these, 3 (S457G, L773F, and V810I) were nonsynonymous. These amino acid changes are located outside the binding interface between MERS-CoV spike protein and human DPP4 receptor, suggesting these amino acid differences are unlikely to affect receptor binding. Thus, these dromedary viruses may retain capacity to infect humans, as Chu et al. suggested for dromedary MERS-CoV in Egypt (*4*).

MERS-CoV may be isolated from nasal swab specimans and feces, indicating that both could be possible sources of virus transmission to humans and other animals, but virus detection rates were higher in nasal swab specimens.

Table 2.Longitudinal sampling of MERS-CoV–positive dromedary camel calves on farm A, Al-Hasa, Saudi Arabia*

Calf no.	Sample collection date	Sex/age	RT-PCR result	Titer
13	2013 Dec 30	F/1 y	Positive	<20
	2014 Feb 14	F/1 y	Negative	640
15	2013 Dec 30	F/1 y	Positive	20
	2014 Feb 14	F/1 y	Negative	160
17	2013 Dec 30	F/40 d	Positive	80
	2014 Feb 14	F/3 mo	Negative	1,280
19	2013 Dec 30	F/1 y	Positive	NA
	2014 Feb 14	F/1 y	Negative	320

*MERS-CoV, Middle East respiratory syndrome coronavirus; RT-PCR, reverse transcription PCR.

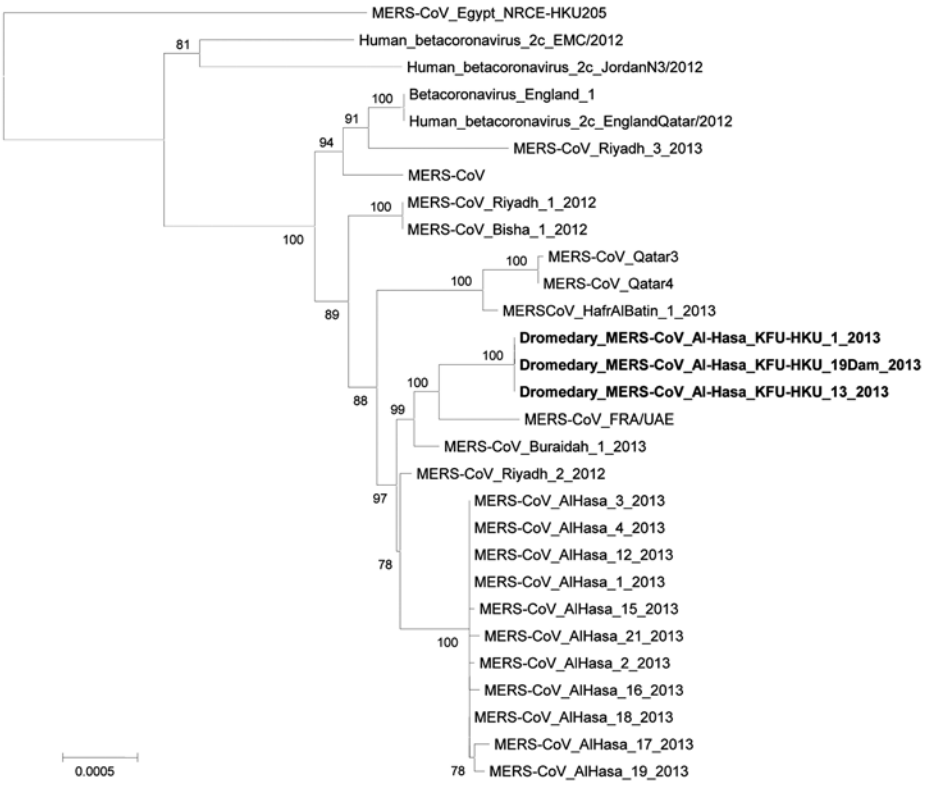

Figure. Phylogenetic tree of Middle East respiratory syndrome coronavirus (MERS-CoV) full genomes (29,901 nt after trimming the ends) or near–full genomes from humans and dromedary camels. The tree was constructed by using neighbor-joining methods with bootstrap resampling of 500 replicates. The most divergent MERS-CoV, Egypt NRCE-HKU205, was used as outgroup. Bold type indicates camel MERS-CoV genomes from this study. GenBank accession numbers of genome sequences included in this study are KJ477102, KF600652, KF600630, KF600651, KF186567, KF600627, KF186564, KF600634, KF600632, KF600644, KF600647, KF600645, KF186565, KF186566, KF745068, KF600620, KF600612, KC667074, KC164505, KF192507, KF600613, KF600628, KF961222, KF961221, KC776174, and JX869059. Scale bar indicates nucleotide substitutions per site.

Our preliminary data suggest that preexisting MERS-CoV antibody might not completely protect against re-infection; however, this question needs more investigation.

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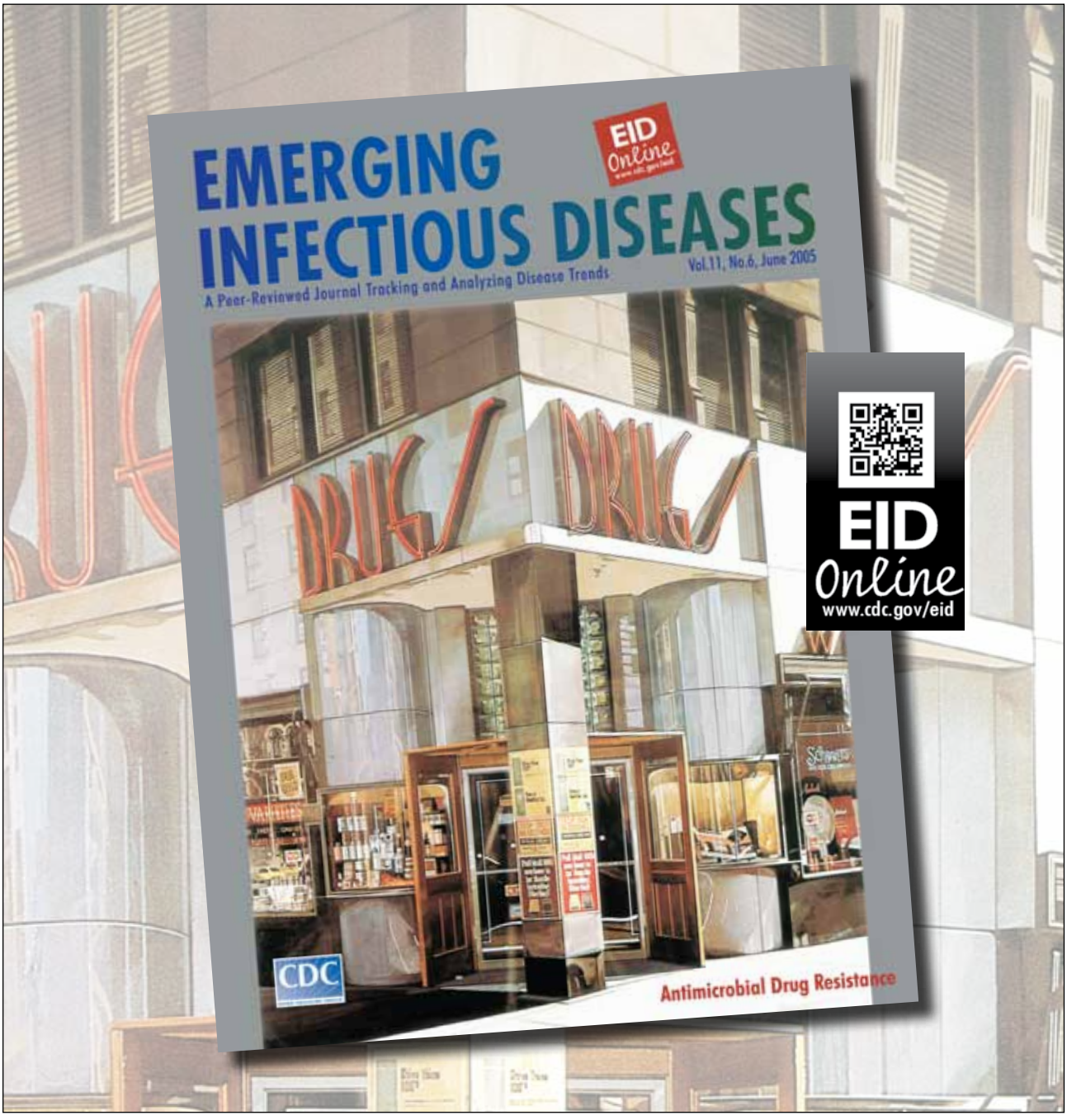

Carbapenemase-producing *Klebsiella pneumoniae* and Hematologic Malignancies

To the Editor: Until a few years ago, the most frequent microbiologically documented cause of severe bloodstream infections among patients with hematologic malignancies was gram-positive bacteria (*1*). However, over the years, gram-negative bacteria have become the main infectious cause of death among patients with hematologic malignancies, and rates of different phenotypes associated with antimicrobial drug resistance are increasing (*2*). This trend could be the result of increasing empirical use of antimicrobial drug therapy and prophylaxis and use of new, more effective antimicrobial drugs. In particular, over the past few years at our hospital (Agostino Gemelli Teaching Hospital, Rome, Italy), we have observed a progressive increase in bloodstream infections caused by *Klebsiella pneumoniae* carbapenemase–producing *K. pneumoniae* (KPC-Kp), which are responsible for a dramatic new scenario.

We reviewed records of all patients affected by hematologic malignancies who were admitted to the hospital hematology department from January 2009 through December 2012 and in whom a bloodstream infection caused by gram-negative bacteria developed. A KPC-Kp bloodstream infection was defined as a bloodstream infection documented on the basis of blood culture positivity (at least 1 specimen) for a KPC-Kp strain and clinical signs of systemic inflammatory response syndrome. The Vitek 2 system (bioMérieux. Firenze, Italy) was used for isolate identification and antimicrobial drug susceptibility testing; PCR and sequencing, as previously described, was used to identify bla_{KPC} genes (*3*). Antibiograms were reported 72–120 hours (median 76 hours) after onset of bloodstream infection. Death was considered attributable to infection for patients who died within 2 weeks after the first positive blood culture and for whom other potential causes of death could be excluded.

During the study period, we detected 147 bloodstream infections caused by gram-negative bacteria, 38 (25%) of which were caused by *K. pneumoniae*; of these, 26 (18%) were caused by KPC-Kp. We did not identify any episodes of recurrent KPC-Kp bloodstream infection. We did document a progressive, exponential increase in infections caused by KPC-Kp. No KPC-Kp cases were documented until 2009, and cases increased from only 1 case in 2010 to 12 cases in 2012 (Table). The incidence of KPC-Kp among all gram-negative causes of bloodstream infections increased significantly from 2009–2010 (1/69, 1.4%) to 2011–2012 (25/78, 32.1%) ($p<0.0001$). Most patients with KPC-Kp bloodstream infection had acute myeloid leukemia (14, 53.8%); others had non-Hodgkin lymphoma (4, 15.4%), acute lymphoid leukemia (3, 11.6%), Hodgkin lymphoma (2, 7.8%), myeloproliferative disease (1, 3.8%), myelodysplastic syndrome (1, 3.8%), or aplastic anemia (1, 3.8%). At time of bloodstream infection onset, 19 (73.1%) of 26 patients were markedly neutropenic ($<500 \times 10^9$ neutrophils/mL for >10 days); almost half (12/26, 46.1%) of these patients experienced complete remission during the course of consolidation therapy or were receiving initial chemotherapy. Among KPC-Kp isolates, 80.8% were susceptible to colistin, 69.2% to tigecycline, and 65.4% to gentamicin. The overall KPC-Kp bloodstream infection–attributable mortality rate was 57.6% (15/26), which was significantly higher than that for bloodstream infections caused by gram-negative bacteria other than KPC Kp (17/121, 14%; $p<0.0002$) and for bloodstream infections caused by non–KPC-Kp (2/12, 16.7%; $p = 0.02$) (Table).

Despite tailoring of antimicrobial drug therapy to antibiogram results, the KPC-Kp bloodstream infection–attributable mortality rate was high. For ≈50% of patients, therapy consisted of combinations of ≥2 antimicrobial drugs with in vitro activity against the KPC-Kp isolate. Outcomes are reportedly better after this therapy than after monotherapy (*3*,*4*).

In our opinion, the high mortality rate related to KPC-Kp bloodstream infections in patients with hematologic malignancies could be related to various factors. First, patients with hematologic malignancies usually receive antimicrobial drugs recommended for the management of fever in immunocompromised patients with cancer but rarely receive empirically administered drugs active against KPC-Kp bloodstream infections. The delay in appropriate antimicrobial treatment reportedly has a strong negative effect on patient outcomes (*5*). Second, KPC-Kp isolates may not be susceptible to the antimicrobial drugs generally considered

Table. Prevalence and attributable mortality rate of BSI, Rome, Italy, 2009–2012*

	Any gram-negative bacteria		Non-KPC–producing *K. pneumoniae*		KPC-producing *K. pneumoniae*	
Year	BSI, no.	Deaths, no. (%)	BSI, no.	Deaths, no. (%)	BSI, no.	Deaths, no. (%)
2009	30	5 (16.6)	1	0	0	0
2010	39	7 (17.9)	2	0	1	1 (100)
2011	41	9 (24.3)	5	1 (20)	13	7 (53.8)
2012	37	11 (29.7)	4	1 (25)	12	7 (58.3)
Total	147	32 (21.7)	12	2 (16.6)	26	15 (57.6)

*BSI, bloodstream infections; KPC, *Klebsiella pneumoniae* carbapenemase.

as the therapy of choice for such infections. In our study, the rates of nonsusceptibility to colistin, tigecycline, and gentamicin were 19%, 31%, and 35%, respectively. Third, many patients have severe clinical conditions caused by hematologic malignancy and other concurrent conditions (e.g., renal failure, heart disease).

In conclusion, in areas where KPC-Kp is endemic, progress in treating hematologic malignancies could be slowed by the emergence of severe KPC-Kp infections. In these settings, the early identification of patients likely to be colonized and/or infected by KPC-Kp strains represents a major step toward preventing and containing the spread of these strains among hospitalized patients. Policies on empirical treatment might need to be revised, depending on the possibility of serious infections caused by carbapenem-resistant *Enterobacteriaceae.*

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Neisseria meningitidis Serogroup W135 Sequence Type 11, Anhui Province, China, 2011–2013

To the Editor: *Neisseria meningitidis* colonizes the nasopharynx of humans and can cross the epithelial barrier of the nasopharynx, causing septicemia, meningitis, or both (*1*,*2*). In Anhui Province, China, there has been a previously high risk for epidemic cerebrospinal meningitis. Before 2012, all meningococcal diseases were caused by *N. meningitidis* serogroups A, B, and C, and the unique sequence type (ST) 4821 clone of serogroup C was first identified in this region during 2003–2004 (*3*).

No widespread epidemics of cerebrospinal meningitis and no *N. meningitidis*–associated deaths have occurred in Anhui since bivalent meningococcal vaccines against serogroups A and C were first used in 2003 (*4*). During 2011–2013, however, 15 infections caused by *N. meningitidis* serogroup W135 ST11, which belongs to a hyperinvasive lineage (*5*), were reported in Hefei, Anhui Province. Two of the cases (1 each in 2012 and 2013) were fatal and occurred in patients who denied having recently traveled, which suggests that the clone may have spread in an endemic fashion. The 2 patients also had no history of vaccination with tetravalent polysaccharide vaccine (serogroups A/C/Y/W). The other 13 cases occurred in close contacts of the patients who died.

The fatal cases of serogroup W135 infection were in 14- and 17-year-old boys. One of the boys had dropped out of school and worked in a hotel. He sought medical care for a headache with sudden onset, vomiting, and high fever (temperature 40°C). The other boy was a junior college student. At hospital admission, he had vomiting, diarrhea, and high fever (temperature 39°C).

According to the Chinese surveillance system, meningococcal disease is reported by local hospitals to the local Center for Disease Control and Prevention and then to the provincial Center for Disease Control and Prevention, where specific measures are taken to control and prevent the disease. Serogroup W135 infection in the 2 boys in Hefei was identified and reported by different hospitals. Both boys reported that they had not traveled outside Hefei in the 2 months before illness onset or had any contact with persons with meningococcal disease. A total of 61 close contacts were identified for the boys.

Despite treatment, the 2 boys died of disseminated intravascular coagulation and multiple organ failure. Cerebrospinal fluid and blood specimens

were cultured on chocolate agar for 24 h; results showed bacterial growth consistent with the features of *Neisseria* spp. Four isolates were identified as serogroup W135 by using specific antiserum (Remel, Lenexa, KS, USA).

Throat swab specimens were collected from the 61 close contacts of the 2 boys; all contacts were asymptomatic. Gram staining and biochemical tests confirmed the presence of *N. meningitidis* in 13 of the 61 samples, and slide agglutination, using specific antiserum, showed that all 13 isolates from contacts were serogroup W135.

Pulsed-field gel electrophoresis (PFGE) (*6*), multilocus sequence typing, outer membrane protein gene (*porA*) variant region subtyping, and antimicrobial drug susceptibility tests were used to characterize the 17 isolates. PFGE patterns (pulse types 1 and 2) for the isolates were indistinguishable and shared >96% similarity with the dominant patterns, including patterns for isolates from Guangdong, Guangxi, and Jiangsu Provinces, China (Figure). All isolates had the same multilocus ST (ST11) and PorA subtype (P1.5,2), which belong to the multilocus ST11/electrophoretic type 37 complex (*7*). Results of Kirby-Bauer testing showed that all 17 isolates were resistant to sulfamethoxazole and sensitive to penicillin, ampicillin, ceftriaxone, cefotaxime, meropenem, minocycline, chloramphenicol succinate, and rifampin. Resistance to ciprofloxacin, which, to our knowledge, had not previously been reported for *N. meningitidis* from mainland China, was shown for 70.6% (12/17) of the isolates.

Since 2000, *N. meningitidis* W135 ST11 disease has become a serious problem worldwide (*8*,*9*). In mainland China, serogroup W135 ST11 cases have recently been reported in Guangdong, Guangxi, Zhejiang, Jiangsu, and Henan Provinces (*10*), but no deaths were reported until 2012, when the first of the 2 boys died in Anhui Province (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/7/13-1138-Techapp1.pdf). According to the PFGE, multilocus sequence typing, and PorA typing results, the dominant clonal complex detected in Hefei matches the dominant type recently detected in other Chinese provinces. Pathogenicity characteristics of the isolates from Hefei and epidemiologic investigations indicate that the *N. meningitidis* W135 ST11 clone has emerged in Hefei, raising the possibility of its introduction into other regions.

Meningococcal polysaccharide vaccines A and C are being used for routine vaccination in China. However, meningococcal diseases caused by *N. meningitidis* serogroups other than A and C, especially those belonging to hyperinvasive lineages, are an emerging problem that must be addressed. The 2 fatal meningitis cases in Hefei highlight the need for further epidemiologic surveillance to monitor the incidence of meningococcal disease caused by serogroup W135 and the need for better public health strategies to control the disease.

Testing of the 17 isolates from Hefei for antimicrobial drug susceptibility indicated that sulfamethoxazole is not effective against serogroup W135 ST11 infection and that 70.6% of isolates were resistant to ciprofloxacin. Our findings indicate that changes in the molecular and epidemiologic characteristics of *N. meningitidis* in China should be monitored to enhance our ability to respond to emerging meningococcal disease.

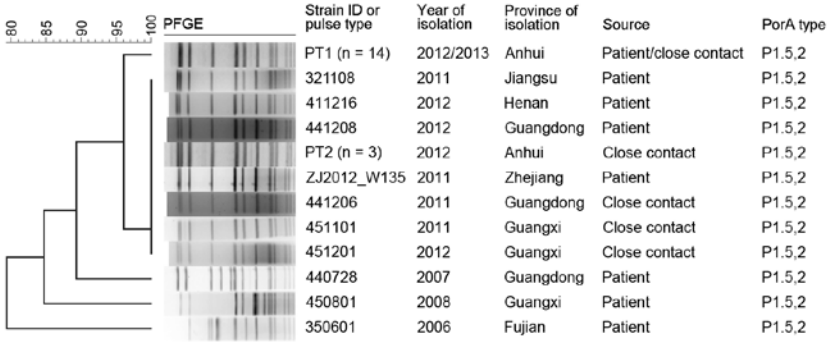

Figure. Pulsed-field gel electrophoresis (PFGE) pattern–based cluster analysis of 27 *N. meningitidis* serogroup W135 isolates from China: 17 isolates were collected from persons in Hefei City, Anhui Province, and 10 were collected from persons from other provinces in China. Clustering was performed by using the Dice coefficient and an optimization setting of 1.2%. The dendrogram was generated by using the unweighted pair group method with averages. All isolates belong to the multilocus sequence type 11/electrophoretic type 37 complex.

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Early Public Response to Influenza A(H7N9) Virus, Guangzhou, China, May 30–June 7, 2013

To the Editor: On May 15, 2013, surveillance in live poultry markets (LPMs) identified an influenza A(H7N9) virus–infected chicken in Guangzhou, the capital of Guangdong Province, China. During May 30–June 7, 2013, we conducted a population-based survey in Guangzhou to investigate changes in the public's buying behavior at LPMs and to determine the public's attitude toward potential implementation of specific interventions against avian influenza in LPMs. Behaviors and attitudes in 3 residential areas of Guangzhou were compared: urban districts, Conghua (a semirural area), and Zengcheng (a semirural area where subtype H7N9 infection had been detected in an LPM on May 15, 2013). These locations were chosen to compare possible urban–rural differences in live poultry exposure, as observed in an earlier study (*1*), and to assess the effect of epidemic proximity on the public's attitude and behavior.

Study participants were recruited by using the Mitofsky–Waksberg 2-stage sampling method (*2*). First 120, 60, and 60 telephone prefixes in urban districts, Conghua, and Zengcheng, respectively, were randomly selected. Then for each prefix, telephone numbers were randomly generated and called until 5 households were successfully recruited. Within each household, the person whose birthday was closest to the interview date and who was ≥15 years of age was invited to participate in the telephone interview. Using a standardized questionnaire, we collected demographic information and information on behavior related to buying live poultry from LPMs, attitudes toward measures for reducing avian influenza transmission in LPMs, and perceived risk for infection from LPMs.

Of 1,930 persons recruited, 1,196 (62.0%) completed the interview. Information on age was missing for 19 persons, so they were excluded; thus, a total of 1,177 persons were included in the analysis. Responses from the 3 residential areas were generally comparable, with the exception that respondents from urban districts reported higher levels of education and personal income (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/7/13-1155-Techapp1.pdf). Compared with the overall Guangzhou population (*3*), the respondents were slightly better educated and less likely to be single (online Technical Appendix).

We used logistic regression models (*4*) adjusted for age, sex, and education level to calculate the percentages and 95% CIs related to buying live poultry from LPMs, attitudes, and risk perception in each area and for the sample as a whole. During the 2 months before the survey, ≈33.5% (95% CI 29.7%–37.5%) of the sampled households bought live poultry from LPMs at least once a week (Table). The number of households that bought live poultry on a weekly basis was substantially lower in Zengcheng than in urban areas. After the epidemic in Zengcheng was announced, 59.1% (95% CI 55.1%–63.0%) of all respondents reported buying less poultry or having completely stopped buying live poultry. Compared with respondents in the other 2 areas, Zengcheng respondents were more likely to report a reduction in buying (Table).

Most respondents expressed support for the policy of introducing 1 or 2 monthly market rest days in Guangzhou, but only 21.1% (95% CI 18.1%–24.4%) agreed with complete closure of LPMs (Table). Zengcheng respondents were more likely to agree on closure of LPMs. Approximately

Table. Public attitudes and behaviors in response to influenza A(H7N9) virus, Guangzhou, China, May 30–June 7, 2013*

Characteristic	% (95% CI) persons with characteristic			
	Urban districts, n = 594	Semirural districts		Total, N = 1,177
		Conghua, n = 283	Zengcheng, n = 300	
Household purchase of live birds from LPMs				
Weekly to monthly	21.2 (17.6–25.2)	29.7 (23.8–36.5)	19.7 (14.7–25.8)	21.6 (18.5–25.0)
At least weekly	34.1 (29.7–38.7)	29.5 (23.2–36.6)	**23.3 (18.1–29.6)**	33.5 (29.7–37.5)
Buying less/stopped buying because of A(H7N9) epidemic	58.7 (54.0–63.3)	56.3 (49.3–63.2)	**71.6 (64.8–77.5)**	59.1 (55.1–63.0)
Attitude (agree/strongly agree) toward control measures				
Introducing 1 or 2 market rest days per month	88.7 (85.4–91.4)	90.5 (85.9–93.7)	91.2 (87.1–94.0)	89.7 (87.6–91.5)
Closing LPMs	20.8 (17.3–24.7)	16.2 (12.0–21.4)	**31.1 (25.4–37.3)**	21.1 (18.1–24.4)
Perception (agree/strongly agree) of risk from LPMs				
Live animals sold in markets are a risk to human health	34.0 (29.8–38.5)	33.3 (26.9–40.4)	38.2 (32.0–44.7)	34.3 (30.7–38.1)
Poor market hygiene is main cause of avian influenza transmission	68.5(64.0–72.7)	73.1 (66.9–78.6)	68.3 (61.7–74.4)	69.0 (65.2–72.5)
Likelihood (likely/very likely) of getting sick as a result of buying live birds from LPMs	18.7 (15.5–22.4)	**9.9 (6.8–14.1)**	**30.0 (23.8–37.0)**	19.1 (16.3–22.3)

*All values are weighted by population age and sex and adjusted by sample age, sex, and education level, using logistic regression models. Boldface indicate that attitudes/perceptions in Conghua or Zengcheng were significantly different ($p<0.05$) from those in urban districts. LPMs, live poultry markets.

one third of the respondents agreed that live animals sold in LPMs posed risks to human health, and more than two thirds agreed that avian influenza transmission was due to poor market hygiene. However, only 19.1% (95% CI 16.3%–22.3%) of the respondents perceived that they would be likely/very likely to get sick from buying live poultry from LPMs. Perceived risk from buying was highest in Zengcheng and lowest in Conghua (Table).

Although there were no cases of human A(H7N9) infection in Guangzhou at the time of study, >50% of respondents reported a reduction in buying live poultry from LPMs after the A(H7N9) epidemic in China was officially announced (*5*). Information on A(H7N9) virus may have motivated a population-level change in live poultry buying habits; however, such change is likely to be temporal (*6*). Detection of A(H7N9) virus infection in poultry may have further raised risk awareness and prompted behavioral change in the local area (*7*). However, respondents seemed to prefer measures such as monthly market rest days in LPMs rather than the more extreme measure of complete LPM closure. This finding suggests that the progressive introduction of measures to control avian influenza risk in LPMs would be more acceptable to the public. Although the risk from sales of live animals in LPMs was generally ignored, poor market hygiene was commonly accepted as a cause of avian influenza virus transmission. The public should be educated about the risk from sales of live animals in markets.

This study was limited by its reliance on self-reported data, making results potentially subject to social desirability bias. In addition, stratified rather than simple random sampling was used to recruit participants, so the sample may not be representative of the general population of Guangzhou. Furthermore, the study was conducted before any human cases of A(H7N9) infection were confirmed in Guangzhou, so the results may not apply to other parts of China with confirmed human cases. The results should, however, guide public health responses in regions neighboring epidemic areas.

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Socioeconomic Status and Campylobacteriosis, Connecticut, USA, 1999–2009

To the Editor: *Campylobacter* is the second most common bacterial cause of foodborne gastrointestinal illnesses in the United States and the leading cause of these illnesses in Connecticut (*1*). It is also the leading identifiable cause of Guillain-Barré syndrome in the United States and all industrialized countries in which it has been studied (*2*). According to the Foodborne Disease Active Surveillance Network (FoodNet), campylobacteriosis incidence in the United States is increasing (*1*). Clarification of the epidemiology of campylobacteriosis is needed to control and prevent infection.

Socioeconomic status (SES) measures have not been explored in the United States as determinants for *Campylobacter* infection. Although individual SES measures are not routinely collected in FoodNet, street address of patient residence is. Following the recommended method of the Public Health Disparities Geocoding Project (*3*), we used census tract–level poverty as an SES measure for analysis. We attempted to geocode patient residences for all campylobacteriosis cases reported in Connecticut during 1999–2009 and to categorize them into 4 groups on the basis of percentage of residents in the census tract living below the federal poverty line: 0–<5%, 5%–<10%, 10%–<20%, and ≥20%. The average annual age-adjusted (on the basis of 2000 US Census data for Connecticut) incidence rate was calculated for each of 4 census tract-level neighborhood SES (i.e., neighborhood poverty) categories for all years combined and for 3 periods (1999–2002, 2003–2005, and 2006–2009). In addition, age group-specific rates were calculated for case-patients in the 4 SES categories. We used the χ^2 test for trend to assess the statistical significance of observed gradients of incidence across SES levels.

We geocoded 5,708 (95.9%) of the 5,950 campylobacteriosis cases reported during 1999–2009 to census tract level. The average annual crude incidence rate was 15.9 per 100,000 population; average age-specific incidence ranged from 9.4 in the 10–19-year age group to 18.1 in the ≥50-year age group. We found a strong dose-response relationship between higher campylobacteriosis incidence and higher neighborhood SES. Average annual age-adjusted incidence was 10.1 (95% CI 9.1–11.1) for the lowest SES group (≥20% below poverty), 11.9 (95% CI 11.0–12.9) for the 10%–<20% group, 14.8 (95% CI 14.0–15.7) for the 5%–<10% group, and 16.9 per 100,000 (95% CI 16.3–17.4) for the highest SES group (0–<5% below poverty) ($p<0.001$ by$\chi^2$for trend). A strong SES gradient was also consistent and significant ($p<0.001$ by χ^2 for trend) for each of the 3 periods.

Incidence within age groups by neighborhood SES level is shown in the Figure. For all age groups ≥10 years, incidence of campylobacteriosis increased as neighborhood SES increased ($p<0.001$ for each category by χ^2 for trend). However, for children 0–<10 years of age, the socioeconomic gradient seen in teenagers and adults reversed direction; incidence increased as neighborhood SES decreased ($p<0.001$ by χ^2 for trend). Because only 51% of case reports included information on race and ethnicity, we were unable to examine whether SES gradients occurred within each major racial/ethnic group in Connecticut.

Previous studies using similar area-based methods in Denmark; Manitoba, Canada; Queensland, Australia; and Scotland also found an association between *Campylobacter* infection incidence and higher area-based SES (*4*–*7*). A true higher prevalence of major campylobacteriosis risk factors among patients with a higher SES

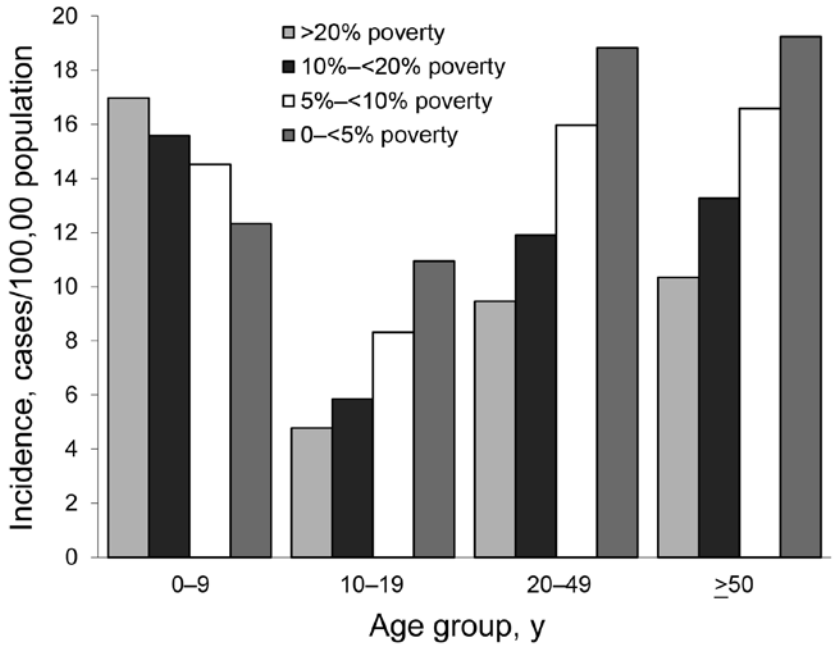

Figure. Average annual incidence rates for *Campylobacter* infection, by age group and neighborhood poverty level, Connecticut, 1999 -2009. Census tract groupings were determined by percentage of residents living below the federal poverty level on the basis of data from the 2000 US Census.

might explain these findings, but these results could also indicate surveillance artifacts if persons at higher SES levels are more likely to seek health care and have an organism-specific diagnosis made. We believe the former hypothesis is more likely for several reasons. First, major risk factors for adult campylobacteriosis at FoodNet sites are international travel and eating out at restaurants (*8*). We examined these factors in Connecticut by using the 3 FoodNet population surveys (*9*) that occurred during the study period (2000–2001, 2002–2003, and 2006–2007) and found that these factors were associated with higher SES (K. Bemis, unpub. data). Second, we found that higher incidence in children <10 years of age was associated with lower SES, a finding that would not be expected if children living in poorer neighborhoods were less likely to receive a diagnosis of campylobacteriosis. Last, we examined Connecticut-specific data from the same 3 FoodNet population surveys (*9*) and found that high-income adults who had diarrhea were no more likely than those with lower incomes to visit a healthcare provider and have a stool specimen taken (K. Bemis, unpub. data). The finding that children living in poorer census tracts were at higher risk than those in higher SES areas could conceivably reflect a higher rate of exposure to *Campylobacter* spp. in the home. However, this hypothesis needs verification. In addition, studies in other parts of the United States are needed to corroborate this study's findings.

We conclude that *Campylobacter* control efforts, at least in Connecticut, should take into consideration the groups with highest age-specific, SES-related incidence. Area-based SES measures should be more widely used when analyzing surveillance data.

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Legionnaires' Disease Caused by *Legionella pneumophila* Serogroups 5 and 10, China

To the Editor: Legionnaires' disease is a systemic infection caused by gram-negative bacteria belonging to the genus *Legionella*. The primary clinical manifestation is pneumonia. *Legionella* spp. are typically found in natural and artificially hydrated environments.

Legionella pneumophila is the species responsible for ≈90% of human cases of infection. *L. pneumophila* is divided into 15 serogroups, among which serogroup 1 is the most prevalent disease-causing variant (*1*). In contrast, rare cases are caused by other serogroups. We describe a case of Legionnaires' disease caused by co-infection with *L. pneumophila* serogroups 5 and 10 and the genotype characteristics of these strains.

The case-patient was a 77-year-old man who had chronic hepatitis B for 50 years, ankylosing spondylitis for 40 years, and chronic cholecystitis for 5 years. On September 17, 2012, he was admitted to Wuxi People's Hospital (Wuxi, China) for treatment after a continuous cough for 15 days and a high fever for 2 days. At admission, the patient had a blood pressure of 130/65 mm Hg, a pulse rate of 102 beats/minute, and a body temperature of 37.4°C, which increased to 38.4°C four hours later. Laboratory tests showed a leukocyte count of 9,200 cells/μL (88.7% neutrophils) and a C-reactive protein level of 31 mg/L in serum. Lung inflammation was identified by computed tomography. The result of a urinary antigen test for *L. pneumophila* serogroup 1 (Binax, Portland, ME, USA) was negative. Bronchoalveolar lavage was performed, and fluid was collected for bacterial culture and molecular analysis.

Real-time PCRs were performed with primers specific for the 5S rRNA gene of the genus *Legionella* (*2*) and the *L. pneumophila*-specific *mip* gene (*3*). *Legionella* colonies isolated from bronchoalveolar lavage fluid grew on buffered-charcoal yeast extract agar. Nine *Legionella*-like colonies were isolated, and all showed positive results by PCRs. The colonies were identified as *L. pneumophila* serogroups 2–14 by using the *Legionella* latex test (Oxoid, Basingstoke, UK). Among these colonies, 5 were identified as *L. pneumophila* serogroup 5, and 4 were identified as serogroup 10 by using a monoclonal antibody (Denka Seiken, Tokyo, Japan). Environmental investigations were conducted in the patient's house and hospital room, but *L. pneumophila* serogroup 5 and 10 were not detected in any of the locations tested.

Pulsed-field gel electrophoresis (PFGE) (*4*) was used to investigate the 9 *L. pneumophila* strains. Two PFGE patterns that were 94% similar were observed; each pattern represented 1 serogroup. The PFGE patterns were compared with those of a reference database of *L. pneumophila* for China. All *L. pneumophila* in the database, including 41 strains isolated from the city in which the patient resided in 2012, had patterns different from those of the 9 strains.

Two clinical *L. pneumophila* strains of different serogroups were further analyzed by sequence typing (*5*,*6*). Sequence type (ST) indicated that allele numbers for *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA* genes were 6, 10, 15, 28, 21, 7, and 207 for serogroup 5 strains and 6, 10, 15, 10, 21, 40, and 207 for serogroup 10 strains. By querying the ST database for *L. pneumophila* (http://www.ewgli.org), we found that both profiles were new and assigned these 2 strains the numbers ST1440 (serogroup 5) and ST1439 (serogroup 10). STs of these 2 isolates differed from each other by only 2 alleles (3 nt in the *mip* gene and 1 nt in the *proA* gene), which suggested that the isolates might be more closely related to each other than suggested by serologic analysis.

Human infections with *L. pneumophila* serogroups 5 and 10 have been rarely reported (*1*,*7*). Our study confirms human infection with 2 *L. pneumophila* serogroups that did not involve serogroup 1. Results for this case-patient also indicated that a negative urinary antigen test result should not be a reason for ruling out Legionnaires' disease because the urinary antigen kit used detects only *L. pneumophila* serogroup 1 antigen. *L. pneumophila* serogroups 5 and 10 are probably underrecognized pathogenic serogroups. Culture and molecular analysis should be performed to obtain an accurate diagnosis. Rare co-infections with *L. pneumophila* serogroup strains have been identified by culture methods (*8*,*9*).

The cases reported previously and in this study indicate that co-infections with different serogroups are more common than currently recognized and that multiple colonies should be

tested for accurate epidemiologic investigations. Qin et al. reported that pathogenic *Legionella* strains of different species, serogroups, and genotypes were isolated from the same hot spring water samples (*10*). This finding suggests that co-infections with different *Legionella* strains may occur under certain conditions.

In China, Legionnaires' disease is usually ignored in the differential diagnosis of pneumonia because most clinicians lack experience with this disease. This case highlights the need to familiarize physicians with diagnostic methods for identifying *Legionella* pneumonia in clinics in China and for further epidemiologic surveillance to monitor this disease and improve public health disease control strategies.

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Human Exposure to Tickborne Relapsing Fever Spirochete *Borrelia miyamotoi*, the Netherlands

To the Editor: *Borrelia miyamotoi* is a relatively novel tickborne relapsing fever spirochete, and is a different species than *B. burgdorferi* sensu lato, the causative pathogen of Lyme borreliosis (*1*). *B. miyamotoi* was first isolated in 1995 from *Ixodes persulcatus* ticks in Japan, after which it was detected in ticks in North America, Europe, and Russia (*1*,*2*). *B. miyamotoi* infections among humans were first reported in Russia in 2011 (*3*), and in 2013 in the United States (*4*). Recently, the first patient infected with *B. miyamotoi* was reported in the Netherlands (*5*). Conditions reported to be associated with *B. miyamotoi* infection were systemic, including malaise and fever, meningoencephalitis, and neurologic symptoms. Because of the nature of these manifestations and because regular diagnostic tests for *B. burgdorferi* will most probably not detect *B. miyamotoi* infections (*3*,*5*), *B. miyamotoi* infections may remain undiagnosed. Nevertheless, the relationship between *B. miyamotoi* infection and illness is not very well established; the case-patients reported, including the patient in the Netherlands, were usually hospitalized, severely ill, and often immunocompromised (*3*–*5*). The extent to which *B. miyamotoi* causes infection and disease in immunocompetent persons is unknown. As a first step to indicate the population at risk for infection, we investigated human exposure to *B. miyamotoi* in the Netherlands.

To do this, we assessed the *B. miyamotoi* infection rate of ticks that had bitten humans. Earlier studies included ticks collected through flagging an area (*1*,*2*); our study provides specific information about the infection rate of ticks feeding on humans. The ticks were collected from persons who reported their tick bites on the website http://www.tekenradar.nl. After removal of the ticks from the skin, the ticks were submitted to the National Institute of Public Health and the Environment. For 1,040 ticks gathered during April–June 2012, we determined tick species, stage of development, and gender by microscopic examination.

We defined the degree of engorgement in 4 categories from unengorged (score 0) to fully engorged (score 3), as visually determined. To isolate DNA, we boiled the ticks with engorgement scores of 0–1 in ammonium hydroxide (*6*); for ticks with engorgement scores of 2–3, we used the QIAGEN (Valencia, CA . USA) blood and tissue DNA-extraction kit (*7*). We used a *B. miyamotoi*–specific real-time PCR based on the flagellin gene for detection of the bacteria (*5*). Quantitative PCR-positive tick lysates were tested with a conventional PCR, which amplifies a fragment of glycerophosphodiester phosphodiesterase (*glpQ*) gene, to confirm the outcome (*5*). These PCR products were sequenced and were identical to *B. miyamotoi* sequences filed in GenBank (AB824855). We determined the presence of *B. burgdorferi* DNA with a duplex quantitative PCR using fragments of the outer membrane protein A gene and the flagellin B gene as targets (*7*).

All 1,040 ticks were identified as *Ixodes ricinus*, the most common tick that transmits *B. burgdorferi* in northern Europe (*8*). We detected *B. miyamotoi* DNA in 37 ticks (3.6%) using real-time PCR targeting the flagellin gene, which was confirmed for 32 ticks (3.1%) in the conventional PCR targeting the *glpQ* gene. (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/20/7/13-1525-Techapp1.pdf). In 9 of the 37 ticks positive for *B. miyamotoi*, *B. burgdorferi* was also detected. Similar to *B. burgdorferi*, the risk of transmission of *B. miyamotoi* is likely to become higher if ticks become engorged with blood; 23 of the 37 (62.2%) *B. miyamotoi*–infected ticks were somewhat engorged (score 1–3) and thus had such an increased risk for transmission. All *glpQ* sequences of the detected *B. miyamotoi* isolates were identical to the sequence detected in the sample from the patient reported in the Netherlands by Hovius et al. (*5*). *B. burgdorferi* DNA was detected in 190 ticks (18.3%) compared with 11.8% detected in a study that included ticks collected through flagging (*9*).

Ticks included in the study were submitted from all parts of the country; B. miyamotoi– and B. burgdorferi–positive ticks were found in almost every region (Figure). Of the ≈1 million tick bites per year in the Netherlands (*10*), an estimated 36,000 were by ticks that were infected with *B. miyamotoi,* and 183,000 were by ticks infected with *B. burgdorferi*. This substantial human exposure to *B. miyamotoi* and the reported cases in Russia, the United States, and, recently, the Netherlands (*3*–*5*) raises the question to what extent exposure to *B. miyamotoi* leads to human disease in the general population. These results call for the development of sensitive and specific serologic and molecular tests for *B. miyamotoi* to identify possible patients, which will lead to a better understanding of the clinical spectrum of *B. miyamotoi*–induced disease.

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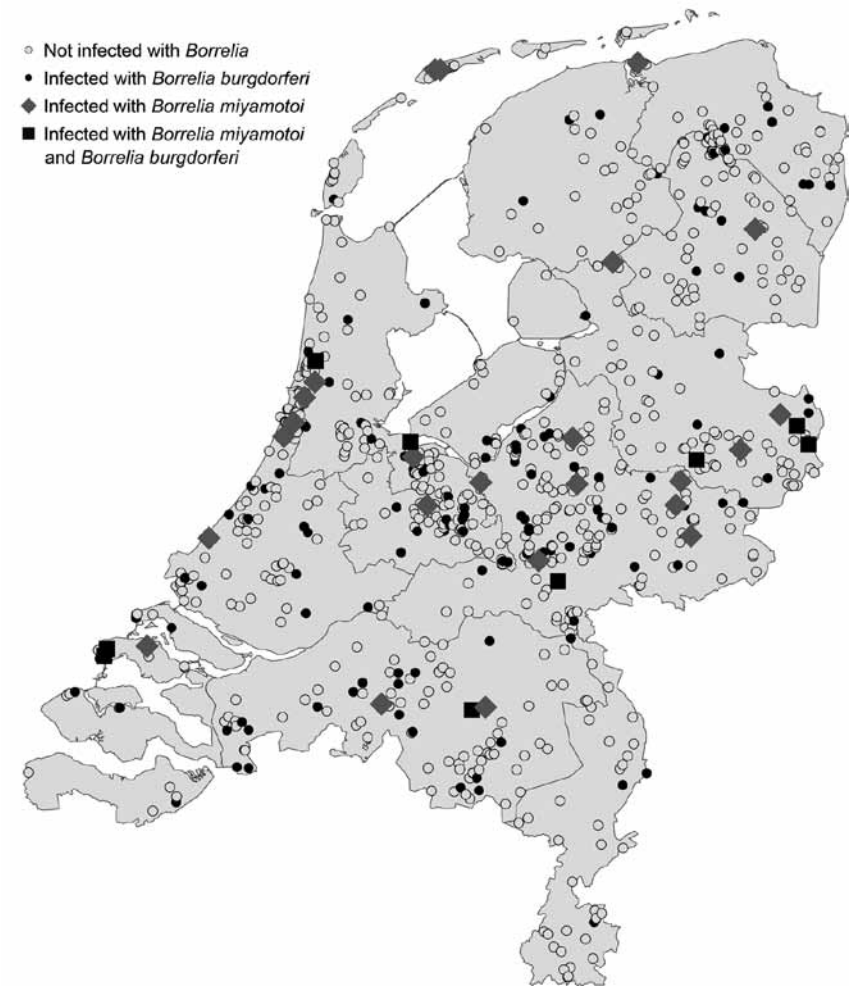

Figure. Locations of ticks collected through the website http://www.tekenradar.nl in the Netherlands during summer 2012,. Ticks included in the study were submitted from all parts of the country; ticks positive for *Borrelia miyamotoi* and *B. burgdorferi* were found in almost every region.

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Carbapenemase-producing Bacteria in Patients Hospitalized Abroad, France

To the Editor: The emergence and rapid worldwide dissemination of carbapenemase-producing bacteria (CPB), especially carbapenemase-producing *Enterobacteriaceae* (CPE), have prompted public health authorities to reconsider prevention strategies to control the spread of these organisms (*1*–*5*). In France, national guidelines recommend systematic screening for commensal CPE and glycopeptide-resistant enterococci (GRE) in all patients admitted to hospitals who have been hospitalized in other countries during the preceding 12 months (*6,7*) (repatriated patients), independently of whether transfer was direct from hospital to hospital (DT) or not (NDT). These guidelines also recommend implementation of presumptive patient isolation and contact precautions on admission (*6,7*). We conducted a 33-month survey at Hôpital Européen Georges Pompidou (HEGP), a university teaching hospital in Paris, of CPE and GRE in repatriated patients; we also investigated incidence of extended-spectrum β-lactamase (ESBL)–producing *Enterobacteriaceae* and carbapenemase-producing *Acinetobacter baumannii* and *Pseudomonas* spp. in the same patient group.

During November 2010–July 2013, a total of 541 patients who had previously been hospitalized in a total of 71 other countries were admitted to HEGP. Rectal swab specimens were taken from 510 patients; 82 (16.1%) were DT, 415 (81.4%) were NDT, and 13 (2.5%) had an unclear history of transfer. Median patient age was 61 (range 12–98) years; 70% of patients were male. Results of screening by using antibiotic-containing Luria Bertani broths for enrichment and plating on selective media were negative for 354 (69.4%) of the 510 patients surveyed; 33 (6.5%; 16 DT, 17 NDT) patients were colonized with ≥1 CPB and/or GRE and 123 (24.1%; 22 DT, 99 NDT, 2 unclear) with ESBL producers only. More specifically, 19.5% (16/82) of DT patients and 4.1% (17/415) of NDT patients were colonized with CPB and/or GRE ($p<10^{-5}$ by χ^2 test); 26.8% (22/82) of DT patients and 23.9% (99/415) of NDT patients were colonized with ESBL producers only ($p = 0.67$). Characteristics of the 33 patients carrying CPB and/or GRE are shown in the Table. Of all isolates, 191 produced ESBLs only.

Rates of resistance for ESBL-producing *Enterobacteriaceae* and CPE were, respectively, 53.1% and 57.1% to gentamicin, 16.7% and 32.1% to amikacin, 77.1% and 82.1% to nalidixic acid, 63% and 75% to levofloxacin, and 70.3% and 75% to ciprofloxacin. The *Pseudomonas* spp. and *A. baumannii* isolates were also multidrug resistant; all isolates were colistin susceptible.

Among the 33 colonized patients, 13 (39.4%) were not infected; 1 of the uninfected patients died. Seven patients were infected with ≥1 CPB (health care–related in 2 patients, 1 of whom died), 4 patients with ESBL-producing *Enterobacteriaceae* (health care–related in 1 patient, who died), and 9 patients with other bacteria (health care–related in 4 patients, 1 of whom died). No patients were infected with GRE. Overall, 60.6% of colonized patients were infected and 12.1% died; 35% (7/20) of the infections were health care–related (3 urinary tract device–related infections, 2 cases of ventilator-associated pneumonia, 1 infection at the site of a portacath, and 1 case of cellulitis).

Almost 25% of the repatriated patients carried ESBL-producing *Enterobacteriaceae* (mostly CTX-M-15 producers; online Technical Appendix, http://wwwncd.cdc.gov/EID/article/20/7/13-1638-Techapp1.pdf); 6.7% carried CPB and/or GRE. By comparison, during the study period, only 10.8% of 2,314 systematically screened patients in the medical and general surgery intensive care units at HEGP (repatriated patients excluded) carried ESBL-producing *Enterobacteriaceae*; 1 carried *vanA Enterococcus faecium* (data not shown). For patients with no record of hospitalization abroad, no CPE isolates were found; other bacterial isolates included 1 *vanA E. faecalis*, 13 *vanA E. faecium* (all known from previous outbreaks), 4 OXA-23–producing *A. baumannii*, and 4 VIM- and 1 IMP-producing *P. aeruginosa*.

Of the repatriated patients, 19.5% of DT patients (vs. 4.1% of NDT) and 23.9% (7 DT, 4 NDT) of those who were transferred to medical and general surgery intensive care units (ICUs) were CPB and/or GRE carriers. This finding highlights the role of severe underlying disease or injury and recent antimicrobial drug treatment. Among ICU patients, 3 died, most likely from underlying conditions, findings in line with the observation that carriage of or infection with multidrug-resistant bacteria is not the only predictor of death (*8*). Most of the 28 CPE isolates were resistant to fluoroquinolones and aminoglycosides except amikacin; 21 carried OXA-48–type genes, 7 of which were non-ESBL producers and were detected only around an ertapenem disk on Drigalski agar (Bio-Rad, Marnes-la-Coquette, France). All CPB, irrespective of species, showed imipenem hydrolysis in a recently described test (*9*) that was shortened and simplified by incubating colonies directly in antibiotic solution.

Although time-consuming and certainly perfectible, implementation of strict control measures to limit CPB and GRE spread (*6,7*) seems justified, a conclusion supported by the occurrence, since November 2010, of just 1 cross-transmission–linked CPB outbreak in an ICU at HEGP (after urgent intervention for cardiac arrest). Of particular concern

Table. Clinical and laboratory data on 33 patients hospitalized in France who were previously hospitalized in other countries and were carrying carbapenemase-producing bacteria, glycopeptide-resistant enterococci, or both*

Patient no.	Year of initial hospital admission	Patient transfer status	Country of hospitalization	Species of infection	β-lactamase content		Glycopeptide resistance gene
					ESBL	Carbapenemase	
1†	2010	DT	Egypt	*E. coli*	Pos	OXA-48	
2†	2010	DT	Thailand	*A. baumannii*	Pos	OXA-23	
3†	2010	DT	Iraq	*A. baumannii*	Neg	OXA-23	
				E. faecium			*vanA*‡
4	2010	NDT	USA	*E. faecium*			*vanA*
5	2011	NDT	Morocco	*K. pneumoniae*	Neg	OXA-48	
				K. pneumoniae	Neg	OXA-48	
6†	2011	DT	Senegal	*K. pneumoniae*	Pos	OXA-48	
7	2011	DT	Congo	*E. faecium*			*vanA*‡
8†	2011	NDT	Benin	*A. baumannii*	Neg	OXA-23‡	
9	2011	NDT	Kuwait	*P. aeruginosa*	Neg	VIM-2	
10†	2011	NDT	Kuwait	*K. pneumoniae*	Neg	OXA-48	
11†	2011	NDT	Kuwait	*P. aeruginosa*	Neg	VIM-2	
12†	2011	NDT	Kuwait	*E. faecium*			*vanA*
13†	2011	DT	Libya	*K. pneumoniae*	Pos	OXA-48	
14†	2011	DT	Libya	*E. coli*	Pos	OXA-48	
				K. pneumoniae	Pos	OXA-48	
				A. baumannii	Neg	OXA-23	
				E. faecium			*vanA*
15	2011	NDT	Saudi Arabia	*E. faecium*			*vanA*
16†	2011	NDT	Pakistan	*E. faecium*			*vanA*
17	2011	NDT	Italy	*A. baumannii*	Neg	OXA-23	
				K. pneumoniae	Neg	KPC-3	
18†	2011	DT	Spain	*K. pneumoniae*	Neg	OXA-48	
19	2011	NDT	Israel	*K. pneumoniae*	Neg	KPC-3	
20†	2012	DT	Egypt	*A. baumannii*	Neg	NDM-1	
				A. baumannii	Neg	NDM-1	
				P. putida	Neg	VIM-2	
21†	2012	DT	Tunisia	*E. coli*	Pos	OXA-48	
				K. pneumoniae	Pos	OXA-48	
				K. pneumoniae	Neg	OXA-48	
22†	2012	NDT	Tunisia	*A. baumannii*	Neg	OXA-23	
23†	2012	DT	India	*E. coli*	Neg	NDM-1	
				M. morganii	Pos	NDM-1	
				P. aeruginosa	Neg	VIM-2	
24†	2012	NDT	Cambodia	*E. coli*	Neg	NDM-4‡	
				E. coli	Pos	OXA-48‡	
25†	2012	DT	Sri Lanka	*E. coli*	Pos	OXA-48‡	
				K. pneumoniae	Pos	OXA-181‡	
26	2013	NDT	Algeria	*E. coli*	Neg	OXA-48	
27†	2013	NDT	Algeria	*E. coli*	Neg	OXA-48	
28	2013	DT	Tunisia	*A. baumannii*	Neg	NDM-1	
29†	2013	DT	Libya	*K. pneumoniae*	Pos	OXA-48	
30	2013	DT	Libya	*K. pneumoniae*	Pos	OXA-48	
				K. pneumoniae	Pos	OXA-48	
31	2013	NDT	India	*E. coli*	Pos	OXA-181	
				E. coli	Neg	NDM-7‡	
				K. pneumoniae	Neg	NDM-7‡	
32	2013	NDT	Georgia	*P. aeruginosa*	Pos	VIM-2‡	
33†	2013	DT	Montenegro	*E. faecium*			*vanA*‡

*ESBL, extended-spectrum β-lactamase; DT, direct transfer from hospital abroad to HEGP; *E. coli, Echerichia coli*; Pos, positive; *A. baumannii, Acinetobacter baumannii*; Neg, negative; *E. faecium, Enterococcus faecium*; NDT, nondirect transfer (hospitalized abroad within 12 mo before transfer to HEGP); *K. pneumoniae, Klebsiella pneumoniae*; *P. aeruginosa; Pseudomonas aeruginosa*; *P. putida, Pseudomonas putida*; *M. morganii, Morganella morganii*.
†Patient carrying an ESBL producer in addition to the carbapenemase-producing bacteria and/or glycopeptide-resistant enterococci.
‡Resistance gene not reported previously in the country of initial hospitalization.

is the high proportion of OXA-48–producing isolates in persons with no documented link to repatriation in France (*10*). This finding could be explained in part by the historical and demographic relationships between France and North Africa, where prevalence of OXA-48 is high, reflected in results from patients repatriated from that part of the continent.

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Zoonotic Filariasis Caused by Novel *Brugia* sp. Nematode, United States, 2011

To the Editor: Zoonotic brugian filariasis is an incidental infection of humans with *Brugia* spp. nematodes that primarily parasitize nonhuman vertebrates, rarely humans (*1*–*3*). In contrast to classical lymphatic filariasis caused by *B. malayi* and *B. timori*, which are found in Asia, most zoonotic *Brugia* infections have been reported from the northeastern United States (*2*,*3*) or South America (*3*). We report a case of symptomatic brugian infection in a New York City resident who had not traveled to the Eastern Hemisphere.

In 2011, a 53-year-old White man first noted tenderness and swelling behind his penis and in his right groin after having fallen 3 months earlier. The tenderness was relieved by nonsteroidal antiinflammatory drugs, but the swelling continued; an oral antimicrobial drug, prescribed for presumed cellulitis, produced no improvement. At the time of examination, the patient had no fever or other signs or symptoms. Only a 3.0-cm × 3.0-cm firm, nonfixed right inguinal nodule without warmth or tenderness was noted. Laboratory findings were remarkable for total leukocytes of 6.4×10^9, eosinophilia (12%, 600 cells/mm^3), decreased hemoglobin level (10.0 g/dL), and low hematocrit of 31.2%. An excisional biopsy sample revealed intralymphatic adult nematodes with viable-appearing microfilaria (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/20/7/13-1654-Techapp1.pdf).

The patient had been born and raised in Champlain, Illinois, and had resided in the Bronx, New York, since 1979; he had no history of travel to filariasis-endemic regions. Characteristics of the adult worms and microfilaria were most consistent with those of *Brugia* spp., which was surprising because classical brugian lymphatic filariasis seems to be limited to Asia (*B. malayi*) and Indonesia (*B. timori*) (*4*,*5*). However, the adult filariae were smaller than expected for *B. malayi* or *B. timori* nematodes, prompting consideration of zoonotic filariasis (*1*,*6*). The adult worms and microfilaria seemed to be viable, although zoonotic *Brugia* spp. in histologic

sections often appear degenerated (*1*,*2*,*6*). The diameters of the adult worms were similar to those reported from South America (females 90–100 μm, males 50 μm) (*7*,*8*) rather than those from North America (females 35–75 μm, males 32–52 μm) (*1*). Peripheral blood was repeatedly negative for microfilaria. Serum sent to the Centers for Disease Control and Prevention (Atlanta, GA, USA) for ELISA testing for *B. malayi* anti-filarial IgG 4 showed optical density of 0.13, below the ELISA cutoff for filariasis.

Because micromorphologic information was not adequate for species identification, paraffin-embedded biopsy specimens were submitted for molecular testing. Genomic DNA extracted from paraffin-embedded tissue with the QIAamp DNA–formalin-fixed, paraffin-embedded tissue procedure was amplified by using the primer sets DiBu-F(5′ GCTAGATATGCTACCAACAAAA-3′)/ITS1 R(5′-CTCAATGCGTCTGCAATTCGC-3′) and BuF2-(5-CATTTATGCTAGATATGCTACCAAC-3′)/ITS1-R. The products were fractionated on 2% agarose gel and stained with ethidium bromide. The internal transcribed spacer (ITS) 1 PCR product (182 bp) was automatically sequenced by using the same primers used for PCR. Lasergene software (DNASTAR, Madison, WI, USA) was used to align the sequences obtained with *Brugia* spp. sequences deposited in GenBank; detailed sequence comparison identified the isolate as a novel *Brugia* (Nematoda: Onchocercidae) species closely related to *B. pahangi* and *B. malayi* (Figure). The ITS-1 sequence was submitted to the EMBL Nucleotide Sequence Database (accession no. HE856316).

Removal of an affected lymph node without additional treatment is often considered sufficient treatment for zoonotic filariases. However, for the patient reported here, persistence of inguinal swelling prompted a repeat biopsy 4 months later; the specimen again demonstrated reactive follicular hyperplasia, although no parasites were seen. Because the patient's initial clinical signs and subsequent persistent adenopathy were reminiscent of unilateral lymphadenitis, lymphangitis, and induration that are typical of *B. malayi* or *B. timori* filariasis, and the microfilariae in the original biopsy sample appeared to be viable, we empiricially prescribed a standard dosage of oral doxycycline for 6 weeks, followed by single doses of ivermectin at 400 μg/kg and 800 mg albendazole. The patient has been well, without further adenopathy or eosinophilia, for >2 years. Because adult filariae can live for >10 years, the place of acquisition cannot be stated with certainty.

The prevalence of zoonotic infection with *Brugia* spp. nematodes is unknown. Many reported cases are asymptomatic or diagnosed incidentally during evaluation for persistent adenopathy (*1*–*3*). Conversely, differentiation of zoonotic from classical filariasis is unlikely in disease-endemic areas; most cases published since the initial 1962 case report (*1*) occurred in the United States. Most case-patients were from the Northeast,

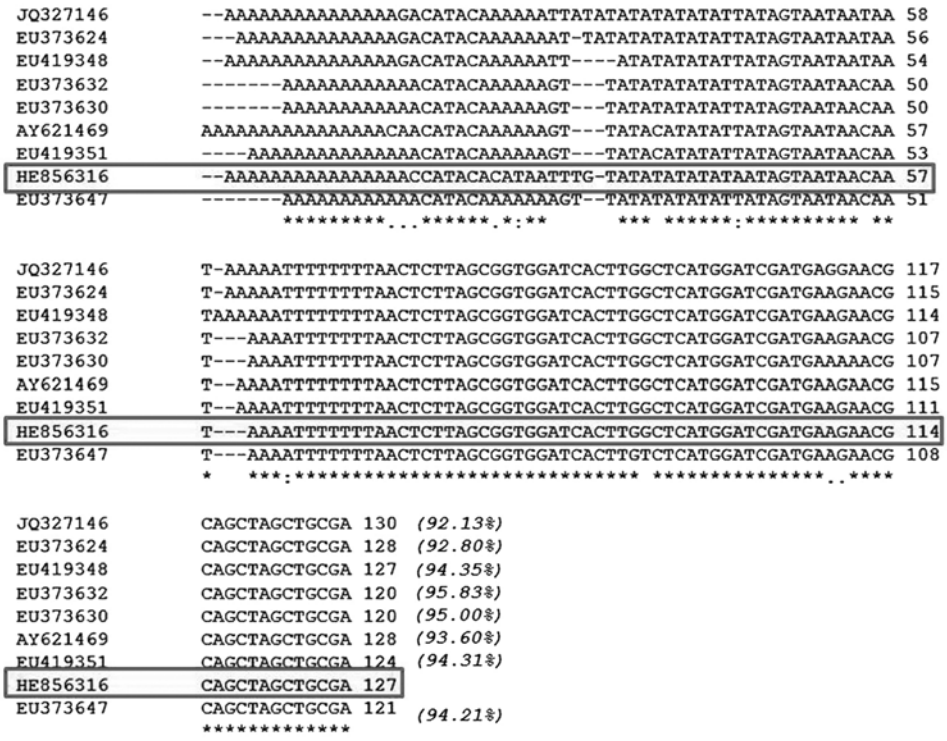

Figure. Pile-up of partial ribosomal DNA sequences from *Brugia* NY strain (HE856316) and from other related *Brugia* spp. strains and clones, *B. malayi* BM28 (JQ327146), *B. malayi* C27Cat5 (EU373624), *B. pahangi* C61CAT5 (EU419348), *B. pahangi* C14Cat6 (EU373632), *B. pahangi* C7Cat6 (EU373630), *B. pahangi* Bp-1 (AY621469), *B. pahangi* C46CAT5 (EU419351), and *B. pahangi* C27Cat7 (EU373647). Boxes indicate the *Brugia* NY strain (HE856316); asterisks (*) indicate conserved residues; periods (.) indicate nucleotide changes; colons (:) indicate nucleotide changes just in the *Brugia* NY isolate; hyphens (-) are included in the sequences to maximize the comparisons among the 9 DNA molecules. Italicized numbers in parentheses indicate the percentage of similarity with the *Brugia* NY isolate.

including New York (8 cases), Massachusetts, Pennsylvania, Connecticut, and Rhode Island (3 cases each) (*1*,*2*); single cases have been identified in Michigan, Ohio, North Carolina, Oklahoma, New Jersey, Louisiana, Florida, and California (*1*,*2*). Four other cases have been reported: 3 in South America (Colombia, Brazil, Peru) (*3*,*7*,*8*) and 1 in Africa (Ethiopia) (*9*). Only a few *Brugia* species have been identified, including *B. leporis*, found in rabbits in the northeastern United States (*1*,*10*); *B. beaveri*, found in raccoons and bobcats in the southern United States; and *B. guyanensis*, found in coatimundi and other vertebrates in South America (*8*). Definitive identification with molecular techniques will better identify causative species and help clarify many of the ecologic and epidemiologic questions surrounding zoonotic filarial infections.

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Candida auris–Associated Candidemia, South Africa

To the Editor: We noted the report by Chowdhary et al. (*1*) and report *Candida auris* as a causative agent of candidemia in South Africa, with an estimated prevalence of 0.3% (N.P. Govender et al., unpub. data). First isolated in 2009, *C. auris* is an emerging species associated with clinical disease (*2*–*6*). We analyzed 4 isolates submitted to the National Institute for Communicable Diseases (Johannesburg, South Africa) from 4 patients with candidemia who had been admitted to different public- and private-sector hospitals from October 2012 through October 2013.

Identification of the isolates was undertaken by using ChromAgar *Candida* medium (Mast Diagnostics, Merseyside, UK), Vitek-2 YST (bioMérieux, Marcy l'Etoile, France), API 20C AUX (bioMérieux), and sequencing of internal transcribed spacer (ITS) and D1/D2 domains of the ribosomal RNA gene (*7*), followed by microbroth dilution susceptibility testing (*8*). All isolates were misidentified as *C. haemulonii* and *Rhodotorula glutinis* by Vitek-2 YST and API 20C AUX assays, respectively (Table).

Similar to the findings of Chowdhary et al., all isolates assimilated *N*-acetyl-glucosamine (*1*). With the use of the CBS-KNAW database, pairwise sequence alignment of ITS region showed 99% sequence homology to Kuwait isolates, and alignment of D1/D2 domain showed 98% homology to the Kuwait/India isolates (*9*). In a neighbor-joining phylogenetic tree based on ITS sequences, South Africa isolates formed a cluster with India and Kuwait isolates (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/20/7/13-1765-Techapp1.pdf).

Table. Identification and antifungal susceptibility results of 4 *Candida auris* isolates from 4 male patients with candidemia, South Africa, October 2012–October 2013*

Isolate ID	Patient age, y	Hospital unit	Vitek-2 YST†	API 20C AUX†	DNA sequence analysis‡	MIC								
						AMB	FLX	VRC	POS	ITC	5FC	CAS	MFG	AFG
208	85	High-care	*C. haemulonii*	*Rhodotorula glutinis*	*C. auris*	1	>256	0.5	0.03	0.12	0.12	0.25	0.06	0.25
209	60	Medical ICU	*C. haemulonii*	*R. glutinis*	*C. auris*	0.5	>256	1	0.06	0.12	0.12	0.12	0.06	0.12
224	73	Burn	*C. haemulonii*	*R. glutinis*	*C. auris*	1	>256	2	0.06	0.25	0.12	0.25	0.12	0.25
293	27	Trauma ICU	*C. haemulonii*	*R. glutinis*	*C. auris*	1	64	0.25	0.015	0.06	0.06	0.03	0.06	0.06

*AMB, amphotericin B; FLX, fluconazole; VRC, voriconazole; POS, posaconazole; ITC, itraconazole; 5FC, flucytosine; CAS, caspofungin; MFG, micafungin; AFG, anidulafungin.
†bioMérieux, Marcy l'Etoile, France.
‡Sequence data for the 4 isolates have been deposited in GenBank, accession nos. KJ1236762–KJ126765 and KJ126758–KJ126761 for the internal transcribed spacer and D1/D2 regions, respectively.

Fluconazole MICs were high for all isolates (Table). Isolates 209 and 224 showed reduced voriconazole susceptibility with MICs of 1 μg/mL and 2 μg/mL, respectively, which is above the epidemiologic cutoff value for 11 *Candida* species (*10*). Isolates were susceptible to amphotericin B and echinocandins at low MICs Clinical data were available for 1 patient (online Technical Appendix Table). Two *C. haemulonii* isolates were identified during laboratory-based sentinel surveillance for candidemia in South Africa; the ITS region of one isolate was sequenced and the isolate identified as *C. auris* (N.P. Govender, pers. comm.). In this study, *C. auris* was misidentified by routinely used tests and was accurately identified by sequencing, in keeping with previous findings (*1*,*3*,*4*,*6*).

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Prevalence and Drug Resistance of Nontuberculous Mycobacteria, Northern China, 2008–2011

To the Editor: Nontuberculous mycobacteria (NTM), defined as members of *Mycobacterium* species other than those in the *M. tuberculosis* complex or *M. leprae*, are mostly considered to be opportunistic pathogens (*1*). However, many NTM can and do cause disease in immune-competent hosts. Pulmonary infection by NTM can be a source of diagnostic uncertainty, especially in locations such as in China, where acid-fast staining of sputum samples is the mainstay of diagnosis for tuberculosis (*2*). NTM are also relatively resistant to many of the first- and second-line drugs used to treat tuberculosis, thus making accurate diagnosis and drug-susceptibility testing critical to clinical management of NTM infections (*3*). The medical and public health communities have been concerned about increasing prevalence of NTM infection in China, and 2 recent surveys, 1 from Shanghai and another from a rural population in Shandong Province, gave somewhat conflicting reports of the prevalence of these infections (*4*,*5*). We therefore decided to conduct a survey of NTM isolates in Beijing from the National Tuberculosis Clinical Laboratory of the Beijing Chest Hospital. We also tested isolates from specimens collected in this laboratory against an extended drug susceptibility panel to determine which drug regimens would be most useful in therapy for various NTM infections.

During January 2008–December 2011, sputum samples collected from 3,714 patients attending the Beijing Chest Hospital with suspected pulmonary tuberculosis were positive for mycobacterial spp. Among the surveillance population, 92% were from northern China, including 13 provinces and the 2 major urban conurbations of Beijing and Tianjin. From our survey, the Han ethnic group accounted for 82% of patients, and 61% of total patients were from urban, rather than rural, areas. Most (59%) of the patients were male, and 40% were attending the hospital for re-treatment of pulmonary tuberculosis; mean age was 51 ± 20 years. Of these mycobacterial isolates, 95 (2.6%) were positive for NTM; NTM were identified during initial screening for resistance to *p*-nitrobenzoic acid. We identified the strains to species level by sequencing the internal transcribed spacer region of the 16S-23S rRNA and 16S rRNA genes (*6*), which is able to discriminate between even closely related species such as *M. chelonae* and *M. abscessus* (*7*).

Of the 95 NTM isolates, 38 (40%) were *M. intracellulare* and 28 (29%) were *M. abscessus* (Table). Five additional species were also identified: *M. fortuitum* (8%), *M. gordonae* (8%), *M. kansasii* (7%), *M. avium* (5%), and *M. parascrofulaceum* (1%). A survey performed recently in Shandong Province also identified *M. intracellulare* as the most common isolate (*4*), but in that study, it represented 52 (81%) of 64 cases. By contrast, 2 previous surveys found *M. chelonae* to be the most commonly isolated species (20% and 27% of isolates) (*5*,*8*). However, none of the isolates from our study were *M. chelonae*. Differences in isolates may represent the representative patient population from which they were derived; *M. chelonae* was most commonly isolated from hospitals in southern China (*5*,*8*). The most common NTM species found in eastern Asia was *M. avium* complex, in keeping with findings from our study (*9*). Documenting another trend, the International Union Against Tuberculosis and Lung Disease reported that *M. fortuitum* was the most frequently encountered species in Turkey (33.9%), the Czech Republic (17.5%), Portugal (16.5%), and other countries in Europe (*10*).

Drug susceptibility testing (DST) was performed by the proportion method according to the WHO Guidelines for the Programmatic Management of Drug-resistant Tuberculosis, 2011 Update (http://whqlibdoc.who.int/publications/2011/9789241501583_eng.pdf). We tested 3 first-line anti-tuberculosis drugs (rifampin, isoniazid, and ethambutol) and 7 second-line agents (streptomycin, capreomycin, amikacin, protionamide, para-amino salicylic acid, ofloxacin, and levofloxacin) (Table). If a patient had multiple positive NTM isolates, DST was performed on the last isolate. In agreement with other studies (*4*,*5*), ethambutol remained the most useful agent against NTM; its overall resistance rate among isolates tested was 42%. Ranking of second or third agents, however, should be guided by species identification and DST. For example, levofloxacin appears to be a good choice for *M. kansasii, M. gordonae*, or *M. fortiutum* infections (overall resistance rate 22%), but a poor choice against *M. avium* complex infections (overall resistance rate 95%). The second most prevalent species in our study (28% of isolates), *M. abscessus*, was resistant to the test drugs in >90% of cases, highlighting the difficulties associated with treatment for some NTM infections.

Our study suggests that there has been no substantial increase in the prevalence of NTM in respiratory isolates from persons in northern China. Most of the isolates show substantial and extensive drug resistance, providing major therapeutic challenges for clinicians, especially if patients are treated as they would be for drug susceptible tuberculosis. To guide therapy, both species-level identification and DST of NTM isolates should be performed. Our data suggest that testing the efficacy of some second-line agents, in particular, fluoroquinolones, may be beneficial in identifying further options for therapy.

Table. Species and drug-resistance profiles of 95 nontuberculous mycobacteria strains, northern China, 2008–2011*

Drugs	No. (%) resistant strains in *Mycobacterium* spp.							Total
	M. intracellulare	*M.abscessus*	*M.fortuitum*	*M. gordonae*	*M.kansassi*	*M. avium*	*M.parascrofulaceum*	
INH	37 (97.37)	28 (100)	7 (87.5)	6 (75)	3 (42.86)	5 (100)	1 (100)	87 (91.58)
RIF	34 (89.47)	28 (100)	7 (87.5)	2 (25)	0	5 (100)	1 (100)	77 (81.05)
EMB	4 (10.53)	26 (92.86)	7 (87.5)	1 (12.5)	0	2 (40)	0	40 (42.11)
SM	38 (100)	28 (100)	7 (87.5)	4 (50)	6 (85.71)	5 (100)	1 (100)	89 (93.68)
CPM	31 (81.58)	26 (92.86)	4 (50)	1 (12.5)	2 (28.57)	3 (60)	1 (100)	68 (71.58)
AK	31 (81.58)	25 (89.29)	4 (50)	1 (12.5)	1 (14.29)	4 (80)	0	66 (69.43)
PTO	25 (65.79)	27 (96.43)	6 (75)	4 (50)	0	4 (80)	1 (100)	67 (70.53)
PAS	38 (100)	28 (100)	7 (87.5)	8 (100)	7 (100)	4 (80)	1 (100)	93 (97.89)
OFLX	38 (100)	28 (100)	3 (37.5)	3 (37.5)	1 (14.29)	5 (100)	1 (100)	79 (83.16)
LVFX	36 (94.74)	28 (100)	3 (37.5)	2 (25)	0	5 (100)	1 (100)	75 (78.95)
Total	38 (40)	28 (29.47)	8 (8.42)	8 (8.42)	7 (7.37)	5 (5.26)	1 (1.05)	95 (100)

*INH, isoniazid; RIF, rifampin; EMB, ethambutol; SM, streptomycin; CPM, capreomycin; AK, amikacin; PTO, protionamide; PAS, para-aminosalicylic acid; OFLX, ofloxacin; LVFX, levofloxacin.

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Rotavirus Epidemiology in Bangui, Central African Republic, 2008[1]

To the Editor: Infection with group A rotavirus is among the leading causes of gastroenteritis in children, especially in sub-Saharan Africa (*1*). Data with regard to the incidence of rotavirus-A disease in the Central African Republic are limited (*2*). To estimate the prevalence of rotavirus-A disease among young children before introduction of rotavirus-A vaccine in Bangui, the capital of the Central African Republic, we performed a prospective study during February–September 2008. The target sample size, based on an expected 20% prevalence of rotavirus diarrhea and a 5% significance level, was 250 cases. All patients were children 0–5 years of age, who were hospitalized for acute diarrhea at the Complexe Pédiatrique, Bangui, the main hospital for children in the Central African Republic, and all had an illness that met the World Health Organization definition of a suspected case of rotavirus-A gastroenteritis (http://www.who.int/nuvi/surveillance/RV_Case_Defs.pdf). After informed consent and epidemiologic and clinical data had been obtained, a fecal specimen was collected from each child. Samples were transported to the Institut Pasteur de Bangui, where they were tested for rotavirus-A antigen by using the VIKIA Rota-Adeno test, (VIKIA Rota-Adeno; bioMérieux SA, Lyon, France). Results were immediately reported to the referring physician.

Rotavirus-A G-type (virus protein [VP] 7) and P-type (VP4) genotyping were performed by using previously described 2-step amplification methods (*3*). Extracted double-stranded RNA was denatured at 97°C for 5 min, and VP7 and VP4 were amplified by reverse transcription PCR (RT-PCR) by using consensus primers 9Con1-L/VP7R (*3*) and Con3/Con2 (*3*), respectively, and the One-Step RT-PCR kit (QIAGEN, Inc., Valencia, CA, USA) according to the manufacturer's instructions. RT-PCR was conducted by using a GeneAMP PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) with the following thermocycling profile: 30 min at 42°C; 15 min at 95°C; 35 cycles of 30 s at 94°C, 30 s at 42°C, and 45 s at 72°C; followed by a final 7-min extension at 72°C. G-typing used primer 9Con1-L in combination with primers 9T1–1, 9T1-Dg, 9T-2, 9T-3P, 9T-4, and 9T-9B; P-typing used primer Con3 in combination with primers 1T-1, 1T1-VN, 2T-1, 3T-1, 4T-1, 5T-1, and 1T1-Wa. Genotyping reactions were analyzed by use of electrophoresis on a 3% agarose gel.

Data were analyzed by using Stata 11 software (StataCorp, College Station, TX, USA). Prevalence was expressed in percentages. The χ^2 test was used to analyze categorical variables and testing by the Yates correction, as appropriate. A 95% confidence interval was calculated, and $p<0.05$ was considered significant. Ethical and administrative permissions were obtained from the National Committee of Ethics of the Central African Republic; the Complexe Pédiatrique, Bangui; and the Central African Republic Government Ministries.

A total of 250 infants and young children with diarrhea (159 male and 91 female, mean age 8.2 months) were enrolled in this study. Results obtained by the VIKIA Rota-Adeno test revealed that 100 (40%) of these children were infected with rotavirus-A, mostly male children (61/100, $p<0.5$). The proportions of rotavirus-A infection in children <9 months of age and those ≥10 months of age were 37.3% (62/166) and 45.2% (38/84), respectively ($p = 0.2$). Rotavirus-A infections were more prevalent during February–March (67/108, 62.0%) than during April–September (33/142, 23.2%) ($p<10^{-6}$). Because data were collected for only 8 months, annual rotavirus-A prevalence might have been underestimated or overestimated, a possible limitation of the study.

Among the 100 ROTAV-A–positive -patients, 32 samples were randomly selected for genotyping. Among these samples, type G1 predominated (62.8%, 22/32); among P genotypes, type P[8] predominated (50%, 16/32), followed by P[6] (25%, 8/32). The predominant genotypic combination was G1P[8] (43.7%, 14/32) and G1P[6] (25%, 8/32) (Table).

Despite the limitations of a short study period and low number of genotyped strains, this study reports useful information. It reveals that 40% of children hospitalized with acute diarrhea at Complexe Pédiatrique, Bangui, were infected with rotavirus-A during the study period, which included the end of the rotavirus-A gastroenteritis season. Most patients were <9 months of age. These results are similar to those found in the 1980s study at Complexe Pédiatrique, Bangui (*2*), which were that the major serotype/genotype was G1 (71.3%), followed by G2 (15.4%) and G3 (13.3%) (*4*). After 25 years, the predominant circulating genotypes are G1P[8]

Table. Genotyping results for 32 human rotavirus group A–positive samples, Bangui, Central African Republic, 2008

G genotype	P genotype		
	P[4]	P[6]	P[8]
G1	0	8	14
G2	3	0	0
G9	0	0	2
Not typeable	3	0	2

[1]This work was presented as a poster at the 7th African Rotavirus Symposium, Preconference Symposium, at the International African Vaccinology Conference, Lagoon Beach Hotel, Cape Town, South Africa; November 8–11, 2012.

and G1P[6] along with G2P[4] and G9P[8]. Our study results are similar to those of recent studies conducted in other African countries (*5*–*8*) and confirm results of studies that found that the same genotypes circulated in western Cameroon in 2003, albeit at different percentages (*4*,*9*).

Our study provides relevant data about the genotypes of rotavirus-A from children in the Central African Republic, 25 years after the most recent study (*2*). These data represent baseline information that will help with monitoring for potential changes in genotype prevalence after the introduction of rotavirus-A vaccine in the Central African Republic.

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Genome Analysis of Mayaro Virus Imported to Germany from French Guiana

To the Editor: Mayaro virus (MAYV), a mosquito-borne New World alphavirus of the family *Togaviridae*, causes a febrile arthralgia syndrome resembling dengue and chikungunya fever. The virus is maintained in a natural cycle involving nonhuman primates and *Haemagogus* spp. mosquitoes in tropical rainforest areas of South America (*1*). After an incubation time of 7–12 days following an infectious mosquito bite, rash, fever, headache, and arthralgia develop in patients, followed by restoration to their original conditions after several weeks (*1*).

Outbreaks of Mayaro fever have been reported from the Amazon region (*1,2*). There are increasing reports of travel-related infections imported from South America to Europe and the United States (*3*–*7*). We describe an acute MAYV infection in a German traveler who returned from French Guiana. Full-length MAYV genome amplification was performed on virus obtained from a serum sample of the patient.

In August 2013, a 44-year-old woman (bookkeeper) came to an outpatient clinic with fever (temperature ≤38.7°C), chills, a mild headache, severe fatigue, highly painful swelling of small finger joints, and pain in both feet. Symptoms appeared 2 days before when she experienced aches in her wrists and left forefoot. Four days before, the patient had returned from a 2.5-week visit to French Guiana, where she traveled with her partner and caught butterflies. She had conducted these activities during her holidays for the past 5 years, mostly in spring or autumn. In July 2013 at the end of the rainy season, she had many mosquito bites, especially on her hands, despite use of repellents and bed nets.

Physical examination showed a body temperature of 38°C, throat enanthema, generalized macular exanthema, and slightly swollen and tender interphalangeal joints of the hands and feet. Her medical history was unremarkable, and her partner was asymptomatic. Laboratory tests showed reference values for hemoglobin concentration; platelet count; and levels of liver enzymes, creatinine, and antinuclear and anti–citrulline peptide antibodies. C-reactive protein level was increased (24.2 mg/L; reference value <5 mg/L), and serum lactate dehydrogenase level was slightly increased (4.4 μkat/L; reference value <4.12 μkat/L). Leukopenia (2.4 G/L; reference value 4.0–10.0 G/L) was present, which intensified the next day (2.0 g/L). The leukocyte count returned to a reference value 8 days after disease onset and the patient fully recovered.

Malaria, dengue fever, and rickettsiosis were excluded by using several tests. Blood cultures obtained on day 2 after disease onset remained sterile, and a viral infection was suspected. Follow-up investigation on day 16 of illness showed an increased IgG titer (80) against chikungunya virus (by indirect immunofluorescence assay; reference value <1:20) (*6*) but no IgM titer. Additional tests for alphaviruses were then performed on the same sample, and indirect immunofluorescence assay showed an IgM titer of 2,560 and an IgG titer of 10,240 (reference value <20) (*6*) against MAYV. Results of serologic tests were negative for Venezuelan equine encephalitis virus, Eastern equine encephalitis virus, and Oropouche virus. IgM (80) and IgG (160) titers for antibodies against Ross River virus were low.

An acute MAYV infection was strongly suspected and a stored serum sample from day 2 underwent generic reverse transcription PCR (RT-PCR) for alphaviruses with primers VIR2052F (5′-TGGCGCTATGATGAAATCTGGAATGTT-3′) and VIR2052R (5′-TACGATGTTGTCGTCGCCGATGAA-3′) (*8*) and quantitative MAYV real-time RT-PCR (in-house) with primers MayaroF (5′-CCTTCACACAGATCAGAC-3′), MayaroR (5′-GCCTGGAAGTACAAAGAA-3′), probe labeled with 6-carboxyfluorescein (FAM) and black hole quencher 1 (BHQ-1) MayaroP (5′-FAM-CATAGACATCCT-GATAGACTGCCACC-BHQ1 3′) by using the AgPath-ID One-Step RT-PCR Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The generic RT-PCR for alphaviruses showed a positive result, and direct sequencing of the amplicon showed a MAYV-specific sequence. The serum sample had an MAYV viral load of 1.24×10^7 copies/mL when in vitro–transcribed RNA from a reference plasmid was used as a quantification standard.

Attempts to isolate MAYV in cell culture were not successful. Therefore, the serum sample was used to obtain the complete MAYV genome sequence by using primers designed from multiple alignments of the MAYV genomes obtained from databases. (Primer sequences used are available on request.) The complete MAYV genome (strain BNI-1, KJ013266) was amplified from the serum sample, and phylogenetic analysis of a 2-kb genomic fragment showed that strain BNI-1 belonged to genotype D (*9*) and is closely related to strains circulating in Brazil (Figure, http://wwwnc.cdc.gov/EID/article/20/7/14-0043-F1.htm).

In 2 clinic-based syndromic surveillance studies in South America, 0.8%–3% of febrile episodes were caused by MAYV infection (*2,10*). In travelers, MAYV infections were acquired in tropical rainforest or wildlife conservation areas (*7*) and were sometimes associated with insect-hunting activities (*5*). Successful complete genome amplification of MAYV strain BNI-1 from a clinical sample might help identify regions in the MAYV genome that undergo rapid mutations caused by the isolation process in cell culture and improve phylogenetic and functional genome analysis. Moreover, the viral load in our patient was high enough for efficient transmission of MAYV to a susceptible mosquito vector (S. Becker, pers. comm.). Thus, in disease-endemic regions, patients with an acute MAYV infection should be protected from mosquito bites during the first week of disease to prevent spread of the virus.

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This letter is dedicated to the late Ursula Herrmann (1927–2014), for making this study possible.

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Diphtheria-like Disease Caused by Toxigenic *Corynebacterium ulcerans* Strain

To the Editor: Toxigenic *Corynebacterium ulcerans* is an increasingly reported cause of diphtheria in the United Kingdom and is often associated with a zoonotic origin (*1*,*2*). Here, we report a case of diphtheria caused by toxigenic *C. ulcerans* in a woman, 51 years of age, from Scotland, UK, who was admitted to a hospital in August 2013 with a swollen, sore throat and a gray-white membrane over the pharyngeal surface. The patient had returned from a 2-week family holiday in the state of Florida, United States, before the admission and also reported recent treatment of a pet dog for pharyngitis. The patient was believed to have been vaccinated against diphtheria during childhood. She was immediately admitted to an isolation ward and treated with a combination of clindamycin, penicillin, and metronidazole.

Microscopic examination of the throat biofilm (collected by using a swab) showed gram-positive bacilli; swab samples from the exudative membrane and throat produced small, black colonies indicative of *Corynebacterium* spp. on Hoyle medium. Further efforts to identify the strain by using VITEK MS and VITEK2 ANC card systems (bioMérieux, Marcy l'Etoile, France) to evaluate the swab samples suggested that the infection was caused by either *C. ulcerans* or *C. pseudotuberculosis* (50% CI). The isolate detected from this process was sent to the *Streptococcus* and Diphtheria Reference Unit, Public Health England, Colindale, UK, and was confirmed to be a toxigenic *C. ulcerans* strain that we designated RAH1. Throat swab samples were collected from family members of the patient and were negative for *C. ulcerans*. The family dog was not tested for presence of the organism, although it is known that *C. ulcerans* infections are often of a zoonotic nature (*1*,*2*). After treatment, the patient made a full recovery.

Toxigenic *C. ulcerans* can produce both diphtheria-like and Shiga-like toxins (*3*); to identify the genetic basis of toxin production and other potential virulence factors in this strain, a whole genome sequencing approach was applied to the isolate. The genome was sequenced by using an Ion PGM System (Thermo Fischer Scientific, Loughborough, Leicestershire, UK) and resulting reads (2,965,044 reads, ≈90× coverage. Data are available on GenBank SRA: high-throughput DNA and RNA sequence read archive (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=search_obj, accession no.: SRR1145126) and were mapped onto the published genome sequences of a Shiga-like toxin–producing clinical isolate 809, asymptomatic canine strain BR-AD22 (*3*), and diphtheria-like toxin–producing strain 0102 (*4*). Most of the previously identified virulence genes (*3*,*4*) were present in the patient isolate (Table). The *tox* gene, encoding diphtheria toxin, was present, which verified the diphtheria-like disease in the patient. The *rbp* gene, responsible for Shiga toxin–like ribosome-binding protein, was absent. However, strain RAH1 also possessed the venom serine protease gene (*vsp2*), which, in *C. ulcerans* strain 809, has been implicated in the increased virulence in humans. The *tox* gene was present in a prophage that showed similarities to ΦCULC809I (*3*) and ΦCULC0102-I (*4*). Genome-based phylogenetic analysis of the RAH1 strain (ClonalFrame analysis [*5*]) and strains 809, BR-AD22, and 0102 indicates a much wider phylogenetic diversity of *C. ulcerans* strains than previously appreciated (data not shown).

This case raises the issue of waning vaccine protection in older patients and suggests that toxin-mediated corynebacterial disease remains a threat to public health. The declining costs of next-generation sequencing and availability

Table. Virulence genes associated with *Corynebacterium ulcerans* present in strain RAH1 isolated from patient with diphtheria-like disease, 2013, United Kingdom*

Gene	Strains	Strain RAH1	Potential function
tox	0102	P	Diphtheria-like toxin
rbp	809	A	Shiga toxin–like ribosome binding protein
cpp	809, BR-AD22, 0102	P	Corynebacterial protease CP40, protective antigen against caseous lymphadenitis
pld	809, BR-AD22, 0102	P	Toxic phospholipase D
spaF	809, BR-AD22, 0102	P	Surface-anchored protein, pilus tip protein
spaE	809, BR-AD22, 0102	P	Surface-anchored protein, minor pilin subunit
spaD	809, BR-AD22, 0102	P	Surface-anchored protein, major pilin subunit
spaC	809, BR-AD22, 0102	P†	Surface-anchored protein, pilus tip protein
spaB	809, BR-AD22, 0102	P	Surface-anchored protein, minor pilin subunit
rpfI	809, BR-AD22, 0102	P	Resuscitation-promoting factor interacting protein
cwlH	809, BR-AD22, 0102	P	Cell wall–associated hydrolase
nanH	809, BR-AD22, 0102	P	Neuraminidase, glycosyl hydrolases
vsp1	809, BR-AD22	P	Venom serine protease
vsp2	809	P	Venom serine protease
tspA	809, BR-AD22	P	Trypsin-like serine protease

*P, present; A, absent.
†≈700 bp deletion.

of easy-to-handle bioinformatics tools emphasize the suitability of deep-sequencing technology for rapid diagnostics and for the development of high-resolution genotyping. It is time for the wider introduction of this technology into public health investigations.

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Death of Woman with Peripartum Influenza B Virus Infection and Necrotizing Pneumonia

To the Editor: Pregnant women are at increased risk for severe influenza-related complications (*1*). Bacterial pneumonia with Panton-Valentine leukocidin-producing (PVL) *Staphylococcus aureus* is infrequently described in the literature as occurring concurrently with influenza B virus infection (*2–4*). Additionally, only 2 occurrences of peripartum PVL-methicillin-resistant *S. aureus* (MRSA) pneumonia have been described (*5*,*6*). We report a case of influenza B virus and PVL-MRSA coinfection during pregnancy.

In December 2012, a previously healthy pregnant woman, 38 years of age, at 37 weeks' gestation and in active labor, sought treatment in a New York hospital reporting 2 days of fever, productive cough, shortness of breath, and pleuritic chest pain. Household contacts included children with influenza-like illness. The patient had declined influenza vaccination while receiving prenatal care. On arrival, examination showed that her temporal temperature was 101.6°F, blood pressure was 122/71 mm Hg, pulse was 121 beats per minute, respiratory rate was 40 breaths per minute, and oxygen saturation was 89% on room air; bilateral inspiratory crackles were heard on lung auscultation. Rapid influenza screening of a nasopharyngeal swab sample by using ELISA was negative for influenza A and B viruses. Culture of the patient's nares was positive for MRSA colonization. Laboratory evaluation showed leukopenia of 1500/mL, and although imaging was limited by the patient's lead apron, a chest radiograph demonstrated bibasilar opacities (Figure, panel A).

The differential diagnosis for this patient included influenza pneumonia, community-acquired pneumonia, and MRSA pneumonia; treatment with oseltamivir, ceftriaxone, vancomycin, and azithromycin was started. Because of impending respiratory failure, she was admitted to the Medical Intensive Care Unit where mechanical ventilation was initiated and she underwent a spontaneous vaginal delivery of a live male infant. The patient's condition deteriorated and progressed to severe acute respiratory distress syndrome with multiple organ failure and required substantial inotropic support. Subsequent laboratory studies showed the following results: leukocyte count 400/mL, lactate 4.2 mmol/L, pH 7.16, $PaCO_2$ 36 mm Hg, PaO_2 68 mm Hg, HCO_3 12 mmol/L, and oxygen saturation of 87% at 1.0 FiO_2. Repeat imaging demonstrated diffuse infiltrates in all lung fields (Figure, panel B). Because the patient responded poorly to treatment, vancomycin was discontinued and linezolid was started.

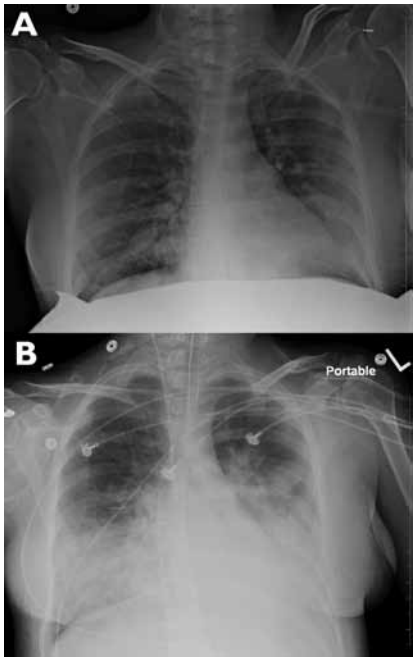

Figure. Course of influenza B virus infection and necrotizing pneumonia in peripartum woman, 2012, New York, USA. A) Chest radiograph at time of admission. B) Chest radiograph 1 day later, demonstrating progression of pneumonia.

Despite lung recruitment maneuvers and inhalation of nitric oxide, the patient remained hypoxemic. Extracorporeal membrane oxygenation was initiated and the patient was transferred to another institution.

After transfer, culture of 1 peripheral blood sample obtained at admission identified MRSA, and viral culture of the patient's nasal swab sample isolated influenza B virus. Genetic testing of the MRSA isolate identified a PVL-producing USA300 *spa*1 clone carrying staphylococcal cassette chromosome *mec* type IV. The patient died 2 weeks later from overwhelming sepsis. The neonatal course was notable for a birth weight of the infant of 2,825 g and Apgar scores of 5 and 8 at 1 and 5 minutes, respectively. He was intubated and transferred to the Neonatal Intensive Care Unit with an arterial cord blood pH of 6.78 and base deficit of 16 mmol/L. Nasal swab culture isolated methicillin-sensitive *S. aureus*. Viral culture of endotracheal aspirate was negative for influenza A and B viruses. Blood cultures were sterile. He received vancomycin for 1 week and was discharged home to the family on day 8 of life.

This case emphasizes the potential lethality of respiratory complications related to seasonal influenza. Colonization of the patient's nares with MRSA, possibly PVL-producing, may have predisposed her to a bacterial co-infection, consequentially increasing her risk for death from influenza (*1*). *S. aureus* clones USA300 and USA400 are emerging causes of community-acquired pneumonia in healthy adults and are leading to a rise in co-infections with influenza and MRSA. These 2 infections have been shown to act synergistically in animal models to induce a rapidly progressive necrotizing pneumonia associated with severe leukopenia (*7*). This is unlike classic secondary bacterial pneumonia, which typically occurs in a biphasic course with influenza (*2*).

Although methicillin susceptibility does not influence the mortality rate of PVL-*S. aureus* pneumonia (*8*), antibiotic drugs should be administered early and selection should reflect local resistance patterns. When making the diagnosis, physicians should recognize that the sensitivity of rapid influenza diagnostic tests is low and should not be relied on when a high level of clinical suspicion exists (*1*). Despite trivalent vaccine correspondence with circulating influenza B virus in 5 of 10 influenza seasons during 2001–2011 (*9*), vaccination against seasonal influenza is still the most effective way to prevent this potentially fatal condition. Availability of a quadrivalent influenza vaccination, introduced for the 2013–14 influenza season, should improve future incidence of influenza B virus infection. Because PVL-MRSA colonization is becoming more prevalent (*10*), necrotizing pneumonia must be considered in critically ill patients during influenza season.

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MERS-Related Betacoronavirus in *Vespertilio superans* Bats, China

To the Editor: Middle East respiratory syndrome coronavirus (MERS-CoV), a novel lineage C betacoronavirus, was first described in September 2012, and by April 16, 2014, the virus had caused 238 infections and 92 deaths in humans worldwide (*1*). Antibodies against MERS-CoV in dromedary camels were recently reported (*2*), as was the full genome of MERS-CoV from dromedary camels (*3*). Finding the natural reservoir of MERS-CoV is fundamental to our ability to control transmission of this virus to humans (*4*).

We report a novel lineage C betacoronavirus identified from *Vespertilio superans* bats in China. The full-length genome of this betacoronavirus showed close genetic relationship with MERS-CoV. Together with other evidence of MERS-CoV–related viruses in bats (*5–8*), our findings suggest that bats might be the natural reservoirs of MERS-related CoVs.

In June 2013, we collected anal swab samples from 32 *V. superans* bats from southwestern China. A small proportion of each sample was pooled (without barcoding) and processed by using virus particle–protected nucleic acid purification and sequence-independent PCR for next-generation sequencing analysis with the Illumina (Solexa) Genome Analyzer II (Illumina, San Diego, CA, USA). Redundant reads were filtered, as described (*9*), from the raw sequencing reads generated by the genome analyzer and then aligned with the nonredundant protein database of the National Center for Biotechnology Information (ftp://ftp.ncbi.nlm.nih.gov/blast/db/) by using BLAST (http://blast.ncbi.nlm.nih.gov). The taxonomy of these aligned reads was parsed by using MEGAN 4 (http://ab.inf.uni-tuebingen.de/software/megan/).

On the basis of the BLAST results, 8,751,354 sequence reads 81 nt in length were aligned with the protein sequences of the nonredundant protein database: 72,084 of the reads were uniquely matched with virus proteins. Of these 72,084 reads, 32,365 were assigned to the family *Coronaviridae*, primarily to lineage C of the genus *Betacoronavirus*, and found to share 60%–97% aa identity with MERS-CoV.

The MERS-CoV–related reads were extracted and assembled by using SeqMan software from the Lasergene 7.1.0 program (DNASTAR, Madison, WI, USA), resulting in a draft CoV genome. Reverse transcription PCR selective for the partial RNA-dependent RNA polymerase (RdRp) gene of this novel lineage C betacoronavirus suggested that 5 of the 32 samples (≈16%) were positive for the novel betacoronavirus, and the PCR amplicons shared >98% nt identity with each other. Using a set of overlapped nested PCRs and the rapid amplification of cDNA ends method, we determined the full-length genome of 1 strain of this *V. superans* bat–derived betacoronavirus (referred to as BtVs-BetaCoV/SC2013, GenBank accession no. KJ473821).

The betacoronavirus strain had a genome length of 30,413 nt, excluding the 3′poly (A) tails, and a G+C content of 43.1%. Pairwise genome sequence alignment, conducted by the EMBOSS Needle software (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) with default parameters, suggested that the genome sequence of BtVs-BetaCoV/SC2013 showed 75.7% nt identity with that of human MERS-CoV (hCoV-MERS); this shared identity is higher than that for other lineage C betacoronaviruses (from bats and hedgehogs) with full genomes available. hCoV-MERS showed 69.9% nt identity with bat CoV (BtCoV) HKU4-1, 70.1%

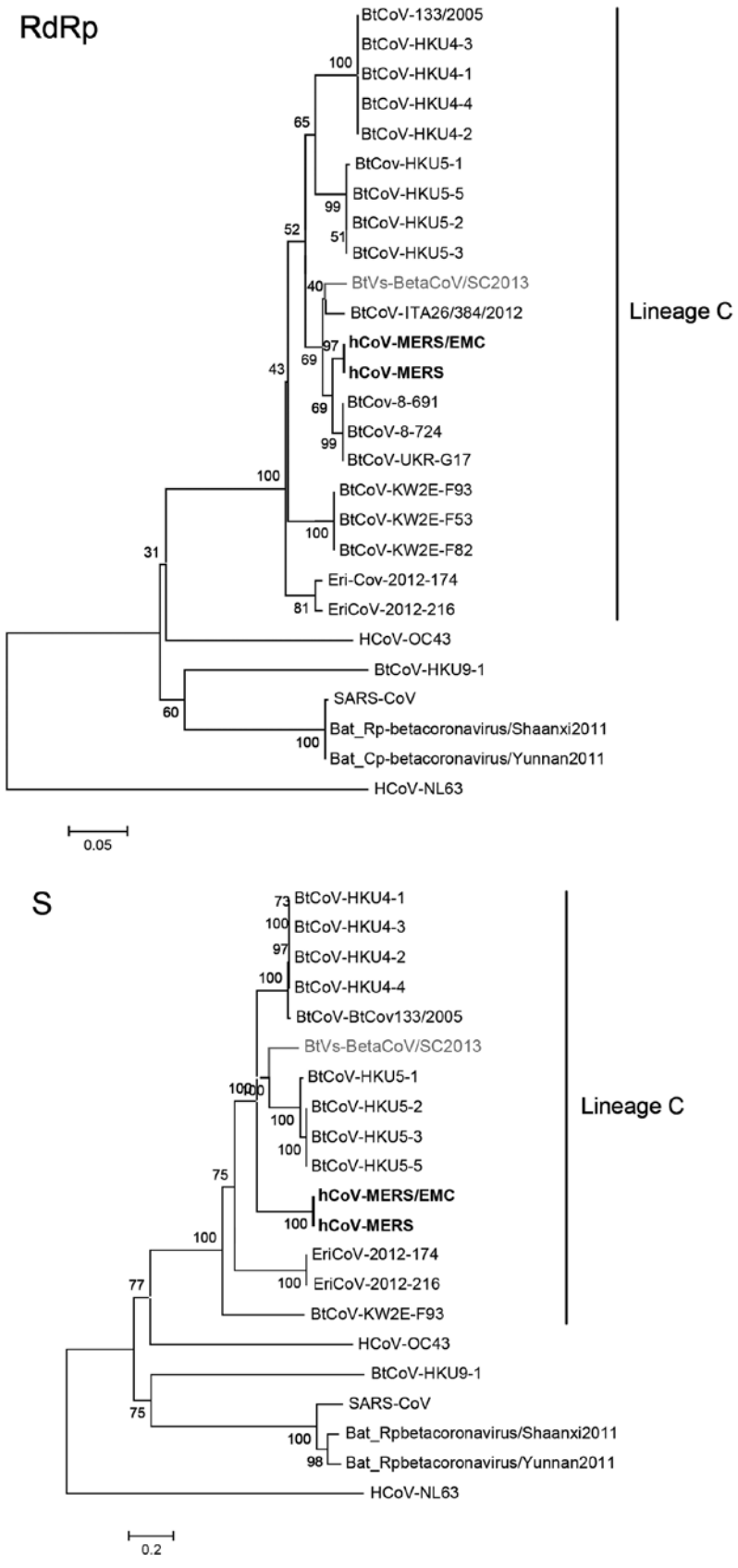

Figure. Phylogenetic trees based on the deduced amino acid sequences of the partial RNA-dependent RNA polymerase (RdRp; an 816-nt sequence fragment corresponding to positions 14817–15632 in human Middle East respiratory syndrome coronavirus [hCoV-MERS; KF192507]) and complete spike (S) protein. The novel virus is shown in gray, and hCoV-MERS is shown in bold. The following coronaviruses were used (GenBank accession numbers are shown in parentheses): severe acute respiratory syndrome coronavirus (SARS-CoV; NC004718), Bat Rp-coronavirus/Shaanxi2011(JX993987), Bat Cp-coronavirus/Yunnan2011(JX993988), Bat coronavirus HKU9-1 (BtCoV-HKU9-1; EF065513), BtCoV-133/2005(NC008315), BtCoV-HKU4-1 (EF065505), BtCoV-HKU4-2 (EF065506), BtCoV-HKU4-3 (EF065507), BtCoV-HKU4-4 (EF065508), BtCoV-HKU5-1 (EF065509), BtCoV-HKU5-2 (EF065510), BtCoV-HKU5-3 (EF065511), BtCoV-HKU5-5 (EF065512), BtCoV-ITA26/384/2012 (KF312399), BtCoV-KW2E-F82 (JX899382), BtCoV-KW2E-F93 (JX899383), BtCoV-KW2E-F53 (JX899384), BtCoV-8–724 (KC243390), BtCoV-8–691 (KC243391), BtCoV-UKR-G17 (KC243392), Human betacoronavirus 2c EMC/2012 (hCoV-MERS/EMC; JX869059), hCoV-OC43 (NC005147), hCoV-NL63 (NC005831), Betacoronavirus ErinaceusCoV/2012-174 (EriCoV-2012-174; KC545383), and EriCoV-2012-216 (KC545386). Scale bars indicate genetic distance estimated by using WAG+G model for the RdRp and WAG+G+F model for the S protein implemented in MEGA5 (http://www.megasoftware.net).

nt identity with BtCoV-HKU5-1, and 69.6% nt identity with hedgehog CoV EriCoV-2012–174.

Compared with those lineage C betacoronaviruses, which had an 816-bp partial RdRp sequence fragment available, BtVs-BetaCoV/SC2013 shared 96.7 % aa identity with hCoV-MERS. *Pipistrellus* BtCoVs found in Europe (BtCoV-8-724, BtCoV-8-691, BtCoV-UKR-G17) shared 98.2 % aa identity with hCoV-MERS, and *Eptesicus* BtCoV found in Italy (BtCoV-ITA26/384/2012) and other lineage C betacoronaviruses shared 96.3 % aa and <95% aa identity, respectively, with hCoV-MERS.

To clarify the evolutionary relationship between BtVs-BetaCoV/SC2013 and other lineage C betacoronaviruses, we performed phylogenetic analyses based on the deduced RdRp and the spike, envelope, membrane, and nucleocapsid proteins by using MEGA5 (http://www.megasoftware.net) (Figure; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/7/14-0318-Techapp1.pdf). For RdRp and the envelope, membrane, and nucleocapsid proteins, BtVs-BetaCoV/SC2013 always clustered with hCoV-MERS with short branch lengths, reflecting their high sequence similarities.

In the spike protein phylogenetic tree, BtVs-BetaCoV/SC2013 clustered with a clade defined by BtCoV-HKU5, with which it shares 74.8% aa identity. The spike proteins of hCoV-MERS form a sister clade of the clade defined by HKU5 BtCoVs and BtVs-betaCoV/SC2013, and the spike proteins share 69.0% aa identity with BtVs-betaCoV/SC2013. Spike proteins of BtVs-BetaCoV/SC2013, HKU5 BtCoVs, HKU4 BtCoVs, and hCoV-MERS, rather than EriCoV-2012-174, EriCoV-2012-216, and BtCoV-KW2E-F93, form a super clade. Spike protein is the critical factor for receptor recognition, binding, and cellular entry of CoVs in different host species (*10*), which may explain why the spike proteins in our study were relatively conserved within the same host species.

We identified a novel lineage C betacoronavirus from a *V. superans* bat and determined its full-length genome sequence. This novel betacoronavirus represents one of the most MERS-like CoVs that have been identified in bats as of the end of March 2014. The full-length genome sequence of the novel virus showed a closer genetic relationship with hCoV-MERS and camel MERS-CoV than with any other fully sequenced lineage C betacoronaviruses previously identified in bats or hedgehogs. Further studies of CoVs from more bat species worldwide may, therefore, help provide additional clues to the origins of pathogenic hCoV-MERS.

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Stability of Middle East Respiratory Syndrome Coronavirus in Milk

To the Editor: Middle East respiratory syndrome coronavirus (MERS-CoV) was first diagnosed in humans in 2012. Human-to-human transmission of MERS-CoV has been limited, and the transmission route is still unclear. On the basis of epidemiologic studies, involvement of an animal host has been suggested (*1*). Dromedary camels have been identified as a possible intermediate host on the basis of MERS-CoV antibodies and detection of MERS-CoV viral RNA in respiratory swab samples (*1*–*3*). Furthermore, MERS-CoV genome sequences obtained from dromedary camels clustered with MERS-CoV sequences obtained from humans linked to the same farm (*2*). Nonetheless, most persons with MERS-CoV did not report any direct contact with dromedary camels; therefore, how MERS-CoV zoonotic transmission occurs is unclear. MERS-CoV replicates in cell lines originating from a wide variety of different hosts, which suggests the potential for a broader reservoir species range then currently recognized (*4*). However, unlike in dromedary camels, no serologic evidence pointing toward MERS-CoV infection has been found in goats, sheep, and cows (*1*).

Contamination of dairy products has been associated with transmission of bacteria and viruses. Shedding of infectious tick-borne encephalitis virus in milk was detected after experimental infection of goats, and the consumption of raw milk has been associated with tick-borne encephalitis virus clusters (*5*). Similarly, cattle can be infected with foot-and-mouth disease through consumption of raw contaminated milk (*6*).

Here, we investigate the stability of MERS-CoV in dromedary camel milk, goat milk, and cow milk at different temperatures. MERS-CoV strain Jordan-N3/2012 was diluted in unpasteurized milk or nonsupplemented Dulbecco modified Eagle medium (DMEM, GIBCO, Grand Island, NY, USA) to a final median 50% tissue culture infectious dose of $10^{5.5}$/mL. We placed 1-mL aliquots in screw-cap tubes (Sarstedt, Nümbrecht, Germany) at either 4°C or 22°C and stored them at –80°C at 0, 8, 24, 48, and 72 hours post dilution (hpd) in quintuplicate. Infectious virus titers were determined by endpoint titration on Vero E6 cells in triplicate (*7*). When MERS-CoV was stored at 4°C, the geometric mean of infectious virus titers decreased over 72 hours; we found they decreased 37% (95% CI 0%–62%) in dromedary camel milk, 64% (95% CI 26%–82%) in goat milk, 56% (95% CI 0%–92%) in cow milk, and 80% (95% CI 70%–86%) in DMEM. At 0–72 hpd, virus titers decreased significantly only in goat milk ($p = 0.0139$, 1-tailed paired t test) and DMEM ($p = 0.0311$) but not in dromedary camel milk ($p = 0.1414$) or cow milk ($p = 0.2895$). Samples stored at 22°C showed a greater loss of infectivity than did samples stored at 4°C. Infectious virus titers decreased to <15% when samples were stored at 22°C for 48 hours (loss of 88% [95% CI 67%–96%] for dromedary camel milk, 99% [95% CI 98.6%–99.8%] for goat milk, 98% [95% CI 95%–99%] for cow milk, and 97% [95% CI 87%–99%] for DMEM). This decrease was significant by student 1-tailed paired t test analysis comparing $t = 0$ and $t = 48$ hpd ($p<0.05$). However, despite the reduction in virus titer, viable virus could still be recovered after 48 hours. Pasteurization of raw milk can prevent foodborne disease outbreaks caused by a variety of pathogens. We heat-treated dromedary camel, cow, goat milk, and DMEM samples for 30 min at 63°C, after which no infectious virus could be recovered (Figure).

CoV survival has been studied in phosphate-buffered saline and minimal essential media and, like MERS-CoV,

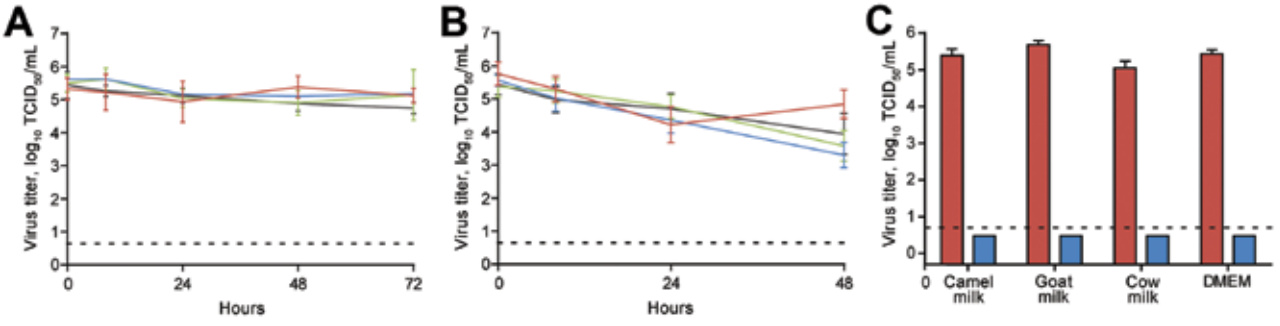

Figure. Viability of MERS-CoV in milk. MERS-CoV strain Jordan-N3/2012 was diluted in milk from dromedary camels, goats, or cows or in DMEM to a final $TCID_{50}$ of $10^{5.5}$/mL and stored at either 4°C (A) or 22°C (B). MERS-CoV titer was determined at 0, 8, 24, 48, and 72 hours postdilution in quintuplicate. C) Milk containing MERS-CoV was pasteurized by heating 1-mL aliquots of diluted virus at 63°C for 30 min in triplicate. Gray indicates unpasteurized; Black indicates pasteurized. Infectious virus titers were determined by endpoint titration on Vero E6 cells in triplicate. Dotted line depicts the detection limit of the assay. MERS-CoV, Middle East respiratory syndrome coronavirus; $TCID_{50}$, 50% tissue culture infective dose; DMEM, Dulbecco modified Eagle medium. Error bars indicate geometric mean titers with 95% CIs.

human coronaviruses–229E and -OC43 and severe acute respiratory syndrome–CoV were able to survive in suspension at room temperature for several days (*8*,*9*). Moreover, severe acute respiratory syndrome–CoV was completely inactivated after heat treatment at 60°C for 30 min (*9*).

Human-to-human transmission of MERS-CoV is inefficient, and the transmission route has not yet been revealed. The predominant detection of MERS-CoV by quantitative PCR in nasal swab samples suggests the virus causes upper respiratory tract infection in dromedary camels (*3*). Which route or combination of routes is responsible for its zoonotic transmission is unclear, and foodborne transmission should not be excluded. Residents of the Arabian Peninsula commonly drink unpasteurized milk. Our results show that MERS-CoV, when introduced into milk, can survive for prolonged periods. Further study is needed to determine whether MERS-CoV is excreted into the milk of infected dromedary camels and, if so, whether handling or consuming contaminated milk is associated with MERS-CoV infection. Recently Nipah virus was transmitted experimentally by drinking , which resulted in respiratory tract rather than intestinal tract infection (*10*). A similar transmission mechanism for MERS-CoV could result in contamination of the oral cavity and subsequent infection of the lower respiratory tract. Pasteurization of milk can prevent foodborne transmission (*9*). We showed that heat treatment decreased infectious MERS-CoV below the detection limit of our titration assay, and this might function as a relatively easy and cost-effective measure to prevent transmission.

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Carbapenemase-producing Organism in Food, 2014

To the Editor: Carbapenem antimicrobial drugs are the line of defense against multidrug-resistant gram-negative bacterial infections. The global emergence of carbapenemase-producing organisms is a public health emergency because these enzymes confer resistance to nearly all β-lactam drugs and are often associated with multidrug or pandrug resistance (*1*). Alarmingly, reports of carbapenemase-producing organisms from environmental and animal sources, including food animals, are increasing (*1*). Recently, clinical isolates of *Salmonella enterica* serotype Kentucky that produce VIM-2 and OXA-48 were reportedly isolated from patients in France with a travel history to Africa and the Middle East, suggesting foodborne transmission of carbapenemase producers (*2*).

Table. Antimicrobial drug susceptibility of a VIM-2 producing *Pseudomonas fluorescens*–like organism isolated from food (squid), Saskatoon, Canada, January 2014

Antimicrobial drug	MIC
Ampicilin	>32
Amoxicillin + clavulanic acid	>32
Cefoxitin	>32
Ceftiofur	>8
Ceftriaxone	>64
Azithromycin	16
Chloramphenicol	16
Tetracycline	≤4
Naladixic acid	16
Ciprofloxacin	0.06
Gentamicin	≤0.25
Kanamycin	16
Streptomycin	≤32
Sulfisoxazole	32
Trimethoprim + sulfamethoxazole	0.5
Ertapenem*	>32
Tigecycline*	0.125
Colistin*	3

*MICs determined by Etest; all others were determined by broth microdilution.

To the best of our knowledge, before this report no foodborne carbapenemase-producing organisms had been identified in Canada and the United States, although the scope of antimicrobial drug resistance surveillance programs is limited to major agricultural products (poultry, beef, and pork) (*3*,*4*). In our modern, ethnically diverse societies, niche-market meat products, including imported foods, are becoming increasingly common. Worldwide dissemination of the *Klebsiella pneumoniae*, VIM, OXA, and New Delhi metallo-b-lactamase type carbapenemases among humans has been facilitated by intercontinental passenger travel, but the role of the global food trade in this dissemination has not been investigated (*5*,*6*). We describe a carbapenemase-producing organism isolated from a squid purchased from the seafood section of a food store.

Among other items, the squid was purchased from a Chinese grocery store in Saskatoon, Canada, in January 2014 as part of a drug-resistance surveillance pilot study. Although no country-of-origin labeling was available for inspection, the store owner reported that, according to the distributor, this squid originated in South Korea. An organism with 95.5% sequence identity to *Pseudomonas fluorescens* was isolated on Mueller-Hinton agar with 2 μg/mL meropenem and identified by partial sequencing of the *cpn*60 gene (GenBank accession no. KJ606641). Although the organism was not extensively resistant, it was resistant to all β-lactam drugs tested including ertapenem (Table). PCR amplification and sequencing confirmed that this organism contained VIM-2 carbapenemase (GenBank accession no. KJ625238).

The presence of carbapenemase-producing organisms in the food supply is alarming. Although this organism may not be a pathogen, its contribution to the resistome and the potential for lateral gene transfer to clinically relevant bacteria is certainly a cause for concern. This finding indicates that the risk for exposure to carbapenemases extends beyond persons with particular travel histories, previous antimicrobial drug use, or hospitalization and into the general public. There is an urgent need for expanded resistance surveillance for carbapenemase-producing organisms and their resistance plasmids in food products that are not captured under current programs.

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Chlamydial Infection: A Clinical and Public Health Perspective

C.M. Black, editor

S. Karger AG , Basel, Switzerland; 2013

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Pages: 162; Price: US $138.00 (hard cover), $166.00 (online)

Chlamydial Infection: A Clinical and Public Health Perspective, a relatively short textbook, focuses on *Chlamydia trachomatis*, the most common bacterial agent of sexually transmitted infections. In the introduction, Black provides some definitions, a historical background, and a table summarizing the main clinical syndromes associated with urogenital serovars. Regrettably, this introduction does not mention the increasingly recognized role of urogenital chlamydial infections in miscarriage and infections caused by other members of the Chlamydiales order, such as *C. pneumoniae* and *Waddlia chondrophila*. Such mention would be especially useful because understanding the biology and evolution of *C. trachomatis* also relies partially on research performed on other chlamydiae, as is discussed nicely in the genomics chapter by T.E. Putman and D.D. Rockey.

A basic science chapter, "*Chlamydia trachomatis* Pathogenicity and Disease," highlights the importance of *C. trachomatis* surface protein and host genetics in the immunopathogenesis of chlamydial infection. This chapter by Deborah Dean is enjoyable to read, highly informative, and represents an extended review with 292 references. The remaining 8 chapters emphasize epidemiology, clinical presentation, antimicrobial drug susceptibility, antibiotherapy, and vaccines. These chapters, written by field experts, provide precise practical recommendations about screening and diagnosis of, and treatment approaches to, urogenital chlamydial infection and about caring for sexual minority groups, such as men who have sex with men.

Overall, this textbook is an excellent reference for epidemiologists working on sexually transmitted infections, for clinicians interested in that field, and for doctoral students starting their research on *C. trachomatis*. However, given its relative conciseness, Black's textbook is unlikely to meet the expectations of basic researchers working on the evolution, cell biology, and/or molecular microbiology of *Chlamydia*.

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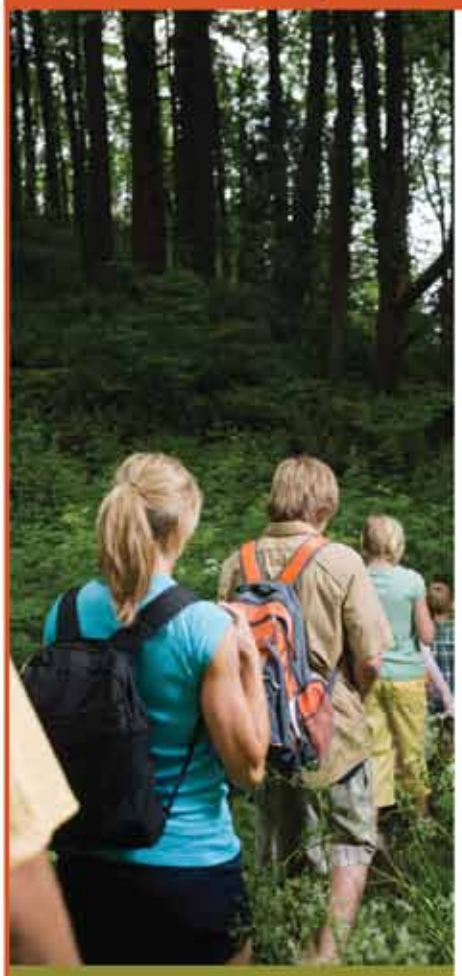
Correction: Vol. 19, No. 3

In the article Multidrug-Resistant Tuberculosis, Somalia, 2010–2011 (I. Sindani et al.), author Amal Bassili's affiliation with the Medical Research Institute, Alexandria University, Alexandria, Egypt, was omitted. The article has been corrected online (http://wwwnc.cdc.gov/eid/article/19/03/12-1287_article.htm).

Ticks and Lyme Disease



For more information about Lyme disease, visit http://www.cdc.gov/Lyme

How to prevent tick bites when hiking and camping

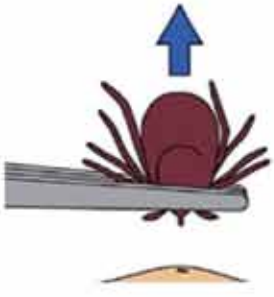
Ticks can spread disease, including Lyme disease. Protect yourself:

- Use insect repellent that contains 20 - 30% DEET.
- Wear clothing that has been treated with permethrin.
- Take a shower as soon as you can after coming indoors.
- Look for ticks on your body. Ticks can hide under the armpits, behind the knees, in the hair, and in the groin.
- Put your clothes in the dryer on high heat for 60 minutes to kill any remaining ticks.

How to remove a tick

1. If a tick is attached to you, use fine-tipped tweezers to grasp the tick at the surface of your skin.
2. Pull the tick straight up and out. Don't twist or jerk the tick—this can cause the mouth parts to break off and stay in the skin. If this happens, remove the mouth parts with tweezers if you can. If not, leave them alone and let your skin heal.
3. Clean the bite and your hands with rubbing alcohol, an iodine scrub, or soap and water.
4. You may get a small bump or redness that goes away in 1-2 days, like a mosquito bite. This is not a sign that you have Lyme disease.

Note: *Do not put hot matches, nail polish, or petroleum jelly on the tick to try to make it pull away from your skin.*



If you remove a tick quickly (within 24 hours) you can greatly reduce your chances of getting Lyme disease.

National Center for Emerging and Zoonotic Infectious Diseases
Division of Vector Borne Diseases | Bacterial Diseases Branch

CS232422D

Eileen Pestorius (b. 1939) *The Alamo* (2014) (detail) Watercolor on paper (12.75 in x 24 in/32.385 cm x 60.96 cm) Courtesy of the artist

After the Resistance: The Alamo Today

Byron Breedlove and Murray L. Cohen

San Antonio de Valero is a former Roman Catholic mission and fortress where the Battle of the Alamo occurred from February 23 until March 6, 1836. Now known simply as the Alamo, this compound has been damaged, ransacked, and renovated many times before work on its present configuration as a museum in downtown San Antonio, Texas, USA, was completed in 1968.

Texas artist Eileen Pestorius used watercolors to capture the setting on a still, spring day. In her painting, trees and grass frame the mission; a flagstone pathway leads to the entrance, and thin clouds swirl overhead in a hazy blue sky. A Texas flag hangs limply on this calm day. The stonework looks weathered, even warm in the bright sunlight; a large wooden door flanked by pairs of columns invites exploration, as does an archway that juts from the right and seems to lead to a garden.

Although located in a bustling urban setting, the modern day Alamo seems a tranquil, restive place suited for relaxation or contemplation. It takes some effort to associate the Alamo that Pestorius has rendered with the garrison known as an iconic symbol of heroic fighting and resistance.

The story is well known. During the 1836 battle, a Mexican force numbering in the thousands and led by General Antonio Lopez de Santa Anna besieged the Alamo. The vastly outnumbered defenders—200 men, including the frontiersman Davy Crockett, commanded by Colonel James Bowie and Lieutenant Colonel William Travis—valiantly held the compound for 13 days before the Mexicans

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breached the mission, killing nearly everyone. On April 21, 1836, when the outnumbered Texan militia commanded by General Sam Houston defeated Santa Anna's troops at San Jacinto, the rallying cry was "Remember the Alamo!" Houston's surprise attack proved such a tactical advantage that he routed the larger force in just 18 minutes, effectively ending the Texas Revolution and leading to treaties that established the independent Republic of Texas.

Our often-used metaphor of humans waging "war" on infectious diseases and on the microbes that cause them invites comparison with the Battle of the Alamo. Through various antibiotics, we reduced the number of deaths caused by infections and, bolstered by successes, assumed we would win this war.

In reality, we live in a microbial world where we are the invaders. The Board on Global Health and Institute of Medicine noted that "On reflection, perhaps it would be wise to reconsider the frequently used metaphor of humans being 'at war with microbes.' It is absurd to believe that we could ever claim victory in a war against organisms that outnumber us by a factor of 10^{22}, that outweigh us by a factor of 10^{8}, that have existed for 1,000 times longer than our species, and that can undergo as many as 500,000 generations during 1 of our generations."

The Centers for Disease Control and Prevention estimates that in 2013, antibiotic resistance threats caused more that 2 million illnesses and 23,000 deaths in the United States and that in 2011, those threats were responsible for an estimated $20 million in excess health care costs, 8 million additional hospital days, and $35 million in societal costs. Many factors, including overuse and misuse of antibiotics, global climate change, human encroachment into more remote, less hospitable places, modern factory farming and food production practices, and rapid and accessible global mobility have heightened our vulnerability. Since the 1980s, nearly 40 new pathogens have been identified as human disease threats, and 12% of known human pathogens have been classified as either emerging or remerging.

The cycle of many of these emerging microbes can be seen with methicillin-resistant *Staphylococcus aureus* infections moving from being hospital associated to community acquired and now to a ubiquitous supply-chain associated. *Clostridium difficile* seems headed on the same path.

Successfully controlling drug-resistant microbes requires not just greater vigilance with our infection control tools, but it also requires developing and deploying creative and aggressive tactics. Changing our tactics against drug-resistance microbes involve keeping pathogens out of our supply chains to schools, hospitals, and workplaces, and keeping sick workers at home. New guidelines from the Society for Healthcare Epidemiology of America, for example, provide recommendations to reduce the role that health care personnel attire plays in the cross-transmission of pathogens.

The scenario of a postantibiotic era of infectious diseases that looks like the preantibiotic era that preceded penicillin and vaccines is an alarming scenario. Sir Alexander Fleming even warned about antibiotic resistance in his 1945 Nobel Prize speech. Now is the time to incorporate new strategies into our battle plans and prepare to fight against overwhelming odds.

About the Artist

Eileen Pestorius, MA, is a painter and art instructor who lives in Austin, Texas. She is docent emerita at Blanton Museum of Art at the University of Texas and signature member of the Texas Watercolor Society. She leads in-residence painting clinics in France and Italy. Ms. Pestorius is the past president of the Waterloo Watercolor Group and was an adjunct faculty member at Austin Community College. She earned her BA degree in English from Nazareth College and her MA degree in art education from the University of Texas at Austin.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Leptospirosis-Associated Hospitalizations, United States, 1998–2009

Global and Local Persistence of Influenza (H5N1) Virus

Rapid Whole-Genome Sequencing for Surveillance of *Salmonella enterica* Serovar Enteritidis

Human Exposure to Live Poultry and Psychological and Behavioral Responses to Influenza A(H7N9), China

Human Infections with *Borrelia miyamotoi*, Japan

Geographic Distribution of MERS Coronavirus among Dromedary Camels, Africa

Rapid Detection, Complete Genome Sequencing, and Phylogenetic Analysis of Porcine Deltacoronavirus

Novel Reassortant Influenza A(H5N8) Viruses in Domestic Ducks, Eastern China

Borrelia crocidurae Infection in Acutely Febrile Patients, Senegal

Severe Murine Typhus with Pulmonary System Involvement

Pulmonary Infection and Colonization with Nontuberculous Mycobacteria, Taiwan, 2000–2012

Levofloxacin-Resistant *Haemophilus influenzae*, Taiwan

Isolation of MERS Coronavirus from a Dromedary Camel, Qatar, 2014

Antibodies against MERS Coronavirus in Dromedary Camels, Kenya, 1992–2013

Detection of East Central South African Genotype of Chikungunya Virus in Myanmar, 2010

Cocirculation of Dengue and Chikungunya Viruses, Al Hodayda,Yemen, 2012

Severe Fever with Thrombocytopenia Syndrome Virus, Zhejiang Province, China, 2013

New Introductions of Enterovirus 71 Subgenogroup C4, France, 2012

Precursor of Avian Influenza A(H7N9) Viruses in a Child with Mild Manifestation, China, 2013

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

Upcoming Infectious Disease Activities

2014

July 27–August 1, 2014
IUMS 2014
International Union of Microbiological Societies
Montreal, Canada
http://www.montrealiums2014.org

September 5–9, 2014
ICAAC 2014
Interscience Conference on Antimicrobial Agents and Chemotherapy
Washington, DC
http://www.icaac.org

October 8–12, 2014
ID Week 2014
Philadelphia, PA
http://www.idweek.org/

October 31–November 3, 2014
IMED 2014
Vienna, Austria
http://imed.isid.org

November 2–6, 2014
ASTMH
American Society of Tropical Medicine and Hygiene
63rd Annual Meeting
New Orleans, LA, USA
http://www.astmh.org/Home.htm

November 15–19, 2014
APHA 142nd Annual Meeting & Expo
New Orleans, LA
http://www.apha.org/meetings/AnnualMeeting

November 30–December 4, 2014
ASLM2014 International Conference
Cape Town International Convention Centre, South Africa
http://www.aslm2014.org/

2015

March 8–11, 2015
ICEID
International Conference on Emerging Infectious Diseases
Atlanta, GA

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Lessons for Control of Heroin-Associated Anthrax in Europe from 2009–2010 Outbreak Case Studies, London, UK

CME Questions

1. Your patient is a 29-year-old male heroin user who injects heroin directly into a vein and who presents with a lesion near an injection site. According to the study by Abbara and colleagues, which of the following statements about clinical manifestations of heroin-associated anthrax in the 2009–2010 UK outbreak is correct?

A. Clinical manifestations of users who "skin pop" are similar to those of cutaneous anthrax
B. Clinical manifestations were restricted to localized abscesses or inflammatory lesions at the injection site
C. Features differentiating anthrax-associated soft tissue infection from that caused by other bacteria include the degree of surrounding edema, excessive bleeding at surgery, and no clear demarcation between affected and unaffected tissue
D. Coagulopathy and kidney failure were not reported

2. According to the study by Abbara and colleagues, which of the following statements about the transmission and epidemiology of heroin-associated anthrax in the 2009–2010 UK outbreak is correct?

A. Mode of transmission is from an infected user to others via droplet inhalation
B. After the 2009–2010 UK outbreak, no additional cases of heroin-associated anthrax were reported
C. Seroprevalence of anthrax in the heroin-injecting population is clearly determined
D. Antibiotics used for serious soft tissue infection cover anthrax, which may cause significant underrecognition of this new presentation of heroin-associated anthrax

3. According to the study by Abbara and colleagues, which of the following statements about lessons learned from the 2009–2010 UK outbreak of heroin-associated anthrax would most likely be correct?

A. The outbreak makes a strong case for ongoing vigilance, use of serologic testing in diagnosis, and serologic surveillance schemes to determine and monitor anthrax exposure rates among people who inject drugs
B. Safe and specific disposal of clinical waste is not relevant to this outbreak
C. Multidisciplinary management is not likely to improve outcome
D. Vigilance is not needed outside of Europe

Activity Evaluation

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

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Epidemiology of Influenza Virus Types and Subtypes in South Africa, 2009–2012

CME Questions

1. You are consulting for a hospital in South Africa regarding treatment of patients with severe acute respiratory illness (SARI). According to the surveillance study by Cohen and colleagues, which of the following statements about differences in characteristics of patients hospitalized with SARI by infection with different influenza types and subtypes is correct?

A. Patients with influenza A were more likely to be infected with HIV than patients with influenza B
B. Multivariate analysis showed significant differences between influenza A and B in co-infection with another virus, longer duration of hospitalization, and fever
C. Findings from this study make it easy to distinguish influenza A from influenza B clinically and to treat appropriately
D. Adjusted odds ratio for HIV infection was 1.4 for patients with influenza B vs those with influenza A

2. According to the surveillance study by Cohen and colleagues, which of the following statements about characteristics of patients hospitalized with SARI in South Africa by infection with different influenza types and subtypes during the first and second influenza A(H1N1)pdm09 waves is correct?

A. A lower proportion of patients infected during the first A(H1N1)pdm09 wave were 5 to 24 years old compared with patients in the second wave
B. During the first influenza A(H1N1)pdm09 wave, 19% of patients were 5 to 24 years old
C. The age distribution during the second wave did not have a bimodal distribution
D. HIV did not affect age distribution for the second wave

3. According to the surveillance study by Cohen and colleagues, which of the following statements about case fatality and severity rates among infections with different types and subtypes and between the first and second influenza A(H1N1)pdm09 waves would most likely be correct?

A. Influenza B had a significantly higher case fatality and severity rate than influenza A
B. The first influenza A(H1N1)pdm09 wave had a significantly higher case fatality and severity rate than the second wave
C. Case fatality rate was 2.8% for influenza A(H3N2), 1.5% for influenza A(H1N1)pdm09, and 3.9% for influenza B
D. Influenza B has a significantly higher rate of admission to intensive care, mechanical ventilation, oxygen requirement, and prolonged duration of hospitalization than influenza A

Activity Evaluation

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

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

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

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Instructions to Authors

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

Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

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

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

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

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

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

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.