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Foodborne Infections

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January 2013

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Still Life with Two Figures (1622)
Oil on canvas
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North Carolina Museum of Art, Raleigh,
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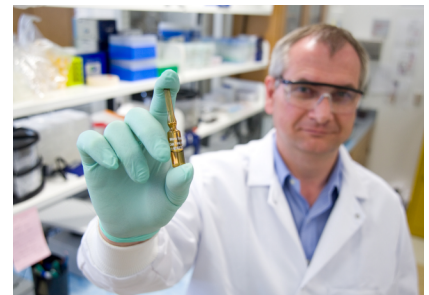
Listeriosis Outbreaks and Associated Food Vehicles, United States, 1998–2008

Emily J. Cartwright, Kelly A. Jackson, Shacara D. Johnson, Lewis M. Graves,
Benjamin J. Silk, and Barbara E. Mahon

Listeria monocytogenes, a bacterial foodborne pathogen, can cause meningitis, bacteremia, and complications during pregnancy. This report summarizes listeriosis outbreaks reported to the Foodborne Disease Outbreak Surveillance System of the Centers for Disease Control and Prevention during 1998–2008. The study period includes the advent of PulseNet (a national molecular subtyping network for outbreak detection) in 1998 and the *Listeria* Initiative (enhanced surveillance for outbreak investigation) in 2004. Twenty-four confirmed listeriosis outbreaks were reported during 1998–2008, resulting in 359 illnesses, 215 hospitalizations, and 38 deaths. Outbreaks earlier in the study period were generally larger and longer. Serotype 4b caused the largest number of outbreaks and outbreak-associated cases. Ready-to-eat meats caused more early outbreaks, and novel vehicles (i.e., sprouts, taco/nacho salad) were associated with outbreaks later in the study period. These changes may reflect the effect of PulseNet and the *Listeria* Initiative and regulatory initiatives designed to prevent contamination in ready-to-eat meat and poultry products.

Listeria monocytogenes is transmitted predominantly through contaminated food (*1*). Listeriosis includes a spectrum of clinical illnesses ranging from febrile gastroenteritis to potentially fatal bacteremia and meningitis in groups at higher risk for invasive disease, including older adults and persons with certain medical conditions (*2,3*). Although pregnant women infected with *L. monocytogenes* typically experience a mild influenza-like illness or an asymptomatic infection, pregnancy-associated listeriosis can result in fetal loss, preterm delivery, invasive neonatal infection, and infant death.

The Centers for Disease Control and Prevention (CDC) estimates that 1,662 invasive infections with *L. monocytogenes* occur annually in the United States, causing 1,520 hospitalizations and 266 related deaths (*4*). Population-based surveillance demonstrated a 24% decrease in the crude incidence of laboratory-confirmed listeriosis from 0.41 to 0.31 cases per 100,000 population during 1996–2003 (*5*). Since 2003, the incidence of listeriosis has remained stable, with rates ranging from 0.25 to 0.32 cases per 100,000 population during 2004–2009. The 6-year average rates of hospitalization and death were 0.26 hospital admissions and 0.05 deaths or fetal losses per 100,000, respectively (*6*). Millions of US dollars in health care expenditures and quality-adjusted life years are lost to invasive listeriosis annually (*7*).

Foodborne transmission of listeriosis was first recognized conclusively after an outbreak in Canada in 1981 that was associated with consumption of contaminated coleslaw (*1*). In the United States, the first recognized foodborne listeriosis outbreak occurred in 1983 and was associated with pasteurized milk (*8*). During 1983–1998, outbreaks of foodborne listeriosis associated with Mexican-style cheese (*9*) and shrimp (*10*) were subsequently documented; a single case was also attributed to turkey frankfurters (*11*).

PulseNet, the national molecular subtyping network for enteric bacterial disease surveillance, was established in 1998 (www.cdc.gov/pulsenet). *L. monocytogenes* isolates from patients are sent to state public health laboratories for standardized pulsed-field gel electrophoresis (PFGE); the PFGE patterns are then uploaded to a central database (PulseNet) for national comparisons (*12*). When ≥2 *L. monocytogenes* isolates with indistinguishable PFGE pattern combinations are uploaded within a 120-day period, this cluster is evaluated. An investigation is initiated if the upload rate for this pattern combination is greater than the historical background or if other epidemiologic indicators suggest a common source.

Invasive listeriosis has been a nationally notifiable disease in the United States since 2001. Although most listeriosis cases are sporadic (i.e., not associated with a recognized cluster of illness), the detection of a listeriosis outbreak is a critical opportunity to prevent additional ill-ness and death by removing a contaminated vehicle from the food supply. In addition, outbreak investigations often provide information about transmission of *L. monocytogenes* that can be used to improve food safety (*13*). However, epidemiologic investigations of listeriosis clusters are challenging because they are typically detected as a small number of geographically dispersed case-patients (some of whom may have died), and the incubation period can be lengthy, making patients' recall of food exposures difficult (*14*).

Following a 2003 Council of State and Territorial Epidemiologists position statement, the *Listeria* Initiative was launched in 2004 to address these concerns (www.cdc.gov/listeria/surveillance.html). The *Listeria* Initiative encourages state and local health department officials to routinely interview all patients with culture-confirmed listeriosis as soon as they are reported by using a standardized, extended questionnaire to collect food histories. Concurrently, clinical isolates are submitted to public health laboratories for PFGE subtyping and submission to PulseNet, and PFGE results are linked to epidemiologic information in the *Listeria* Initiative database. When a cluster is identified in PulseNet, *Listeria* Initiative data related to that cluster can be reviewed quickly to identify common food exposures. The *Listeria* Initiative also facilitates case–case studies by comparing exposures reported by cluster-associated cases with information from listeriosis cases that are not associated with the cluster. The effectiveness of the case–case approach has been illustrated repeatedly, for example, during the investigation of large, multistate outbreaks associated with delicatessen turkey meat and cantaloupe (*15,16*).

This report summarizes single-state and multistate listeriosis outbreaks reported to CDC during 1998–2008. We describe characteristics of the outbreaks and affected patients to summarize outbreak trends, *L. monocytogenes* serotype distribution, and implicated foods.

Outbreak Identification and Characterization

To identify all listeriosis outbreaks reported during 1998–2008 in the United States, we reviewed data from the CDC Foodborne Disease Outbreak Surveillance System (FDOSS). FDOSS is a national surveillance system through which state, local, tribal, and territorial health departments voluntarily submit to CDC reports of outbreaks by using a standardized form (CDC form 52.13) (*17*). In 1998, FDOSS surveillance activities were enhanced through the use of an electronic data collection form and other activities to increase reporting. For each outbreak, FDOSS captures information on etiology, food vehicle, outbreak size, duration, geographic location, setting, and selected outcomes (i.e., number of illnesses, hospitalizations, and deaths). Aggregated age group and sex data are also reported. A listeriosis outbreak was defined as ≥2 listeriosis cases linked to a

common source by a public health investigation. A listeriosis outbreak was considered confirmed if the same serotype of *L. monocytogenes* was isolated from ≥2 patients exposed to either epidemiologically implicated food or food from which the same serotype was isolated. Outbreaks were considered to be multistate if exposure to the implicated food occurred in >1 state.

Hospitalization (number of hospitalized cases/total cases) and case-fatality rates (CFRs) (number of deaths/total cases) were calculated for the study period. When >50% of demographic data were missing, remaining data were not analyzed. Outbreak duration was calculated as the number of days between the dates of illness onset of the first and the last reported cases. To define early (1998–2003) and late (2004–2008) study periods, 2004 was selected as a cutoff point because it coincides with the launch of the *Listeria* Initiative. Serotyping of outbreak-associated *L. monocytogenes* isolates was performed by CDC (*18*). Serotype information was matched to each outbreak reported in FDOSS. In addition, we conducted a systematic literature review of published reports of listeriosis outbreaks by using 5 electronic databases (PubMed, Embase, Web of Science, Toxnet, and CAB Direct) and medical subject headings "*Listeria monocytogenes*," "*Listeria* infections," "listeriosis," "disease outbreaks," and "foodborne diseases." When discrepancies were identified between published reports and FDOSS, the published data were used.

Outbreaks and Associated Food Vehicles

During 1998–2008, a total of 26 listeriosis outbreaks were reported to FDOSS; 24 were confirmed (Figure 1; Table 1). Eight (33%) of these outbreaks were described in the published literature (*14,15,19–24*). The 24 confirmed outbreaks resulted in 359 illnesses, 215 hospitalizations, and 38 deaths. The 11-year hospitalization rate was 60%, and the CFR was 11% (Table 2). Among 16 outbreaks with available data, the median duration was 42 days (range 1–389 days). Seven (29%) multistate outbreaks were reported, and 33 states reported cases in multistate outbreaks (Figure 2). Nine states reported single-state outbreaks. New York reported more listeriosis outbreaks than any other state (n = 6).

Information on the age distribution of ill persons was available for 17 (71%) of the 24 outbreaks. These 17 outbreaks included 238 patients (66% of all illnesses associated with the 24 outbreaks), including 10 (4%) infants, 120 (50%) persons 1–49 years of age, and 108 (45%) ≥50 years of age. Information on sex was available for 19 (79%) of the 24 outbreaks. Among 296 patients with sex reported, 160 (54%) were female (Table 2).

Although most of the outbreaks occurred in community settings (i.e., foods consumed in private homes, commercial establishments, or both), 2 outbreaks were attributed to
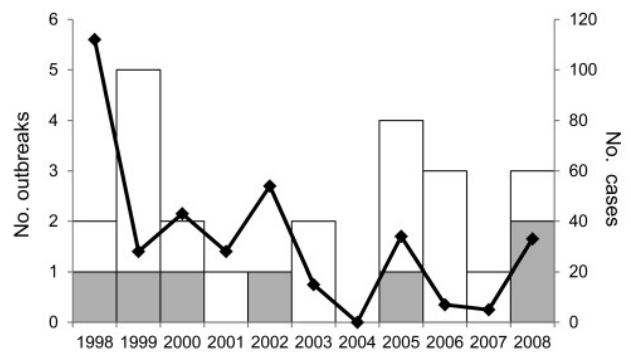
Figure 1. Reported listeriosis outbreaks by single-state or multistate status and total number of outbreak-associated cases, Foodborne Disease Outbreak Surveillance System, United States, 1998–2008 (n = 24 outbreaks). White bar sections indicate single-state and multistate outbreaks, gray bar sections indicate multistate outbreaks, and black line indicates total ill.

foods prepared and served in hospitals. A food vehicle was implicated in 20 (83%) of the 24 confirmed listeriosis outbreaks (Table 2). Six (25%) outbreaks were attributed to delicatessen (deli) meats. Three (13%) outbreaks, including the largest outbreak (n = 108 cases) during the study period, were associated with frankfurters. Mexican-style cheese (queso fresco or queso blanco) was implicated in 4 (17%) outbreaks. Three outbreaks involved Mexican-style cheese made from unpasteurized milk, and 1 outbreak was associated with cheese made from pasteurized milk (*24*). Outbreaks caused by Mexican-style cheese primarily affected women (84%); most (73%) patients were 1–49 years of age.

Two outbreaks involved other dairy products, including cheese made from pasteurized sheep's milk and flavored, pasteurized milk. Environmental investigations conducted during these 2 dairy-associated outbreaks (*20,24*) found that milk had been adequately pasteurized. Thus, postpasteurization contamination was believed to be the contributing factor for both outbreaks. Other implicated food vehicles included taco/nacho salad, tuna salad, and sprouts.

L. monocytogenes serotype information was available for 20 (83%) of the 24 confirmed listeriosis outbreaks (Table 3). Serotype 4b caused the largest number of outbreaks (n = 10) and outbreak-associated cases (n = 218). Serotype 4b was also associated with the highest hospitalization rate (70%) and the highest CFR (13%). Serotype 1/2a was responsible for 8 (40%) outbreaks and 119 (33%) cases. Serotype 1/2b was least common, causing 2 (10%) outbreaks that resulted in 5 (1%) outbreak-associated cases and no deaths (Table 3).

In the early study period (1998–2003), 13 listeriosis outbreaks (4 multistate and 9 single-state) were reported,

Table 1. Reported listeriosis outbreaks (n = 24) by year, Foodborne Disease Outbreak Surveillance System, United States, 1998–2008*

Study period, year†	Multistate	Duration, d	Total no. cases‡	No. hospitalizations	No. deaths	Listeria monocytogenes serotype	Food vehicle (reference)
Early							
1998	Yes	389	108	101	14	4b	Frankfurters (19)
	No	NA	4	NA	NA	NA	Frankfurters
1999	No	NA	6	NA	NA	NA	Unknown
	No	NA	4	NA	NA	1/2a	Frankfurters
	No	NA	5	5	1	NA	Deli meat
	Yes	NA	11	NA	NA	1/2a	Pâté
	No	NA	2	2	1	NA	Deli meat
2000	No	122	13	13	0	4b	Mexican-style cheese (22)
	Yes	151	30	29	4	1/2a	Deli meat (14)
2001	No	3	28	0	0	1/2a	Deli meat (21)
2002	Yes	100	54	NA	8	4b	Deli meat (15)
2003	No	NA	3	NA	NA	4b	Unknown
	No	NA	12	12	1	4b	Mexican-style cheese
Late							
2005	No	32	6	6	0	4b	Unknown
	No	7	3	3	0	1/2b	Grilled chicken
	Yes	37	13	13	1	1/2a	Deli meat
	No	36	12	12	0	4b	Mexican-style cheese
2006	No	7	2	1	1	4b	Unknown
	No	2	2	0	0	1/2b	Taco or nacho salad
	No	1	3	2	1	4b	Cheese
2007	No	163	5	5	3	4b	Milk (20)
2008	No	47	5	5	3	1/2a	Tuna salad (23)
	Yes	351	20	2	0	1/2a	Sprouts
	Yes	150	8	4	0	1/2a	Mexican-style cheese (24)

*NA, no data available.
†No listeriosis cases were reported in 2004.
‡Includes laboratory-confirmed and epidemiologically linked cases.

and in the late study period (2004–2008) 11 (3 multistate and 8 single-state) were reported (Figure 1). Information on outbreak duration was available for 5 (38%) of 13 early study period outbreaks and for all late study period outbreaks. Among outbreaks with information available, those reported in the early study period were generally larger than those in the late study period (median 11 cases [range 2–108 cases] vs. 5 cases [range 2–20 cases]) and longer (122 days vs. 36 days). Among the 11 outbreaks with a food vehicle reported in the early study period, 5 were associated with deli meats and 3 with frankfurters. Novel food vehicles (sprouts and taco/nacho salad) were reported only in the late study period.

Conclusions

Changes in characteristics of outbreaks reported to CDC during 1998–2008 highlight the successes and continued challenges of listeriosis prevention. During the study period, an average of 2.2 confirmed outbreaks per year were reported. In contrast, an average of 0.25 outbreaks per year was reported during the 20 years before the study period (i.e., the pre-PulseNet era) (Figure 3). We theorize that this 9-fold increase in the number of listeriosis outbreaks reported is predominantly attributed to enhanced detection through PulseNet. Furthermore, outbreaks in the late study period were generally shorter and smaller than those reported in the early study period. This finding suggests that

Table 2. Characteristics of 24 listeriosis outbreaks by implicated food categories, Foodborne Disease Outbreak Surveillance System, United States, 1998–2008*

Food category	No. outbreaks	Total no. cases†	No. (%) hospitalized	No. deaths (CFR, %)	Age group, y, no. (%)‡ <1	1–49	>50	No. (%) female‡
Deli meats	6	132	49 (37)	15 (11)	NA	NA	NA	40 (45)
Frankfurters	3	116	101 (87)	14 (12)	0	52 (46)	60 (54)	57 (49)
Other meats	2	14	3 (21)	0	NA	NA	NA	2 (67)
Mexican-style cheese	4	45	41 (91)	1 (2)	8 (18)	32 (73)	4 (9)	37 (84)
Other dairy products	2	8	7 (88)	4 (50)	1 (13)	1 (13)	6 (75)	4 (50)
Salad/other	3	27	7 (26)	3 (11)	0	7 (26)	20 (74)	16 (59)
Unknown	4	17	7 (41)	1 (6)	0	1 (13)	7 (88)	4 (50)
Overall	24	359	215 (60)	38 (11)	10 (4)	120 (50)	108 (45)	160 (54)

*CFR, case-fatality rate; NA, not available.
†Includes laboratory-confirmed and epidemiologically linked cases.
‡Percentages were calculated on the basis of available information on age distribution (17 outbreaks) and sex (19 outbreaks) for 238 and 296 patients, respectively.

the combined application of PulseNet and the *Listeria* Initiative was useful for timely outbreak detection and investigation (*14*).

Participation in the *Listeria* Initiative, measured by the number of states reporting ≥1 cases and the percentage of cases reported among all nationally notified cases, has increased steadily from 2004 (no. states reporting = 10, percentage of cases reported = 15%) to 2010 (no. states reporting = 42; percentage of cases reported = 71%) (CDC, unpub. data). The *Listeria* Initiative has contributed to numerous investigations of outbreaks, including those attributed to pasteurized milk, tuna salad served in a hospital, Mexican-style cheese, and hog head cheese (a meat jelly made from swine heads and feet) (*20,23–25*).

Changes in the size and duration of listeriosis outbreaks occurred during a time when the national incidence of invasive listeriosis decreased by 24% during 1996–2003 (*5*), and then reached a plateau during 2004–2009 (*6*). Enhanced regulatory and industry efforts that stemmed from outbreak investigations (*15*) likely contributed in great part to the observed decrease in invasive listeriosis cases during this earlier time. Notably, 8 of 9 outbreaks associated with frankfurters or deli meat occurred early in the study period. The marked reduction in outbreaks associated with ready-to-eat (RTE) meat and poultry mirrored a decrease in the occurrence of *L. monocytogenes* contamination in RTE meat and poultry, from 1.0% to 8.1% in the 1990s to 0.3% in 2010 (*26*).

The reduction in *L. monocytogenes*–contaminated RTE meat and poultry likely demonstrates the effect of several federal regulatory initiatives. First, the US Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) required in 1999 that hazard analysis and critical control point systems be reassessed to ensure that meat and poultry processing establishments in the United States were adequately addressing risk for infection with *L. monocytogenes*. Second, in 2003, USDA-FSIS issued the *Listeria* Rule, which encourages establishments to use combinations of postlethality treatments (i.e., after the product has been cooked), such as inclusion of antimicrobial drugs and bacterial growth inhibitors, to prevent contamination (*27*). Most large producers of frankfurters and fully cooked sliced meat and poultry products, such as deli meat, use ≥1 such interventions (USDA-FSIS, un-
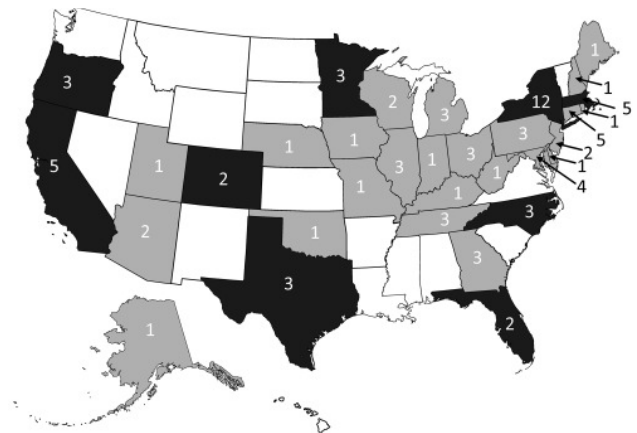
Figure 2. Distribution of single-state and multistate outbreaks of listeriosis, 1998–2008, Foodborne Disease Outbreak Surveillance System, United States, 1998–2008 (n = 24 outbreaks). Dark gray indicates single-state and multistate outbreaks, and light gray indicates multistate outbreaks. Values indicate total outbreaks in each state. The grand total of outbreaks indicated in each state is >24 because of multistate outbreaks.

pub. data); establishments that opt to forgo them are subject to requirements for more frequent product and production surface sampling (*28*). Third, USDA-FSIS implemented a random sampling program in 2004 for *L. monocytogenes* in production establishments; an additional risk-based, verification sampling program with compliance incentives was started in 2005 (*29*). Establishments where *L. monocytogenes* is detected undergo intensive sampling (intensified verification testing) as part of an assessment of the ability of the establishment to manufacture products eligible to bear the mark of inspection (*30*). Although an outbreak of listeriosis associated with deli meat (hog head cheese) occurred in 2010 (*25*), the processing establishment was subject only to state, not federal, regulation because it did not distribute its product outside the state.

In contrast, listeriosis outbreaks from dairy products showed no decrease in frequency throughout the study period. Unpasteurized (raw) milk poses a relatively high risk for listeriosis on a per-serving basis. Mexican-style cheese made from raw milk contributed to at least 1 dairy-associated listeriosis outbreak in the study period (*22*). Although certain cheeses made from raw milk that are aged for >60

Table 3. Characteristics of 24 listeriosis outbreaks by *Listeria monocytogenes* serotype, Foodborne Disease Outbreak Surveillance System, United States, 1998–2008*

Serotype	No. outbreaks	Total no. cases†	Median duration, d (range)	No. cases/outbreak	No. (%) hospitalized	No. deaths (CFR, %)
4b	10	218	68 (1–389)	22	152 (70)	28 (13)
1/2a	8	119	99 (3–351)	15	53 (45)	8 (7)
1/2b	2	5	5 (2–7)	3	3 (60)	0
Unknown	4	17	NA	4	7 (41)	2 (12)

*NA, not available.
†Includes laboratory-confirmed and epidemiologically linked cases.
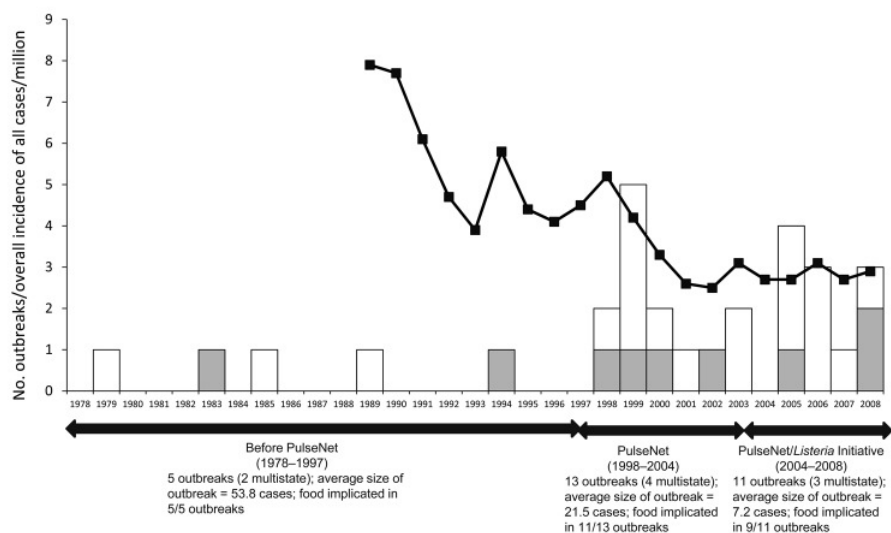
Figure 3. Incidence of all cases (per million) and outbreaks of listeriosis, 1978–2008, United States. White bar sections indicate single-state outbreaks, gray bar sections indicate multisite outbreaks, and black line indicates incidence per million. Data were obtained from the Foodborne Diseases ActiveSurveillance Network (FoodNet) and the Foodborne Disease Outbreak Surveillance System. Data are as of June 2010.

days can be sold under Food and Drug Administration regulations (21 CFR Section 133), outbreaks of bacterial, enteric diseases associated with aged cheeses have occurred (*31*), and microbiologic studies have demonstrated the viability of bacterial pathogens in cheeses made from raw milk that have aged ≥60 days (*32*). In addition to outbreaks associated with cheese made from raw milk, listeriosis outbreaks from pasteurized dairy products (*20*) also occurred during the study period. Although pasteurization is known to adequately eliminate *L. monocytogenes* from dairy products, postpasteurization contamination can occur and was the likely source of contamination in at least 1 of the listeriosis outbreaks (*24*) during the study period and at least 4 additional listeriosis outbreaks associated with Mexican-style cheese since the study period (*33*). To prevent postpasteurization contamination, it is necessary to maintain strict separation of prepasteurized and postpasteurized dairy products and strict sanitation in the postprocessing environment.

The outbreaks associated with Mexican-style cheese primarily affected women 1–49 years of age, which includes the reproductive years. FDOSS does not collect information on ethnicity or pregnancy status, but other surveillance data indicate that pregnant Hispanic women have disproportionately higher rates of listeriosis (*6*). Investigations of outbreaks associated with Mexican-style cheese have informed recommendations for pregnant women to avoid consumption of unpasteurized dairy products, including Mexican-style cheeses.

The Food Safety Modernization Act provides the Food and Drug Administration with greater authority to prevent and control outbreaks of listeriosis and other foodborne infections through mandatory prevention controls and safety standards, more stringent tools for inspection and com-

pliance, and an enhanced ability to remove contaminated foods from the food supply (e.g., mandatory recalls). However, dairy products imported from countries with different regulatory controls, homemade products or products made by unlicensed manufacturers, and products contaminated after pasteurization continue to pose regulatory and enforcement challenges (*22*). Therefore, additional prevention and education efforts are still needed to effectively protect susceptible populations.

Several novel food vehicles, including sprouts and taco/nacho salad, were implicated in listeriosis outbreaks described in this review. Three additional novel vehicle outbreaks have since been reported. In 2010, hog head cheese was associated with 8 listeriosis cases in Louisiana (*25*), and celery in a diced chicken salad was associated with an outbreak of 10 cases among hospitalized patients in Texas (*34*). Whole cantaloupe from a single farm was associated with a large, multistate outbreak (n = 147 cases) in 2011, and caused the most deaths (n = 33) from an outbreak of foodborne illness in the United States in nearly 90 years (*16*). Outbreaks that were linked to food vehicles with multiple ingredients are noteworthy because identifying a particular ingredient that is contaminated can be challenging (e.g., celery in chicken salad, taco/nacho salad). Preventing *L. monocytogenes* contamination of food is difficult; the bacteria proliferate under acidic conditions, high salt concentrations, and low temperatures. We expect that future outbreak investigations will continue to identify new food vehicles that can transmit *L. monocytogenes* to humans and identify new opportunities for consumer prevention education, industry interventions to reduce contamination, and regulatory control policies.

We found that serotype 4b caused more outbreaks, more outbreak-associated cases, and resulted in higher rates

of hospitalization and deaths than serotypes 1/2a and 1/2b. Studies have shown that most isolates from food belong to serogroup 1/2, but most epidemic listeriosis is caused by serotype 4b (*35,36*). In addition, a study in Denmark suggested that illness caused by serogroup 4 is associated with a higher CFR than serogroup 1/2 (*37*). Recent research on *L. monocytogenes* virulence mechanisms has shown that certain mutations in the *inlA* gene, which encodes for internalin (a membrane-anchored protein responsible for *L. monocytogenes* invasion into nonprofessional phagocytes), can attenuate virulence (*38,39*). These virulence-attenuating mutations are prevalent in serogroup 1/2 but have not been observed in serotype 4b isolates (*40*). Further work is needed to bridge our understanding of *L. monocytogenes* virulence to epidemiologic trends in sporadic and outbreak-associated listeriosis.

There is wide variation in outbreak reporting practices among FDOSS users. For example, New York reported the largest number of listeriosis outbreaks during the study period, but active, population-based surveillance do not suggest that the incidence of invasive listeriosis was higher in New York than in other states (*5*). These findings may reflect variable population sizes and composition and differences in resources for investigating and reporting outbreaks among states, but more information is needed to fully understand these differences. Variation in the quality and completeness of FDOSS reports can limit conclusions drawn from the data. For example, demographic information was missing for nearly 30% of the outbreaks we analyzed. Because FDOSS uses the same reporting form for all pathogens, variables of specific interest for listeriosis, such as underlying medical conditions, ethnicity, age categories for older adults, pregnancy status, and clinical outcomes, are not available unless the reporting agency submits supplemental information. For example, the fact that 2 outbreaks occurred in hospital settings suggests that the medical fragility of the outbreak populations was a major contributor to the outbreaks. Although clinical information is not collected in FDOSS, there is a recognized need to improve food safety in hospitals (e.g., avoiding service of higher risk foods to immunocompromised patients) (*6,23*).

Listeriosis outbreak investigations are crucial to prevent additional illness, hospitalization, and death. They also provide a unique source of information to improve our understanding of *L. monocytogenes* infections and transmission and to identify gaps in industry and regulatory measures to safeguard against contamination of the food supply. The changes we observed in characteristics of listeriosis outbreaks during 1998–2008 illustrate the contributions of PulseNet and the *Listeria* Initiative for outbreak detection and investigation and subsequent effects of industry and regulatory efforts to prevent similar contamina-

tion from reoccurring. In particular, we found that reports of outbreaks caused by frankfurters and deli meats have become less frequent. Also, outbreaks have generally become smaller in size and shorter in duration.

Nevertheless, listeriosis outbreaks continue to cause considerable illness and death and associated costs. For example, the 2011 outbreak associated with whole cantaloupe (*16*) demonstrated that large outbreaks can still occur and that work remains to identify and prevent new sources of contamination. Repeated occurrences of outbreaks caused by dairy products, especially Mexican-style cheese, also signal the need for additional work. Although invasive listeriosis is rare (average annual incidence for 2004–2009 was 0.27 cases/100,000 population) (*6*), US subpopulations at increased risk are growing in size, including adults ≥65 years of age and Hispanics (2010 Census estimates of 40.3 and 50.5 million, respectively). Therefore, a concerted effort from regulatory, industry, and public health authorities will be required to reduce the overall incidence of listeriosis below the Healthy People 2020 goal of 0.2 cases per 100,000 population (www.healthypeople.gov).

Dr Cartwright is an Infectious Disease fellow at Emory University in Atlanta, Georgia. She was an Epidemic Intelligence Service Officer in the Division of Foodborne, Waterborne and Environmental Diseases at CDC when this study was conducted. Her research interests are using epidemiology to understand infectious diseases in community and health care settings, to reduce disease transmission, and to evaluate public health interventions.

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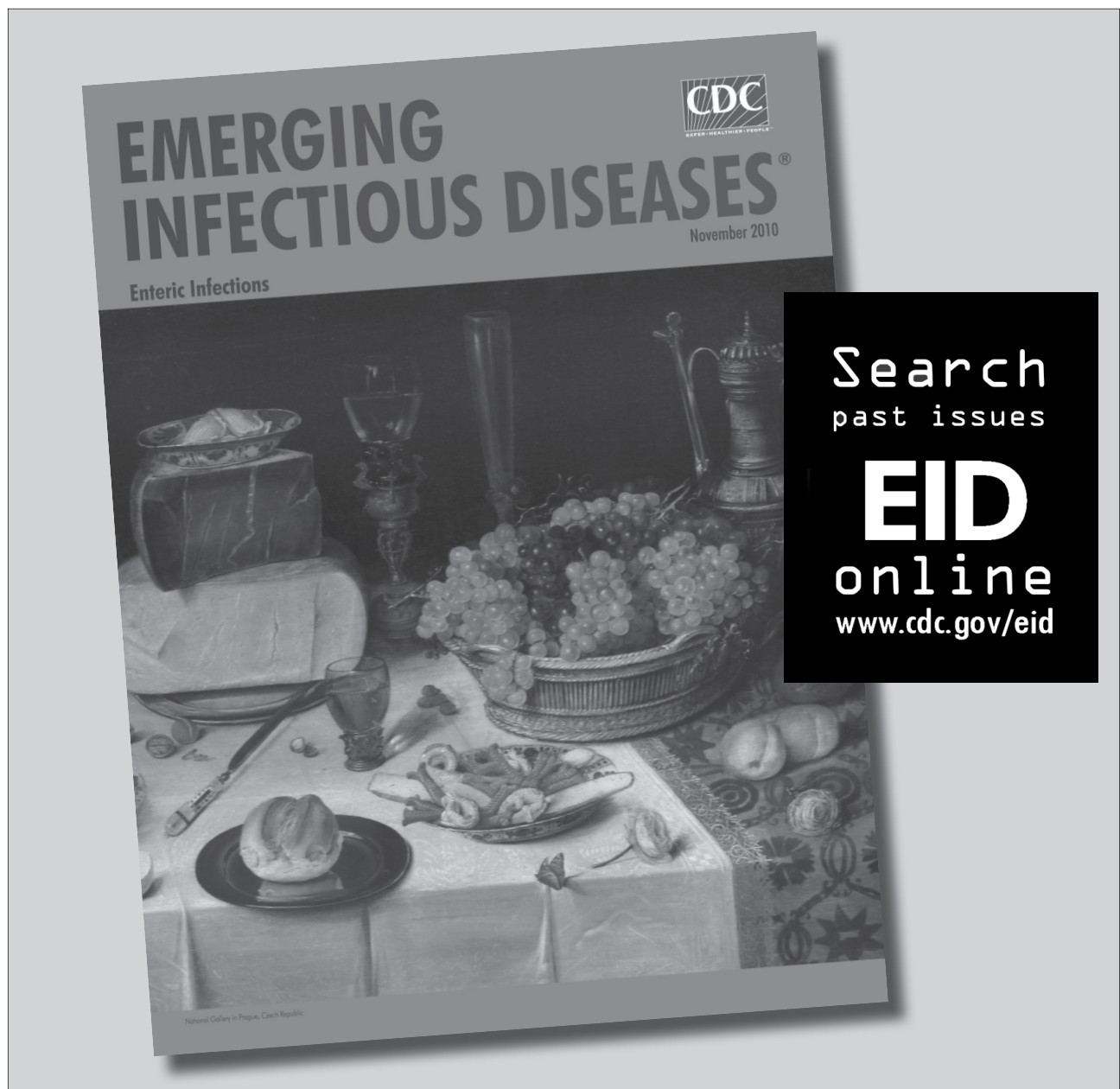
Staphylococcal Infections in Children, California, USA, 1985–2009

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We conducted a retrospective, observational, population-based study to investigate the effect of staphylococcal infections on the hospitalization of children in California during 1985–2009. Hospitalized children with staphylococcal infections were identified through the California Office of Statewide Health Planning and Development discharge database. Infections were categorized as community onset, community onset health care–associated, or hospital onset. Infection incidence was calculated relative to all children and to those hospitalized in acute-care facilities. A total of 140,265 records were analyzed. Overall incidence increased from 49/100,000 population in 1985 to a peak of 83/100,000 in 2006 and dropped to 73/100,000 in 2009. Staphylococcal infections were associated with longer hospital stays and higher risk for death relative to all-cause hospitalizations of children. The number of methicillin-resistant *Staphylococcus aureus* infections increased, and the number of methicillin-susceptible *S. aureus* infections remained unchanged. Children <3 years of age, Blacks, and those without private insurance were at higher risk for hospitalization.

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The increasing prevalence of antimicrobial drug–resistant staphylococcal infections is a threat to public health in the United States. Methicillin-resistant *Staphylococcus aureus* (MRSA) is now a frequent cause of skin, soft tissue, and invasive *S. aureus* infections requiring hospitalization in the United States (*1–6*).

The characteristics of staphylococcal infection in children in particular communities have been described (*7–9*). However, there are fewer population-based studies, and little is known about changes in MRSA transmission among children in California. We conducted a retrospective observational study to determine the incidence of and trends in staphylococcal infection–associated hospitalizations among children in California during 1985–2009.

Methods

Study Population

The California Office of Statewide Health Planning and Development (OSHPD) maintains a hospital discharge database that includes demographic and clinical information on patients discharged from hospitals. In compliance with the California Health Safety Code (Section 128735), non-federal hospitals in the state report all patient discharges to OSHPD. We extracted records from the OSHPD database for children (persons <18 years of age) who were admitted to and discharged from an acute-care facility during January 1, 1985–December 31, 2009, with a primary diagnosis of staphylococcal infection or 1 of ≤24 other diagnoses. To reduce multiple counting of the same event, we excluded data for children transferred to another acute-care facility within 2 days of admission. International Classification of Diseases, Ninth Revision (ICD-9), Clinical Modification or Diagnosis Related Group (DRG), codes were used to extract medical records and to further classify extracted records (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/pdfs/11-1740-Techapp.pdf). Infections were categorized as community onset (CO), CO health care–associated (CO-HCA), or hospital onset (HO) (online Technical Appendix Table 2). Information obtained included age, sex, race, hospital length of stay (LOS), expected source of payment, diagnosis, and medical procedures. Age-related data were analyzed by age group (Table 1). We refer to babies hospitalized at ≤30 days of age as neonates, independent of the age at which they received a diagnosis of staphylococcal infection or were discharged. No attempt was made to identify patients or access their medical records.

Race

Until 1994, race was specified in OSHPD data as White, Black, Hispanic, Native American, Asian (including Pacific Islanders), Other, or Unknown. Starting in 1995, Hispanic was removed from the options for race, and an ethnicity field, including the following options, was introduced: Hispanic, Non-Hispanic, and Unknown. Because the ethnicity field was not available during the entire study period, we combined race and ethnicity into 5 race categories: White, Black, Hispanic, Asian, and Other. The Hispanic category included all OSHPD data for patients with race or ethnicity identified as Hispanic. Unlike the OSHPD database, for which ≈5% of the records specify Other/Unknown race, the US Census Bureau does not allow for Other/Unknown race. Thus, when estimating incidence rates by race, we did not include data for patients with race identified as Other.

Social Status

The only OSHPD variable associated with socioeconomic status was expected source of pay. Thus, data for this variable were combined in a 2-level variable (private insurance, yes/no) and used as a surrogate for social status.

Statistical Analysis

The incidence of staphylococcal infection was calculated relative to estimates of the population of children in California (obtained from the California Department of Finance) and to the number of children admitted to California acute-care facilities during 1985–2009, not including children who were transferred to a different acute-care facility within 2 days of admission or children admitted with codes for nonpathological diagnoses, indicating normal newborns or deliveries. Admission years were considered from January through December. Children hospitalized in 2009 and discharged in 2010 are not included in the database, thus biasing downward the estimates of incidence of disease and mean LOS for 2009. To account for this bias, we designed an extrapolation process (online Technical Appendix), which was used in determining all 2009 estimates.

We analyzed 4 primary outcomes: 1) risk for hospitalization with a staphylococcal infection; 2) risk that hospitalized children would be diagnosed with a staphylococcal infection; 3) LOS; and 4) risk for death in children with a diagnosis of staphylococcal infection. Risks were estimated separately for each independent variable and by multivariable logistic regression with the covariates sex; age at admission; race; insurance status; year of admission; and, in the case of death, LOS and complexity of the disease (Tables 1, 2). For the population-based logistic regression, a yes/no "hospitalized with staphylococcal infection" outcome variable was artificially introduced. The value "yes" was assigned by year and demographic attribute to the number of children hospitalized for staphylococcal infection; the value "no" was assigned to the remaining children (e.g., in 1985, a total of 449 White boys <1 year old were assigned the value "yes," and the other White boys <1 year old were assigned the value "no").

Table 1. Incidence of children hospitalized and length of stay for hospitalized children with staphylococcal infections, California, USA, 1985–2009*

Sociodemographic variable and infection category	% Total population of children, N = 140,265	Incidence of children hospitalized among		LOS, d, median (IQR)	% Children who died†
		General population, per 100,000 children	Hospitalized children, per 1,000 children		
Overall	100	62	14	7 (4–21)	2.5
Sex					
M	57	69	15	7 (4–21)	2.5
F	43	56	14	7 (4–21)	2.6
Race					
White	37	60	13	7 (3–18)	2.3
Black	10	88	16	8 (4–25)	2.9
Hispanic	42	63	15	7 (4–21)	2.4
Asian	6	40	13	7 (4–21)	2.9
Age at admission					
<30 d (neonates)	25	452‡	8	28 (7–69)	4.5
31–91 d	6		21	7 (4–17)	3.2
92–365 d	12		21	6 (3–13)	2.3
1–2 y	15	83	22	5 (3–10)	1.5
3–5 y	9	34	17	6 (3–13)	1.5
6–9 y	9	26	17	6 (3–13)	1.5
10–13 y	10	28	19	7 (4–14)	1.6
14–17 y	14	40	16	6 (3–14)	2.0
Expected source of payment					
Private insurance	41	NA	12	7 (3–20)	2.4
Other§	59	NA	16	8 (4–21)	2.6
Staphylococcal infection category					
Uncomplicated¶	72	45	10	6 (3–14)	1.3
Pneumonia only	6	4	1	14 (7–37)	5.7
Septicemia only	20	12	3	14 (6–45)	5.5
Complicated#	2	1	<0.5	38 (16–92)	7.3

*LOS, length of stay; IQR, interquartile range; NA, not applicable.
†For differences >0.2% in the proportion of children that died, p<0.0001 (χ^2 test); p = 0.04 for the differences in the proportion of deaths for Hispanic vs. White children.
‡Combined value for all children <1 year of age.
§Medi-Cal, Medicare, other government sources, charity, and none.
¶No code for septicemia or pneumonia identified.
#>2 staphylococcal codes identified, including >1 code for septicemia or pneumonia.

Results

Because of the size of the study population, even small differences are highly statistically significant. Therefore, we omitted most p values from the text. We used SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA) to perform statistical analyses.

Discharge records for 140,265 children in California satisfied inclusion criteria for the study. During 1985–2009, the overall incidence of staphylococcal infections among children in California was 63 cases/100,000 children; incidence was calculated as the mean of the yearly incidences weighted by the yearly population size. Yearly incidences ranged from 49 cases/100,000 children in 1985 to a high of 83/100,000 in 2006, followed by a decrease to 73/100,000 in 2009. The increased incidence of hospitalized children with staphylococcal infections during 2002–2006 mirrored an increase in the number of hospitalized children with cellulitis. The decreased incidence of hospitalizations for cellulitis in subsequent years lagged after the decrease in the incidence of hospitalizations for staphylococcal infections (Figure 1). During 1985–2004, the incidence of all-cause hospitalizations among children decreased; incidence has increased only slightly since then (Figure 1). This decrease resulted in even sharper increases in the incidence of staphylococcal infections among hospitalized children (from 8.2 cases/1,000 children in 1985 to 23.3/1,000 in 2006, decreasing to 19.9/1,000 in 2009).

Demographic information for hospitalized children with staphylococcal infection is shown in Figure 2. All groups except neonates demonstrated an increase in infection during 2002–2006. The increase was most pronounced in White and Black children (60%); the smallest increase was among Asian children (40%).

Table 1 shows the incidence of staphylococcal infection, by demographic characteristic, among the population and among hospitalized children; Table 2 shows the odds ratios (ORs) for the risk of infection estimated while controlling for year of admission and demographic characteristics. Compared with children 1–2 years of age, those <1 year of age were at high risk for hospitalization with staphylococcal infection (OR 5.6) (Table 2). Black children were 1.5× more likely and Asian children 0.6× less likely than White children to be hospitalized with staphylococcal infection (Tables 1, 2). However, among hospitalized children, the risk of acquiring staphylococcal infection was low for neonates compared

Table 2. Risk for staphylococcal infection, by demographic characteristic, for children, California, USA, 1985–2009*

Demographic characteristics	Risk of infection, OR† (95% CI)	
	General population of children	Hospitalized children
Sex		
M	1.25 (1.23–1.26)	1.07 (1.06–1.09)
F	Reference	Reference
Race		
White	Reference	Reference
Black	1.46 (1.43–1.49)	1.05 (1.04–1.07)
Hispanic	0.94 (0.93–0.95)	0.96 (0.95–0.97)
Asian	0.63 (0.62–0.65)	Dropped
Age at admission		
$\leq$30 d	5.64 (5.55–5.73)‡	0.39 (0.39–0.40)
31–91 d		0.95 (0.92–0.97)
92–365 d		0.98 (0.96–1.00)
1–2 y	Reference	Reference
3–5 y	0.42 (0.41–0.43)	0.80 (0.78–0.81)
6–9 y	0.31 (0.31–0.32)	0.81 (0.79–0.92)
10–13 y	0.34 (0.33–0.35)	0.90 (0.88–0.92)
14–17 y	0.48 (0.47–0.49)	0.76 (0.74–0.77)
Expected source of payment		
Private insurance	NA	0.78 (0.77–0.79)
Other§	NA	Reference

*OR, odds ratio; NA, not applicable.
†ORs were calculated by using backward logistic regression (stay criteria p<0.01) with the variables listed in the table and year of admission.
‡Combined value for all children <1 year of age.
§Medi-Cal, Medicare, other government sources, charity, and none.

with children 1–2 years of age (OR 0.4); for infants 1–12 months of age, the risk was approximately equal to that for older children. Lack of private insurance was the single most dominant factor associated with increased risk for staphylococcal infection among hospitalized children (OR 1.3) (Table 2); the association with race was much lower (ORs of 1.05, 0.96, and 1.0 for Black, Hispanic, and Asian race, respectively, compared with White race) (Table 2).

Length of Hospital Stay

Hospital stays ranged from 0 to 2,067 days (median 7 days, mean 20.7 ± 0.1 days); for 10% of the hospitalizations, LOS was >60 days. LOS varied by sociodemographic factor (Table 1). Children hospitalized in the first month of life had long LOSs (median 28 days). Children without private insurance had longer LOSs (median 8 days) than children with private insurance (median 7 days).

Extremely long LOSs were mostly among premature infants. Neonates hospitalized within 2 days of birth made up 16% of the study population, but they represented 65% of the children hospitalized for ≥3 months. When we excluded neonates from the analysis, LOS was shorter and the 10%–90% interval was 2–31 days (median 6 days, mean 13.9 days). However, even after we excluded neonates from analysis, LOS was still considerably longer for hospitalized children with staphylococcal infections than for children hospitalized for any other cause (median LOS 2 days, mean 4.9 days). During 1985–2002, the mean LOS for hospitalized children with staphylococcal infections increased from 17.3 to 26.7 days, but by 2009, the mean LOS decreased to 17.7 days (Figure 1).

Outcomes

Among all children with a diagnosis of staphylococcal infection, 82.6% had a routine discharge, 10.4% required additional low- or medium-intensity care on discharge, 4.4% were transferred to another acute care facility, and 2.5% died. Using a multivariable model controlling for year of admission, LOS, age at admission, sex, race, and insurance status, we compared the risk for death among children with and
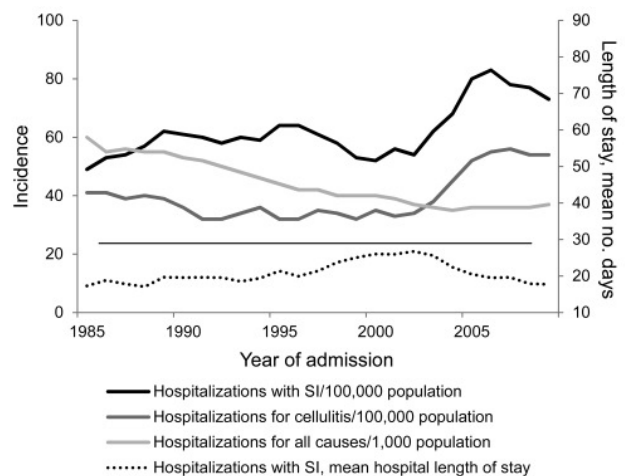
Figure 1. Hospitalization trends for children ≤17 years of age, California, USA, 1985–2009. The incidence of hospitalizations and mean length of stay for children with staphylococcal infection (SI) are compared with the incidences of hospitalizations for cellulitis (Diagnosis Related Group [DRG] 279 or Medicare Severity–DRG 602–603) and for all-cause hospitalizations of children. The horizontal line separates the incidence graphs, which are to be read against the left axis, and the graph for length of stay, which is read against the right axis.
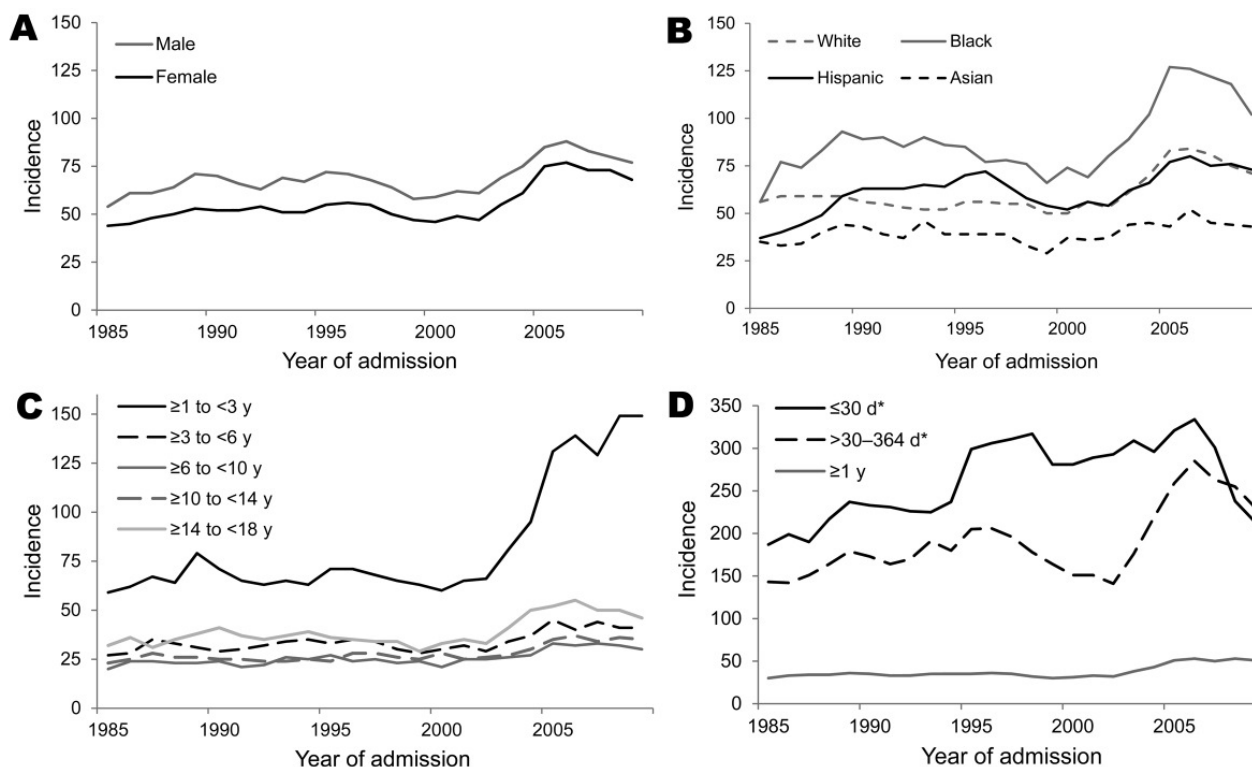
Figure 2. Hospitalization trends for children ≤17 years of age with staphylococcal infection, California, USA, 1985–2009. Data are no. of patients/100,000 population, except as indicated for children <1 year of age. A) Trends by sex. B) Trends by race. C) Trends by age group, age ≥1 year. D) Trends for infants (children ≤364 days of age) compared with trends for children 1–17 years of age; *number/100,000 children <1 year of age.

those without a diagnosis of staphylococcal infection: the risk was higher for children with staphylococcal infection (OR 2.1, 95% CI 2.0%–2.2%). Among hospitalized children with staphylococcal infections, the risk for death was associated with the complexity of the disease and with some demographic factors (Table 1). Most relationships remained unchanged in multivariable analysis that included LOS and the factors presented in Table 1. The exceptions were higher risk of death for children of Asian race, but not Hispanic or Black race, compared with White race (OR 1.2, 95% CI 1.1%–1.4%) and not having private insurance (OR 1.6, 95% CI 1.5%–1.9%). For hospitalized children with staphylococcal infections, the risk of death increased from 2.4% in 1985 to 3.5% in 2002; the risk decreased beginning in 2003 and remained at <2% during 2006–2009.

Temporal Trends in Incidence

Among the entire population of children in California, the yearly incidence of staphylococcal infection increased from 1985 to 2009 (Figure 1). The increase was not uniform: incidence decreased during 1996–2000, followed by a large increase during 2002–2006. Overall, 38% of

the records for hospitalized children with staphylococcal infection had an ICD-9-CM code for skin and soft tissue infection (SSTI), and 67% of those records had an ICD-9-CM code for cellulitis. The proportion of hospitalized children with staphylococcal infection who also had an ICD-9-CM code for cellulitis remained at <20% through 2002 and then rose to 44.4% in 2009. The rise in hospitalizations for staphylococcal infections corresponded to a 2002–2007 rise in hospitalizations for cellulitis (from 34 to 56/100,000 children), as classified by DRG (Figure 1). The percentage of records including DRG-classified cellulitis and staphylococcal infection increased from 13%–19% during 1985–2002 to 40%–44% during 2005–2009. During this period, all-cause hospitalizations for children in California did not increase (Figure 1).

The increased incidence of staphylococcal infections during 2002–2005 was seen across all racial and age groups, except neonates. The steepest increase was seen among Black children and children 1 month–2 years of age; the increase was less pronounced among Asian children (Figure 2). The rise in incidence among children 1 month–2 years of age was observed in all races.
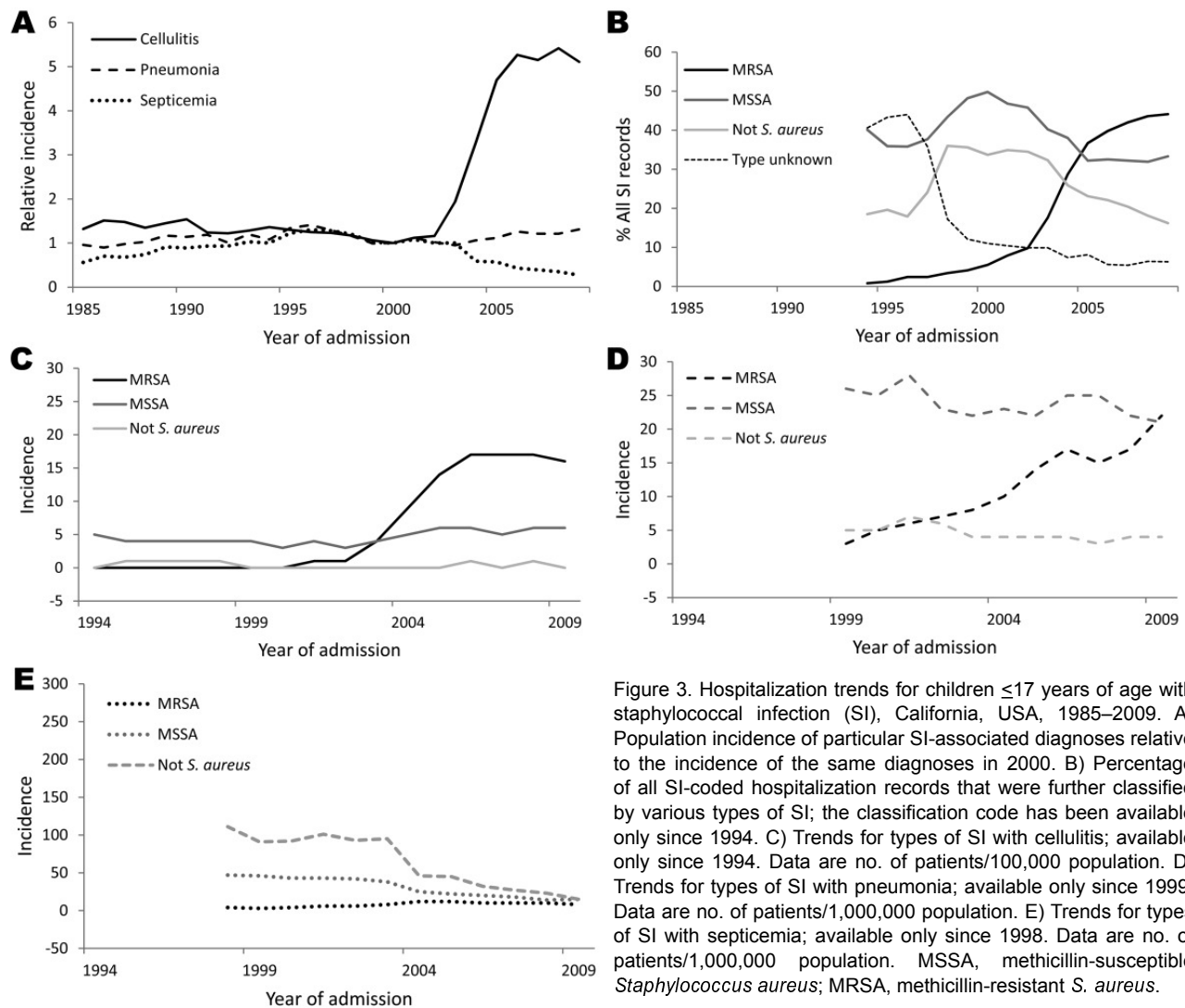
Figure 3. Hospitalization trends for children ≤17 years of age with staphylococcal infection (SI), California, USA, 1985–2009. A) Population incidence of particular SI-associated diagnoses relative to the incidence of the same diagnoses in 2000. B) Percentage of all SI-coded hospitalization records that were further classified by various types of SI; the classification code has been available only since 1994. C) Trends for types of SI with cellulitis; available only since 1994. Data are no. of patients/100,000 population. D) Trends for types of SI with pneumonia; available only since 1999. Data are no. of patients/1,000,000 population. E) Trends for types of SI with septicemia; available only since 1998. Data are no. of patients/1,000,000 population. MSSA, methicillin-susceptible *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*.

Diagnostic Categories of Infections

The increase in the incidence of hospitalizations for staphylococcal infections, which began in 2002, was driven by diagnoses of MRSA infection and cellulitis (Figure 3). In 1994, codes were introduced to distinguish between MRSA and methicillin-sensitive *S. aureus* (MSSA) infections (online Technical Appendix). During the late 1990s, as the proportion of identified infections increased, the proportion of records with infections of unknown type dropped sharply (Figure 3, panel B). During 2000 and 2009, >90% of the hospitalization records identified an infection type: the proportion of MSSA infections decreased, and the incidence of MRSA-related hospitalizations increased >10-fold (from 3 to 35/100,000 children). Hospitalizations for MRSA cellulitis accounted for 46% of the MRSA-related hospitalizations; MRSA pneumonia and septicemia accounted for 6% and 4.5%, respectively (Figure 3, panels C–E).

Infections in Neonates versus Older Children

The population incidence of staphylococcal infection–related hospitalizations was higher for neonates than older infants and children (Figure 2, panel D). This incidence is the number of cases of staphylococcal infection in neonates relative to the number of babies born that year; it increases 12-fold if estimated relative to the number of babies who were <1 month old. However, the risk for a staphylococcal infection in all-cause hospitalized neonates was much lower than the risk in all-cause hospitalized older babies (Tables 1, 2). During 2002–2006, a sharp increase in the incidence of staphylococcal infections occurred for all age groups except neonates, and the decrease that occurred in 2007–2009 was faster among neonates than other age groups (Figure 2d). Compared with staphylococcal infections in older children, those in neonates were associated with longer LOSs and poorer clinical outcomes (Table 1) and with reduced likeli-

hood of being caused by *S. aureus* (42% vs. 76%) or MRSA if caused by *S. aureus* (21% vs. 32%). Coagulase-negative staphylococci were assumed to be the cause of some non–*S. aureus* staphylococcus infections.

Community-onset versus Hospital-onset Infection, 1996–2009

Because of the differences in types of staphylococcal infections (noted above), we excluded infections in neonates from this analysis. The demographics for children with CO, CO-HCA, and HO staphylococcal infections are shown in Table 3.

The incidences of hospitalization for CO, CO-HCA, and HO MSSA and MRSA infections are shown in Figure 4, panels A, B. Throughout the study period, »10% of MSSA cases were HO infections, and the incidence of

CO infections was always higher than that for CO-HCA infections. While the incidence of MSSA infections changed little during 2000–2009, the incidence of MRSA infections increased dramatically. HO MRSA infections peaked in 2005, increasing 4.1-fold from 2000 to 2005; CO-HCA and CO infections peaked in 2006, increasing 5.3- and 27.7-fold, respectively, from 2000 to 2006. Until 2001, CO-HCA infections accounted for >50% of MRSA-related hospitalizations. By 2005, >70% of MRSA-related hospitalizations were CO infections, and only 25% were CO-HCA infections. The proportion of HO MRSA infections dropped from 18% in 1996 to <5% in 2005–2009.

A high proportion of CO MRSA infections was associated with SSTIs (Table 4). CO staphylococcus–related septicemia, pneumonia, and musculoskeletal infections were more often associated with MSSA (Table 4).

Table 3. Staphylococcal infections in non-neonate children, by onset location, California, 1996–2009*

Variable	All, N = 66,141	CO	CO-HCA	HO
% of all records	100	48	41	11
Overall incidence, no./100,000 population	49	24	20	5
Demographic data				
Sex				
M	56	46	42	12
F	44	51	39	10
Race				
White	34	46	43	10
Black	10	51	39	10
Hispanic	46	48	40	11
Asian	6	49	38	12
Age at admission				
31–91 d	6	52	33	16
92–365 d	16	57	32	11
1–2 y	21	58	34	9
3–5 y	12	41	49	10
6–9 y	12	41	49	10
0–13 y	14	42	47	11
14–17 y	19	43	45	12
Expected source of payment				
Private insurance	39	48	41	11
Other†	61	48	41	11
Outcome				
% Death	1.7	0.1	2.0	7.5
% Normal discharge	84.2	92	81	65
Mean (SE) LOS, d	12.5 (0.1)	5.3 (<0.1)	13.6 (0.1)	40.7 (0.5)
Median (interquartile range) LOS, d	6 (3–13)	4 (3–6)	8 (4–15)	28 (15–51)
Infection category and type				
Staphylococcal infection category				
Uncomplicated‡	79	91	71	61
Pneumonia	6	2	8	11
Septicemia	13	5	19	25
Complicated§	2	1	3	4
Type of staphylococcal infection				
Unknown	11	8	13	14
Other than *Staphylococcus aureus*	19	10	25	37
MSSA	42	43	42	36
MRSA	29	40	20	13

*Data represent the association of particular attributes with the particular locations of infection onset: 1) first column, for demographic attributes and category/type of infection, data are the % of all the records; 2) columns 2–4, for demographic attributes, the data represent the distribution of an attribute among CO, CO-HCA, and HO onset cases; and 3) columns 2–4, for category/type of infection, data are the % of all the records with CO, CO-HCA, or HO onset. CO, community onset; CO-HCA, CO–health care associated; HO, hospital onset; LOS, length of stay in hospital; MSSA, methicillin-sensitive *S. aureus*; MRSA, methicillin-resistant *S. aureus*.
†Medi-Cal, Medicare, other government sources, charity, and none.
‡No code for septicemia or pneumonia identified.
§ ≥2 staphylococcal codes identified, including ≥1 code for septicemia or pneumonia.

Discussion

The results of this population-based study, spanning 25 years, show major changes in the incidences and clinical manifestations of hospitalizations related to staphylococcal infection in a large and diverse population of children. The yearly incidence of pediatric hospitalizations for staphylococcal infections remained stable during 1985–2002 and then increased substantially from 2003 forward; the change was driven by an increase in MRSA infections. Other investigators have identified similar trends in smaller numbers of patients over a shorter time (10–12). The increase in incidence paralleled an increase in admissions for cellulitis and peaked in 2006. The decrease in staphylococcus infection–related hospitalizations among children during 2006–2009 corresponded with a decrease in hospitalizations for MRSA-related cellulitis. California OSHPD data for children and adults confirm that the number of hospitalizations for MRSA infection stabilized during 2005–2007 (13); our study shows that the trend is sustained. A recent observational study of Department of Defense healthcare beneficiaries of all ages showed a decline in rates of CO and HO MRSA–related bacteremia and in the proportion of CO MRSA–related SSTIs beginning in 2006 (14).

The incidence of admissions of children with staphylococcal infections (MSSA, MRSA, and non–S. aureus staphylococci infections) was lower in our study than in other studies (14 cases/1,000 admissions vs. 20.8–35.8/1,000 during 2002–2007 in another study [10]). The time covered in our study was the main factor driving the lower overall incidence of infections in hospitalized patients; the very low incidence of MRSA infections before 2002 lowered overall incidence. Other factors that possibly contributed to the differences between study findings include differences in patient populations, differences in S. aureus colonization and susceptibility patterns, and variability in clinical practices. The OSHPD database includes data for children hospitalized at all nonfederal hospitals in California, few of which are solely for children. Some doctors may have a referral bias and send children with chronic medical conditions and a higher risk of acquiring a staphylococcal infection to a children's hospital.

In our study population, the incidence of hospitalization with a staphylococcal infection was highest among boys, Black children, and, in particular, children <3 years of age (both in the general and in hospital populations). These groups have been found to be at higher risk for MRSA infections (15–17). Compared with older children, neonates and infants are at higher risk for serious invasive disease when contracting a bacterial infection and, therefore, are more likely to be hospitalized. This increased likelihood of being hospitalized for a bacterial infection may have contributed to an increased population incidence of staphylococcal infections identified in this younger age
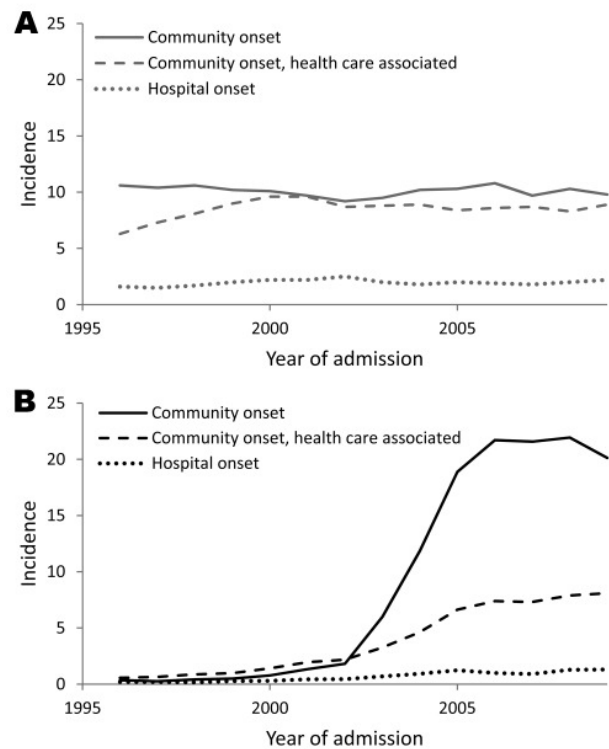
Figure 4. Hospitalization trends for children 1 month to 17 years of age with *Staphylococcus aureus* infection, by infection onset, California, USA, 1996–2009. Data are no. of patients/100,000 population. A) Incidence of methicillin-susceptible *S. aureus*. B) Incidence of methicillin-resistant *S. aureus*.

group of children. The number of all-cause hospitalizations of neonates was higher than that for children in other age groups, partly explaining the relative decreased hospital incidence of staphylococcal infection in this age group. Compared with their privately insured peers, children without private insurance had a higher incidence of hospitalization for staphylococcal infections, longer LOSs, and higher risk for dying. It is unclear why the incidence of hospitalization was higher and LOS was longer for uninsured children, but similar findings have been noted in other analyses of childhood diseases (18).

Children who received a diagnosis of staphylococcal infection had longer LOSs and had a greater risk of dying than those who did not receive a diagnosis of staphylococcal infection; we could not determine what role the infection played in the cause of death or extended LOS. In a previous study, we found that the risk for acquiring a staphylococcal infection increased incrementally the longer a patient was hospitalized (19); this finding likely reflects the key role that underlying medical conditions have in increasing LOS and risk for death. Among non-neonate children, the highest risk for death in children with staphylococcal infection was in those with HO infection (7.5%);

Table 4. Medical conditions associated with community-onset staphylococcal infections in non-neonate children, California, 1996–2009*

Medical condition	Type of infection†			
	All, N = 31,893	MRSA, n = 12,667	MSSA, n = 13,605	Other, n = 3,073
Skin/soft tissue infection				
Records with diagnosis, %	71	91	66	36
Cellulitis, %	84	90	80	63
Deaths, no.	1	0	1	0
Mean (SE) LOS, d	4.5 (<0.1)	4.3 (<0.1)	4.6 (<0.1)	5.5 (0.2)
Median (IQR) LOS, d	3 (2–5)	3 (2–5)	3 (2–5)	4 (2–6)
Sepsis, shock, or bacteremia				
Records with diagnosis, %	12	4	12	29
Deaths, no.	15	1	6	1
Mean (SE) LOS, d	8.0 (0.2)	10.9 (0.4)	8.3 (0.2)	6.6 (0.3)
Median (IQR) LOS, d	6 (4–9)	8 (5–13)	6 (4–10)	5 (3–7)
Respiratory infection				
Records with diagnosis, %	19	13	18	30
Deaths, no.	31	2	19	1
Mean (SE) LOS, d	7.1 (0.1)	6.9 (0.2)	7.3 (0.2)	6.6 (0.3)
Median (IQR) LOS, d	5 (3–8)	4 (3–7)	5 (3–8)	4 (3–7)
Musculoskeletal infections				
Records with diagnosis, %	12	6	20	6
Deaths, no.	0	0	0	0
Mean (SE) LOS, d	9.0 (0.1)	11.0 (0.4)	8.4 (0.1)	9.4 (0.7)
Median (IQR) LOS, d	7 (4–10)	7 (5–13)	6 (4–9)	6 (4–10)

*MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *S. aureus*; LOS, length of stay in hospital; IQR, interquartile range
†A particular staphylococcal type could not be determined for 11% of the records; thus, added together, numbers in the MRSA, MSSA, and Other staphylococci groups do not equal the number in All types of staphylococcal infection group. Diagnostic groups are not mutually exclusive; therefore, percentages in columns total >100.

children with CO-HCA and CO staphylococcal infections had a 2.0% and 0.1% risk, respectively. The percentage of death in non-neonates hospitalized with a staphylococcal infection peaked at 2.5% in 2002 and decreased to 1.0% in 2009. The decrease may be partly explained by the relative increase of staphylococcal infections that are CO MRSA SSTIs, which carried a very small risk for death, although deaths continued to decrease even when CO MRSA infections started to decline.

More than half of non-neonates with a diagnosis of staphylococcal infection had CO-HCA or HO infections, and most had MSSA infections (42% and 36%, respectively), followed by MRSA infections (20% and 13%, respectively) and infections with other staphylococci (25% and 37%, respectively). The continued predominance of invasive MSSA infections among children with CO-HCA conditions has been confirmed by others (*8*). Infections with coagulase-negative staphylococci, common pathogens in patients with health care–related conditions, represent some of the infections caused by other staphylococcal (i.e., non-MRSA and non-MSSA) infections (*20*). Despite an increase in the number of hospitalized children with MRSA pneumonia and septicemia, the overall incidence of MRSA invasive disease was low. Children with CO-HCA or HO infections were more likely to have a diagnosis of invasive disease (pneumonia or septicemia) compared with children with CO disease. This clinical pattern is consistent with previously described patterns (*7,16,21,22*). Most CO non-neonatal hospitalizations in this study were due to SSTIs, and MRSA was coded more frequently than MSSA after 2002.

The incidence of HO staphylococcal infection increased until 2005 and has since stabilized. This finding may reflect implementation of more stringent infection-control procedures, including those in neonatal intensive care units. Other investigators have attributed a recent decrease in the national prevalence of inpatient MRSA infections to enhanced infection-control strategies, including active surveillance testing (*23*).

Limitations of this study include inherent concerns regarding the accuracy of ICD-9 coding (*24*); miscoding could have occurred. It is possible that MRSA infections were incorrectly reported in some cases because the code used to indicate resistance to methicillin can also be used to indicate resistance to other antimicrobial drugs in the penicillin family. As awareness of MRSA infections increased, it is likely that diagnostic testing for MRSA increased, leading to an apparent increase in incidence. We did not review medical records for patients identified with staphylococcal infection, and clinical and microbiologic diagnoses could not be verified. The incidence data presented here represent hospitalizations reported to a large state database, not numbers of individual patients. We attempted to correct for possible overcounting by excluding children transferred from 1 acute care institution to another within 2 days of admission. The incidence in nondecennial years was calculated by using state population estimates, which are subject to a small degree of error, pending revisions based on 2010 census data.

This analysis represents one of the largest population-based studies of staphylococcal infection–related hospitalizations among children. The substantial increase in

childhood hospitalizations with staphylococcal infection in California was driven by an increase in CO MRSA SSTIs. The highest incidence of hospitalizations with staphylococcal infection was among children <3 years of age, Black children, and children without private insurance. We also found that the incidence of hospitalizations with staphylococcal infection declined during the last 3 years of the study, corresponding to a decrease in the number of diagnoses of MRSA SSTIs. The reasons for these declines are unknown, but they could include changes in the epidemiology or strains of circulating S. *aureus* or changes by physicians in the management of uncomplicated MRSA infections. It is also possible that extensive MRSA-related prevention education efforts by local health departments are having an effect. Additional studies are needed to determine whether this trend in declining numbers of hospitalizations with staphylococcal infection will be sustained and to provide a better understanding of the epidemiologic or biologic factors or public health and infection-control interventions that may have contributed to the decline in MRSA hospitalizations.

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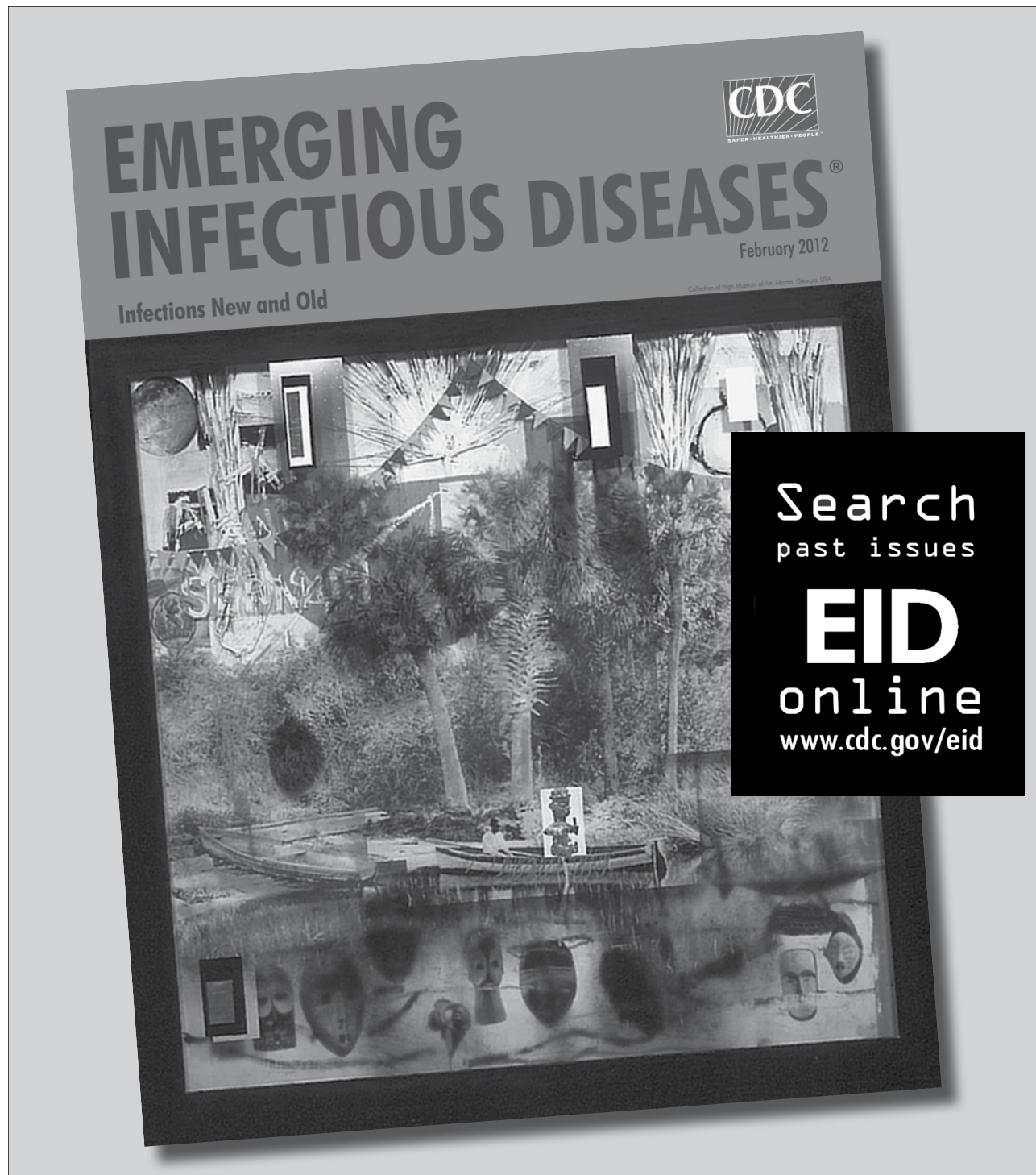
Pneumocystis jirovecii Genotype Associated with Increased Death Rate of HIV-infected Patients with Pneumonia

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Pneumocystis jirovecii dihydropteroate synthase (DHPS) mutations have been associated with failure of sulfa prophylaxis; their effect on the outcome of patients with *P. jirovecii* pneumonia (PCP) remains controversial. *P. jirovecii* DHPS polymorphisms and genotypes were identified in 112 cases of PCP in 110 HIV-infected patients by using PCR single-strand conformation polymorphism. Of the 110 patients observed, 21 died; 18 of those deaths were attributed to PCP. Thirty-three percent of the PCP cases involved a *P. jirovecii* strain that had 1 or both DHPS mutations. The presence or absence of DHPS mutations

had no effect on the PCP mortality rate within 1 month, whereas *P. jirovecii* type 7 and mechanical ventilation at PCP diagnosis were associated with an increased risk of death caused by PCP. Mechanical ventilation at PCP diagnosis was also associated with an increased risk of sulfa treatment failure at 5 days.

*P*neumocystis jirovecii causes severe pneumonia in immunocompromised patients, including HIV-infected persons, transplant recipients, patients receiving high-grade chemotherapy for hemato-oncologic diseases, and persons with autoimmune diseases who are treated with immunosuppressive drugs. Cotrimoxazole, the combination of sulfamethoxazole and trimethoprim (SMX/TMP), is the drug of choice for prevention of and treatment for *Pneumocystis* pneumonia (PCP). SMX/TMP targets enzymes involved in the biosynthesis of folic acid, dihydropteroate synthase (DHPS), and dihydrofolate reductase.

Several investigators have reported an association between failure of prophylaxis when using sulfa drugs and substitutions of 2 aa within the putative sulfa binding site of DHPS at positions 55 (Thr to Ala, mutation M1) and 57 (Pro to Ser, M2) (*1–4*). These mutations were observed either as single (M1 or M2) or double (M3) mutation. This association strongly suggested that *P. jirovecii* DHPS mutations conferred a level of sulfa resistance sufficient to cause failure of anti-PCP prophylaxis. However, the mutations might have also conferred a clinically substantial resistance to sulfa treatment for overt PCP.

To investigate the issue, many studies have analyzed the effect of the mutations on the outcome of PCP. About half of those studies did not detect any association between the mutations and an increased risk of death caused by PCP (*5–8*) or a decreased response to sulfa drugs (*3,9–11*). Conversely, other studies detected an association with a poor outcome (*12,13*): sulfa treatment failure (*14,15*); more severe symptoms and need of assisted ventilation (*13*); or a trend for a worse prognosis (*16*). Thus, the effect of these mutations on PCP outcome is unclear and justifies investigation to improve PCP treatment and prognosis.

The possibility of other parameters influencing PCP outcome has also been explored. *P. jirovecii* genotype Ne of the internal transcribed spacers (ITSs) of the nuclear rRNA operon has been associated with milder disease (*17*), failure of PCP prophylaxis (*18*), and failure of PCP treatment (*9*). One ITS genotype observed in Australia was associated with reduced severity of PCP (*13*). Specific *P. jirovecii* genotypes defined by single-nucleotide polymorphisms in 3 loci were associated with low or high burden during the course of PCP (*19*). In comparison, some studies did not detect any associations between *P. jirovecii* genotypes, including Ne genotype, and several clinical parameters, such as severity

and survival at 3 months (*15,20*). These observations suggested that some *P. jirovecii* genotypes might be more virulent or resistant to drugs, but further studies are needed to provide better understanding of the issue.

We previously examined *P. jirovecii* DHPS polymorphisms in clinical specimens of 158 immunosuppressed patients from 5 hospitals in the city of Lyon in France (*7*). We detected an association between DHPS mutation M2 and failure of prophylaxis when pyrimethamine/sulfadoxine was used but not between DHPS mutations and death caused by PCP. In this study, we further analyzed the proportion of the organisms harboring DHPS mutations (36%) and of death attributed to PCP (20%) among these 158 patients. We investigated in more detail the effect of DHPS mutations on PCP prognoses, taking into account more clinical parameters. Moreover, to test the hypothesis of variable virulence of some *P. jirovecii* genotypes, we identified those present in the specimens. Because the disease signs and symptoms vary considerably between HIV-infected and HIV-uninfected patients, we limited our analyses to the HIV-infected patients.

Patients, Materials, and Methods

Patients and Specimens

The specimens consisted of 112 bronchoalveolar lavage (BAL) samples obtained from 110 HIV-infected patients with confirmed PCP who were hospitalized in 5 university hospitals in Lyon, France. These 110 patients were a subset of the 158 patients analyzed (*7*) who were HIV-infected and who had a medical chart complete enough to support the analyses performed in the present study (Tables 1, 2). Two of the patients had second cases of PCP separated from their initial infections by 5 and 12 months, respectively; each was treated as an independent observation. BAL specimens were collected during April 1993 and December 1996 and were stored at –20°C before analysis. The 112 cases represented 47% of the PCP cases that occurred during this period in the 5 hospitals.

Characteristics of Patients

Specific information on demographic and clinical characteristics, treatment regimens, and PCP outcome were obtained from patients' medical charts. Death within 1 month after the date of PCP diagnosis was attributed to PCP when the physician recorded it as the primary cause of death in the medical chart and on the death certificate. Failure of sulfa treatment (SMX/TMP or dapsone) was defined by occurrence of ≥1 of the following events within 5 days after PCP diagnosis: a change of drug treatment because of lack of clinical improvement, worsening of clinical features or gas exchange parameters, addition of corticosteroids, new need of mechanical ventilation,

Table 1. *Pneumocystis jiroveci* dihydropteroate synthase genotype distribution according to clinical parameters of 112 cases of pneumonia in 110 HIV-positive patients from 5 university hospitals in Lyon, France*

Characteristic	DHPS genotype			p value
	Wild type, n = 75	M2, n = 17	M3, n = 20	
Age at PCP diagnosis, y				
1–40	41	10	14	0.62
41–60	31	7	5	
61–80	3	0	1	
Sex				
M	62	14	19	0.38
F	13	3	1	
Diagnosis year				
1993	6	2	0	0.58
1994	24	3	4	
1995	20	5	6	
1996	25	7	10	
CD4 cell count, median cells/μL†				
0–50	42	15	11	0.05
51–100	17	0	4	
>100	10	0	2	
First-line treatment				
SMX/TMP	59	14	15	0.90
Pentamidine or atovaquone	14	3	5	
Others	2	0	0	
SMX/TMP allergy				
Yes	4	0	3	0.27
No	71	17	17	
Corticotherapy at PCP diagnosis				
Yes	18	1	3	0.25
No	57	16	17	
Mechanical ventilation at PCP diagnosis				
Yes	11	0	3	0.26
No	64	17	17	
P. jirovecii SSCP type 1	3	6	1	<0.001
7	3	0	6	
10	2	1	1	
Others	30	3	3	
Co-infected	37	7	9	
Outcome within 1 month				
Favorable	61	16	14	0.39
Death attributed to PCP	12	1	5	
Death not attributed to PCP	2	0	1	

*Data derived from Fisher's exact test. DHPS, dihydropteroate synthase; M, mutation; SSCP, single-strand conformation polymorphism; PCP, *P. jirovecii* pneumonia; SMX/TMP, sulfamethoxazole and trimethoprim.
†Data missing for 11 patients.

or death attributed to PCP. Start-of-therapy dates were available for 81 patients. Therapy was started during 1 of 3 periods: before the day on which PCP was diagnosed (n = 34, 42%); the day on which PCP was diagnosed (n = 37, 46%); or after that day (n = 10, 12%). Therapy was started most frequently 1 or 2 days before the day on which BAL was obtained (n = 26, 77% of the 34 patients whose therapy was started before PCP was diagnosed). All chart abstractions were performed without knowledge of *P. jirovecii* and DHPS genotyping results. Informed consent was obtained from all patients. Study protocols and patient consent forms were approved by the institutional review board.

DHPS and Genotyping

DNA extraction from BAL specimens and DHPS binding site genotyping by using the PCR–single-strand conformation polymorphism (SSCP) technique were done as described (*7*). Four DHPS SSCP patterns were observed, each corresponding to 1 of the 4 known DHPS alleles (M1, M2, M3, and wild type). *P. jirovecii* present in BAL specimens were typed as described by using PCR-SSCP of 4 variable genomic regions (*21*). The variable regions analyzed were ITS1 of the nuclear rDNA operon, the intron of the nuclear 26S rRNA gene, the variable region of the mitochondrial 26S rRNA gene, and the region surrounding the intron no. 6 of the β-tubulin gene. In the PCR-SSCP technique, each allele is identified by a specific SSCP pattern made of 2 DNA bands, each corresponding to 1 of the 2 single strands of the allele.

Statistical Analysis

Because of small sample sizes, we used Fisher exact tests for general association to compare proportions

Table 2. *Pneumocystis jiroveci* SSCP genotype distribution according to clinical parameters of 112 cases of pneumonia in 110 HIV-positive patients from 5 university hospitals in Lyon, France*

| Characteristic | *P. jirovecii* SSCP type | | | | | p value |
	1, n = 10	7, n = 9	10, n = 4	Others, n = 36	Co-infected, n = 53	
Age at PCP diagnosis, y						
1–40	8	5	2	22	28	0.89
41–60	2	4	2	13	22	
61–80	0	0	0	1	3	
Sex						
M	3	1	1	6	6	0.44
F	7	8	3	30	47	
Diagnosis year						
1993	1	0	0	2	5	0.90
1994	3	2	2	8	16	
1995	2	2	1	9	17	
1996	4	5	1	17	15	
CD4 cell count, median cells/μL†						
0–50	7	5	3	23	30	0.87
51–100	0	2	1	6	12	
>100	1	1	0	5	5	
First-line treatment						
SMX/TMP	7	3	3	26	49	0.001
Pentamidine or atovaquone	2	6	1	9	4	
Others	1	0	0	1	0	
SMX/TMP allergy						
Yes	0	2	0	3	2	0.27
No	10	7	4	33	51	
Corticotherapy at PCP diagnosis						
Yes	0	4	0	7	11	0.17
No	10	5	4	29	42	
Mechanical ventilation at PCP diagnosis						
Yes	0	2	0	2	10	0.19
No	10	7	4	34	43	
Outcome within 1 month						
Favorable	9	5	4	28	45	0.44
Death attributed to PCP	1	4	0	6	7	
Death not attributed to PCP	0	0	0	2	1	

*Data derived from Fisher's exact test. SSCP, single-strand conformation polymorphism; PCP, *P. jirovecii* pneumonia; SMX/TMP, sulfamethoxazole and trimethoprim.
†Data missing for 11 patients.

relative to the numbers presented in Tables 1 and 2. We estimated the 2-month survival curves after PCP by using the Kaplan-Meier method and compared those curves by using log-rank tests. Cox proportional hazards method was used to identify independent prognostic factors associated with survival. The risk factors were selected a priori based on theoretical considerations; because no deaths occurred among women, we did not include gender in the variables. Also, the variable CD4 cell count was not included because it had 1) numerous missing values (n = 11, including 3 deaths attributed to PCP), and 2) no significant association with death attributed to PCP in bivariate analysis (p = 0.09). We used logistic regression analysis to identify factors associated with sulfa treatment failure; odds ratios with 95% CIs are reported. We tested the proportionality assumption using the nonzero slope test based on the scaled Schoenfeld residuals, and checked the colinearity (mean variance inflation factor = 1.16). All statistical analyses were conducted by using STATA version 11.1 (StataCorp LP, College Station, TX, USA).

Results

Specimens and Patients

One hundred twelve BAL specimens collected from 110 HIV-infected patients in whom PCP was diagnosed were included in the study. Two of the patients had second cases of PCP. Two patients also had tuberculosis, and 1 had histoplasmosis; each of the 3 recovered from PCP. The cohort ranged in age from 4–69 years (median 37 years); 94 (85%) were men. The most common risk factor for HIV was homosexuality (41%); the next most common risk factor was intravenous drug use (8%). CD4 counts at the diagnosis of PCP were documented in the medical charts of 101 patients and ranged from 0 to 390/μL (median 24). In 2 cases, patients had CD4 cell counts >200 cells/μL (242 and 390).

Detection of DHPS Alleles by using PCR-SSCP

We previously genotyped the *P. jirovecii* DHPS binding site from the 112 BAL specimens by PCR

amplification of a region of 318 bp, then SSCP (*7*). To simplify the analyses, specimens that contained a mixture of wild type and mutant DHPS genotypes (n = 11) were classified in the corresponding mutant category. For most of the cases, the patients harbored *P. jirovecii* with wild type DHPS (n = 75, 67.0%). Seventeen (15.2%) episodes involved a M2 mutant DHPS genotype, and 20 (17.8%) involved a M3 allele. The overall proportion of cases in which the patient had mutant DHPS was 33.0% (n = 37). There was a variation of this proportion from 25% to 57% among the 5 hospitals, but the difference did not reach statistical significance (*7*). No significant differences (p = 0.05) were found between patients who harbored *P. jirovecii* with DHPS mutations and those who did not for all the demographic or clinical characteristics analyzed.

Genotyping by Using PCR-SSCP

Twenty-seven *P. jirovecii* genotypes were identified among the 112 BAL specimens by using the multitarget PCR-SSCP typing method (*21*). Fifty-nine (52.7%) specimens contained a single *P. jirovecii* type, 47 (42.0%) specimens contained 2 types, and 6 (5.3%) specimens contained more than 2 types that could not be identified. The 5 most prevalent genotypes were type 1 (n = 29 occurrences), type 7 (n = 15), type 10 (n = 12), type 2 (n = 10), and type 6 (n = 10). The 2 patients who had 2 cases each of PCP were infected with different types for each case, suggesting possible de novo infection for each case, rather than reactivation of the type that caused the first case.

Associations between DHPS and *P. jirovecii* SSCP Genotypes and between Each Genotype and Clinical Factors

Possible associations between DHPS and *P. jirovecii* SSCP genotypes and between each DHPS and *P. jirovecii* SSCP genotype and the clinical factors were investigated for the 112 PCP cases (Tables 1, 2). Specimens co-infected with >2 *P. jirovecii* SSCP types were gathered into a specific category, and the 3 most prevalent types among specimens containing a single genotype were considered separately. The sample was stratified into 5 groups: type 1, type 7, type 10, other types, and co-infected specimens. The DHPS mutations were not distributed evenly among the *P. jirovecii* SSCP type (p<0.001, by Fisher exact test) (Table 1), type 7 being associated with genotype M3 (66.7% of the types 7). The DHPS mutations were also unevenly distributed among the CD4 cell count groups (p = 0.05); mutation M2 was associated with <50 cells/μL (100%). Finally, the first-line treatment varied significantly according to the *P. jirovecii* SSCP type (p = 0.001) (Table 2), type 7 being most often treated with pentamidine or atovaquone rather than SMX/TMP (66.7%).

Predictors of Death Attributed to PCP

Of the 112 observed cases in this study, 21 (18.8%) patients died within 1 month. PCP was identified as the cause of death for 18 patients. Because of the small number of observations, the categories *P. jirovecii* type 10 and others types were further grouped with co-infected specimens, forming 3 groups: type 1, type 7, and other types. Bivariate analyses revealed that *P. jirovecii* SSCP type 7 and the need for mechanical ventilation at PCP diagnosis were possible predictors of death attributed to PCP (log-rank test, p = 0.08 and p<0.0001, respectively) (Figure). No effect of the DHPS mutated alleles on the PCP mortality rate was observed within 1 month (p = 0.35). Because type 7 was associated with DHPS mutation M3 and with first-line treatment with pentamidine or atovaquone, we included these variables in a multivariable analysis (Table 3). This analysis showed that type 7 and the need for mechanical ventilation at PCP diagnosis were significantly associated with an increased risk of death caused by PCP (relative hazard = 4.2, 95% CI 1.0–17.9, p = 0.05, and relative hazard = 5.0, 95% CI 1.8–13.5, p = 0.002, respectively) (Table 3). Similar results were obtained when the DHPS genotype variable was removed from the model.

Predictors of Sulfa Drug Treatment Failure

To treat most cases, the patients received a sulfa drug as first-line treatment: for 88 (78.6%) cases, patients were treated with SMX/TMP, and for 2 (1.8%) cases, patients were treated with dapsone. Adverse effects of SMX/TMP in 5 patients led to a therapy change that excluded them from the analysis. Among the remaining 85 cases, 30 patients (35.3%) did not respond to the sulfa treatment at 5 days. Multivariate analysis of the same variables as for the analysis of predictors of death caused by PCP, except the first-line treatment, showed that an increased risk of failure of sulfa treatment was associated with the need for mechanical ventilation at PCP diagnosis (odds ratio 34.2, 95% CI 3.6–321.3, p = 0.002).

Discussion

Analysis of the parameters of 112 PCP cases in HIV-infected patients involving a high proportion of *P. jirovecii* DHPS mutations and deaths attributed to PCP did not show an association between these mutations and a worse PCP outcome. This observation contrasts with studies that reported some negative influence of these mutations on PCP prognosis (*12–15*) but converges with results of other studies reporting no effect (*3,5–11*). The lack of standardization of the outcome parameters analyzed in the different studies, the confounders used for adjustment, and the small number of cases in some investigations might explain these conflicting conclusions. Nevertheless, a strong effect would have
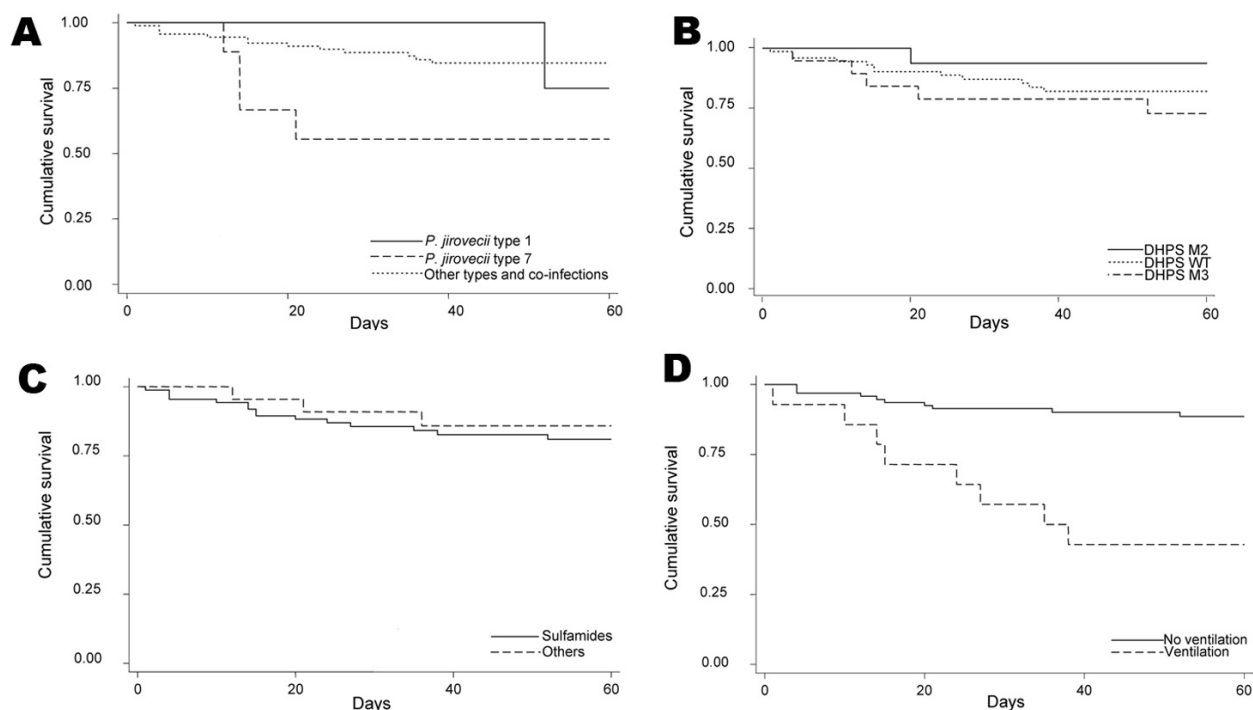
Figure. Kaplan-Meier survival plots for 4 variables of 112 *Pneumocystis* pneumonia cases. A) p = 0.08. B) p = 0.35. C) p = 0.60. D) p = 0.0001. DHPS, dihydropteroate synthase.

been identified even in studies with restricted sample sizes, suggesting that if these mutations have any effect on PCP outcome, it is small. Studies in the model organisms *Saccharomyces cerevisiae* (*22*) and *Escherichia coli* (*23*) strongly suggested that DHPS mutations confer some level of sulfa resistance to *P. jirovecii*. However, these reports did not speculate whether this level is sufficient to provoke clinical failure of sulfa treatment.

P. jirovecii type 7 was independently associated (i.e., nonnull partial correlation) with an increased risk for death caused by PCP. However, this type was also associated with DHPS mutation M3 (66.7% of the single infections), suggesting that the combination of the 2 parameters might have been necessary for its pathogenicity, even if mutation M3 alone was not a predictor of death caused by PCP. Consequently, we cannot exclude the role of mutation

Table 3. Multivariate analysis of risk factors for death attributed to PCP among 112 cases in 110 patients from 5 university hospitals, Lyon, France

Risk factor	Death attributed to PCP		Adjusted RH (95% CI)†	p value
	Yes	No		
P. jirovecii PCR-SSCP type				
7	4	5	4.2 (1.0–17.9)	0.05
Others	14	89	1.0 (Reference)	
Mechanical ventilation at PCP diagnosis				
Yes	8	6	5.0 (1.8–13.5)	0.002
No	10	88	1.0 (Reference)	
DHPS genotype				
M3	5	15	1.1 (0.4–3.4)	0.82
M2	1	16	0.6 (0.08–5.1)	0.66
Wild type	12	63	1.0 (Reference)	
Mean age at PCP diagnosis, y	38.5	40.6	1.0	0.95
First-line treatment				
Others	3	19	0.4 (0.1–2.0)	0.29
Sulfonamides	15	75	1.0 (Reference)	

*PCP, *Pneumocystis jirovecii* pneumonia; RH, relative hazard; SSCP, single-strand conformation polymorphism; DHPS, dihydropteroate synthase; M, mutation.
†RH for death attributed to PCP after adjustment for the 5 variables presented in the table. The global p value of the test for proportionality was 0.55, and no individual test was statistically significant at the 0.05 level.

M3 for the increased virulence of *P. jirovecii* PCR-SSCP type 7. Type 7 was also associated with first-line treatment with pentamidine or atovaquone rather than SMX/TMP, suggesting that use of these less effective drugs might have contributed to an increased number of deaths of patients infected with type 7. However, the proportion of deaths attributed to PCP among these patients was higher with SMX/TMP than with pentamidine or atovaquone (2 of 3 vs. 2 of 6 patients, respectively). *P. jirovecii* PCR-SSCP type 7 has not been reported to have a higher virulence than the other types. *P. jirovecii* ITSs type B2a1, as well as other genotypes, were suggested to have higher and modified virulence (*9,13,17–19*).

We observed that the need for mechanical ventilation at PCP diagnosis was associated with an increased risk for death caused by PCP, as was failure of sulfa treatment. An association of this comorbidity factor with an increased risk for death has already been reported (*24,25*). Several other clinical parameters were reported to be predictors for death caused by PCP in HIV-infected patients (*26*), and a scoring tool was recently proposed (*27*). Host polymorphisms within receptors involved in the immune response have also been reported to be related to *P. jirovecii* infection (*28,29*). This study confirms that certain *P. jirovecii* genotypes might have different pathogenic traits. Further studies of PCR-SSCP type 7 could help understanding of *P. jirovecii* virulence and drug resistance by clinicians and public health professionals.

Dr Rabodonirina is a parasitologist and a mycologist at the Hospices Civils de Lyon, as well as a lecturer at Lyon University. Her research interests include diagnosis, epidemiology, and drug resistance of opportunistic infections.

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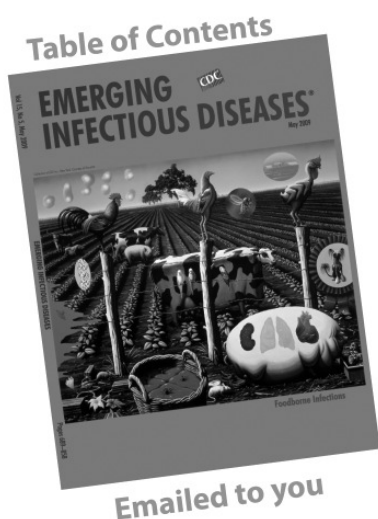
Infections with Spore-forming Bacteria in Persons Who Inject Drugs, 2000–2009

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Since 2000 in the United Kingdom, infections caused by spore-forming bacteria have been associated with increasing illness and death among persons who inject drugs (PWID). To assess temporal and geographic trends in these illnesses (botulism, tetanus, *Clostridium novyi* infection, and anthrax), we compared rates across England and Scotland for 2000–2009. Overall, 295 infections were reported: 1.45 per 1,000 PWID in England and 4.01 per 1,000 PWID in Scotland. The higher rate in Scotland was mainly attributable to *C. novyi* infection and anthrax; rates of botulism and tetanus were comparable in both countries. The temporal and geographic clustering of cases of *C. novyi* and anthrax into outbreaks suggests possible contamination of specific heroin batches; in contrast, the more sporadic nature of tetanus and botulism cases suggests that these spores might more commonly exist in the drug supply or local environment although at varying levels. PWID should be advised about treatment programs, injecting hygiene, risks, and vaccinations.

Clostridium and *Bacillus* spp. produce spores that can be found in soil, dust, human and animal intestines, and aquatic environments; these spores can remain viable for long periods (*1*). Spores can contaminate illicit drugs or drug-injecting equipment. If injected intravenously, intramuscularly, or subcutaneously, spores can germinate and produce potent neurotoxins or histotoxins that cause illness and death (*2*). In persons who inject drugs (PWID), these organisms often initially cause localized infections; however, the toxins they produce can result in severe systemic illness, which usually becomes apparent within a week after infection.

Infections with spore-forming bacteria in PWID have historically been more common in the United States than in Europe. By the 1950s, injection drug use accounted for most cases of tetanus in New York (*3,4*), and wound botulism associated with injecting black tar heroin was first described in California just over 2 decades ago (*5*). In contrast, such infections have occurred more recently in Europe; in the United Kingdom, for example, few infections had been reported before 2000 (*1*). Nevertheless, a recent article noted that 367 infections with spore-forming bacteria among PWID in Europe were reported during 2000–2009 (*6*). Although high rates of these infections were reported in northwestern Europe (United Kingdom, Norway, and Ireland), few cases have been reported elsewhere in Europe. The reasons for this marked regional variation within Europe remain unclear but might reflect drug trafficking routes, the type of drugs injected locally, and/or differences in local injecting practices (*6*).

In addition to the varied extent of these infections among PWID across Europe, some regional variation within the United Kingdom has been noted (*7*) but not fully explored. To further explore this variation, we compared the regional rates of infection and death caused by a small number of aerobic and anaerobic spore-forming bacteria among PWID in Scotland and England over a 10-year period beginning in 2000. The availability of detailed epidemiologic data on cases in England and Scotland enabled us to examine regional and temporal trends and demographic patterns. Information about differences in drug-injecting populations and practices that might be associated with infection could be used to prevent future infections.

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Materials and Methods

Case Ascertainment

We collated information about reported cases of infection with *Clostridium botulinum* (botulism), *C. tetani* (tetanus), *C. novyi*, and *Bacillus anthracis* (anthrax) among PWID in England and Scotland with dates of onset from January 2000 through December 2009. Information about suspected cases of botulism or tetanus was obtained from voluntary or statutory notifications to the Health Protection Agency and Health Protection Scotland; reports included information about possible risk factors. Corresponding samples were sent to the Foodborne Pathogens Reference Unit, the Special Pathogens Reference Unit, or the Anaerobic Reference Laboratory for the detection of toxin and microbiological confirmation. Confirmation criteria have been described (*8,9*). Clinical, demographic, and risk factor information was obtained from a questionnaire administered to patients by clinicians or microbiologists. Information about cases of *C. novyi* infection and anthrax were obtained from reports and documentation of the respective outbreaks (*7,10–12*); case definitions are described in these reports. The analyses presented here are limited to definite and probable *C. novyi* infections and confirmed anthrax cases.

Data Analysis

To derive infection rates, we used regional estimates of the number of PWID in England (2004–05 fiscal year) and Scotland (2006), closest to the midpoint of the 10-year period (2000–2009) (*13,14*). Both sets of estimates of PWID populations were derived by log–linear modeling of capture–recapture data. Numbers of infections were tabulated by region (England) and National Health Service Board area (Scotland), and rates per 1,000 PWID were calculated.

Numbers of infections were also tabulated by sex, and median age of case-patients was calculated. To compare demographics, we compared the sex distribution and median age of our study population with that derived from national surveys of PWID in England and Scotland (these data were not available from the capture–recapture PWID estimates described above) undertaken in years closest to the midpoint of the 10-year period. For England, we used data from the 2005 Unlinked Anonymous Monitoring Survey of PWID (*15*) and, for Scotland, the 2008–2009 Needle Exchange Surveillance Initiative (*16*). These 2 surveys aimed to recruit representative samples of PWID in contact with specialist services; the numbers of PWID participating in these surveys who had injected in the preceding 4 weeks were 1,740 and 1,772, respectively. We compared national survey respondents and case-patients in terms of sex and age by using χ^2 tests (or Fisher exact tests

when there were <5 persons in a given tabular cell) and Wilcoxon rank tests, respectively.

Results

During January 1, 2000–December 31, 2009, a total of 295 infections caused by spore-forming bacteria (157 botulism, 33 tetanus, 92 *C. novyi*, and 13 anthrax) were reported among PWID in England and Scotland; the overall infection rate was 1.83 cases per 1,000 PWID. Two thirds (199) of these cases were reported in England and one third (96) in Scotland, corresponding to rates of 1.45 and 4.01 per 1,000 PWID, respectively (Table 1).

The number of reported cases varied over time (Figure 1). The *C. novyi* infections and anthrax cases were clustered in 2000 and 2009, respectively, and most tetanus cases occurred during 2003–2005. By contrast, botulism was reported in all years; the annual number of cases varied from 3 to 41.

Infection rates varied by health region. In England, rate of infection varied from 0.68 cases per 1,000 PWID for the West Midlands to 2.02 for the East of England (Figure 2); rates were also high for the East Midlands, London, and the North West (1.7, 1.9, and 1.7 cases/1,000 PWID, respectively). In Scotland, rates ranged from zero in 3 rural areas with small populations of PWID (Ayrshire and Arran, Borders, and Highlands) to 7.7 per 1,000 PWID in Greater Glasgow and Clyde; rates were also high in Grampian (3.6 cases/1,000 PWID) and Fife (3.9 cases per /1,000 PWID).

In terms of specific infections, the rate of botulism was slightly higher for England than for Scotland, although this difference was not statistically significant (1.0 vs. 0.8 cases/1,000 PWID, p = 0.232), and rates of tetanus were similar for both countries (0.20 vs. 0.21/1,000 PWID, p = 0.962). In contrast, rates of *C. novyi* infections and anthrax were markedly higher for Scotland than for England (2.5 vs. 0.2 cases/1,000 PWID, p<0.001; and 0.5 vs. 0 cases/1,000 PWID, p<0.001, respectively). *C. novyi* infections were particularly concentrated in Greater Glasgow and Clyde (5.6 cases/1,000 PWID) and in the North West region of England (0.7 cases/1,000 PWID). Higher than average rates of botulism were reported in the East of England region (1.8 cases/1,000 PWID) and in Grampian (2.0 cases/1,000 PWID).

When we compared the demographic characteristics of case-patients with those of PWID participating in the 2 national surveys, we found that the proportion of female patients with tetanus, *C. novyi* infection, and anthrax was higher (38%–60%) than the proportion of female PWID in the community (24%–26%) (Table 2). These differences were statistically significant for *C. novyi* infections in England and Scotland (p = 0.011 and p<0.001, respectively) and for tetanus cases in England. In England, the median age of PWID with botulism, tetanus, and *C. novyi* infection

Table 1. Cases of infection with spore-forming bacteria and rates of infection among PWID, by health region, England and Scotland, 2000–2009*

| Health region | No. PWID† | No. cases (rate/1,000 PWID) | | | | Total no. cases (rate/1,000 PWID, 95% CI) |
		Botulism	Tetanus	*Clostridium novyi* infection	Anthrax	
England	137,141	139 (1.01)	28 (0.20)	32 (0.23)	0	199 (1.45, 1.22–1.65)
East of England	9,418	17 (1.81)	0	2 (0.21)	0	19 (2.02, 1.45–3.04)
East Midlands	11,796	15 (1.27)	4 (0.34)	1 (0.08)	0	20 (1.70, 1.48–1.91)
London	17,909	28 (1.56)	4 (0.22)	2 (0.11)	0	34 (1.90, 1.42–2.10)
North East	8,959	6 (0.67)	1 (0.11)	0	0	7 (0.78, 0.66–0.99)
North West	22,089	14 (0.63)	7 (0.32)	16 (0.72)	0	37 (1.68, 1.47–1.97)
South East	13,778	9 (0.65)	3 (0.22)	5 (0.36)	0	17 (1.23, 0.95–1.41)
South West	17,444	23 (1.32)	3 (0.17)	1 (0.06)	0	27 (1.55, 1.38–1.69)
West Midlands	14,734	4 (0.27)	5 (0.34)	1 (0.07)	0	10 (0.68, 0.59–0.74)
Yorkshire and the Humber	21,014	23 (1.09)	1 (0.05)	4 (0.19)	0	28 (1.33, 1.23–1.41)
Scotland‡	23,933	18 (0.75)	5 (0.21)	60 (2.51)§	13 (0.54)	96 (4.01, 3.43–4.29)
Ayrshire and Arran	2,373	0	0	0	0	0
Borders	201	0	0	0	0	0
Dumfries and Galloway	486	0	1 (2.06)	0	0	1 (2.06, 1.49–2.70)
Fife	1,270	2 (1.57)	0	2 (1.57)	1 (0.79)	5 (3.94, 3.27–4.64)
Forth Valley	786	1 (1.27)	0	0	0	1 (1.27, 1.04–1.52)
Grampian	3,056	6 (1.96)	2 (0.65)	3 (0.98)	0	11 (3.60, 2.83–4.48)
Greater Glasgow and Clyde	8,862	8 (0.90)	1 (0.11)	50 (5.64)	9 (1.02)	68 (7.67, 5.74–9.17)
Highland	734	0	0	0	0	0
Lanarkshire	1,649	0	0	2 (1.21)	2 (1.21)	4 (2.43, 1.92–4.46)
Lothian	3,262	0	1 (0.31)	0	0	1 (0.31, 0.23–0.40)
Tayside	1,254	1 (0.80)	0	0	1 (0.80)	2 (1.59, 1.26–1.97)

*PWID, persons who inject drugs.
†References *13,14*.
‡Regions of mainland Scotland only.
§Region not known for 3 of the 60 case-patients who had *C. novyi* infection.

ranged from 33 to 37 years; this age range was higher among those with botulism than among the PWID participating in the Unlinked Anonymous Monitoring Survey (37 vs. 32 years; p<0.001). In Scotland, the median ages of PWID with botulism, *C. novyi* infection, and anthrax were comparable to the median age of PWID from the community sample; whereas, the median age was higher for PWID infected with tetanus (47 vs. 33 years), although not significantly so (p = 0.065).

Of the 295 reported case-patients, 52 (18%) are known to have died. Of these, 8 (5%) died of botulism, 2 (6%) died of tetanus, 36 (39%) died of *C. novyi* infection, and 6 (46%) died of anthrax.

Discussion

Over the decade beginning in 2000, almost 300 severe infections caused by spore-forming bacteria were reported among PWID in England and Scotland; 52 of these patients died. The distribution of the cases varied markedly between these countries. In Scotland, the number of cases was excessive relative to the estimated population of PWID when compared with England; this excess, however, is mainly attributable to an excess of *C. novyi* infections and anthrax cases. In contrast, rates of botulism and tetanus for Scotland were lower than and comparable with, respectively, those for England.

In the United Kingdom, microbiological testing has usually been unable to confirm the presence of these

bacterial species in seized or surrendered heroin (*2*), although, in 2009, *C. botulinum* was isolated from 1 sample of heroin seized in Scotland (K.A. Grant, pers. comm.). Nevertheless, it is generally recognized that the infections discussed here have resulted from contaminated heroin, which might have become contaminated during processing, transport, or storage. In the United Kingdom, 90% of heroin used originates in Afghanistan, where the opium is produced and—increasingly since 2002—converted to heroin. Heroin from Afghanistan usually travels over land, passing through several countries before entering the European Union and reaching the United Kingdom (*17,18*). The conditions in which heroin is processed, transported, and stored are uncertain; because these activities are illegal, they all probably make the drug vulnerable to inadvertent contamination with bacterial spores, for example, from soil or dust. Contaminated heroin is thought to have been the source of *B. anthracis* infection in a drug injector in Norway in 2000 (*19,20*) and in the more recent outbreak among PWID in Europe (*12*). Another source of potential contamination is drug adulterants (cutting agents), which are widely used to dilute and increase the bulk of illicit drugs (*21*). Although most infections probably resulted from upstream (before it reaches the end user) contamination of heroin, spores on the soiled hands of users and dirty needles could be inoculated during the injection process (*22*). This mode of infection remains unproven, although signs of tetanus were observed
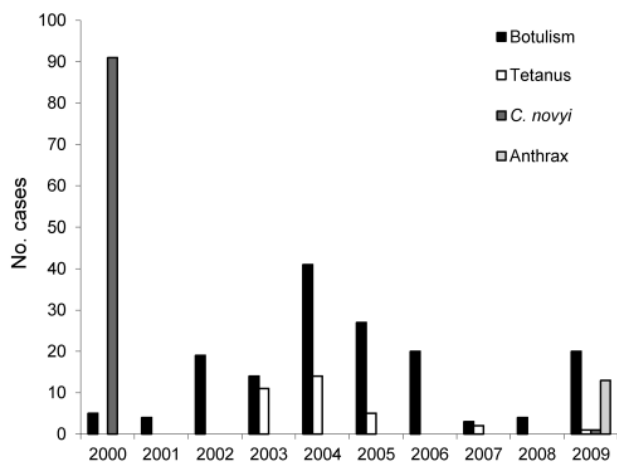
Figure 1. Annual numbers of cases of botulism, tetanus, *Clostridium novyi* infection, and anthrax among persons who inject drugs, England and Scotland, 2000–2009.

by Arthur Nicolaier in 1884 after he injected garden soil containing *C. tetani* (at that point unnamed) into animals (*23*), and clostridial infections after injection through dirt-covered hides have been reported (*24*).

Although the presence of bacterial spores is a necessary prerequisite for infection, several other factors might influence the development and geographic patterns of infections. The clustering of cases of *C. novyi* infection; anthrax; and, to a lesser extent, tetanus into outbreaks suggests that the contamination might have affected specific batches of heroin. By contrast, the botulism cases were generally more sporadic (albeit with some clustering) (*25,26*), suggesting that *C. botulinum* and, to a lesser degree, *C. tetani* spores might be more commonly present in the drug supply or in the local environment but at varying levels of contamination. Different drug supply routes serving eastern and western England and Scotland (*12*) might account for some of the geographic patterns and are consistent with the excessive *C. novyi* infections among PWID in Greater Glasgow and Clyde (western Scotland) and the North West region of England and with the higher rates of botulism among PWID in the East of England and Grampian (eastern Scotland).

Practices such as skin or muscle popping (intentionally or accidentally injecting into skin or muscle) (*10,27,28*) or the use of large amounts of citric acid to dissolve heroin can damage soft tissue, leading to necrosis and providing a suitable environment for anaerobic bacteria, such as *Clostridium* spp., to thrive. Older age (a proxy for a longer injecting career) and female sex have been associated with infections and injuries at injecting sites (*29,30*), which are associated with difficulty accessing veins. These persons might resort to injecting into the skin or muscle.

Geographic variation in these practices might explain some of the variations seen in this study. PWID across England and Scotland might be regularly exposed to botulism and tetanus spores, but the levels of infection might be higher in some areas where skin or muscle popping is more common. This finding is consistent with the high proportion of women and the older median age among PWID with clostridial or *B. anthracis* infections described here in comparison with the wider population of PWID in England and Scotland. The emergence of these infections as a major public health issue in the United Kingdom and Ireland (*6*) over the past decade might reflect the changing characteristics of the drug-using population, an aging cohort of users resulting from the marked increase in injection drug use during the 1980s and 1990s (*31*). With regard to tetanus, variation could also reflect differences in the levels of effective immunization among PWID.

This analysis captures only the anthrax cases reported before the end of December 2009; however, the anthrax outbreak continued into 2010 and resulted in a total of 52 confirmed cases, including 5 in England (*12,32*). The risk factors for anthrax might differ from those for the other infections/diseases because anthrax is the only disease considered here that is caused by an aerobic bacterium. Furthermore, we cannot exclude the possibility of inhalational anthrax in some of the case-patients who reported smoking heroin (*11,12*). We considered only confirmed cases of anthrax in this analysis; however, the inclusion of probable cases (although it would have increased the numbers and rates) most likely would not have changed
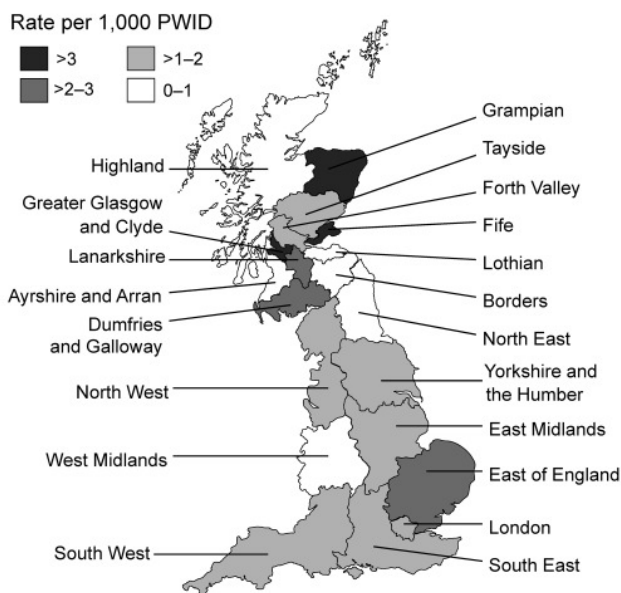
Figure 2. Rates of infection with spore-forming bacteria (*Clostridium botulinum*, *C.tetani*, *C. novyi*, and *Bacillus anthracis*) among persons who inject drugs (PWID), by health region, England and Scotland, 2000–2009.

Table.2. Sex distribution and median age of PWID with botulism, tetanus, *Clostridium novyi* infection, and anthrax among a community sample of PWID, England and Scotland, 2000–2009*

	England						Scotland					
PWID	No.†	Female, %	p value‡	No.†	Median age, y (IQR)	p value§	No.†	Female, %	p value‡	No.	Median age, y (IQR)	p value§
Community¶	1,732	24		1,711	32 (11)		1,763	26		1,772	33 (10)	
With botulism	139	30	0.157	128	37 (13)	<0.001	18	11	0.186	13#	31 (5)	0.670
With tetanus	28	46	0.006	28	35 (19)	0.067	5	60	0.112	5	47 (17)	0.065
With *C. novyi* infection	32	44	0.011	32	33 (11)	0.654	60	52	<0.001	57#	30 (8)	0.086
With anthrax		NA			NA		13	38	0.297	13	35 (9)	0.093
Total with infection	199	32	0.002	188	35.5 (13.5)	<0.001	96	43	<0.001	88	31 (9)	0.232

*PWID, persons who inject drugs; IQR, Interquartile range; NA, not applicable.
†For the community samples, information about sex of 8 (England) and 9 (Scotland) persons was missing, and information about age for 29 persons (England) was missing.
‡Based on the χ^2 test or Fisher exact test (where tabular cell counts were <5 for the difference in proportion of female case-patients with the respective infections and the community sample of PWID).
§Based on the Mann-Whitney test for the difference in median age between the case-patients with the respective infection(s) and the community sample of PWID.
¶Data from the Unlinked Anonymous Monitoring Survey 2005 (*15*) for England (n = 1,740) and from the Needle Exchange Surveillance Initiative 2008/2009 (*16*) for Scotland (n = 1,772).
#Complete data on age were available for 13/18 botulism case-patients and 57/60 C. *novyi* case-patients.

our findings with regard to demographic characteristics, given that probable and confirmed cases were similar in terms of age (mean 34 vs. 35 years, respectively) and sex (29% vs. 30% female, respectively) (*12*).

Because infections might go unreported or be misdiagnosed, the data presented here potentially underestimate the actual numbers of infections among PWID in England and Scotland. For tetanus and botulism, little toxin is required to cause symptoms; therefore, in combination with a reported history of injection drug use, index of clinical suspicion should be high (*33–35*). However, tetanus cases are underreported because some clinicians are not familiar with this rare disease (*36*). Misdiagnosis of infection might also account for underreporting because the symptoms of other illnesses can resemble those of the infections of interest in this study (e.g., Guillain-Barré syndrome vs. botulism) (*33,34*). In addition, if an injection site infection is treated promptly with broad spectrum antimicrobial drugs before tissue samples are collected, microbiological confirmation might not be possible (*7*).

Another limitation of this study is associated with estimates of the size of the PWID population. The estimates from Scotland and England were produced by using indirect methods by the same team but were based on different data sources and definitions. Moreover, estimates produced by indirect methods are difficult to validate. For example, the national study used here estimated 17,909 PWID in London (*13*), but another study estimated >30,000 PWID in London for 2000–2001 (*37*).

Because the quality and safety of illicit heroin is not monitored or controlled, sporadic cases and outbreaks of illness associated with spore-forming bacteria among PWID might continue. Persons who use heroin should be encouraged to seek treatment for their dependency. Health care professionals should educate PWID who continue to inject about injecting hygiene, the risks from specific injecting practices that have been associated with these infections, the need to ensure that their tetanus vaccinations are up to date, and the need to seek care if they have symptoms of an injection-related infection. Public health professionals should continue to be vigilant to ensure prompt detection of outbreaks and so permit the rapid dissemination of advice.

Acknowledgment

Ms Palmateer is an epidemiologist with Health Protection Scotland. Her work focuses on the epidemiology and prevention of bacterial and viral infections, primarily hepatitis C virus infections, among PWID.

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Automated Biosurveillance Data from England and Wales, 1991–2011

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Outbreak detection systems for use with very large multiple surveillance databases must be suited both to the data available and to the requirements of full automation. To inform the development of more effective outbreak detection algorithms, we analyzed 20 years of data (1991–2011) from a large laboratory surveillance database used for outbreak detection in England and Wales. The data relate to 3,303 distinct types of infectious pathogens, with a frequency range spanning 6 orders of magnitude. Several hundred organism types were reported each week. We describe the diversity of seasonal patterns, trends, artifacts, and extra-Poisson variability to which an effective multiple laboratory-based outbreak detection system must adjust. We provide empirical information to guide the selection of simple statistical models for automated surveillance of multiple organisms, in the light of the key requirements of such outbreak detection systems, namely, robustness, flexibility, and sensitivity.

The past decade has witnessed much interest in real-time outbreak detection methods for infectious diseases, driven by worries about the possibility of large-scale bioterrorism, public concern about emerging and reemerging infections, and the increased availability of computerized data (1–3). More prosaically, outbreaks of commonly occurring pathogens, notably, those causing infectious intestinal disease, remain a serious public health issue, causing an appreciable number of deaths and imposing a substantial drain on public health resources in many countries (4,5).

In England and Wales, automated laboratory surveillance of infectious diseases has been undertaken since the early 1990s. Laboratory surveillance is based on counts of laboratory isolates of infectious pathogens, usually classified for epidemiologic purposes by subtype or phage type. The organism reports come mainly from samples sent to hospital laboratories or to specialist laboratories when additional typing is required, as for salmonellae.

This automated system was designed to supplement the frontline investigator-led outbreak detection methods used by national and regional epidemiologists, with the primary aim of identifying geographically distributed outbreaks that may have escaped local detection. In a typical week, several hundred different pathogens are reported; the automated system provides a back-up and the assurance that the entire database is routinely scanned. The output comprises a short list of organisms with potential outbreaks for review, ranked according to an exceedance score that measures the degree of statistical aberrance. The statistical methodology of the system was described previously (6) and has since been applied in Scotland (7) and in several other European countries (8).

Much research on statistical methods of prospective outbreak detection has been aimed at identifying unusual clusters of 1 syndrome or disease (9–12), and some work has focused on multivariate surveillance methods (12). However, little research has been directed toward developing outbreak detection methods that are suited to large, multiple surveillance systems involving thousands of different organisms, such as the system used in England and Wales.

We are reviewing the statistical methods used in the England and Wales system. The first stage of this review, reported here, has been to carry out a detailed analysis of the data accumulated over the 2 decades since 1991. We aimed to document some of the generic features of surveillance data and their imperfections across the range of organisms of interest and to identify the key problems confronting automated outbreak detection systems. Specifically, we endeavored to answer 2 key questions: How diverse are the patterns displayed by the range of

organisms monitored? How complex must a statistical algorithm be to handle this diversity?

Data and Methods

Data

The data were provided by the Health Protection Agency (www.hpa.org.uk) from their LabBase surveill-ance database. This is a computerized database that receives details of all organisms reported by participating laboratories (the numbers of which vary from week to week) in England and Wales. The data are routinely subjected to intensive de-duplication checks at the time of report. A single report contains a data trail that starts with the date of collection of the first specimen and ends with the date at which the complete identification of the organism is entered into the database. The delay between the first specimen date (hereafter referred to as specimen date) and the date of final entry (referred to as report date) is a key feature of all systems of laboratory surveillance. The outbreak detection system operates on the basis of report dates (current and historical); the alternative is to operate on the basis of specimen dates, which requires explicit modeling of the delay distribution.

The outbreak detection system runs automatically every weekend, processing the previous week's reports. Thus, the time unit of analysis is by the week unless otherwise specified. We obtained weekly counts of all infectious disease organisms reported to the Health Protection Agency between week 1, 1991, and week 52, 2011, by date of report and date of specimen collection. In years with 53 weeks, the week 53 count was added to the week 1 count of the following year. To mitigate the effect of delays at the end of the series, only isolates with specimen dates through week 26 of 2011 were used in the analyses. All analyses are by week of specimen collection unless otherwise specified.

Data Processing

Calculating rates and other organism-specific statistics is complicated by the fact that it is not possible to distinguish between genuine zeroes, corresponding to organisms looked for but not found, and missing values that arise when organisms are not sought. It is highly likely that some organisms that were identified toward the end of the study period would not have been identified by the tests that were performed a decade or so earlier. Rates and trends calculated without taking any account of this feature would be biased. To reduce this bias, we recoded all leading sequences of zeroes as missing. However, this in turn introduces a selection bias, because every time series would then start with a nonzero count. To mitigate this, we reduced the first nonzero count by 1.

Statistical Models

The statistical models are described informally; a technical account is provided in the online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/12-0493-Techapp.pdf). To summarize the mean frequencies, trends, and seasonality of each organism, we used log-linear models structured as follows:

log (average count in week t) = baseline + trend at week t + seasonality at week t

We fitted a range of such log-linear models to the data, incorporating a smooth long-term trend component and monthly seasonality for each series of organism counts (*13,14*). A key aim was to identify a simple family of models that adequately represents all organisms. The simplest model is the Poisson model, for which the variance of the count in week t equals its average value. The model is convenient and easy to automate, but restrictive. We therefore considered other convenient but less restrictive models where

Variance of count in week t = dispersion × average count in week t (1)

Models of this form are called quasi-Poisson. The dispersion in Equation 1 is a constant specific to each organism. In a Poisson model, the dispersion is equal to 1. When the dispersion is >1, more variability is thereby allowed.

We also investigated the negative binomial model. This model satisfies equation 1 but also allows a greater degree of skewness (that is, asymmetry around the mean) than the Poisson model. For the negative binomial model,

Skewness of count in week t = antilog [constant – 0.5 × log (average count in week t)] (2)

where the constant in equation 2 is nonnegative. For the Poisson model, this constant is zero: equation 2 allows greater positive skewness.

Model Evaluation: Relationships between Mean, Variance, and Skewness

We sought a simple family of models that adequately describes all organisms, rather than a well-fitting model for any particular organism. Formal goodness-of-fit tests were not used because they can be unreliable with sparse data. Our criterion was that the relationships between mean, variance, and skewness should be adequately described. To display these relationships for each organism, we subdivided the data into 41 half-years, dropped weeks 52 (or 53) and 1 (which are atypical, as noted above), and de-seasonalized the data. We then calculated the mean, variance, and skewness in each half-year.

For each organism, we investigated the validity of equation 1 by plotting the log of the variance of the weekly counts against the log of the average weekly count in the 41 half-years. If equation 1 holds, the points should lie on

a straight line with slope 1. We obtained the histogram of these slopes; a narrow spread around 1 suggests that the quasi-Poisson model is adequate.

Similarly, we investigated the validity of equation 2 by plotting the skewness of the weekly counts against the log of the average weekly counts. If equation 2 holds, the points should lie on the curve determined by this equation, for which the coefficient of the log of the average weekly count is −0.5. We obtained these coefficients and plotted their histogram; a narrow spread around −0.5 suggests that the negative binomial model is adequate.

Results

We present the results in 5 subsections: global features of the surveillance system; frequency distributions; means, seasonality and trends; dispersion; and relationships between mean, variance, and skewness. Additional details are available in the online Technical Appendix.

Global Features of the Surveillance System

More than 9 million individual isolates were collected with specimen dates from week 1 in 1991 through week 26 in 2011. These isolates were of 3,303 different organism types. Figure 1 shows the time series of counts of organism types and organism isolates by week of specimen collection. The number of types is highly seasonal with summer peaks; such seasonality is less apparent for individual isolates because of the large number of distinct, rare enteric infections. Also apparent are troughs at weeks 52 and 1, representing lower activity over the Christmas period.

The strong upward trends shown in Figure 1 represent a genuine increase in numbers of isolates and organism types over time, rather than an increase in the number of reporting laboratories. The numbers of laboratories reporting to the system tended to decline over time (online Technical Appendix Figure 1). When ordered by date of report, the number of laboratories reporting is fewer than when ordered by date of specimen, reflecting batching of reports (some laboratories wait to accumulate isolates before reporting them). This factor is a notable source of additional noise in the surveillance system when considering counts by week of report. This batching will also affect the timeliness of the surveillance system.

On average, the weekly count of isolates is the same, whether ordered by week of specimen or by week of report; this is also true of organism counts. The variation in the differences in counts reflects the variability in delays from specimen collection to reporting, which can be considerable (online Technical Appendix Figure 2).

The distribution of delays between date of specimen and date of report varies from organism to organism, with the median typically in the range of 7–28 days, depending

on the complexity of the laboratory procedures involved. For example, modal delays for salmonellae are increased by the additional subtyping step required. Extreme delays are not uncommon, owing to late submissions or data entry errors (online Technical Appendix Figure 3).

Frequency Distributions

There is huge variation in frequency, seasonality, and trends among the 3,303 organism types reported. Figure 2 exemplifies this variation, even among more common organisms. Most organisms were seldom reported; of the 3,303 recorded organisms, nonzero counts occurred in only 1 week for 637 organisms (19%), in only 2 weeks for 291 (8.8%), and in only 3 weeks for 225 (6.8%). At the other end of the scale, for 30 organisms the count by week of specimen was nonzero for each of the 1,070 weeks (including the 4 occurrences of a 53rd week) spanning the
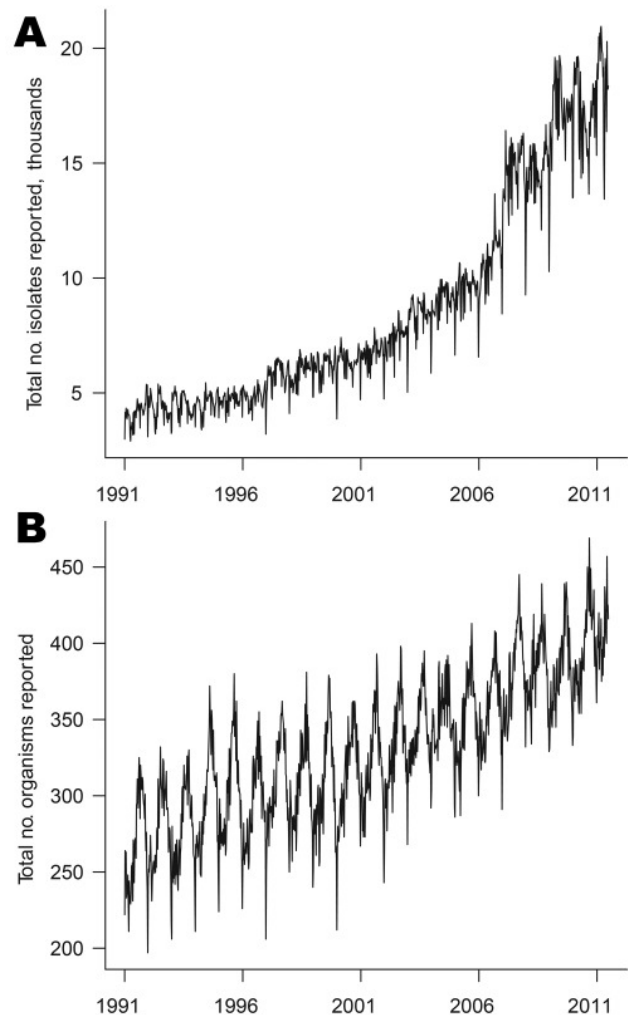
Figure 1. Weekly counts of organisms by date of specimen collection, England and Wales, 1991–2011: A) isolates; B) organism types.
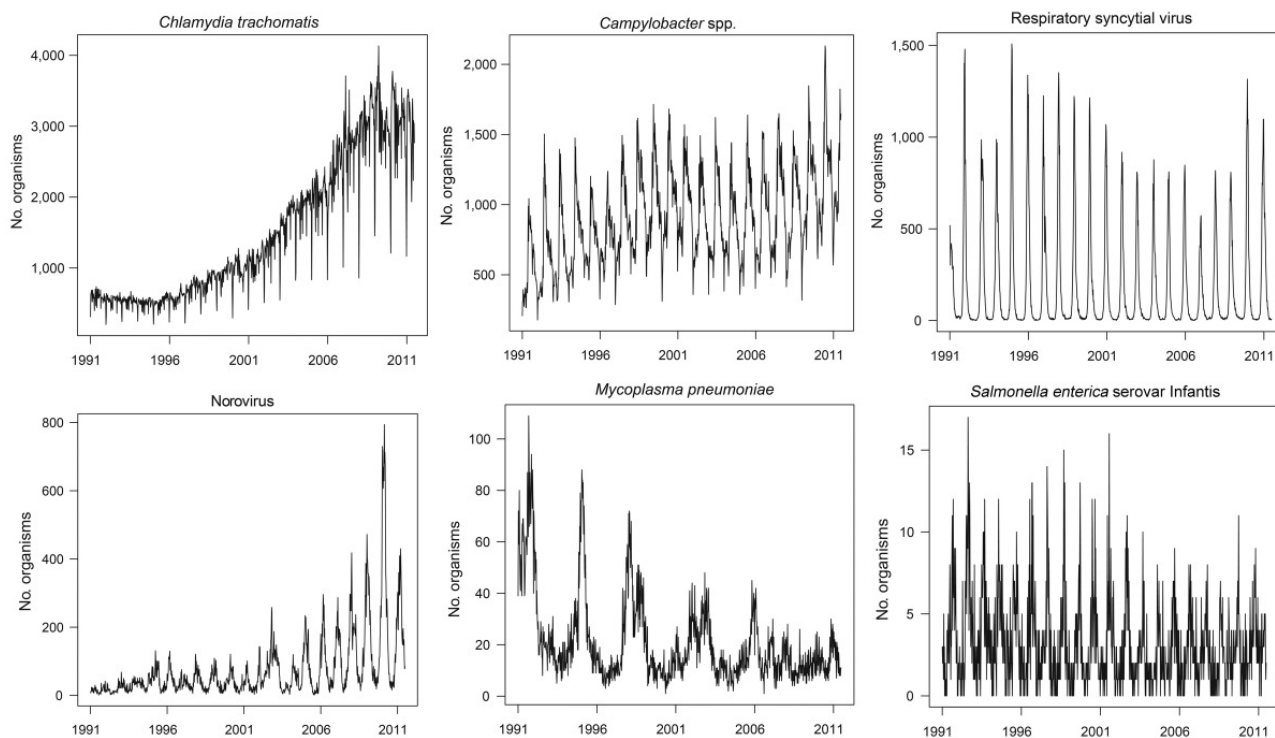
Figure 2. Weekly counts for 6 selected organisms, by date of specimen collection, England and Wales, 1991–2011.

period. These organisms are listed in Table, along with the mean weekly counts by week of specimen.

This variation in the number of nonzero counts is mirrored by the maximum weekly count for each organism. For 90% of all organisms, the weekly maximum was ≤12; 1,651 (50%) had a maximum weekly count of 1. The remaining 10% includes several organisms with maximum weekly counts of several thousand, such as *Chlamydia trachomatis* (maximum 4,133) and *Staphylococcus aureus* (maximum 2,317).

Means, Seasonality, and Trends

The large increase in numbers of organisms reported over time (Figure 1, panel B) suggests that laboratory procedures have changed over time. A total of 2,675 organisms with nonzero counts were left after recoding leading sequences of zeroes. The median weekly count for 2,408 (90%) of these organisms was zero. Figure 3 shows histograms of the mean and standard deviation of weekly counts by organism. The means span 6 orders of magnitude. There is also much variation in standard deviations, the 3 largest being for *C. trachomatis* (982.6), *S. aureus* (637.8), and rotavirus (338.4). The means for these organisms are 1,480, 764, and 303, respectively.

We fitted log-linear models to the 2,254 organisms for which nonzero counts spanned >1 year. The distribution of slope parameters for linear trend is shown in Figure 4, panel

A: 1,107 organisms display some evidence of an increasing trend (positive slopes, of which 655 are significant at the 5% level) and 1,146 of decreasing trend (negative slopes, 683 are significant).

Figure 4, panel B, shows the bar chart of the modal season for the 2,254 organisms analyzed. Every period is modal for some organism, though organisms with summer peaks predominate. However, the seasonal effect was significant at the 5% level for only 723 (32%) of the 2,254 organisms analyzed.

Some organisms displayed evidence of nonconstant seasonality. Rotavirus, for example, which typically peaks in the early months of the year, had slightly earlier peaks in the earlier years of data collection.

Dispersion

For 1,333 (59%) organisms, the dispersion (that is, the ratio of variance to mean, equation 1) is >1, indicating that the variability of weekly counts of that organism is greater than that of a Poisson distribution. There is a general tendency for the dispersion to increase with the mean: the more common the organism, the less appropriate a Poisson model tends to be (online Technical Appendix Figure 4, left panel). For many organisms, the dispersion is greater when calculated from data based on week of report, than when calculated from data based on week of specimen (online Technical Appendix Figure 4, right panel). This extra

Table. Organisms (mean weekly count, by specimen collection week) with nonzero specimen counts in every week from 1991 to mid-2011, England and Wales

Organism name	Mean weekly count
Chlamydia trachomatis	1,480
Campylobacter spp.	899
Staphylococcus aureus	764
Clostridium difficile toxin detection	313
Rotavirus	303
Escherichia coli untyped	267
Staphylococcus coagulase negative	167
Streptococcus pneumoniae	119
Herpes simplex virus untyped	102
Herpes simplex virus type 2	100
Pseudomonas aeruginosa	96
Herpes simplex virus type 1	92
Cryptosporidium spp.	86
Giardia lamblia	86
Clostridium difficile not stated	81
Norovirus	76
Streptococcus group B	57
Mycobacterium tuberculosis	54
Enteroccocus faecalis	52
Klebsiella pneumoniae	50
Streptococcus group A	49
Adenovirus untyped	43
Staphylococcus epidermidis	36
Proteus mirabilis	35
Enterobacter cloacae	34
Cytomegalovirus	29
Streptococcus group G	26
M. pneumoniae	21
Enterococcus faecium	19
Bacteroides fragilis	14

variability likely reflects the extra clustering induced by reporting delays. The increase in the dispersion primarily affects the more common organisms. The mean value of the ratio of the 2 dispersion values is 1.14 (median 1); when restricted to organisms for which the dispersion by specimen date is >1, it is 1.26 (median 1.04).

In some cases, a contributing factor to the extra variation is large systematic variation in diagnostic practice, resulting in large variations in reporting intensity, notably, long runs of zeroes, as with *Helicobacter pylori* (online Technical Appendix Figure 5). However, such patterns appear to be unusual; it is most likely that the extra variation is caused by clustering of cases in time, which can be accommodated relatively simply by a suitable choice of statistical model, to be discussed next.

Relationships between Mean, Variance, and Skewness

Relationships between mean, variance, and skewness were investigated for the 1,001 organisms with dispersion >1 for which nonzero means and variance were obtained for ≥3 half-years. In all cases, the scatterplot of log(variance) against log(mean) was remarkably linear. Figure 5, panel A, shows this relationship for *Cyclospora* spp. (the full set of plots is available from the corresponding author). The full line is the best fit line through the points, and lies some way above the dotted line, which corresponds to the Poisson

model. The dashed line corresponds to a quasi-Poisson model (equation 1). The closeness of the dashed line to the full line suggests that this model is not unreasonable for *Cyclospora* spp.

For 538 (54%) of these 1,001 organisms, the slope of the best-fit line is significantly different from 1, the value corresponding to the quasi-Poisson model, and in 535 of these the slope is >1 (the exceptions are *Providencia stuartii, Mycobacterium bovis* (bacillus Calmette-Guérin strain), and *Neisseria meningitidis* serotype B not further typed). This indicates that there is statistical evidence against the quasi-Poisson in about half the cases. However, departures from the quasi-Poisson model are typically moderate, the slope parameters lying for the most part between 0.9 and 1.7 (and thus reasonably close to 1), as shown in Figure 5, panel B.
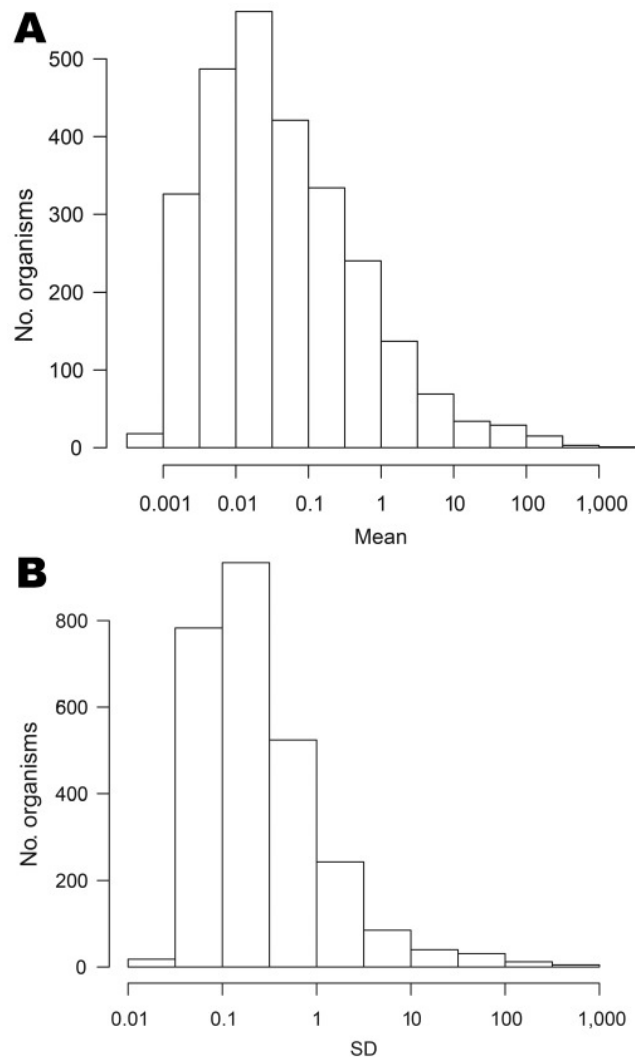
Figure 3. Distributions of mean (A) and SD (B) of weekly counts for all organisms, England and Wales, 1991–2011.
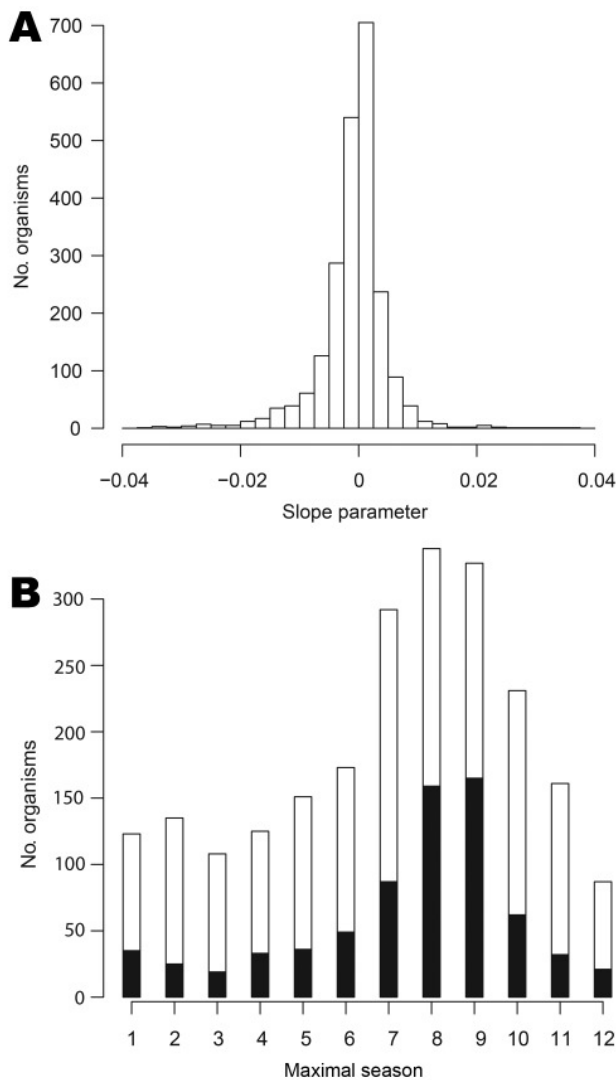
Figure 4. A) Distribution of estimated linear trend parameters (units: per week) for data on 2,250 organisms (excluding 4 organisms with extreme slopes), England and Wales, 1991–2011. B) Stacked bar chart of modal seasonal period for 2,254 organisms. The black bar sections represent organisms for which the seasonal effect is statistically significant.

departures from this reference value were moderate, most slope parameters lying between −0.6 and 0, as shown in Figure 6, panel B.

What these results signify is that the quasi-Poisson model provides an adequate, though far from perfect, account of the week-to-week variability in organism counts for the broad range of organisms considered. The negative binomial model may also provide an adequate representation of these highly heterogeneous data; because this model accounts for the skewness in the data, which the quasi-Poisson model does not, it may provide more
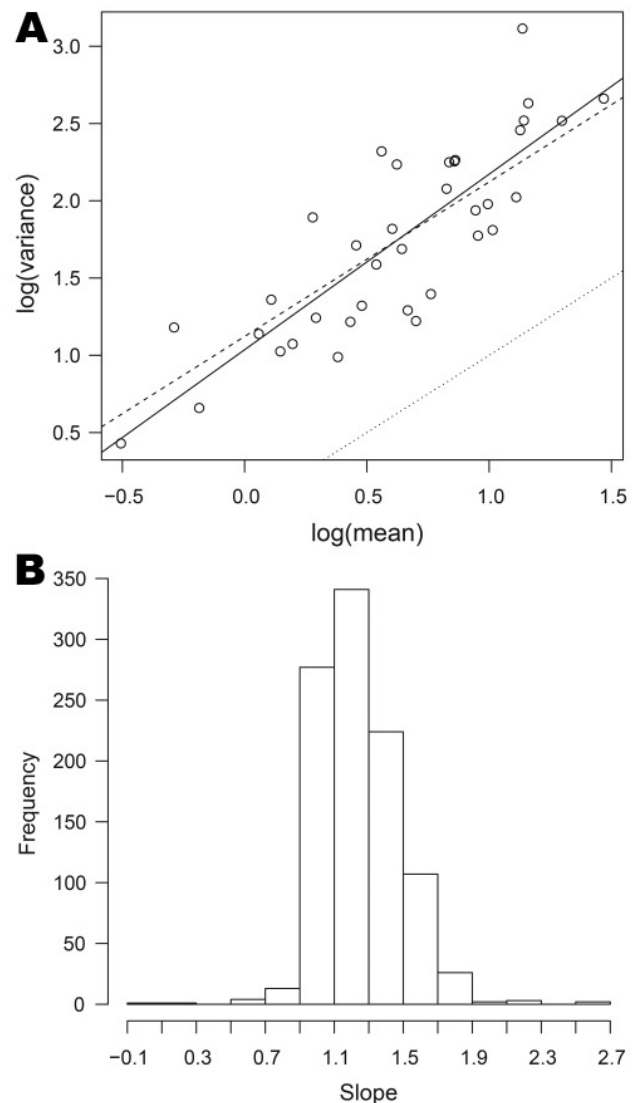
Figure 5. Relationships between mean and variance for data on organisms collected, England and Wales, 1991–2011. A) The log of variance plotted against log of mean for *Cyclospora* spp. The full line is the best fit to the points; the dashed line corresponds to the quasi-Poisson model; the dotted line corresponds to the Poisson model. B) Histogram of the slopes of the best-fit lines for 1,001 organisms; the value 1 corresponds to the quasi-Poisson model (equation 1).

Most organisms, other than the most common, displayed a degree of positive skeweness, that is, long upper tails. The plots of skewness against log(mean), though often broadly exponential, showed more scatter than those of log(variance) against log(mean). Figure 6, panel A, shows the plot for *Cyclospora* spp., the dashed line now corresponding to the negative binomial model (the full set of plots is available from the corresponding author).

For 486 (49%) of the 1,001 organisms, the slope parameter (on the log scale) is significantly different from −0.5, the value corresponding to the negative binomial. For 475 of these it was greater than −0.5. Again,

accurate threshold values, above which counts are declared to be aberrant.

Conclusions

We have undertaken a detailed analysis of the global features of a large surveillance database accumulated during >20 years. Most striking is the variety of temporal patterns, in terms of frequencies, trends, and seasonality. Some valuable general conclusions emerge of direct relevance to the design of outbreak detection systems.

The first stems from the great variation in organism frequency, which stretches over 6 orders of magnitude (from 10^{-3} to 10^3 per week). The sensitivity and specificity of the detection system should remain broadly constant over this range, so that the system performs well for both rare and common organisms. The primary output from a multiple outbreak detection system is likely to be a ranking of aberrances in decreasing order of the statistical evidence underpinning them. The correctness of the ordering is arguably more important than achieving nominal sensitivity and specificity levels, so that attention is focused on the most discrepant organisms. In practice, this means that outbreak detection methods used with multiple surveillance systems must perform robustly and consistently over the range of frequencies expected (or a large part of this range).

A second conclusion is that the systematic components of the statistical outbreak detection models must be able to cope automatically with the idiosyncrasies of individual data series, notably seasonality and trends, without requiring intervention by the user. This necessitates the use of suitably flexible modeling environments, though excessive flexibility can itself cause problems of overfitting. A careful balance needs to be struck: for example, between the detailed modeling required to incorporate seasonal effects, which is crucial for some organisms, while recognizing that such effects are not greatly relevant for many others. In addition, robust numerical algorithms that are guaranteed to work for all but known extreme data configurations are essential.

Third, our analyses provide empirical support for the use of a single, robust algorithm across this range of organisms. The data suggest that the great majority of organisms can adequately—though far from perfectly—be represented by a statistical model in which the variance is proportional to the mean, such as the quasi-Poisson or negative binomial models. Some improvement would nevertheless be possible through the use of more general models in which the variance is proportional to a power of the mean. Such more general distributions, based on birth processes, have been studied (15); further investigation is warranted for application to surveillance data.

These conclusions apply specifically to the use of automated biosurveillance as a second line of defense in
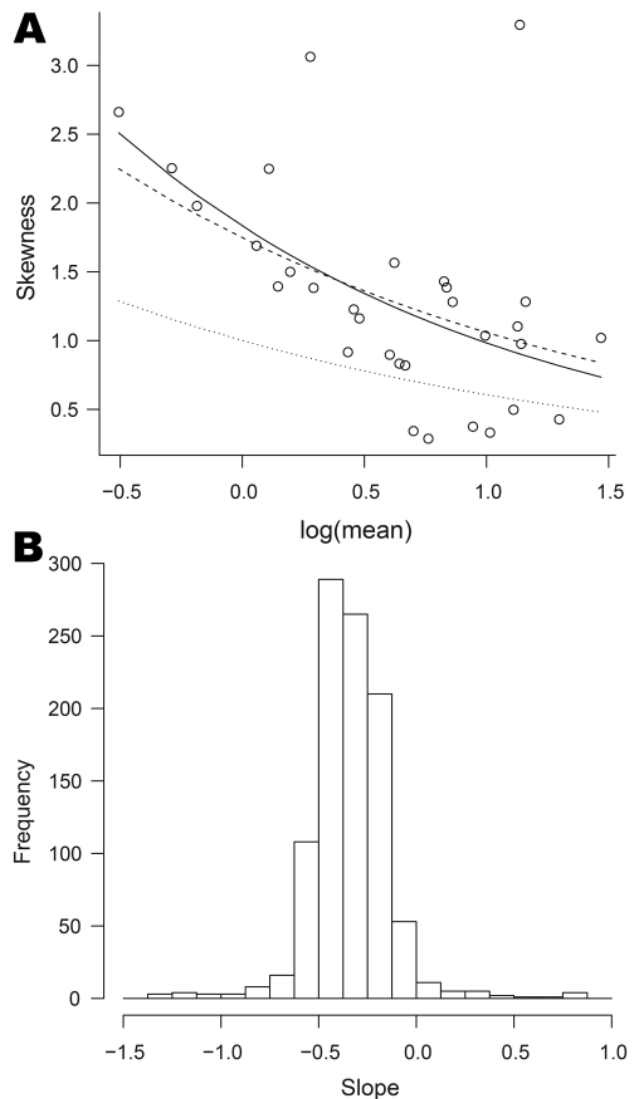
Figure 6. Relationships between mean and skewness for data on organisms collected, England and Wales, 1991–2011. A) Skewness plotted against log of mean for *Cyclospora* spp. The full curve is the best fit to the points; the dashed curve corresponds to the negative binomial model; the dotted curve corresponds to the Poisson model. B) Histogram of the parameters corresponding to the best-fit curves for 1,001 organisms; the value −0.5 corresponds to the negative binomial model (equation 2).

support of investigator-led outbreak detection methods, as implemented in England and Wales. Thus, we seek a system that performs adequately over the entire range of organisms, to be scanned routinely, rather than one that is optimized for a particular organism. We believe that integrating investigator-led and automated surveillance in this way plays best to the strengths of each method.

Each week, the England and Wales detection system flags ≈20 organisms, listed in decreasing order

of aberrance, for further investigation. A proportion of these results are false positive and do not correspond to a genuine outbreak. The remainder are genuine outbreaks, many of which will also have been picked up by the front-line investigator-led network of surveillance specialists, as intended. Occasionally, genuine outbreaks are picked up which have escaped detection by other means. These events often involve pathogens with a wide geographic distribution and relatively high baseline frequency of reporting. Such dispersed outbreaks may be overlooked at the local level, where they often equate to only marginal increases, but nationally may represent noteworthy events. Recent examples include outbreaks of *Salmonella enterica* serotype Enteritidis phage type 14b in 2009 (*16*), *S. enterica* ser. Java in 2010 (*17*), *S. enterica* ser. Montevideo in 2011, and *S. enterica* ser. Poona in 2012.

Our current efforts at improving the system are to reduce the false-positive rate while maintaining sufficient power to detect genuine outbreaks. Some of the key issues to be revisited are treatment of trends, seasonality, and calculation of thresholds, in the light of the findings presented here. Other issues are how to handle past outbreaks and delays between specimen collection and reported identification. The data and experience gained from >20 years' of automated biosurveillance will provide valuable empirical underpinning for such improvements.

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Microevolution of Highly Pathogenic Avian Influenza A(H5N1) Viruses Isolated from Humans, Egypt, 2007–2011

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We analyzed highly pathogenic avian influenza A(H5N1) viruses isolated from humans infected in Egypt during 2007–2011. All analyzed viruses evolved from the lineage of subtype H5N1 viruses introduced into Egypt in 2006; we found minimal evidence of reassortment and no exotic introductions. The hemagglutinin genes of the viruses from 2011 formed a monophyletic group within clade 2.2.1 that also included human viruses from 2009 and 2010 and contemporary viruses from poultry; this finding is consistent with zoonotic transmission. Although molecular markers suggestive of decreased susceptibility to antiviral drugs were detected sporadically in the neuraminidase and matrix 2 proteins, functional neuraminidase inhibition assays did not identify resistant viruses. No other mutations suggesting a change in the threat to public health were detected in the viral proteomes. However, a comparison of representative subtype H5N1 viruses from 2011 with older subtype H5N1 viruses from Egypt revealed substantial antigenic drift.

Outbreaks of highly pathogenic avian influenza (HPAI) A(H5N1) virus infection among poultry in parts of Africa, the Middle East, and Asia have caused sporadic human infections with high case-fatality ratios and a few instances of possible human-to-human transmission (1). In Egypt, HPAI (H5N1) virus was first detected in poultry in February 2006, and in March 2006, the first human infection was detected (2). Surveillance in wild and domestic birds and phylogenetic analyses of viruses from the region indicated that subtype H5N1 virus was probably transported to Egypt by wild birds migrating from the Qinghai Lake region of the People's Republic of China in the fall of 2005 (3). Analyses of the evolution of subtype H5N1 viruses in Egypt in the following years showed the exclusive circulation of clade 2.2 viruses in poultry, indicating no other subtype H5N1 viruses had been introduced (4). Subsequent genetic divergence of the clade 2.2 hemagglutinin (HA) genes in Egypt, however, resulted in a distinct phylogenetic group designated clade 2.2.1, which is enzootic in peridomestic poultry, and a more recently classified sister group known as clade 2.2.1.1, which is mostly found in commercial chicken flocks (4–6).

In 2011, a total of 39 human cases of subtype H5N1 infection and 15 related deaths were reported in Egypt; a total of 158 cases and 55 deaths were reported during 2006–2011, making Egypt the country with the second highest number of human cases after Indonesia (7). Increases in the annual number of human infections and accompanying decreases in case-fatality ratios in Egypt during 2009–2011, compared with those in 2006–2008, led to the hypothesis that the circulating viruses may have acquired distinct virologic properties (8). To address this issue, we studied the genetic and antigenic diversity of subtype H5N1 viruses isolated from humans in Egypt during 2007–2011. Analysis of 90 complete viral genomes was conducted to

[1]These authors contributed equally to this article.

determine 1) the predominant genotype of subtype H5N1 viruses infecting humans and 2) other molecular changes that would suggest altered phenotypic properties, susceptibility to antiviral drugs, or the need for development of new candidate vaccine viruses.

Materials and Methods

Virus Isolation and Sequencing

We extracted RNA from clinical samples from patients with suspected HPAI infection by using the QIAmp Viral RNA Mini Kit (QIAGEN Inc., Valencia, CA, USA), according to the manufacturer's protocol. We performed real-time reverse transcription PCR using the Centers for Disease Control and Prevention's (CDC) primer-probe pairs for the detection of influenza A (matrix gene) or H5a and H5b (HA gene), as described (9). Positive results were confirmed at the Central Public Health Laboratory operated by the Egyptian Ministry of Health and Population and at the US Naval Medical Research Unit No. 3 in Cairo, Egypt. Viruses were isolated from PCR-positive clinical samples in embryonated chicken eggs. All procedures using live virus were conducted in biosafety level 3 facilities with enhancements recommended by the United States Department of Agriculture.

Overlapping amplicons of each gene segment were generated by reverse transcription PCR using subtype H5N1 virus–specific primers (primer sequences are available upon request). We then sequenced the amplicons by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were assembled and edited by using Sequencher 4.9 (Gene Codes Corp., Ann Arbor, MI, USA).

Phylogenetic Analysis and Molecular Characterization

For analysis, we used a total of 59 subtype H5N1 viruses from human clinical specimens (sequenced for this study) and 31 subtype H5N1 viruses from birds (from public databases) collected in Egypt during 2007–2011 (online Technical Appendix 1, wwwnc.cdc.gov/EID/article/19/1/12-1080-Techapp1.xls). In addition, we sequenced 13 HA genes directly from RNA extracted from clinical specimens. Nucleotide sequences were aligned in BioEdit (Ibis Biosciences, Carlsbad, CA, USA) using MUSCLE (10). We used full-length HA sequences for viruses collected during 2006–2011 to generate the clade 2.2.1 and 2.2.1.1 HA gene phylogeny; the sequences comprised 72 genes sequenced by CDC and US Naval Medical Research Unit No. 3 and 56 publicly available HA sequences (Figure 1). Sequences were aligned and trees were built with MEGA5 software (11), using the neighbor-joining method based on a maximum composite likelihood model. The reliability of the trees was estimated by bootstrap resampling analysis (1,000 replications).
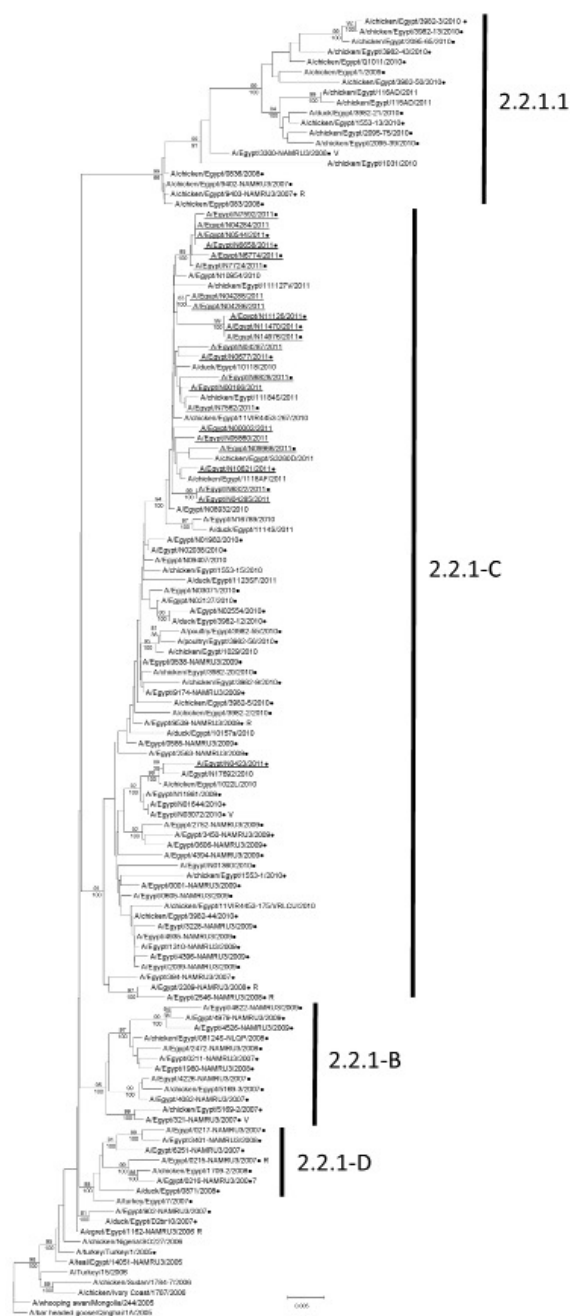
Figure 1. Phylogenetic tree of the influenza A(H5) virus hemagglutinin genes, clade 2.2.1, generated by neighbor-joining analysis. Subgroups of clade 2.2.1 are indicated on the right. Bootstrap values (>79) generated from 1,000 neighbor-joining replicates are shown above branches, and Bayesian posterior probabilities are shown below the branches at relevant nodes. Scale bar represents 0.002 nt substitutions per site. Viruses recommended by the World Health Organization for development of candidate pandemic vaccines are indicated with a V; viruses inoculated into ferrets to raise antiserum for hemagglutinin inhibition assays are indicated with an R. Underlined names denote 23 human viruses collected in 2011. Sequences used for full genome analysis in this study are annotated with a dot.

Additional statistical support for tree topology was assessed by performing Bayesian analyses, using the same datasets as described above and below. Bayesian posterior probabilities were estimated under a Markov Chain Monte Carlo method with 50 million generations implemented in BEAST, using a SRD06 substitution model (*12*). Strain A/turkey/Turkey/1/2005 was used to root all trees. We generated phylogenetic trees in a manner similar to that described above (for HA) for each gene segment from viruses that had full genomes available; representative viruses are shown in online Technical Appendix 2 Figure 1 (wwwnc.cdc.gov/EID/pdfs/12-1080-Techapp2.pdf). Trees for each gene with all sequences analyzed for this study are shown in online Technical Appendix 2 Figure 2.

Viral proteome characterization was conducted after full-length open reading frame nucleotide sequences were aligned using MUSCLE and trimmed to begin at the ATG start codon. For the HA protein sequence analysis, amino acid numbering was based on the mature HA protein sequence after removal of the signal peptide.

Antigenic Analysis

Antigenic characterization was performed by using a panel of ferret antisera in hemagglutination inhibition (HI) tests with turkey erythrocytes, as described (*13*) (online Technical Appendix 2 Table). We used turkey erythrocytes to better resolve antigenic distances between variants identified in this study. All antisera were treated with the receptor-destroying enzyme neuraminidase (NA) from *Vibrio cholerae* (RDE [receptor destroying enzyme]; Denka Seiken Co., Ltd., Tokyo, Japan), according to the manufacturer's recommendation, and pre-adsorbed with turkey erythrocytes.

Antiviral Susceptibility Testing

To determine the antiviral drug concentration required to inhibit 50% of the NA activity, we conducted a fluorescent NA inhibition assay as described (*14*). The assays were conducted under biosafety level 3–enhanced containment at CDC.

Results

Epidemiology of Subtype H5N1 Infections

Most of the 158 persons in Egypt infected with subtype H5N1 virus during 2007–2011 were residents of the Nile Delta region, north of Cairo (Figure 2, Appendix, wwwnc.cdc.gov/EID/article/19/1/12-1080-F2.htm). The cases were geographically distributed in 23 of the 27 governorates in Egypt. As determined by Kandeel et al. (*2*), women ≥15 years of age were at greatest risk for infection and death; 36% of infections and 69% of deaths were in women in this age group (*2*). Risk ratios (RRs) for death

included female sex (RR 2.16, p = 0.002), age ≥15 years (RR 10.26, p<0.0001), and receiving the first dose of oseltamivir >2 days after illness onset (RR 4.15, p<0.0001).

Genomic Phylogeny of Subtype H5N1 Virus Isolates from Humans

We sequenced the complete genomes of 59 subtype H5N1 virus isolates and 13 HA genes sequenced directly from clinical specimens that were obtained from a total of 72 persons in Egypt during 2007–2011. The phylogenetic trees of the HA gene sequences were annotated according to classification criteria established by the World Health Organization (WHO), the World Organisation for Animal Health, and the Food and Agriculture Organization, and clades were further subdivided as reported (*6,8,13*).

The phylogenetic tree of the HA gene showed that the 72 genes from the infected persons (Figure 1, coded dots) have evolved into 4 groups that diverged from the ancestral A/turkey/Turkey/2005-like genes. Three groups identified within clade 2.2.1 were supported by bootstrap values >89 and were designated 2.2.1-B, 2.2.1-C, and 2.2.1-D. Clade 2.2.1.1 formed the fourth group, supported by a bootstrap value of 100. All viruses collected in 2011 (n = 23) were clustered within 2.2.1-C, and 22 of the 23 formed a monophyletic group, supported by a bootstrap value of 94, with an average difference of 26 nt. The rest of the group C viruses, including the Aswan isolate A/Egypt/N0423/2011, originated from a distinct lineage but shared ancestry with the A/Egypt/3072/2010 vaccine candidate virus (Figures 1, 2). Viruses from clade 2.2.1.1 included only 1 isolate from humans, whereas subgroup C of clade 2.2.1 comprised 56 human isolates. The determinants of this difference have not been established.

To detect evidence of genetic reassortment among subtype H5N1 viruses from poultry and zoonotic infections, we compared the topologies of phylogenetic trees from the complete genomes of 90 viruses from Egypt (59 sequenced for this analysis and 31 available in GenBank). The topologies of HA and PB2 trees were identical, indicating an absence of reassortment (online Technical Appendix 2 Figure 1). The other 6 trees indicated that these genes either co-evolved with HA (identical tree topology with high bootstrap support) or had insufficient divergence to support unambiguous evolutionary relationship determinations (low bootstrap support). The exception was the nonstructural (NS) gene of a subgroup C virus, A/Egypt/9174-NAMRU3/2009, which formed a monophyletic group (bootstrap value 95) with several subgroup B viruses isolated in the same year, indicating a possible reassortment of NS gene between these co-circulating subgroups in 2009.

We also assessed the possible transboundary movement of subtype H5N1 viruses by analyzing the phylogenetic relationships between viruses from Egypt (2007–

2011) and other countries. All the genes from viruses from Egypt formed single monophyletic groups, which included isolates exclusively from Egypt (online Technical Appendix 2 Figures 1, 3), and were distinct from other closely related genotype Z viruses, including clade 2.2 viruses previously circulating in other regions of Africa, the Middle East, and Europe (15,16). These results indicated that no additional subtype H5N1 viruses were imported into Egypt after their introduction in 2005/2006. The data also indicate that subtype H5N1 viruses have not reassorted with low pathogenic avian influenza (LPAI) viruses since their initial introduction (online Technical Appendix 2 Figure 1).

Characterization of the Viral Proteome of 2011 Human Isolates

The 10 major viral proteins encoded by the subtype H5N1 genomes were screened to identify variations at sites previously associated with functional traits of public health consequence (Table) (17–24). The multibasic cleavage site common to clade 2.2.1 HA proteins (^{321}PQGE$\underline{K}$RRRKKR$\downarrow$G^{333}) remained unchanged in viruses collected in 2011 from infected humans. As previously noted, viruses belonging to a subset of the clade 2.2.1.1 group collected during 2009–2011 lacked a single basic residue in the HA cleavage site that was present in clade 2.2.1 and 2.2.1.1 cleavage motifs (8).

The HA proteins of the clade 2.2.1 viruses from 2011 also lacked glycosylation at position 154, as observed in older H5N1 viruses from Egypt. However, A/Egypt/N10621/2011 and A/Egypt/N6658/2011 acquired an additional glycosylation site at Asn 119 and 215, respectively, in the globular head of HA. Two signature amino acid changes (a deletion of Ser 129 in the HA gene, and an Ile 151 Thr substitution) were observed in HA proteins of clade 2.2.1 group C viruses collected during 2007–2011; both changes may enhance binding to α-2,6–linked receptors (8,13,25). Additional amino acid comparisons revealed that the HA of the virus from the Aswan region of Egypt lacked the amino acid signatures shared among the viruses collected in 2011 from northern Egypt. However, the HA gene from this virus featured amino acid signatures shared among older group 2.2.1-C viruses isolated in southern and northern Egypt (Table).

Additional polymorphisms of potential relevance in clade 2.2.1-C viruses included the K615R mutation in the polymerase acidic (PA) gene, previously reported to be associated with mammalian host adaptation (20). Several additional polymorphisms were also found within the known functional domains of the internal genes, such as those involved in RNA and/or nucleoprotein binding (17–24) (Table). Two of the isolates (A/Egypt/N6828/2011 and A/Egypt/N7562/2011) possessed a stop codon at position 25 in the polymerase basic 1 (PB1)–F2 coding

region (alternate open reading frame near the 5′ end of the PB1 gene), causing a truncated protein. One of the poultry viruses also possessed a premature stop codon at position 9 in PB1-F2. A single poultry virus, A/chicken/Egypt/083/2008, had only a 22-aa PA-X protein, and all of the human and other poultry viruses had PA-X proteins comprising the full 61-aa sequence. No distinct amino acid signatures were found in the nucleoprotein or NS2 proteins. All of the clade 2.2.1-C viruses and the other clade 2.2.1 and 2.2.1.1 viruses retained PB2 627K and NS1 92E, two mutations imparting host-specific virulence phenotypes in subtype H5N1 viruses, that are also conserved in nearly all clade 2.2-origin viruses isolated from humans and birds (26,27).

Antigenic Characterization of Recent Subtype H5N1 Isolates from Humans

To assess the extent of antigenic drift among recent human subtype H5N1 virus isolates relative to WHO-recommended candidate vaccine viruses, we performed hemagglutinin inhibition (HI) tests using ferret antisera raised against a panel of viruses, including A/Egypt/N03072/2010 (the clade 2.2.1 vaccine candidate recommended by WHO) (28) and isolates from previous years. Antiserum generated against A/Egypt/N03072/2010 revealed HI titers to representative subtype H5N1 isolates from 2011 that differed ≤4-fold, compared with the homologous titer of the candidate vaccine virus (online Technical Appendix 2 Table). Compared with A/Egypt/N03072/2010, most of the analyzed viruses from humans infected in 2011 had 6–8 aa changes in the HA1 protein (online Technical Appendix 3, wwwnc.cdc.gov/EID/article/19/1/12-1080-Techapp3.xls). Relative to the vaccine strain, these viruses shared 3 conserved amino acid differences at positions previously shown to be involved in antibody recognition (D154N and N155D) or receptor binding (V134A). Relative to the homologous titer, HI titers of antiserum to A/Egypt/N03072/2010 were reduced by 8-fold with A/Egypt/N6828/2011 and A/Egypt/N7562/2011 (online Technical Appendix 2 Table). A/Egypt/N6828/2011 possessed a change at putative antigenic site A (A127T) relative to other group C viruses, but there were no other additional amino acid substitutions at known functional sites to support this finding (online Technical Appendix 3).

The HI titers of antisera to earlier clade 2.2.1 viruses (groups A, B, and D) with group C viruses from 2011 were reduced >8-fold relative to the homologous values, indicating substantial antigenic change in recent years. These earlier viruses had many other additional HA1 substitutions in comparison to the group C viruses, including several in or near to antigenic sites B and C (online Technical Appendix 3). In addition, only the group C viruses (including the

Table. Signature amino acids of avian influenza A(H5N1) viruses infecting humans in Egypt since 2007*

Protein, amino acid position	Virus group†						Functional relevance (reference#)
	2.2.1-C	9174‡	2009 variants§	2007–2008 variants¶	A, B, and D	2.2.1.1	
PB2							
80	R	R	R	R	K	K	NP binding site (17,18)
129	N	N	N	N	T	T	
292	M	M	I	I	I	I	No known function
PB1							
384	S	S	S	L	L	L	cRNA binding (19)
PB1-F2							
40	G	G	D/G	D	D	D	No known function
PA							
400	T	T	S	S	S	S	No known function
615	R	R	K/R	K	K	K	615R mammalian host adaptation (20)
HA							
43	N	N	N	N	D	D	Antigenic site C (21)
120	N	N/D	N	N	S	S	No known function
129	Del	Del	Del	Del	S	L/S	Near or adjacent to the receptor binding site 130-loop (21)
151	T	T	T	T	I	I	Antigenic site B and receptor binding (21)
NA							
224	M	M	M	L	L	L	No known function
450	G	G	G	S	S	S	No known function
M1							
95	K	K	K	R	R	R	No known function
168	T	T	I	I	I	I	RNP binding site (22)
207	S	S	N	N	N	N	
M2							
51	F	F	C	C	C	C	No known function
NS1							
48	S	N	S	N	N	N	RNA binding site (23)
198	V	V	I/V	I	I	I/V	Effector domain: inhibition of maturation and exportation of host antiviral mRNAs (23)
229	E	E	K	K	K	K	PDZ ligand motif of HPAI equals ESEV (23,24)

*Hemagglutinin (HA) amino acid numbering was based on the mature HA protein sequence after removal of the signal peptide. All other numbering was relative to the full-length open reading frame of A/goose/Guangdong/1/1996 protein sequences. PB2, polymerase basic 2 gene; NP, nucleoprotein; PB1, PB 1 gene; PB1-F2, alternate open reading frame near the 5′ end of the PB1 gene; PA, polymerase acidic gene; Del, deletion; NA, neuraminidase; M, matrix gene; RNP, ribonucleoprotein; NS, nonstructural gene;PDZ, postsynaptic density protein, *Drosophila* disk large tumor suppressor, and zonula occludens-1 protein; HPAI, highly pathogenic avian influenza ;ESEV, H5N1 PDZ-binding motif amino acid consensus sequence.
†**Boldface** indicates unique amino acid differences found in the various positions along the different genes of 2.2.1-C viruses.
‡Indicates intraclade reassortant virus A/Egypt/9174-NAMRU3/2009.
§A/Egypt/0606-NAMRU3/2009, A/Egypt/2752-NAMRU3/2009, A/Egypt/3450-NAMRU3/2009.
¶Early 2.2.1-C viruses from 2007–2008: A/Egypt/394-NAMRU3/2007, A/Egypt/2546-NAMRU3/2008, A/Egypt/2289-NAMRU3/2008.
#References describing functional significance of mutations.

vaccine virus) shared a deletion of amino acid residue 129, which is near or adjacent to the 130-loop of the HA receptor-binding site (25). The large number of changes at immunodominant sites between the clade 2.2.1.1 viruses and group C viruses, together with a lack of antigenic crossreactivity in 2-way tests, indicate disparate evolutionary paths of these lineages. In agreement with previous findings (13), we showed that sera generated against the clade 2.2.1.1 viruses (e.g., A/chicken/Egypt/9403-NAMRU3/2007 and the A/Egypt/3300-NAMRU3/2008 vaccine candidate) did not crossreact with the group C viruses.

Antiviral Drug Susceptibility

Analyses of putative molecular markers of resistance to antiviral drugs (adamantanes and NA inhibitors)

identified few variations of concern for public health. A/Egypt/4396-NAMRU3/2009 had an Ile-to-Thr mutation at position 223 (I223T) in the NA active site; however, this change did not affect susceptibility of the virus to oseltamivir (50% inhibitory concentration 5.23 nM). A/Egypt/0605-NAMRU3/2009 had a mutation of I117M in the NA, and compared with the sensitive control virus, which lacked this mutation, A/Egypt/0605-NAMRU3/2009 had a 6-fold reduced susceptibility to oseltamivir (16.46 nM vs. 2.68 nM). Four viruses had the V27A mutation in the M2 protein (A/Egypt/ 2289/NAMRU3/2008, A/Egypt/2546/NAMRU3/2008, A/Egypt/N6774/2011, and A/Egypt/N7724/2011), and 1 virus had the S31N mutation (A/Egypt/3300/NAMRU3/2008). These viruses are predicted to be resistant to M2 ion-channel blocking drugs.

Discussion

Since 2009, Egypt has reported a higher number of HPAI (H5N1) virus infections in humans than any other country. At the end of 2011, Egypt had reported 39 (63%) of the total 62 human cases in the world for that year, placing Egypt second only to Indonesia in the number of reported human infections since 2003 (7). Exposure to sick or dead poultry has been reported as the likely source of infection for nearly all human cases in Egypt (29). Most of those exposures were described as occurring in backyard poultry settings (although infection in industrial/commercial settings could not be ruled out if someone worked in or visited these settings) (29). The contrast between the rising numbers of human infections detected each year in Egypt and the declining case-fatality ratios since 2009 led to speculation about the evolution of new virologic properties influencing the epidemiology of subtype H5N1 virus in Egypt (2,8,25). To investigate possible molecular epidemiologic correlates of these trends, we analyzed the complete genomes of viruses isolated from humans in Egypt during 2007–2011.

Phylogenetic analysis of HA genes indicated that subtype H5N1 viruses from clades 2.2.1 and 2.2.1.1 continued to co-circulate in recent years (6). In addition, the HA of clade 2.2.1 viruses was also found to cluster in 1 of 4 distinct monophyletic groups (previously termed groups A–D) (8,13). Of the human infections during 2009–2011, 95% were caused by viruses from a single phylogenetic group, clade 2.2.1-C, whereas infections detected during 2007–2008 involved viruses from 2.2.1-B and -D.

In contrast with the multiple human infections caused by the clade 2.2.1 viruses, only 1 human infection by a clade 2.2.1.1 virus was detected (Figure 1). The predominance of clade 2.2.1-C viruses among zoonotic infections in humans appears to be associated with the persistent circulation of this group of viruses in backyard or peridomestic poultry (30). However, there remains some evolutionary divergence between 2 discrete HA clusters detected in 2011 that may correlate with the geographic separation between the majority of group C genes that originated from the Nile Delta and those from strain A/Egypt/N0423/2011, which was collected from the Aswan governorate in the south of the country. The paucity of human infections with clade 2.2.1.1 viruses, which circulate predominantly in commercial poultry (30), may result from intrinsic viral properties or from husbandry practices that reduce the probability of zoonotic infection. Thus, exposure to subtype H5N1 virus in backyard or peridomestic environments, rather than commercial settings, appears to be correlated with greater risk for zoonotic H5N1 infections.

The genetic variation that was observed among the HA genes of clade 2.2.1 viruses correlated with an increased complexity in the antigenic characteristics of the viruses currently circulating in Egypt. Although all clade 2.2.1 viruses remain crossreactive to sera produced against reference viruses from the same clade, HI assay results indicated that continued variation among A, B, C, and D viruses has resulted in reduced titers of recent 2.2.1-C viruses against antisera to older clade 2.2.1 viruses. It should be noted, that the recent 2011 isolates were antigenically closely related to the proposed WHO candidate vaccine virus, A/Egypt/N03072/2010, indicating a good antigenic match between currently circulating strains and the proposed vaccine.

Although the HA and NA genes play a major role in the transmission of HPAI (H5N1) virus, the internal genes can also modulate pathogenicity and transmissibility of the virus (17–27). To further investigate the evolution of the internal genes of subtype H5N1 viruses from humans and detect possible reassortment (intraclade or other), we performed a systematic analysis of the phylogenetic relationships among all the genes. There was a notable difference in the topology of the phylogenetic tree of the NS gene compared with other internal genes (supported by bootstrap values >80) in that no distinct 2.2.1-B and 2.2.1-D subgroups were evident for the NS gene tree. All the internal genes that did not appear to co-evolve with their surface genes show the closest relationship to the genes of A/turkey/Turkey/2005, indicative of a lack of strong selective pressure. These findings also confirmed previous reports that subtype H5N1 genes have not been introduced from Asia into Egypt since 2006. The phylogenies of all the genes from the viruses in Egypt lacked evidence of reassortment with other avian influenza genes.

The co-circulation of H9N2 and potentially other LPAI A viruses in Egypt has been reported (31); thus, the absence of genetic reassortment between subtype H5N1 and LPAI avian viruses from poultry could be considered unexpected. In contrast to reassortant genotypes that have been identified in other countries, the homogeneous genetic makeup of subtype H5N1 viruses in Egypt may stem from the characteristics of the poultry trade with neighboring countries or from other factors leading to fewer opportunities for co-infections with other avian influenza viruses.

At the protein level, evolution among 2.2.1-C viruses, compared with that among other clade 2.2.1 viruses, has resulted in the fixation of at least 4 substitutions in the HA protein. When found together, 2 of the 4 HA mutations have been implicated as possible host adaptation markers: the deletion of residue 129 and the I151T substitution enabled in vitro binding to α-2,6 sialosides and virulence in mice (25). However, these 2 mutations may also mediate antigenic drift (20). The K615R substitution in the PA proteins of 2.2.1-C viruses represents another potential marker of host adaptation (17). No markers of antiviral drug resistance were found to be conserved in either the NA or M2 of any of the human-derived subtype

H5N1 viruses. Epidemiologic investigations of the human cases of subtype H5N1 infection from which the study isolates were derived could not confirm which patients had received oseltamivir or other antiviral treatments. However, previous analyses of subtype H5N1 viruses isolated from poultry in Egypt were found to have M2 and NA proteins with antiviral resistance markers, indicating that these mutations exist to some extent in viruses circulating within the poultry population (8).

In summary, our findings indicate that the recent subtype H5N1 viruses isolated from human infections originated from poultry. These viruses evolved from a single genotype introduced into Egypt in 2005/2006; there is no evidence of subsequent reassortment with new subtype H5N1 virus genes introduced into Egypt or resident LPAI viruses. The viruses have been observed in all subtype H5N1 infections in humans since 2009 and belong to a subgroup, termed 2.2.1-C, with unique genetic signatures that may contribute to their persistence in poultry. A rationale for linking the observed amino acid changes to the decline in case-fatality ratios since 2009 could not be identified. Systematic analysis of subtype H5N1 viruses in Egypt is critical to better understand the genetic and phenotypic evolution of subtype H5N1 viruses in Egypt and to inform public health programs to reduce the risk for zoonotic infections and prevent or mitigate a potential pandemic.

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Risk Factors for Nipah Virus Infection among Pteropid Bats, Peninsular Malaysia

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We conducted cross-sectional and longitudinal studies to determine the distribution of and risk factors for seropositivity to Nipah virus (NiV) among *Pteropus vampyrus* and *P. hypomelanus* bats in Peninsular Malaysia. Neutralizing antibodies against NiV were detected at most locations surveyed. We observed a consistently higher NiV risk (odds ratio 3.9) and seroprevalence (32.8%) for *P. vampyrus* than *P. hypomelanus* (11.1%) bats. A 3-year longitudinal study of *P. hypomelanus* bats indicated nonseasonal temporal variation in seroprevalence, evidence for viral circulation within the study period, and an overall NiV seroprevalence of 9.8%. The seroprevalence fluctuated over the study duration between 1% and 20% and generally decreased during 2004–2006. Adult bats, particularly pregnant, with dependent pup and lactating bats, had a higher prevalence of NiV antibodies than juveniles. Antibodies in juveniles 6 months–2 years of age suggested viral circulation within the study period.

Nipah virus (NiV) disease emerged in Peninsular Malaysia during September 1998–April 1999 and resulted in 105 human deaths (≈40%) and compulsory culling of 1.1 million pigs (*1*). NiV disease has been successfully controlled in Malaysia. However, elsewhere in Bangladesh and India, 12 NiV outbreaks have since occurred (*2*). Pteropid bats (family Pteropodidae, genus

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Pteropus) were the most likely reservoir host of the virus, and evidence of NiV in these bats had been consistently found in populations across southern Asia (*3–6*) and Africa (*7*) despite an absence of outbreaks in some areas.

Characteristics of bats that promote their competency as a natural host and reservoir for many emerging pathogens from evolutionary, ecologic, sociobehavioral, and immunologic perspectives are progressively being reported (*8–11*). In Malaysia, previous surveillance work suggest 2 pteropid species, *Pteropus hypomelanus* (variable flying fox) and *P. vampyrus* (large flying fox) bats, as reservoir hosts for NiV (*12*). *P. hypomelanus* bats reside on offshore islands along the eastern (n = 14) and western (n = 4) coasts of the peninsula (*13*). NiV was first isolated from pooled urine samples from these bats on the island of Pulau Tioman off the eastern coast of the state of Pahang (*14*). *P. vampyrus* bats, the pteropid species identified during the first NiV disease outbreak location in 1998 (*3*), are anthropogenic-susceptible bats residing in remote and inaccessible areas such as mangroves and dense forests (*15*). *P. vampyrus* bats roost mainly on the mainland but may have focal transitory points on surrounding islands as they travel (*16,17*).

Our recent follow-up work on a cohort of *P. vampyrus* bats (*18,19*) showed a possible NiV recrudescent event leading to horizontal viral transmission to other bats in the colony. The findings further elucidated maintenance and transmission dynamics of the virus within and among roosts, colonies, and the bat metapopulation. In this report, we present results of 2 studies conducted concurrently to determine the geographic extent and prevalence of

NiV-neutralizing antibody for *P. vampyrus* and *P. hypomelanus* bats, and to identify the sexual and reproductive maturity determinants for NiV seropositivity in the wild.

Materials and Methods

Study Design and Study Populations

Cross-sectional Study of NiV (Distribution Study)

During March 2004–May 2007, we sampled *P. hypomelanus* bat colonies from several roost sites in Pulau Kapas and Pulau Perhentian on the northeast coast of Terengganu, Pulau Tioman on the southeast coast of Pahang, and Pulau Pangkor on the northwest coast of Perak. We also sampled *P. vampyrus* bats in the states of Perlis; Perak (Teluk, Memali, and Lenggong); Terengganu (Kampung Alor Lek, Setiu, and Kuala Berang); Pahang (Tanjung Agas, Pasir Panjang, and Ganchong); and Johor (Benut and Kesang) (Figure 1). We determined these sites using published information on pteropid roost locations (15), reports from the Malaysia Department of Parks and Natural Resources authority (PERHILITAN), information from local hunters and residents, and field observations. A complete list of observed roost sites was published by Epstein et. al. (16). Colonies were selected for this study on the basis of observed presence of bats, accessibility for

bat capture, and roost size (e.g., number of bats). A sample size of 35 bats was targeted at each location or for each sampling effort to be able to detect a NiV-seropositive bat given a minimum prevalence of 10% with 10% precision at a confidence level of 95% (20).

Permission for the study was granted by PERHILITAN, and Institutional Animal Care and Use Committee approval was obtained from the Wildlife Trust Institutional Animal Care and Use Committee (New York, NY, USA). A special permit to trap and humanely kill the bats was obtained from PERHILITAN. Bats were captured nonrandomly by using 2 methods: opportunistic sampling of hunted bats (hunters were not solicited or incentivized to hunt bats for this study) and mist nets.

Hunted bats were sampled at the site of hunting, and bats were attributed to the location of the nearest known roost. Blood samples were collected by cardiac puncture, and the kidneys were harvested. The blood, urine, and kidney samples were processed as described (18). Bats captured in mist nets were extracted from the nets immediately after capture and anesthetized before sampling by using medetomidine, a combination of medetomidine/ketamine (21), or isoflurane gas. Blood (3 mL) was collected from the cephalic vein or brachial vein and placed into serum-separator tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA). Sterile cotton swabs were used to collect oropharyngeal and urogenital samples. All samples were
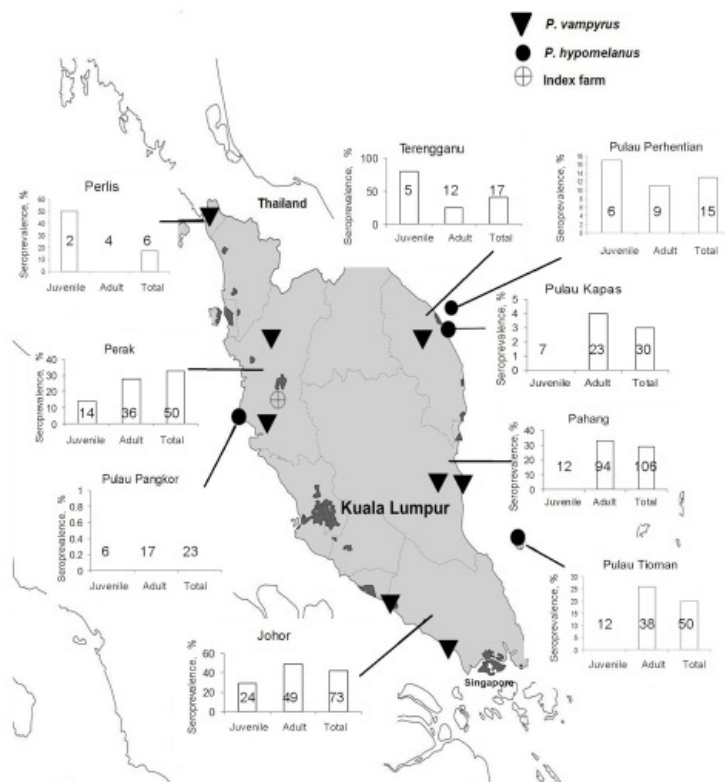
Figure 1. Trapping sites for *Pteropus hypomelanus* and *P. vampyrus* bats and seroprevalence of Nipah virus in 8 sites, Peninsular Malaysia, January 2004–September 2006. Values in the small graphs indicate number of positive samples.

stored in liquid nitrogen at −190°C) and transported to the Veterinary Research Institute in Ipoh, Perak, Malaysia.

For each bat hunted or captured, information on the date of sampling, species, location, sex, reproductive status, and estimated age was recorded. We assigned each bat an age category of adult (secondary sexual characteristics visible/reproductive), juvenile (no observable secondary sexual characteristics, not pregnant, dental wear characteristics), or pup (dependent and attached to its dam) (*3*). We categorized the reproductive status as pregnant, carrying a pup (adult females with attached pup), nursing (adult females without attached pup but with evidence of lactation), or dry (not in any of the previous categories) using published criteria (*18*). The total number of bats sampled for each pteropid species is shown in Tables 1 and 2. Bats captured in mist nets were released at the site of capture after recovery from anesthesia.

We performed descriptive analysis to describe the seroprevalence on the basis of species of bats and locations. Differences among positive antibody titers (range <8–1,025) between species were tested by using the Mann-Whitney U test. Comparison of the seroprevalence rates between the 2 pteropid species was performed by using the χ^2 test. When there were sufficient data to perform the analysis, several factors were examined for its association to NiV seropositivity by using the χ^2 test.

Longitudinal Study on Risk Factors for NiV (Risk Factor Study)

We performed a longitudinal study on a population of *P. hypomelanus* bats in Pulau Tioman in which 50 bats from the same colony were captured by using mist nets and sampled approximately every 6–8 weeks during January 2004–October 2006. Monsoonal rains prevented access to the island of Pulau Tioman during December–February, which extended the interval between some sampling points. Captured bats were tagged by using a thumb band or implantable microchip (Avid Identification Systems, Inc., Norco, CA, USA) and unique identification numbers to ensure that sampling was not repeated on the same bat. The bats were anesthetized by using ketamine and xylazine (*22*). Data, blood, and swab samples were collected as described in the previous section.

We investigated associations between serostatus (positive or negative) and each of the hypothesized risk factors (sex, age, reproductive categories, time) using the χ^2 test or Fisher exact test when the χ^2 test was not appropriate. Our previous study (*18*) suggested a correlation between the serologic status of pups and their dams. Therefore, serologic data for *P. hypomelanus* pups were excluded from further analysis. Logistic regression with generalized estimating equations was used to analyze longitudinal data to control

for the effect of clustering, assuming that bats sampled between various times were from the same population. To compare the categories across sexual and reproductive maturity, we reclassified the data on the basis of sex (male and female) and reproductive maturity (juvenile, adult, dry, pregnant, carrying a pup, and nursing) and renamed the variable the sexual and reproductive maturity factor. The logistic regression analysis includes the sexual and reproductive maturity categories and sampling time for the bats. All hypothesis testing was 2-sided, with α = 0.05, and was performed by using SPSS version 19 (SPSS Inc., Chicago, IL, USA).

Laboratory Analysis

Plasma-neutralizing antibodies against NiV were measured by using the serum neutralization test (SNT) (*23*) with plasma diluted 1:2–1:1,024. A titer ≥8 was considered a positive titer for specific antibody against NiV because serum samples were usually toxic to Vero cells at higher concentrations (i.e., 1:2 or 1:4). Virus isolation was attempted by using rabbit kidney and Vero cells in a biosafety level 3 facility until the third passage before a sample was considered negative (*23*). According to the Malaysian Government Act on Control and Prevention of Infectious Diseases 1988 (revised May 25, 2006), NiV is categorized in risk group 3, which enables the virus to be handled in a biosafety level 3 facility. Any tissue culture with an NiV-like cytopathic effect was confirmed by using a PCR as described (*24*). All laboratory diagnostics (SNT, virus isolation, and PCR) were conducted at the Veterinary Research Institute in Ipoh.

Results

Distribution of NiV

P. vampyrus Bats

NiV-neutralizing antibodies were detected in bats at all locations (Table 1). Overall, 82 (32.8%) of 253 bats were seropositive. We found no significant difference in seroprevalence rates in bats from the 5 states (p = 0.213, by Fisher exact test). NiV-neutralizing antibody titers ranged from <8 to 1,024 (median <8), and among seropositive bats, the median titer was 64. All culture and PCR results were negative. Among pups, juvenile, and adult bats, 0% (0/1), 25% (14/56), and 35.2% (68/193), respectively, were seropositive (Table 1). Age, sex, and female reproductive status were examined for their effects on serostatus of *P. vampyrus* bats. Univariate analysis showed that age and sex were not associated with seroprevalence of NiV. However, among nursing bats, a higher risk for NiV seropositivity was observed than in other adult females (Table 3).

Table 1. *Pteropus vampyrus* bats with Nipah virus–neutralizing antibody titers, Peninsular Malaysia, January 2004–October 2006*

Location	Sex	Age	No.	SNT titer	No. positive	% Seroprevalence (95% CI)
Perlis, n = 6	M	Adult	4	≤8	0	16.7 (1.1–58.2)
		Juvenile	0	NA	NA	
		Pup	0	NA	NA	
	F	Adult	0	NA	NA	
		Juvenile	2	≤8	1	
		Pup	0	NA	NA	
Perak, n = 50	M	Adult	18	≤8–32	3	24 (14.1–37.5)
		Juvenile	11	≤8–64	2	
		Pup	0	NA	NA	
	F	Adult	18	≤8–512	7	
		Juvenile	3	≤8	0	
		Pup	0	NA	NA	
Terengganu, n = 18	M	Adult	6	≤8–128	1	38.9 (20.2–61.5)
		Juvenile	5	≤8–64	4	
		Pup	0	NA	NA	
	F	Adult	6	≤8–16	2	
		Juvenile	0	NA	NA	
		Pup	1	≤8	0	
Johor, n = 73	M	Adult	23	≤8–128	12	42.4 (31.7–53.9)
		Juvenile	16	≤8–128	6	
		Pup	0	NA	NA	
	F	Adult	26	≤8–128	12	
		Juvenile	8	≤8–16	1	
		Pup	0	NA	NA	
Pahang, n = 106	M	Adult	44	≤8–1,024	10	29.2 (21.4–38.5)
		Juvenile	9	≤8	0	
		Pup	0	NA	NA	
	F	Adult	50	≤8–1,024	21	
		Juvenile	3	≤8	0	
		Pup	0	NA	NA	
Overall, N = 253		Adult	193	≤8–1,024	0	32.8 (27.3–38.8)
		Juvenile	66	NA	14	
		Pup	1	NA	68	

*SNT, serum neutralization test; NA, not applicable (no sample).

P. hypomelanus Bats

Of 119 plasma samples collected from island sites, 2 were not used because of inadequate amounts of plasma. Of the remaining 117, 13 (11.1%) were seropositive. NiV antibodies were detected in 13 (13.8%) of 94 bats from islands off the east coast (Pulau Perhentian, Pulau Kapas, Pulau Tioman) of Peninsular Malaysia. Titers ranged from <8 to 256 (median <8); among seropositive bats, the median titer was 32. Samples from Pulau Pangkor (n = 24) were negative for NiV-neutralizing antibodies. All culture and PCR results were negative. Pups were not captured, but among juvenile and adult bats, 0% (0/31) and 11.1% (13/73), respectively, were seropositive (Table 2). Univariate analysis showed that sex was not associated with NiV seropositivity. Stratified data for various reproductive categories were sparse and lacked power for meaningful analysis (Table 3).

Comparison of P. vampyrus and P. hypomelanus Bats

The antibody titer difference between *P. vampyrus* and *P. hypomelanus* bats was significant (p<0.001, by Mann-Whitney U test. The difference in seroprevalence for NiV between *P. hypomelanus* (13/117, 11.1%) and *P. vampyrus* (82/253, 32.8%) bats was significant (χ^2 19.54, p<0.001), and risk for a seropositive reaction to NiV was 3.9× higher for *P. vampyrus* bats than for *P. hypomelanus* bats.

Longitudinal Study of NiV P. hypomelanus Bats

Characteristics of bats sampled are shown in Table 4. All P. *hypomelanus* bat samples had negative culture and PCR results. The overall NiV seroprevalence for *P. hypomelanus* bats from Pulau Tioman was 9.8%. Differences in seroprevalence between sampling times were significant (p<0.001). The highest seroprevalence rate was in 2004, which then waned in 2005 and 2006 (Figure 2).

The seroprevalence of NiV in adult bats (12.8%) was significantly higher than in juveniles (3.7%; p<0.001), and adults were ≈4× more likely to be seropositive (odds ratio 3.9). Seroprevalence was not significantly different between male (11.1%) and female bats (8%; p = 0.258). However, when data for female bats were examined by their reproductive categories, a significant difference (p<0.001) was observed. Bats that were pregnant, carrying a pup, and lactating were 3.9×, 4.8×, and 3.0× more likely
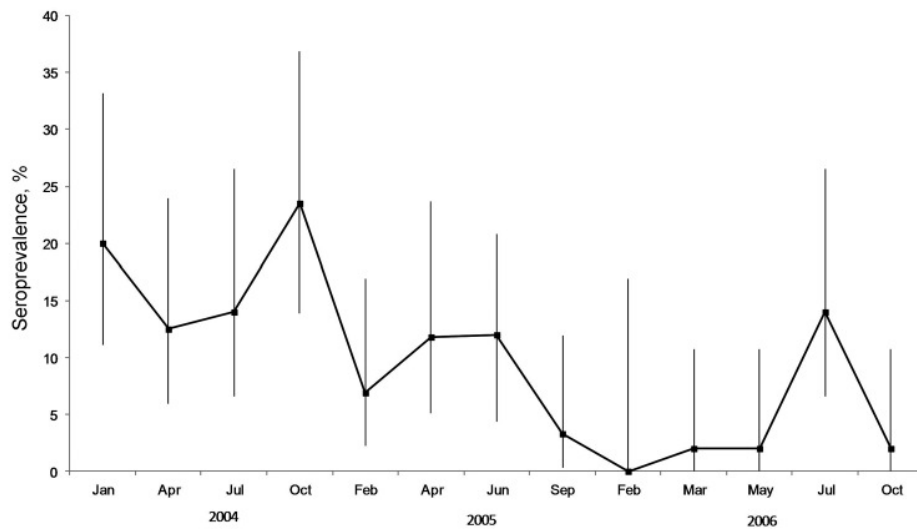
Figure 2. Seroprevalence of Nipah virus among *Pteropus hypomelanus* bats in Pulau Tioman, Peninsular Malaysia, January 2004–September 2006. Error bars indicate 95% CIs.

to be seropositive than adult female bats that did not show these features (Table 5).

Although our data were not sufficiently powered to detect significant differences among various sex and reproductive maturity categories (sample size was not large enough within each category level), when we controlled for the effect of time, the likelihood of seropositivity to NiV among pregnant, carrying, and nursing females remained higher than other female bats, and the highest risk was observed among those females carrying pups. Juvenile female bats were the least likely to have detectable NiV antibodies compared with other bats. Most seropositive cases appeared to cluster in 2004 and decreased toward the end of the study (Table 6).

Anthropic disturbance to roosting sites during our study resulted in dispersal of *P. hypomelanus* bats to smaller ($\leq$20), more disparate cohorts that roosted higher in the trees and finally relocation to another site on Pulau

Table 2. *Pteropus hypomelanus* bats with Nipah virus–neutralizing antibody titers, Peninsular Malaysia, January 2004–October 2006*

Location	Sex	Age	No.	SNT titer	No. positive	% Seroprevalence (95% CI)
Pulau Pangkor, n = 23	M	Adult	13	$\leq$8	0	0 (0–16)
		Juvenile	4	$\leq$8	0	
		Pup	0	NA	NA	
	F	Adult	4	$\leq$8	0	
		Juvenile	2	$\leq$8	0	
		Pup	0	NA	NA	
Pulau Perhentian, n = 15	M	Adult	2	$\leq$8–128	0	13.3 (2.5–39.1)
		Juvenile	1	$\leq$8	1	
		Pup	0	NA	NA	
	F	Adult	7	$\leq$8–64	1	
		Juvenile	5	$\leq$8	0	
		Pup	0	NA	NA	
Pulau Kapas, n = 29	M	Adult	7	$\leq$8	0	3.3 (0.01–18.1)
		Juvenile	0	NA	NA	
		Pup	0	NA	NA	
	F	Adult	15	$\leq$8	1	
		Juvenile	7	$\leq$8–32	0	
		Pup	0	NA	NA	
Pulau Tioman, n = 50	M	Adult	27	$\leq$8–128	6	20 (11.1–33.2)
		Juvenile	4	$\leq$8	0	
		Pup	0	NA	NA	
	F	Adult	11	$\leq$8–256	4	
		Juvenile	8	$\leq$8	0	
		Pup	0	NA	NA	
Overall, N = 117		Adult	73	$\leq$8–256	13	11.1 (6.5–18.2)
		Juvenile	31	NA	0	
		Pup	0	NA	0	

*SNT, serum neutralization test; NA, not applicable (no sample).

Table 3. Univariate analysis of independent variables and Nipah virus serostatus of 2 *Pteropus* bat species surveyed in a cross-sectional survey (without pup data), Peninsular Malaysia, January 2004–October 2006*

Species and risk factor	No. positive/no. tested (%)	p value†	OR (95% CI)
P. vampyrus			
Age			
Juvenile	14/56 (25)	Referent	1.00
Adult	68/193 (35.2)	0.151	1.63 (0.83–3.19)
Sex			
M	38/135 (28.1)	Referent	1.00
F	44/114 (38.6)	0.081	1.6 (0.94–2.73)
Female reproductive status			
Dry	21/58 (36.2)	Referent	1.00
Pregnant	6/14 (42.3)	0.645	1.32 (0.40–4.33)
Carrying a pup	3/7 (42.9)	0.731	1.32 (0.27–6.48)
Nursing	11/17 (64.7)	0.042	3.23 (1.04–9.99)
Juvenile	3/18 (16.7)	0.130	0.35 (0.09–1.36)
P. hypomelanus			
Age			
Juvenile	0/31 (0)	NA	NA
Adult	13/86 (15.1)	NA	NA
Sex			
M	7/58 (12.1)	Referent	1.00
F	6/59 (10.2)	0.744	0.85 (0.26–2.62)
Female reproductive status			
Dry	2/23 (8.7)	Referent	1.00
Pregnant	4/14 (28.6)	0.112	4.20 (0.65–26.89)
Carrying a pup	NA	NA	NA
Nursing	NA	NA	NA
Juvenile	0/22 (0)	NA	NA

*OR, odds ratio; NA, not applicable.
†By χ^2 test, Fisher exact test, or simple logistic regression.

Tioman, ≈6 km from the previous site. We believe roost size affected seroprevalence rates and thus reduced risks for seropositivity, as observed toward the end of the study.

Discussion

All bats captured during the study, including NiV-seropositive bats, appeared healthy, which was consistent with observations from experimental infections of pteropid bats with NiV (*25,26*). Our cross-sectional survey found that seroprevalences of NiV in *P. vampyrus* and *P. hypomelanus* bats were 32.8% and 11.1%, respectively, which differed from results of a study Yob et al. (*12*) after the first NiV outbreak (17% vs. 31%, respectively). Although both studies support the hypothesis that *Pteropus* spp. are the natural reservoir for NiV in Malaysia, we

believe that this difference may be attributed to our study having a larger sample size for each species and a wider geographic sampling scale, as well as potential differences among the diagnostic assays used.

The presence of NiV-seropositive *P. vampyrus* bats across Peninsular Malaysia was expected because satellite telemetry studies by Epstein et al. (*16*) and Breed et al. (*17*) showed that *P. vampyrus* bats are highly mobile, moving beyond state and national borders and making contact with other conspecifics across the region probable. These findings are consistent with the findings of studies of Hendra virus in Australia and NiV in Bangladesh, in which evidence for circulation has been observed across a broad expanse of the home range of each pteropid species (*27–29*). The higher seroprevalence of NiV among *P. vampyrus* bats than among

Table 4. Characteristics of *Pteropus hypomelanus* bats tested for Nipah virus, Pulau Tioman, Peninsular Malaysia, January 2004–October 2006*

Sex	Age group	No.	Reproductive status	No.	SNT titer range	No. (%) seropositive	% Seroprevalence (95% CI)
Male, n = 407	Adult	314	NA	NA	≤8–512	39 (12.4)	10.80 (8.18–14.27)
	Juvenile	81	NA	NA	≤8–32	5 (6.1)	
	Pup	12	NA	NA	≤8	0	
Female, n = 243	Adult	124	Dry	62	≤8–256	4 (6.5)	8.20 (5.30–12.4)
			Pregnant	19	≤8–256	4 (21.1)	
			Attached	20	≤8–256	5 (25)	
			Nursing	23	≤8–128	4 (17.4)	
	Juvenile	113	NA	NA	≤8–64	2 (1.8)	
	Pup	6	NA	NA	≤8–64	1 (16.6)	
Overall, N = 650	NA	650	NA	NA	≤8–512	64	9.80 (7.77–12.39)

*SNT, serum neutralization test; NA, not applicable.

Table 5. Univariate analysis of independent variables and Nipah virus seropositivity (without pup data) in 632 samples from *Pteropus hypomelanus* bats surveyed in Pulau Tioman, Peninsular Malaysia, January 2004–October 2006

Risk factor	No. positive/no. tested (%)	p value*	Odds ratio (95% CI)
Sampling time			
Jan 2004	10/50 (20)	Referent	1.00
Apr 2004	7/50 (14)	0.297	0.57 (0.20–1.64)
Jul 2004	7/50 (14)	0.427	0.65 (0.23–1.88)
Sep 2004	12/51 (6.3)	0.668	1.23 (0.48–3.18)
Feb 2005	4/54 (6.9)	0.052	0.29 (0.08–1.01)
Apr 2005	5/41 (6.9)	0.262	0.53 (0.18–1.59)
Jun 2005	6/50 (12)	0.280	0.54 (0.18–1.63)
Sep 2005	2/61 (3.3)	0.013	0.13 (0.03–0.65)
Feb and Mar 2006†	1/71 (1.4)	0.007	0.05 (0.01–0.45)
May 2006	1/50 (2)	0.019	0.08 (0.01–0.66)
Jul 2006	7/50 (14)	0.427	0.65 (0.22–1.88)
Oct 2006	1/50 (2)	0.019	0.08 (0.10–0.66)
Age			
Juvenile	7/194 (3.7)	<0.001	1.00
Adult	56/438 (12.8)		3.91 (1.75–8.75)
Sex			
M	44/395 (11.1)	0.213	1.00
F	19/237 (8.1)		0.69 (0.39–1.22)
Female reproductive status			
Dry	4/65 (6.5)	Referent	1.00
Pregnant	4/19 (21)	0.077	3.87 (0.86–17.29)
Carrying a pup	5/20 (25)	0.031	4.83 (1.15–20.24)
Nursing	4/23 (17.4)	0.139	3.05 (0.69–13.40)
Juvenile	2/113 (1.8)	0.128	0.26 (0.05–1.47)

*By χ^2 test, Fisher exact, or simple logistic regression.
†Data were combined to enable analysis.

P. hypomelanus bats suggests that exposure to NiV is more common among the *P. vampyrus* bats, which is consistent with higher rates of NiV seroprevalence among juvenile *P. vampyrus* bats than among juvenile *P. hypomelanus* bats. These findings suggest that viral circulation and exposure are probably more common among *P. vampyrus* bats.

We believe that the greater connectivity of *P. vampyus* bats among colonies in the region create a metapopulation structure in which there is more opportunity for virus to circulate by migration, resulting in a higher rate of exposure. Island bats have limited connectivity (*30*). Thus, herd immunity tends to wane over time, creating a relatively lower seroprevalence (*11*). This phenomenon may be illustrated by our survey of *P. hypomelanus* bats on Pulau Pangkor, in which we did not detect any seropositive bats. We sampled 24 bats, which would have enabled us to detect a seropositive bat, given a prevalence >10% with 95% confidence. The fact that we did not detect any seropositive bats suggests a lower seroprevalence, which could also be attributed to the relatively high degree of urbanization on Pulau Pangkor and consequently smaller colonies and a decreased seroprevalence.

The longitudinal study of *P. hypomelanus* bats showed that prevalence of neutralizing antibodies to NiV fluctuated during the study (range 1%–20%) and, although unpredictable, seroprevalence generally waned in the final 2 years (Figure 2). We did not observe any consistent and discernible seasonal seroprevalence pattern. However, other studies have suggested periodic patterns of antibody prevalence that are connected to the reproductive cycle of bats for other viruses such as Hendra virus (*31*), filovirus, and lyssavirus (*8,9*). With the exception of the first NiV outbreak in Malaysia, other studies have linked NiV outbreaks to bat reproductive seasons (*32–34*). We believe that the waning seroprevalence during our study is partly explained by anthropic disturbances that occurred at the original study site in Pulau Tioman, which resulted in relocation and dispersal of the original colony into smaller roost sizes. In addition to waning immunity in bats, smaller roost size decreases viral transmission to other susceptible bats, resulting in decreasing seroprevalence over time. The lack of viral isolation from any sample collected during our studies may have been caused by a low incidence of viral shedding or low viral excretion doses within the colony, which is supported by studies of henipaviruses (*1,4,5,18,35*).

Sex was not a risk factor for NiV exposure, which is consistent with results of other studies (*28,31,36*). However, when we stratified the analysis on the basis of sexual and reproductive maturity of the bats, we found that female bats that were pregnant, had an attached pup, and were lactating had a consistently higher likelihood of exposure to NiV than adult males or dry adult females. Analysis of data from a cross-sectional survey of bats of both species suggests an increasing risk for exposure to NiV when female bats were pregnant or lactating. This finding was strengthened in the longitudinal study because female bats that were pregnant, had an attached pup, or were nursing had a higher risk for

Table 6. Risk factors for Nipah virus seropositivity (without pup data) in *Pteropus hypomelanus* bats sampled in Pulau Tioman, Peninsular Malaysia, January 2004–October 2006*

Variable	β	SE	p value	OR (95% CI)
Time				
Jan 2004	Referent	Referent	Referent	1.00
Apr 2004	−0.58	0.57	0.310	0.55 (0.17–1.72)
Jul 2004	0.52	0.59	0.373	1.69 (0.53–5.43)
Sep 2004	0.38	0.51	0.457	1.46 (0.53–3.97)
Feb 2005	−1.31	0.62	0.034	0.26 (0.07–0.90)
Apr 2005	−1.17	0.78	0.135	0.30 (0.06–1.44)
Jun 2005	−0.44	0.59	0.459	0.64 (0.19–2.07)
Sep 2005	−1.54	0.76	0.045	0.21 (0.04–0.96)
Feb and Mar 2006†	−2.59	1.09	0.018	0.07 (0.00–0.63)
May 2006	−2.13	1.09	0.051	0.11 (0.01–1.00)
Jul 2006	−0.32	0.56	0.564	0.72 (0.24–2.17)
Oct 2006	−2.47	1.08	0.022	0.08 (0.01–0.70)
Sexual and reproductive maturity				
Adult males	Referent	Referent	Referent	1.00
Juvenile males	−0.78	0.52	0.132	0.45 (0.16–1.27)
Dry females	−0.84	0.53	0.114	0.42 (0.14–1.22)
Pregnant females	0.50	0.62	0.421	1.65 (0.48–5.61)
Carrying a female pup	1.24	0.69	0.074	3.48 (0.88–13.6)
Nursing females	0.50	0.63	0.427	1.65 (0.47–5.74)
Juvenile females	−2.34	0.70	0.001	0.09 (0.02–0.38)

*β, estimated coefficient; OR, odds ratio.
†Data were combined to enable analysis.

NiV when we controlled for the potential confounding effect of sampling time (or seasonality).

Among mammals, *Pteropus* spp. bats are known to carry their pups for ≤3 months (*30*) after birth and continue to nurse for ≤4 months after the pup is independent. Pregnancy and lactation are the most metabolically demanding periods of mammalian life (*37*). Therefore, the cumulative stress from reproductive activities, followed by physical exertion from carrying an attached pup to lactation, may have led to the increased risk for NiV infection among this group of bats, consistent with the findings of Plowright et al. (*31*). We speculate that NiV spillover events are most likely to occur during these periods.

Our study has several limitations, including sampling bias, which results from the nonrandom sampling technique used. Sample numbers were often suboptimal because of extreme difficulty in catching pteropid bats. In addition, there are major challenges associated with interpretation of serologic data in wildlife populations. Although we detected differences in the prevalence of neutralizing antibodies between species and increased risk among bats in different reproductive categories, there is still little known about the timing of actual infection or the duration of NiV antibodies in bats. One technique used to overcome this difficulty was to examine age-stratified serologic data, which has been used in similar epidemiologic studies (*31*). *P. hypomelanus* bats reach sexual maturity at ≈12 months of age (*30*) and *P. vampyrus* at 24 months of age (*38*), which enabled us to infer that NiV antibodies in weaned juveniles (≈6–24 months of age) may indicate recent viral circulation. Our previous study among captured *P.*

vampyrus bats demonstrated horizontal transmission of NiV from an adult to juvenile bats within the same colony after a brief shedding episode (*18*). Our finding of juvenile *P. hypomelanus* bats with antibodies to NiV during the longitudinal study support the possibility that virus had been circulating in this population within the lifetime of the juvenile, assuming that the juvenile bats were old enough to have lost maternal antibodies.

Our finding of neutralizing antibodies to NiV in both species of *Pteropus* bats at almost all locations we studied in Malaysia, coupled with isolation of NiV from these bats by our group and another group, strengthened the theory that NiV is enzootic in both *Pteropus* bat species, and that these species serve as the natural reservoir for NiV in Malaysia. Longitudinal surveys of *P. hypomelanus* bats suggest size and colony density may cause lower seroprevalence, and female bats that were pregnant, carrying a pup, and lactating generally had higher rates of NiV exposure than males or nonpregnant adult females, which lend further support to the hypothesis that infection rates may be higher during periods of pregnancy and lactation. Further study of NiV infection and shedding rates in pteropid bats will help elucidate seasonal and intracolonial viral dynamics.

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Invasive Pneumococcal Disease after Routine Pneumococcal Conjugate Vaccination in Children, England and Wales

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We assessed known risk factors, clinical presentation, and outcome of invasive pneumococcal disease (IPD) in children 3–59 months of age after introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) in England and Wales. During September 2006–March 2010, a total of 1,342 IPD episodes occurred in 1,332 children; 14.9% (198/1,332) had comorbidities. Compared with IPD caused by PCV7 serotypes (44/248; 17.7%), comorbidities were less common for the extra 3 serotypes in the 10-valent vaccine (15/299; 5.0%) but similar to the 3 additional PCV13 serotypes (45/336; 13.4%) and increased for the 11 extra serotypes in 23-valent polysaccharide vaccine (PPV23) (39/186; 21.0%) and non-PPV23 serotypes (38/138; 27.5%). Fifty-two (3.9%) cases resulted from PCV7 failure; 9 (0.7%) case-patients had recurrent IPD. Case-fatality rate was 4.4% (58/1,332) but higher for meningitis (11.0%) and children with comorbidities (9.1%). Thus, comorbidities were more prevalent in children with IPD caused by non-PCV13 serotypes and were associated with increased case fatality.

In September 2006, the United Kingdom introduced the 7-valent pneumococcal conjugate vaccine (PCV7) into the national childhood immunization program for receipt at 2, 4, and 13 months of age (*1*). At the same time, a 12-month catch-up campaign was initiated that offered 2 vaccine doses to 2–8-month-old infants and 1 dose to 12–24-month-old children (*1*). The program rapidly achieved high vaccine coverage (*2*) and was highly effective (*3*), resulting in a rapid reduction in invasive pneumococcal disease (IPD) caused by the serotypes in PCV7 (PCV7-IPD; serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F), particularly in children

<2 years of age, for whom PCV7-IPD decreased by 98% by 2009–10 (*4*). Because conjugate vaccines induce high antibody levels that reduce carriage in vaccinated children and, therefore, transmission of *Streptococcus pneumoniae* to others, PCV7-IPD also declined by ≥75% in older age groups through indirect protection (herd immunity) (*4*). Moreover, although this reduction was offset by an increase in IPD caused by serotypes not included in PCV7 (serotype replacement disease), IPD decreased 34% overall across all age groups during 2009–10 (56% in children <2 years of age) (*4*). The introduction of a 13-valent vaccine (PCV13) in April 2010 provided protection against 2 of the key replacing serotypes, 7F and 19A (*5*); however, concern remains about the potential for further replacement disease with non-PCV13 serotypes.

After PCV7 introduction, the Health Protection Agency (HPA) collected detailed clinical information about all laboratory-confirmed IPD cases in children eligible for PCV7 in England and Wales. To predict the potential long-term effect of higher-valent vaccines on childhood IPD, an understanding is needed of the characteristics of children in whom IPD developed and of the infecting serotypes during the PCV7 period. We describe the distribution of known risk factors, clinical features, and outcome of illness in children with IPD in the cohort eligible for PCV7 in England and Wales.

Methods

IPD Surveillance

The HPA conducts enhanced surveillance for IPD in England and Wales (*4*). The HPA routinely collects computerized hospital laboratory reports of invasive pneumococcal isolates and actively requests referral of isolates to its national Reference Laboratory for

serotyping. The laboratory reports are regularly reconciled with serotype data into a single dataset. We report on all IPD case-patients 3–59 months of age in the birth cohorts eligible for PCV7 (children born since September 2004) in whom IPD was diagnosed during the 43-month period spanning September 4, 2006–March 31, 2010 (before PCV13 introduction). The HPA has approval under Patient Information Advisory Group Section 60 of the Health and Social Care Act 2001 to process confidential patient information for public health purposes (www.legislation. hmso.gov.uk/si/si2002/20021438.htm).

In children with >1 IPD episode, only the first episode was included in the analysis except where repeat IPD episodes were described. Vaccination status was obtained by telephone from the child's general practitioner, followed by a questionnaire to the general practitioner and/or pediatrician requesting clinical information, including known risk factors for IPD (6). Incomplete questionnaires and questionnaires that were not returned after a reminder letter was sent were followed up by telephone.

Definitions

An IPD case was defined as culture of *S. pneumoniae* from a normally sterile site or, for culture-negative cases, detection of pneumococcal DNA in cerebrospinal or pleural fluid. Meningitis was defined as *S. pneumoniae* identified in cerebrospinal fluid through culture and/or PCR or clinical and/or radiologic features of meningitis with *S. pneumoniae* isolated from blood culture. Lower respiratory tract infection (LRTI) was defined as *S. pneumoniae* in empyema fluid or in blood with radiologic and/or clinical diagnosis of pneumonia. Septicemia was defined as *S. pneumoniae* cultured in blood with no distinctive clinical syndrome. Repeat samples from sterile sites within 30 days from the same person were regarded as part of the same episode.

PCV7 vaccine failure was defined as PCV7-IPD occurring at least 14 days after 2 doses in children <12 months of age or after 1 dose in children $\geq$12 months of age (irrespective of the number of previous PCV7 doses). The 3 extra serotypes in 10-valent PCV (PCV10) were 1, 5, and 7F; the additional 3 PCV13 serotypes were 3, 6A, and 19A; and the additional 11 serotypes in the 23-valent polysaccharide vaccine (PPV23) were 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F. Fatal cases were followed up by requesting a hospital discharge summary and a postmortem report where appropriate. Death caused by IPD was defined as *S. pneumoniae* identified from a normally sterile site 1) before death, with clinical, laboratory, and/or radiologic evidence of invasive bacterial infection; or 2) at postmortem examination, with histopathologic evidence of invasive bacterial infection and/or a report by the histopathologist that the pathogen contributed to the death.

Data Analysis

Data were exported to Stata version 11.0 (StataCorp, College Station, TX, USA) for analysis. Mid-year population estimates were obtained from the Office for National Statistics (www.statistics.gov.uk). Continuous variables that did not follow a normal distribution were described as median and interquartile ranges (IQR) and compared by using the Mann-Whitney U test. Proportions were compared by using the χ^2 test or Fisher exact test, as appropriate.

Multivariable logistic regression was used to calculate the adjusted odds ratio (aOR) and 95% CIs for 1) comorbidity with increasing age in years at disease onset, after adjustment for time since PCV7 introduction and sex; 2) comorbidity with vaccination status among PCV7-IPD cases after adjustment for age and time since PCV7 introduction; and 3) specific clinical features (e.g., meningitis, LRTI) as binary outcome variables and infecting pneumococcal serotype groups as explanatory variables after adjustment for sex, comorbidities, time since PCV7 introduction, and vaccination status. We also used a multivariable logistic regression model to evaluate risk factors for death; explanatory variables were age and time since PCV7 introduction as continuous variables and sex, vaccination status, infecting serotype group, comorbidities, and clinical features as categorical variables. Multinomial logistic regression was used to study the association between comorbidities and serotype groups after adjustment for age, sex, vaccination status, and time since PCV7 introduction.

Results

During September 2006–March 2010, a total of 1,342 IPD cases occurred in 1,332 children 3–59 months of age. Median age at disease onset was 14.5 months (IQR 9.0–26.6 months);198 (14.9%) of the 1,332 patients had underlying comorbidity (Table 1), which, after adjustment for study year and sex, increased with age (aOR 1.17, 95% CI 1.04–1.33, p = 0.013). Malignancy/immunosuppression (56 cases) accounted for approximately one fourth of comorbidities, followed closely by congenital heart disease (36 cases), given that an additional 17/23 (74.9%) children with Down syndrome also had congenital heart disease (Table 2). Septicemia was the main clinical feature, followed by meningitis and LRTI (Table 1). Clinical presentation with meningitis decreased with age, whereas LRTI increased and accounted for more than one third of cases among children 2–5 years of age (Table 1). Comorbidities were present in 8.0% (19/237) of children with LRTI, 11.3% (34/300) with meningitis, 19.9% (154/772) with septicemia, and 17.4% (4/23) with other conditions.

Pneumococcal Serotypes causing IPD

Serotype information was available for 90.6%

Table 1. Characteristics of children with IPD in the cohort eligible for PCV7, England and Wales, September 4, 2006–March 31, 2010*

Characteristic	Age group, mo, no. (%)			
	3–11, n = 507	12–23, n = 446	24–59, n = 379	Total, n = 1,332
Male sex	307 (60.6)	253 (56.7)	220 (58.0)	780 (58.6)
No comorbidity	441 (87.0)	392 (87.9)	301 (79.4)	1,134 (85.1)
Any comorbidity	66 (13.0)	54 (12.1)	78 (20.6)	198 (14.9)
Serotype group				
PCV7	95 (18.7)	106 (23.8)	47 (12.4)	248 (18.6)
Extra 3 PCV10	86 (17.0)	78 (17.5)	135 (35.6)	299 (22.4)
Extra 3 PCV13	134 (26.4)	112 (25.1)	90 (23.7)	336 (25.2)
Extra 11 PPV23	82 (16.2)	62 (13.9)	42 (11.1)	186 (14.0)
Other	55 (10.8)	39 (8.7)	44 (11.6)	138 (10.4)
Not known	55 (10.8)	49 (11.0)	21 (5.5)	125 (9.4)
PCV7-IPD cases				
Year 1	66/142 (46.5)	88/136 (64.7)	15/25 (60.0)	169/303 (55.8)
Year 2	17/104 (16.3)	13/90 (14.4)	13/69 (18.8)	43/263 (16.3)
Year 3	11/114 (9.6)	4/116 (3.4)	7/134 (5.2)	22/364 (6.0)
Year 4 (36–43 mo)	1/92 (1.1)	1/55 (1.8)	12/130 (9.2)	14/277 (5.1)
Comorbidity				
Prematurity	58 (11.4)	44 (9.9)	28 (7.4)	130 (9.8)
Septicemia	272 (53.6)	291 (65.2)	209 (55.1)	772 (58.0)
Meningitis	195 (38.4)	70 (15.7)	35 (9.2)	300 (22.5)
LRTI	23 (4.5)	82 (18.4)	132 (34.8)	237 (17.8)
Other	17 (3.4)	3 (0.7)	3 (0.8)	23 (1.7)
≥1 PCV7 vaccine dose	410 (80.9)	315 (70.6)	309 (81.5)	1,034 (77.6)
Hemolytic uremic syndrome†	8 (1.6)	11 (2.5)	5 (1.3)	24 (1.8)
Antimicrobial resistance				
Penicillin‡	4/191 (2.1)	5/154 (3.2)	4/118 (3.4)	13/465 (2.8)
Erythromycin	9/153 (5.9)	16/129 (12.4)	4/92 (4.3)	29/374 (7.8)
Died and had	27/507 (5.3)	16/446 (3.6)	15/379 (4.0)	58/1,332 (4.4)
No comorbidity	17/437 (3.9)	13/389 (3.3)	10/295 (3.4)	40/1,121 (3.6)
Comorbidity	10/70 (14.3)	3/57 (5.3)	5/84 (6.0)	18/211 (8.5)
Septicemia	7/272 (2.6)	5/291 (1.7)	6/209 (2.9)	18/772 (2.3)
Meningitis	18/195 (9.2)	9/70 (12.9)	6/35 (17.1)	33/300 (11.0)
LRTI	1/23 (4.3)	2/82 (2.4)	3/132 (2.3)	6/237 (2.5)
Other	1/17 (5.9)	0/3 (0)	0/3 (0)	1/23 (4.3)
Serotype group				
PCV7	3/95 (3.2)	6/106 (5.7)	2/47 (4.3)	11/248 (4.4)
PCV10	3/86 (3.5)	0/78 (0)	2/135 (1.5)	5/299 (1.7)
PCV13	8/134 (6.0)	3/112 (2.7)	5/90 5.6)	16/336 (4.8)
PPV23	2/82 (2.4)	5/62 (8.1)	2/42 (4.8)	9/186 (4.8)
Other	7/55 (12.7)	0/39 (0)	3/44 (6.8)	10/138 (7.2)

*IPD, invasive pneumococcal disease; PCV7, 7-valent pneumococcal conjugate vaccine, PCV10, 10-valent pneumococcal conjugate vaccine; PCV13, 13-valent pneumococcal conjugate vaccine; PPV23, 23-valent polysaccharide vaccine; PCV7-IPD cases, IPD caused by the serotypes in PCV7; LRTI, lower respiratory tract infection.
†The responsible serotypes were 19A (13 cases, 52%), 7F (4 cases, 16%), 3 (3 cases, 12%), 1 (2 cases, 8%), 11A (1 case, 4%), and 22A (1 case, 4%).
‡Two isolates from each age group were of intermediate penicillin resistance.

(1,207/1,332) of first episodes (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/pdfs/12-0741-Techapp. pdf). The proportion of PCV7-IPD cases decreased with time since PCV7 introduction in all age groups (Figure; Table 1). During the last 7 months of surveillance, the extra 6 serotypes in PCV13 accounted for 69.0% (191/277) of IPD cases for which the organism was serotyped.

Comorbidities

The proportion of children with comorbidities did not alter with time since PCV7 introduction (Figure). Among PCV7-IPD cases, although a higher proportion of children with comorbidities had been previously vaccinated with ≥1 PCV7 dose (22/44 [50.0%] vs. 69/204 [33.8%]), the difference was not significant after adjustment for age at

disease onset and time since PCV7 introduction (aOR 1.58, 95% CI 0.72–3.47, p = 0.25). However, the proportion with comorbidity differed by serotype group (p<0.001) (Table 3). The difference was due to a low proportion with comorbidity for the extra 3 PCV10 serotypes and high proportion for the additional 11 PPV23 serotypes, as well as non-PPV23 serotypes. This relationship remained significant after adjustment for age, vaccination status, and time since PCV7 introduction by using multinomial logistic regression where serotype group was set as the outcome variable.

Clinical Features

After PCV7 introduction, the proportion of children with LRTI increased, even after adjustment for age at

Table 2. Comorbidities in children with invasive pneumococcal disease, England and Wales, September 4, 2006–March 31, 2010

Comorbidity	Age group, mo, no. (%)			Total, n = 1,332
	3–11, n = 507	12–23, n = 446	24–59, n = 379	
Total	66 (13.0)	54 (12.1)	78 (20.6)	198 (14.9)
Malignancy/immunosuppression	6 (9.1)	12 (22.2)	38 (48.7)	56 (28.3)
Hematologic malignancy	0	1	23	24
Solid-organ malignancy	3	6	7	16
Transplant*	1	1	3	5
Primary immunodeficiency	2	4	3	9
HIV infection	0	0	2	2
Congenital heart disease	22 (33.3)	7 (13.0)	7 (9.0)	36 (18.2)
Down syndrome†	9 (13.6)	5 (9.3)	9 (11.5)	23 (11.6)
Respiratory disease	10 (15.2)	11 (20.4)	6 (7.7)	27 (13.6)
Chronic lung disease	5	5	4	14
Congenital anomaly	5	5	1	11
Severe asthma on oral steroids	0	1	1	2
Gastrointestinal disease	7 (10.6)	4 (7.4)	2 (2.6)	13 (6.6)
Biliary atresia	5	0	1	6
Long-term total parenteral nutrition dependency‡	2	4	1	7
Neurologic disease	7 (10.6)	7 (13.0)	7 (9.0)	21 (10.6)
Cerebral insult/palsy	4	1	4	9
Congenital anomaly	2	4	2	8
Cochlear implant	0	1	1	2
Ventricular-peritoneal shunt	1	1	0	2
Sickle cell disease	2 (3.0)	3 (5.6)	6 (7.7)	11 (5.6)
Renal disease	3 (4.5)	5 (9.3)	3 (3.8)	11 (5.6)
Congenital anomaly	3	1	1	5
Metabolic renal condition	0	1	0	1
Nephrotic syndrome	0	2	2	4
Chronic renal failure	0	1	0	1

*Transplant of the heart (2), liver (1), bone marrow (1), and kidney (1).
†Congenital heart disease also was reported in 6/9, 4/5, and 7/9 children with Down syndrome in the 3 age groups, respectively.
‡Four with postnecrotizing enterocolitis with surgical resection of bowel, 2 with protein-losing enteropathy, and 1 with malabsorption syndrome.

disease onset, sex, comorbidities, and infecting serotype group (aOR 1.19/year, 95% CI 1.01–1.41, p = 0.045). When analyzed by serotype group, after adjustment for sex, comorbidities, age, time since PCV7 introduction, and vaccination status, the 3 extra PCV10 serotypes were less likely to cause meningitis (aOR 0.43, 95% CI 0.26–0.74, p = 0.002) and more likely to cause LRTI (aOR 6.49, 95% CI 2.73–15.5, p<0.001) than were PCV7 serotypes. The observation was the same for the 3 additional PCV13 serotypes (aOR 0.46, 95% CI 0.28–0.74, p = 0.001, and aOR 11.2, 95% CI 4.77–26.3, p<0.001, respectively), and the findings remained significant even after the 3 additional PCV13 serotypes were replaced with serotype 3 only in the logistic regression model (aOR for meningitis 0.16, 95% CI 0.06–0.43, p<0.001; aOR for LRTI 37.0, 95% CI 14.4–95.3, p<0.001). The prevalence of comorbidity in children with serotype 3 IPD was similar to that in children with IPD caused by serotypes 6A and 19A (13/98 [13.3%] vs. 32/238 [13.4%], p = 0.97) although the median age at disease onset was higher (22.6 months [IQR 14.6–36.1 months] vs. 12.1 months [8.6–19.3 months], p<0.001).

Antimicrobial Susceptibility

Results for penicillin susceptibility testing were reported for 465 isolates; 13 (2.8%) exhibited intermediate (6 [1.3%]) or complete (7 [1.5%]) resistance and belonged to serotypes 19A (4 isolates), 19F, 1, 15A, and 9V (1 isolate each) among serotyped isolates (Table 1). Only 1 child with penicillin-resistant IPD had a comorbidity (malignancy), and another had recently returned from southern Europe, but all survived. Results for erythromycin susceptibility testing were reported for 374 isolates, of which 29 (7.8%) were resistant, mainly among serotypes 14 (13 isolates) and 19F (4 isolates). Four isolates were reported as resistant to penicillin and erythromycin. None of these children had comorbidity; 3 developed septicemia, 1 had meningitis, and none died. Penicillin resistance remained stable during the surveillance period, whereas erythromycin resistance declined from 19.4% (21/108) in the first year after vaccine introduction to 1.4% (1/73) in the final year, mainly because of declines in serotypes 14 and 19F.

Vaccine Failure

PCV7-IPD occurred in 248 (20.5%) of 1,207 serotyped cases, and 52 (3.9%) of 1,332 children with IPD had 53 episodes of PCV7 vaccine failure, including 1 fully vaccinated cochlear implant recipient with 2 distinct meningitis episodes 10 months apart. Serotypes 6B (18/53 cases, 34.0%) and 19F (16/53, 30.2%) were responsible for almost two thirds of PCV7 vaccine failures. Case-

patients with PCV7 vaccine failure were more likely to have comorbidities (15/52 [28.8%] vs. 166/1,155 [14.4%] case-patients with known serotype, p = 0.004). Only 1 case-patient with PCV7 vaccine failure, who had immune deficiency, died of pneumococcal meningitis 2 months after receiving a PCV7 catch-up dose in the second year of life.

The additional 11 PPV23 serotypes were responsible for 14% of serotyped IPD cases overall and 22% for those with comorbidity. Of the 78 children ≥2 years of age who had comorbidities and would have been eligible for PPV23, IPD caused by the 11 additional PPV23 serotypes developed in 17 (21.8%). Only 4 (5.1%) of the 78 children had received PPV23 before their IPD episode; IPD caused by a PPV23 serotype developed in 2 of these, 1 with sickle cell disease who had been fully vaccinated with PCV7 and PPV23 and in whom serotype 19A IPD subsequently developed and 1 with severe developmental delay who had received 1 catch-up dose of PCV7 and PPV23 but in whom serotype 6B IPD subsequently developed. The other 2 PPV23-vaccinated children, both with sickle cell disease and fully vaccinated with PCV7 and PPV23, developed serotypes 6A and 23A IPD, respectively.

Repeat IPD Episodes

Nine (0.7%) children had repeat IPD episodes. Eight had 2 episodes each, and 1 had 3 episodes (online Technical Appendix Table 2). Four had known comorbidities (44.4% vs. 14.7% [194/1,323] for the rest of the cohort; p = 0.012), 1 was a PCV7 vaccine failure, and none died.

Case-Fatality Rate

Sixty-two children died, including 4 with multiple comorbidities who died several weeks after recovering from IPD; their deaths were attributed to complications of chronic liver disease (2 children), *Escherichia coli* septicemia (1 child), and group A streptococcal septicemia (1 child). The IPD-attributable case-fatality rate (CFR) was, therefore, 4.4% (58/1,332); almost one third (18/58 [31.0%]) of children who died had comorbidities. Seven (12.1% [1 with comorbidity]) died at home; 8 (13.8% [6 with comorbidities]) died on the way to the hospital; 12 (20.7% [6 with comorbidities]) died in the emergency department; and 31 (53.5% [5 with comorbidities]) died in the intensive care unit. One toddler, in whom *Staphylococcus aureus* meningitis had developed at 7 months of age, died of pneumococcal meningitis at 13 months of age, and IRAK-4 mutation was subsequently diagnosed at postmortem examination.

Of the 51 deaths in children for whom serotype was known, CFR for PCV7-IPD cases was equally distributed among the 7 serotypes, and 10/11 (90.9%) deaths occurred after 4 months of age, of which 9 were in unvaccinated
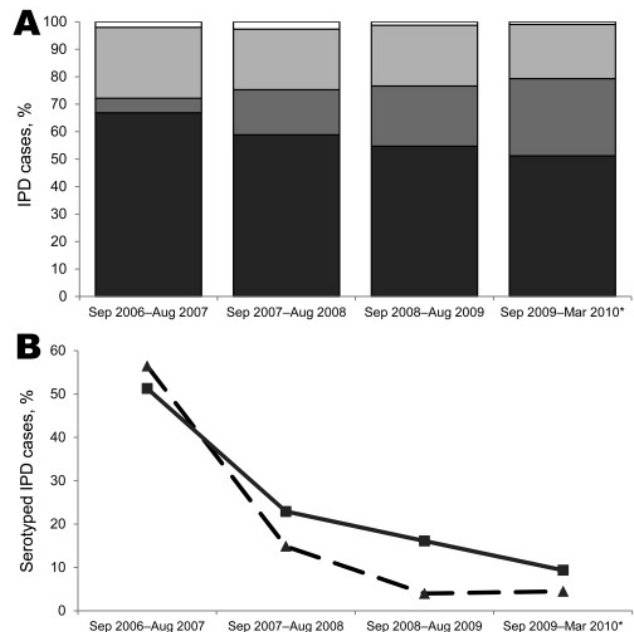
Figure. IPD clinical cases in PCV7-eligible children since PCV7 introduction, England and Wales, September 4, 2006–March 31, 2010. A) Distribution of cases. Black bar sections, bacteremia; dark gray bar sections, lower respiratory tract infection; light gray bar sections, meningitis; white bar sections, other. B) Proportion of serotyped cases caused by PCV7 serotypes in healthy children (dashed line) and children with comorbidities (solid line). The prevalences of comorbidity among IPD cases during the 4 time periods were 13,5%, 17.7%, 17.0%, and 10.7%, and the case-fatality rates were 5.6%, 4.3%, 4.3%, and 3.0%, respectively. *Data included for 7 months only. IPD, invasive pneumococcal disease; PCV7, 7-valent pneumococcal conjugate vaccine.

children and, therefore, potentially vaccine-preventable. CFR did not vary with time since PCV7 introduction (Figure) and was lower for IPD caused by the extra 3 PCV10 serotypes (1.7%) but not statistically significant (Table 1). CFR for serotype 3 IPD (5/98 cases, 5.1%) was similar to the overall IPD CFR in this age group. After adjustment for age, sex, vaccination status, and time since PCV7 introduction in a logistic regression model, infecting serotype group was not associated with death. On the other hand, meningitis (aOR 6.43, 95% CI 3.36–12.3, p<0.001) and presence of comorbidities (aOR 2.70, 95% CI 1.35–5.38, p = 0.005) were significantly associated with death.

Discussion

Comorbidities were identified in 15% of PCV7-eligible children in England and Wales, with malignancy/immunosuppression and congenital heart disease each accounting for one quarter of reported comorbidities. Compared with PCV7-IPD cases, the prevalence of comorbidity was significantly lower for IPD cases caused by the 3 additional PCV10 serotypes and higher for

Table 3. Association between infecting pneumococcal serotype group and presence of comorbidity, England and Wales, September 4, 2006–March 31, 2010*

Serotype group	Comorbidity, no./total (%)	aOR† (95% CI)	p value
All PCV7-IPD cases‡	44/248 (17.7)		
3 extra PCV10 serotypes (1, 5, 7F)	15/299 (5.0) §	0.24 (0.13–0.45) against PCV7	<0.001
3 extra PCV13 serotypes (3, 6A, 19A)	45/336 (13.4)§	0.72 (0.46–1.13) against PCV7	0.15
		3.58 (1.93–6.66) against PCV10‡	<0.001
11 extra PPV23 serotypes	39/186 (21.0)	1.23 (0.76–1.99) against PCV7	0.40
		6.02 (3.16–11.5) against PCV10	<0.001
		1.68 (1.04–2.71) against PCV13	0.033
Remaining non-PPV23 serotypes	38/138 (27.5)	1.76 (1.07–2.89) against PCV7	0.025
		8.57 (4.46–16.5) against PCV10	<0.001
		2.39 (1.46–3.93) against PCV13	0.001
		1.42 (0.85–2.40) against PPV23	0.18

*aOR, adjusted odds ratio; PCV7, 7-valent pneumococcal conjugate vaccine; PCV10, 10-valent pneumococcal conjugate vaccine; PCV13, 13-valent pneumococcal conjugate vaccine; PPV23, 23-valent polysaccharide vaccine.
†Odds ratio adjusted for age, vaccination status, and time since PCV7 introduction by using a multinomial logistic regression with serotype group as the outcome.
‡Serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F.
§Comorbidity was lower for each of the 3 extra PCV10 serotypes, 1 (7/129, 5.4%), 5 (1/17, 5.9%), and 7F (7/153, 4.6%), compared with the additional 3 serotypes in PCV13, 3 (13/98, 13.3%), 6A (12/47, 25.5%), and 19A (20/191, 10.5%).

non-PCV13 IPD cases. Overall CFR was low and independently associated with meningitis and comorbidity but not with infecting serotype group.

The comorbidities in the cohort reported here are typical of children considered at higher risk for IPD (6–9). Other population-based studies that had differing definitions for comorbidities and age ranges have reported comorbidities in 10%–36% of children with IPD (7,10–13). In Massachusetts, USA, 16% of 578 children <18 years of age who had IPD during 2001–2007 had comorbidity (mainly immunosuppression) (9). The US Active Bacterial Core surveillance program identified comorbidity in only 3% of childhood IPD cases before PCV7 introduction in 1998–99, but this proportion increased to 7% in 2006–07 (p = 0.003), with similar increases in adults and the elderly; this finding suggests that the replacing serotypes after the decline in PCV7-IPD might be less virulent and thus more likely to infect vulnerable children with comorbidities (14). The lower prevalence of comorbidities in the Active Bacterial Core surveillance might be explained partly by inclusion of children who had blood cultures performed in an outpatient setting, whereas blood cultures in the United Kingdom are almost always taken in the hospital and, therefore, capture more severe IPD cases (4). Moreover, changes in clinical practice after PCV7 introduction, such as fewer blood cultures from previously healthy children with fever seeking outpatient care, might have contributed to the increased comorbidity prevalence between the 2 periods in the United States (14).

In the cohort reported here, the 3 extra PCV10 serotypes were more likely to affect healthy children. Serotypes 1, 5, and 7 (which are included in both PCV10 and PCV13) are known to be highly invasive and mainly affect previously healthy persons but appear to cause less severe disease, as determined by various clinical severity scores and requirement for intensive care, and have a lower

CFR (15). In children, a meta-analysis of 7 different datasets found that serotypes 1, 5, and 7 were infrequently isolated among carriage strains but had the highest potential for invasive disease, whereas serotypes that were more likely to be carried (e.g., 6B, 19F, and 23F) were less likely to be invasive (16).

A higher prevalence of comorbidity was identified for IPD cases caused by the 11 additional PPV23 serotypes as well as non-PPV23 IPD cases, suggesting that the remaining serotypes after PCV13 introduction in the United Kingdom might be less virulent. In England and Wales, PPV23 uptake for high-risk persons is low, particularly for children (17), and is consistent with the low PPV23 vaccination rates observed among children with comorbidities in the IPD cohort reported here. That such a low proportion of high-risk children were vaccinated with the nationally recommended PPV23 and 2 of the 4 PPV23-vaccinated case-patients had IPD resulting from 1 of the PPV23 serotypes is concerning and merits further investigation on the use of polysaccharide vaccines in high-risk persons.

In keeping with other studies, PCV7 vaccine failure (3.9%) was uncommon (18–20), and nearly one third of case-patients had comorbidities. A recent study in the United States reported 4% of 753 IPD cases diagnosed during a 27-month period as PCV7 vaccine failures, with 37% of the case-patients with vaccine failure having comorbidities (20). In that study, PCV7-vaccinated children with comorbidities were almost 3× more likely to develop PCV7-IPD than were vaccinated children without comorbidities, even after we controlled for various confounders (20). In another case–control study in the United States that involved 782 children 3–59 months of age who had IPD, vaccine effectiveness of ≥1 PCV7 dose against PCV7-IPD was 96% (95% CI 93%–98%) for healthy children and 81% (95% CI 57%–92%) for

children with comorbidities (*21*). Similar estimates of 97% (95% CI 92%–98%) and 88% (95% CI 78%–94%), respectively, were obtained by using the indirect cohort (Broome) method to measure direct protection afforded by PCV7 (*22*). In the United Kingdom, where a 12-month catch-up campaign offering PCV7 to all children <2 years of age was introduced at the same time as routine infant vaccination, vaccine effectiveness estimated by using the indirect cohort method was similar for children with comorbidities and healthy children receiving the nationally recommended immunization courses, albeit with wide confidence intervals for the different comorbidities and immunization schedules (*3*).

In our study and the US studies, serotypes 6B and 19F were predominantly responsible for PCV7 vaccine failures. Similarly, recurrent IPD was reported in only 9 children (0.7%) and, like vaccine failure, was more likely to occur in children with comorbidities. The relatively long duration between episodes and the different infecting serotypes suggest re-infection rather than persistent infection as a consequence of, for example, inadequate or inappropriate therapy. Before routine pneumococcal vaccination, a US population-based surveillance study reporting 318 cases of recurrent IPD identified persons with HIV and children <5 years of age with chronic illness as the 2 main risk groups (*23*). A more recent US study reported recurrent IPD in 90 of 4,067 children with IPD over 12 years, with comorbidities identified in >80% (*24*). Recurrent IPD cases declined significantly after PCV7 introduction, although age at re-infection or proportion with comorbidities during the pre- and post-PCV7 periods did not differ (*24*). Despite the smaller age range and shorter follow-up period, our study supports the low risk for recurrent IPD after PCV7 introduction, even though we are unable to compare with pre-PCV7 rates. Although 5 of 9 children with recurrent IPD had no reported comorbidities, some could have undetected immunologic abnormalities, as was identified in a child with fatal IPD who had IRAK-4 deficiency, for example (*25–27*). Children in whom IPD developed, particularly because of vaccine failure, should be carefully assessed, and children who have a history of >1 serious invasive infection should be investigated for possible immune deficiency along with their close family members because further infections possibly could be prevented through appropriate immunization and/or antimicrobial prophylaxis.

The extensive follow-up of IPD cases, particularly the fatal cases, helped us to more accurately estimate CFR and assess IPD-associated deaths. Overall, IPD-associated CFR was low and independently associated with comorbidity and meningitis. The finding that meningitis was diagnosed in one quarter of mainly healthy children is concerning given the significantly higher CFR and association with the most severe long-term neurodevelopmental complications (reported in >30% of survivors) among pathogens causing bacterial meningitis in children (*28*).

Like all large-scale epidemiologic studies, our study has limitations. Not all hospital laboratories in England and Wales routinely report or submit clinical isolates to the HPA. However, by combining multiple data sources and actively requesting submission of invasive pneumococcal isolates to the HPA national Reference Laboratory for confirmation and serotyping, we believe that our surveillance captures most laboratory-confirmed IPD cases and, because the same surveillance method has been in place since PCV7 introduction, enables comparison of trends over time. The study is also limited by the relatively short follow-up period for identifying vaccine failure cases and recurrent episodes. However, given the significant decline in carriage of PCV7 serotypes (*29*), we are unlikely to see many cases of PCV7-IPD, particularly in young children where vaccine coverage remains high (*30*). The changing pattern of IPD after PCV7 introduction emphasizes the need for continued epidemiologic and molecular surveillance across all age groups. In addition to assessing the role of PPV23 in protecting children with comorbidities, further studies are required to develop new strategies and vaccines with broader coverage to prevent IPD in children who are most susceptible and reduce deaths associated with pneumococcal meningitis.

Dr Ladhani is a pediatric infectious disease consultant and a clinical epidemiologist at the HPA in London. He is currently involved with conducting enhanced national surveillance for a number of vaccine-preventable infections in England and Wales and has a particular interest in the direct and indirect population impact of conjugate vaccines on prevention of invasive bacterial infections.

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Vaccination and Tick-borne Encephalitis, Central Europe

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Tick-borne encephalitis (TBE) is a substantial public health problem in many parts of Europe and Asia. To assess the effect of increasing TBE vaccination coverage in Austria, we compared incidence rates over 40 years for highly TBE-endemic countries of central Europe (Czech Republic, Slovenia, and Austria). For all 3 countries we found extensive annual and longer range fluctuations and shifts in distribution of patient ages, suggesting major variations in the complex interplay of factors influencing risk for exposure to TBE virus. The most distinctive effect was found for Austria, where mass vaccination decreased incidence to ≈16% of that of the prevaccination era. Incidence rates remained high for the nonvaccinated population. The vaccine was effective for persons in all age groups. During 2000–2011 in Austria, ≈4,000 cases of TBE were prevented by vaccination.

Tick-borne encephalitis (TBE) is the most common arthropod-transmitted viral infection of humans in Europe and central and eastern Asia (*1*); each year, >10,000 TBE patients are hospitalized. The role of TBE as a travel-associated disease is probably underestimated (*2,3*). TBE virus is a member of the family *Flaviviridae*, genus *Flavivirus*, and a close relative of the mosquito-transmitted viruses that cause yellow fever, dengue fever, Japanese encephalitis, and West Nile fever (*4*). Three antigenically closely related subtypes are carried primarily by *Ixodes ricinus* (European subtype) and *I. persulcatus* ticks (Siberian and Far-Eastern subtypes) (*5*). In TBE-endemic areas, the virus circulates between ticks and vertebrate hosts (primarily rodents) (*5,6*); humans are dead-end hosts only and do not play any role in the maintenance of TBE

virus in nature. In most instances, transmission to humans occurs by the bites of infected ticks; however, in some TBE-endemic areas, alimentary infections—obtained through consumption of raw milk or milk products from infected goats, sheep, or cattle—are common (*6,7*). Because virus circulation depends on an intricate balance of virus and host factors that are controlled by environmental conditions, TBE-endemic areas do not follow all areas of tick infestation but are restricted to certain regions that are conducive to maintenance of natural virus cycles (*8–11*). In Europe, the most strongly affected countries are southern Germany, Switzerland, Austria, the Czech Republic, Slovakia, Hungary, Slovenia, the Baltic countries, Poland, parts of Scandinavia, and European Russia.

Similar to other flavivirus infections, only a subset of TBE virus infections leads to neurologic diseases such as meningitis, encephalitis, encephalomyelitis, and radiculitis (*12*). On average, the severity of disease increases with patient age (*13*), and case-fatality rates of <1%, 1%–3%, and ≤35% have been reported in Europe, Siberia, and the Far East, respectively (*12*). Effective inactivated whole virus vaccines are produced in Europe (European subtype strain) and Russia (Far-Eastern subtype strain) (*1*), but their usage differs widely among TBE-endemic countries (*14,15*). Experiments with postvaccination serum and direct mouse challenge experiments have shown that vaccines manufactured with 1 subtype will also protect against strains of the other TBE virus subtypes (*16,17*), consistent with their antigenic similarity. A strong upsurge of TBE in Europe in recent years (*6*) has been associated with climatic, ecologic, and human behavioral changes that might increase the risk for virus exposure (*8,18–20*).

To determine the effectiveness of vaccination, we examined incidence of TBE in Austria over 40 years, including 10 years without vaccination followed by 30 years with increasing vaccination coverage. We compared these data with those for the Czech Republic and Slovenia, 2 neighboring central European countries with high TBE incidence rates but comparatively low vaccination rates.

We demonstrate that the strong decline of TBE observed only in Austria resulted from protection by vaccination and that the incidence rate for the nonvaccinated population remained as high as it was during the prevaccination era. The data from all 3 countries reveal a strong degree of annual and longer range variations, which are coincident in some but not all instances.

Materials and Methods

TBE Vaccines and Vaccination Schedules

The TBE vaccines available on the European market are produced by 2 manufacturers: FSME-IMMUN by Baxter AG, Vienna, Austria, and Encepur by Novartis Vaccines, Marburg, Germany. Both vaccines contain purified TBE virus grown in chick embryo cells, inactivated by formalin, and have aluminum hydroxide added as adjuvant (21). In Austria, the recommended basic vaccination schedule consists of 2 vaccinations ≈4 weeks apart followed by a third vaccination after 5–12 months and a fourth vaccination after ≥3 years. For persons <60 years of age, additional booster immunizations are recommended every 5 years; this interval is reduced to 3 years for persons >60 years of age. Both manufacturers also provide vaccines for children; these vaccines contain half of the antigen dose contained in the vaccines for adults (22,23).

Collection of Vaccination Coverage Data for Austria

For Austria, TBE vaccination status data were collected annually by postal surveys conducted by GfK Austria Health Care (Vienna, Austria); 4,000 households (8,500–10,000 household members, representative of different age groups) were surveyed. Data were acquired from written vaccination history for 87% of participants and memory for 13%.

Documentation of TBE Cases

For several decades, Austria, the Czech Republic, and Slovenia have had well-established systems for documenting TBE cases (20,24,25), and, according to the National Reference Laboratories and/or National Public Health Institutes, the principles of the notification system were not changed over the period analyzed in this study. For all 3 countries, incidence rates refer to cases confirmed by laboratory diagnosis. This confirmation is based on TBE virus IgM and IgG ELISA results, which replaced the hemagglutination-inhibition and/or complement fixation assays used until the early 1980s in Austria and the early 1990s in the Czech Republic and Slovenia. In Austria, data are collected by the Department of Virology of the Medical University of Vienna, which serves as a national reference laboratory for TBE virus and other flaviviruses. The documentation includes the history of vaccination; for the purposes of this study, participants were subdivided into groups: those who followed the regular schedule of vaccination and those who had received an undefined number of vaccinations outside the recommended schedule. For a small (5%) proportion of TBE patients, no precise information about vaccination status could be obtained.

Calculation of Field Effectiveness of Vaccination in Austria

Calculation of field effectiveness of vaccination was based on TBE incidence and vaccination coverage for different age groups as described (24). For significance testing, a Monte Carlo procedure was chosen; 10,000 samples were collected under the zero hypothesis of no difference in vaccination effectiveness. p values were determined as the percentiles of the obtained distributions.

Because vaccination status remained undefined for 5% of the 883 TBE patients in Austria during 2000–2011, we analyzed best-case and worst-case scenarios for vaccine effectiveness. For the best-case scenarios of regularly and irregularly vaccinated patients, 45 patients with unknown vaccination history were omitted; for the worst-case scenarios, these patients were assumed to have been regularly or irregularly vaccinated, respectively.

Calculation of Incidence Rates

Population data for the calculation of incidence rates were obtained from Statistics Austria (www.statistik.at/web_en/), the Czech Statistical Office (www.czso.cz/eng/redakce.nsf/i/home), and the Statistical Office of the Republic of Slovenia (www.stat.si/eng/index.asp). For the analysis of time trends, we used a piecewise log-linear model, the joinpoint analysis, to identify possible trend changes during 1972–2011 (Joinpoint Regression Program, version 3.5.2; Statistical Research and Applications Branch, National Cancer Institute, Silver Spring, MD, USA). The model was specified to include a maximum of 5 joinpoints, constrained to be at least 2 years apart and at least 2 years from the start and end of the entire period. The best fitting model was searched under the assumption of a Poisson variation of the number of patients and the population size in midyears as the offset variable. These analyses were conducted separately for the Czech Republic, Slovenia, and Austria. For Austria, these analyses were conducted for the overall incidence and for the incidence among nonvaccinated persons. For the years before 2000, the incidence among nonvaccinated persons was estimated by assuming a vaccine effectiveness of 97%; from 2000 on, the actual number of cases that occurred among nonvaccinated persons was used for the calculation.

Results

Incidence of TBE in Austria, the Czech Republic, and Slovenia

Epidemiologic data on TBE in Austria have been available since 1972; since then, 8,493 cases have been reported through 2011. The corresponding annual incidence rates are displayed in Figure 1, panel A. In the first 10 years, the average incidence was 5.7 cases per 100,000 population (range 3.9–9.0), after which (past 10 years) incidence declined dramatically to an average incidence of 0.9 cases per 100,000 population (range 0.6–1.3). This decline was coincident with the increasing rate of TBE vaccination that started in 1972, reached 88% in 2005 (i.e., 88% of the total population had received ≥1 doses of TBE vaccine), and slightly leveled off to 85% in 2011. Calculation of the incidence rates for the nonvaccinated population (Figure 1, panel A) revealed an average of 6.0 cases per 100,000 population in the past 10 years (comparable to the prevaccination era) and similar extents of annual variation (range 3.9–9.1), demonstrating that the overall decline resulted not from a lower risk for infection but indeed from protection by vaccination.

In the Czech Republic, 18,196 cases were reported during 1973–2011; in Slovenia, 8,129 cases were reported during 1970–2011. Figure 1, panel B, shows a comparison of the annual TBE incidence rates among persons in these 2 countries with those of the nonvaccinated population in Austria. For each of the 3 countries, the patterns vary markedly, and the degree of coincidence of these variations does not seem to follow a distinct annual pattern. For example, the 2 peaks in 1994 and 1996 coincide for Austria and Slovenia but not for the Czech Republic. Conversely, coincident peaks for Slovenia and the Czech Republic were observed in 2006 and 2009; for Austria, they were shifted to the preceding year. However, for all 3 countries, coincident declines (2004, 2007, 2010) and increases (2011) also occurred.

For better visualization of longer range temporal changes in incidence rates, we used a sliding-window representation of the means of 5-year intervals (Figure 1, panel C). As described (*20,25*) and confirmed by joinpoint analysis, strongly increased incidence was noted for the Czech Republic (starting in 1992; Figure 2, panel C) and for Slovenia (starting in 1993; Figure 2, panel D), whereas no such increase was detected for Austria, at least as determined from the incidence rates for the nonvaccinated population (Figure 2, panel B). The sliding-window representation (Figure 1, panel C), however, suggests longer ranging factors that lead to parallel changes of incidence rates for all 3 countries, as exemplified by the relative declines around 1986–1992, 1995–2003, and 2003–2004 and the relative increases around 1993–1999, 2006, and 2009. A clear
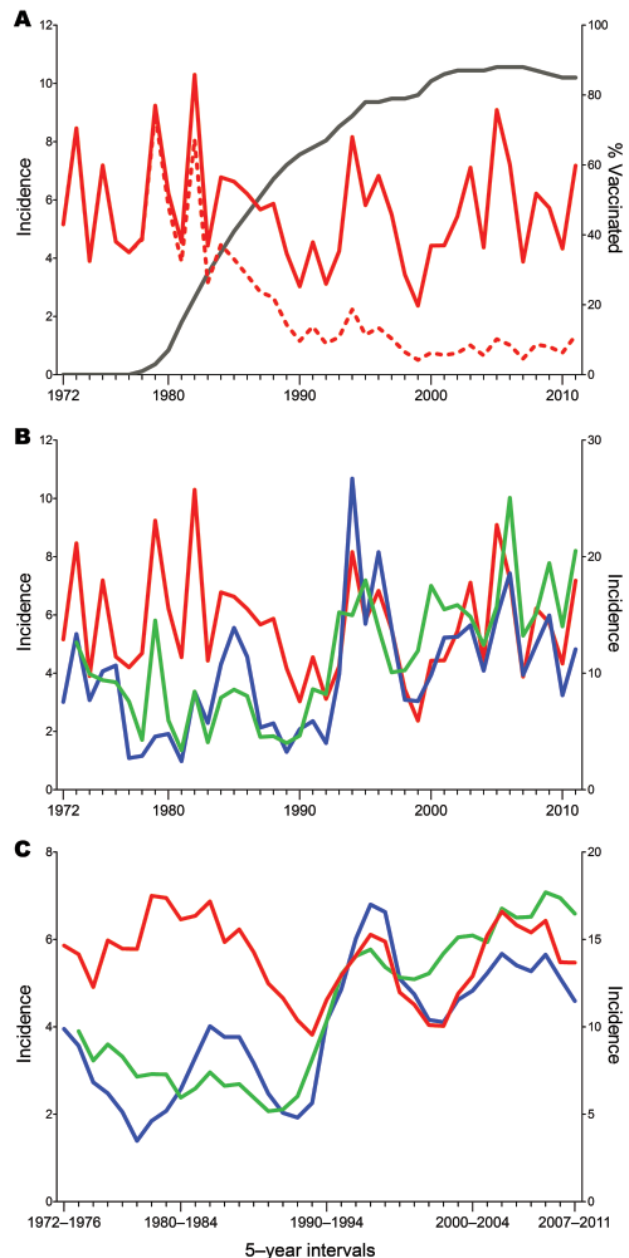
Figure 1. Tick-borne encephalitis (TBE) incidence rates (no. cases/100,000 population), 1972–2011, central Europe. A) Total population (black dashed line) and nonvaccinated population (black solid line) in Austria. The gray line represents the increasing coverage of vaccination, which started in 1978. B) Comparative representation of TBE incidence rates in Austria (black line), Czech Republic (light gray short-dashed line), and Slovenia (dark gray long-dashed line). The incidence scale for Slovenia (right y-axis) differs from that of Austria and the Czech Republic (left y-axis). C) Sliding-window representation of TBE incidence in Austria (black line), Czech Republic (light gray short-dashed line), and Slovenia (dark gray long-dashed line) using means of 5-year intervals. The incidence scale for Slovenia (right y-axis) differs from that of Austria and the Czech Republic (left y-axis). A color version of this figure is available online (wwwnc.cdc.gov/EID/article/19/1/12-0458-F1.htm).
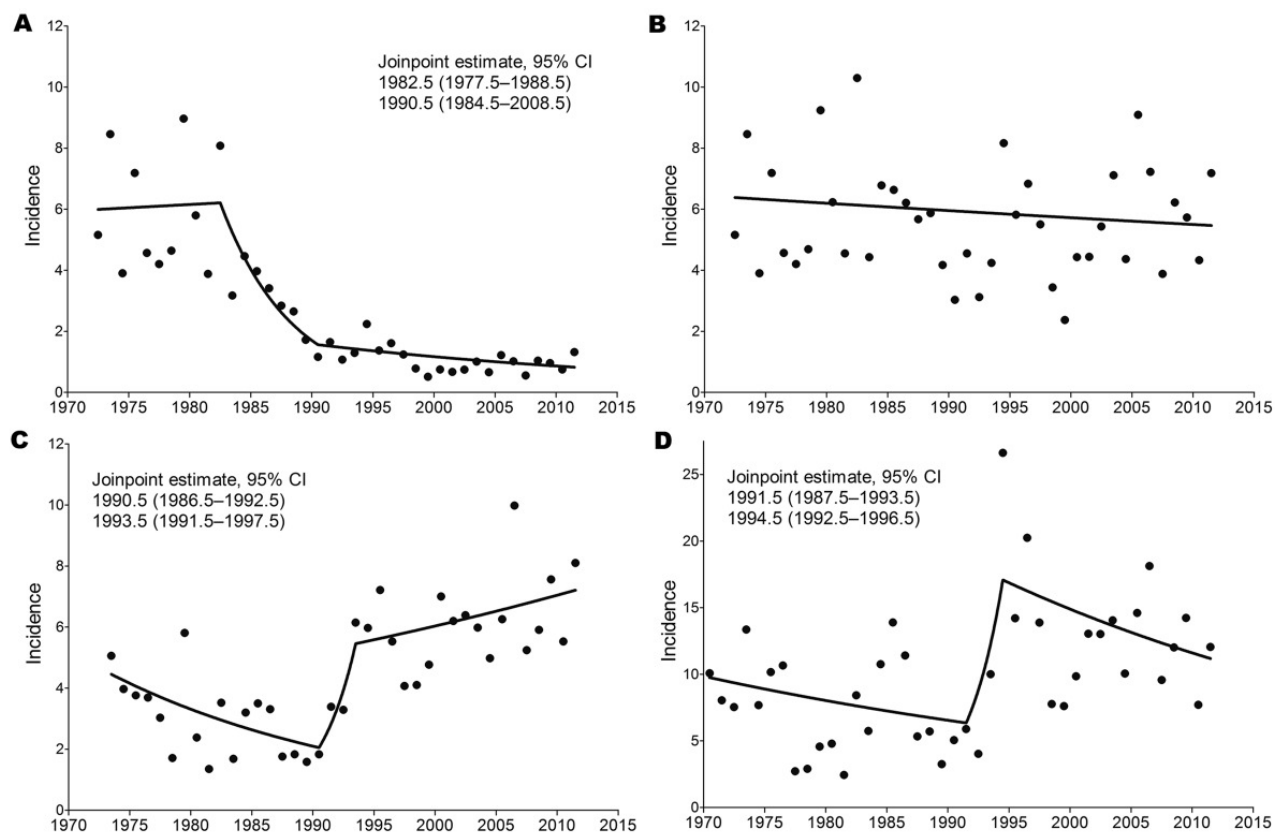
Figure 2. Results of joinpoint analysis of annual incidence rates (no. cases/100,000 population) of tick-borne encephalitis (TBE) in A) Austria (total population), B) Austria (nonvaccinated population), C) Czech Republic, and D) Slovenia. The lines in each panel represent the piecewise log-linear relationship between year and incidence. Estimated joinpoints and their 95% CIs are shown.

discrepancy, however, was observed between incidence rates for Austria and Slovenia and incidence rates for the Czech Republic during 1996–2003.

Age of TBE Patients

The severity of TBE tends to increase as age increases, thereby resulting in a higher proportion of encephalitis cases relative to meningitis cases in older than in younger persons (*13*). Independent of undefined age-specific physiologic characteristics that control disease severity, the risk for virus exposure might differ among age groups, depending on behavioral factors. Analysis of the age distribution of TBE patients in the 3 countries during 1990–1999 and 2000–2010 revealed significant differences (Figure 3). In all 3 countries, incidence rates were highest among elderly persons. During the past decade, incidence rates increased sharply among those 50–80 years of age in the Czech Republic and Slovenia but not in Austria. The substantial TBE incidence rates among children and adolescents in Slovenia and the Czech Republic have virtually disappeared in Austria, although high rates were typical during the prevaccination era (*26*).

Field Effectiveness of Vaccination

Using data on vaccination coverage in Austria and TBE incidence rates for nonvaccinated and vaccinated populations, we calculated the field effectiveness of vaccination for 2000–2011. Patients were stratified into 3 groups: those with a documented history of no vaccination, those vaccinated according to regular schedule (i.e., according to recommendations in Austria), and those vaccinated on an irregular schedule. For some (5%) TBE patients, we were unable to obtain accurate information on their vaccination history and therefore made a worst-case calculation by assuming that all of these patients had been vaccinated according to the recommended schedule. The Table shows that the overall field effectiveness for regularly vaccinated persons is ≈99% under best-case assumptions and 96% under worst-case assumptions. The rate of vaccination failure—usually characterized by a delayed IgM and an anamnestic IgG response (*27*)—was not enhanced among elderly persons, indicating that the vaccine-induced immune response of elderly persons was sufficient to prevent disease in most instances, despite the fact that titers were lower among elderly than among

younger vaccinees (*28*). Irregularly vaccinated persons have a lower degree of protection: 92.5% protection in the best-case and 91.3% in the worst-case scenarios. Vaccine effectiveness seems to be somewhat lower among younger children than among persons ≥15 years of age, but statistical significance is reached only under best-case assumptions after regular vaccination. Because the number of cases in this group was low (only 10 in the entire 12 years: 7 among those 0–6 years of age and 3 among those 7–14 years of age), further stratification yielded extremely wide and overlapping 95% CIs that do not enable meaningful conclusions to be drawn.

Taking TBE incidence rates among nonvaccinated persons as a basis, 5,242 cases would have been expected in the absence of vaccination during 2000–2011. However, because the observed number was only 883, it can be concluded that >4,000 cases have been prevented by vaccination in Austria.

Discussion

A characteristic feature of the epidemiology of TBE is that incidence of TBE among humans in disease-endemic regions can vary dramatically from year to year; in the central European countries analyzed in this study, 2-fold to 3-fold annual variations were found. The reasons for these extensive fluctuations are complex and reflect an intricate interplay of factors that control the natural cycle and transmission dynamics of TBE virus. Climatic and ecologic factors influence the population dynamics of ticks and their vertebrate hosts, such as rodents and larger mammals (*5,6*), thereby affecting the abundance of ticks, especially infected ticks, in different years and different regions. A crucial prerequisite for establishing and maintaining TBE virus in its natural cycle seems to be a microclimate that favors the temporal synchrony of larvae and nymph development in spring and that thus enables transstadial transmission of the virus through cofeeding of infected nymphs and uninfected larvae on the same rodent host (*29,30*). These peculiar requirements also help explain why TBE virus is not endemic to many parts of Europe where Lyme borreliosis, which is transmitted by the same ticks, occurs (*31*). Even in regions where both agents cocirculate, transmission of Lyme borreliosis seems to follow tick habitats wherever they occur, whereas TBE is confined to only a subset of these locations (*9*). In addition to ecologic factors, specific weather conditions and socioeconomic changes can affect persons' outdoor activities, leading to enhanced or diminished risk for exposure to TBE virus–infected ticks (*8,18*). It is, however, unlikely that the increases and decreases in incidence rates observed from year to year in central Europe result from socioeconomic changes because such changes would not be expected to fluctuate so strongly on an annual basis. Also, similar extents of annual
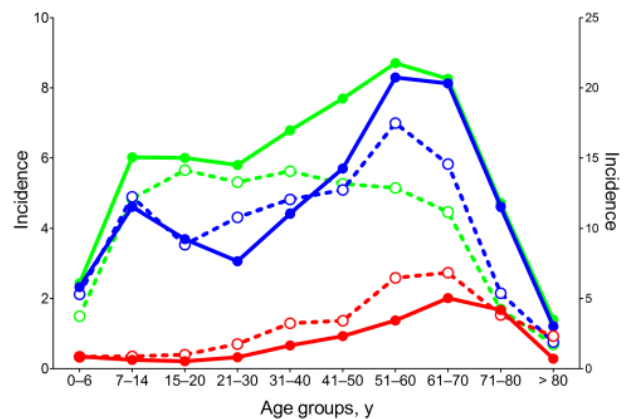
Figure 3. Age distribution of tick-borne encephalitis (TBE) patients during 1990–1999 (dotted lines; open symbols) and 2000–2010 (solid lines; closed symbols) in Austria (black and circles), Czech Republic (light gray and diamonds), and Slovenia (dark gray and rectangles). The incidence scale for Slovenia (right y-axis) differs from that of Austria and the Czech Republic (left y-axis). A color version of this figure is available online (wwwnc.cdc.gov/EID/article/19/1/12-0458-F3.htm).

incidence fluctuations are observed in most other countries where TBE virus is endemic (*32*), suggesting that these are driven primarily by biological and climatic factors.

In addition to the short-term fluctuations, our data analysis also revealed longer range undulations of incidence rates in intervals of ≥5 years, which seemed to be parallel in the 3 countries to a substantial extent (Figure 1, panel C). Except for the strong overall upsurge of TBE cases in the Czech Republic and Slovenia (but not in Austria) starting around 1992, the long-range incidence curves for 1990–2011 are remarkably similar for all 3 countries, suggesting substantial covariation of the underlying risk-for-exposure conditions. The upsurge of TBE in several European countries around 1993 might result from several factors (*8,18*); however, all factors that influence the dynamics of tick development and human outdoor activities would be expected to have similar effects on the incidence of Lyme borreliosis (*5,9,31*). Recent data from Latvia, however, clearly demonstrate an uncoupling of the incidence rates for the 2 diseases (*33*). The decrease of TBE cases after 1998 was not matched by the increasing incidence of Lyme borreliosis or by the unchanged high activity of ticks (*33*). These data, therefore, support the assumption that, in addition to tick vector dynamics and risk for exposure to tick bites in general, the specific factors controlling circulation of TBE virus in its natural hosts (*8*) have a major effect on the long-range characteristics of TBE endemicity. A significant increase was not detected in the overall incidence of TBE in Austria in the 40-year observation period (Figure 1, panel C), in contrast to the upsurges seen in the Czech Republic and Slovenia at the

beginning of the 1990s. However, incidence rates among the nonvaccinated population in Austria might be biased toward numbers that are too low because the risk for exposure is probably disproportionately distributed among vaccinated and nonvaccinated persons. Another factor contributing to this phenomenon could be the switch in diagnostic techniques, from hemagglutination inhibition and complement fixation tests to ELISA.

Among all European countries, vaccination coverage is highest in Austria, where ≈85% of the total population have received ≥1 doses of the vaccine (24). This high vaccination coverage has led to a dramatic decline in the overall incidence of TBE in Austria, in stark contrast to neighboring countries to the north (Czech Republic) (11) and south (Slovenia) (25), which have relatively low vaccination rates (≥1 doses; data from 2009) of ≈16% and ≈12%, respectively (14,15). Similar to what was found in a previous study (24), the effectiveness of the vaccine for preventing disease is high: 96%–99% after regular vaccination and best-case assumptions. Even among persons with a history of irregular vaccinations, the average protection rate is still >90%. Moreover, vaccine effectiveness is excellent among elderly persons, for whom risk for severe forms of TBE and neuropathologic sequelae is highest (13). This high protection rate might be associated with the recent finding that the functional quality of antibodies induced by vaccination is independent of age, although the quantity of antibodies is substantially lower in elderly persons (34). Given the reported data on the immunogenicity of TBE vaccines (22,23) in children, the reason for the slightly lower field effectiveness among children is unclear.

In all 3 countries analyzed in this study, incidence rates for TBE were highest among elderly persons, consistent with increased disease severity and concomitantly lower rates of subclinical infections with increasing age (5,13). In addition, during the past 10 years, incidence rates have tended to shift toward older age groups. This trend has been especially striking in the Czech Republic (Figure 3), where elderly persons, compared with those in other countries, were underrepresented from 1990 through 1999 (and for whom incidence rates were lower than for adolescents and young adults); however, in the past decade, incidence of TBE among elderly persons has sharply increased. This development probably reflects the ever-improving situation—including health status—of retired persons, which results in increased physical outdoor activities and, thus, risk for tick exposure (11). In the Czech Republic and Slovenia, TBE incidence among children and adolescents occurred in distinct peaks, similar to what was observed in Austria during the prevaccination era (26). This peak has completely disappeared in Austria (Figure 3), indicating a high degree of protection, especially among groups of young persons, who have the highest risk for exposure. School vaccination programs probably contributed to this achievement. Nevertheless, because vaccination coverage rates (A. Essl, unpub. data) and protection rates (Table) are similar among persons in different age groups in Austria, one would have expected a higher TBE incidence rate among nonvaccinated children in this country during the past 20 years. The factor or factors responsible for the phenomenon observed are currently unknown and might be associated with differences in age-specific risk for exposure in each of the 3 countries. In summary, the example from Austria indicates that TBE vaccination is an excellent way to prevent disease in all age groups. In this country during 2000–2011, vaccination prevented >4,000 cases of TBE.

Table. Field effectiveness of TBE vaccination, Austria, 2000–2011*						
Scenario, by age group, y	Unvaccinated persons	Regularly vaccinated persons		Irregularly vaccinated persons		
	Incidence†	Incidence†	FE, % (95% CI)	Incidence†	FE, % (95% CI)	
Best-case‡						
0–14	1.62	0.10	94.0 (88.0–97.0)	0.18	88.6 (63.3–96.5)	
15–50	5.41	0.02	99.7 (99.3–99.9)	0.26	95.3 (92.9–96.9)	
51–60	7.60	0.13	98.2 (96.7–99.1)	0.31	96.0 (91.4–98.1)	
≥61	7.52	0.14	98.2 (97.0–98.9)	0.63	91.7 (87.9–94.3)	
Total	5.01	0.07	98.7 (98.2–99.0)	0.37	92.5 (90.3–94.3)	
Worst-case§						
0–14	1.62	0.13	92.2 (87.4–97.0)	0.19	88.0 (62.3–96.2)	
15–50	5.41	0.11	98.1 (97.4–98.8)	0.30	94.5 (91.9–96.2)	
51–60	7.60	0.25	96.8 (95.3–98.4)	0.39	94.9 (89.9–97.4)	
≥61	7.52	0.44	94.4 (92.8–96.1)	0.71	90.5 (86.4–93.3)	
Total	5.01	0.20	96.3 (95.5–97.0)	0.44	91.3 (88.9–93.2)	

*TBE, tick-borne encephalitis; FE, field effectiveness.
†Cases/100,000 population.
‡Persons with TBE but unknown vaccination status were excluded.
§Persons with TBE but unknown vaccination status were considered regularly vaccinated.

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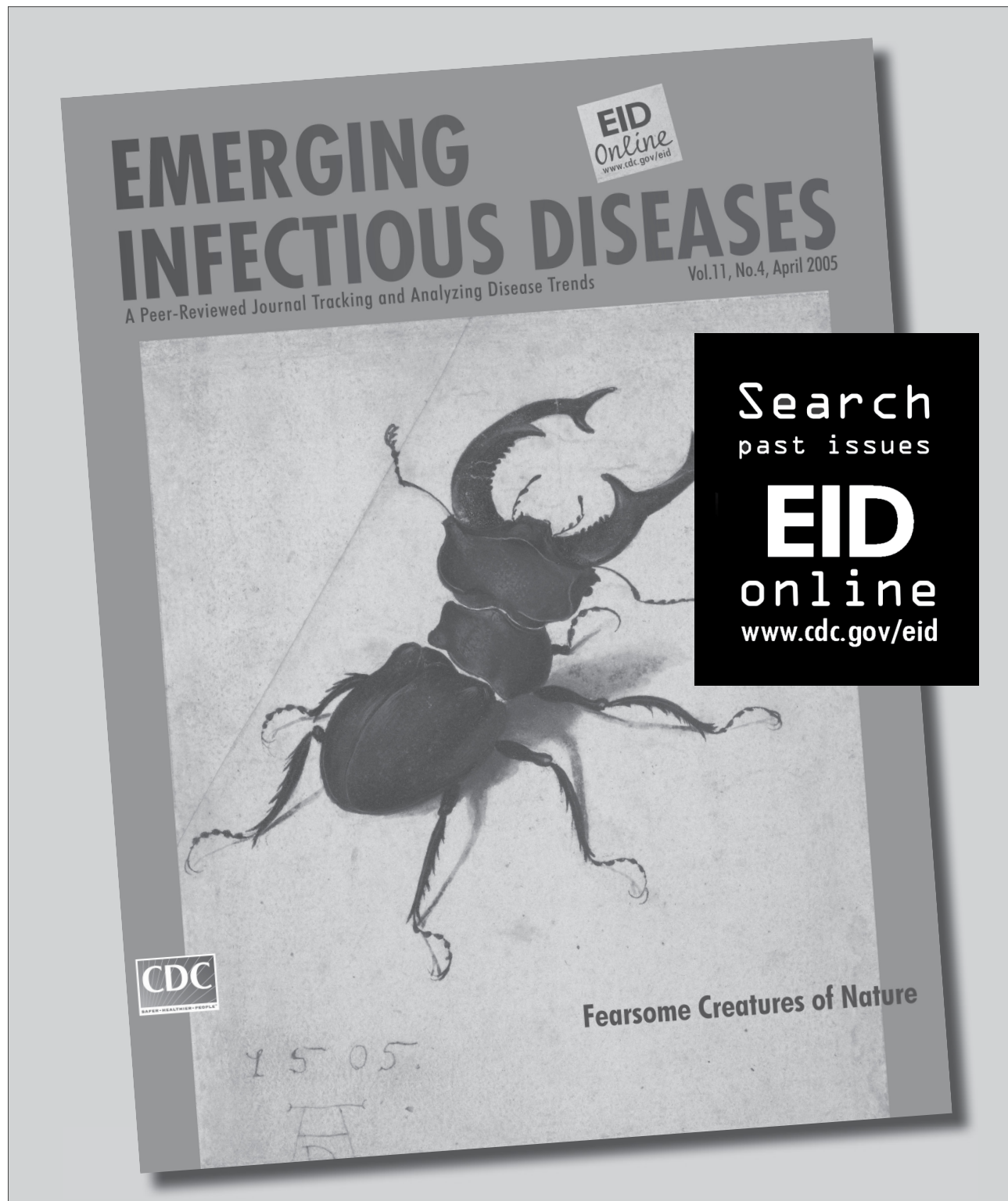
Novel Polyomavirus associated with Brain Tumors in Free-Ranging Raccoons, Western United States

Florante N. Dela Cruz, Jr., Federico Giannitti, Linlin Li, Leslie W. Woods, Luis Del Valle, Eric Delwart, and Patricia A. Pesavento

Tumors of any type are exceedingly rare in raccoons. High-grade brain tumors, consistently located in the frontal lobes and olfactory tracts, were detected in 10 raccoons during March 2010–May 2012 in California and Oregon, suggesting an emerging, infectious origin. We have identified a candidate etiologic agent, dubbed raccoon polyomavirus, that was present in the tumor tissue of all affected animals but not in tissues from 20 unaffected animals. Southern blot hybridization and rolling circle amplification showed the episomal viral genome in the tumors. The multifunctional nuclear protein large T-antigen was detectable by immunohistochemical analyses in a subset of neoplastic cells. Raccoon polyomavirus may contribute to the development of malignant brain tumors of raccoons.

The American Cancer Society estimates that infectious pathogens are associated with up to 20% of all human cancers worldwide. Among oncogenic viruses are those in the Polyomaviridae family, whose members infect an array of vertebrate species, including birds, humans, nonhuman primates, bovids, rodents, and sea lions (1,2). Infection in mammals typically results in persistent asymptomatic infection (3,4). However, natural disease studies of human infection and experimental disease studies suggest that a potential outcome of some polyomavirus (PyV) infections is tumor formation (4–6). Experimental evidence that PyVs are tumorigenic is 50 years old and not debated; PyV-induced tumorigenesis in laboratory animals, by simian virus

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40 (SV40) or by multiple human PyVs, such as JC virus (JCV), is used extensively as a cell transformation model. JCV, for example, induces brain tumors when intracerebrally inoculated in experimental animals (7–11). Furthermore, transgenic mice harboring the viral-encoded large T-antigen (LT-Ag) alone develop tumors of neuroectodermal origin, including malignant peripheral nerve sheath tumors (MPNSTs) and glioblastomas. Evidence that PyVs induce tumors after natural infection is accumulating but more controversial. Studies reliant on molecular detection of tumor-associated virus in isolation, however extensive, are inconclusive because association between PyVs and naturally occurring neoplasms varies and because PyV infections are highly prevalent, yet tumor formation is rare (3,4,12–14). Thus, although PyV-induced oncogenesis in laboratory animals has been a prolific model for the study of the cell cycle and cell transformation, natural infections rarely result in tumor formation, so the steps in cell transformation after natural infection are being revealed more slowly. Recent advances have been made by an accumulation of studies on Merkel cell polyomavirus (MCPyV), which is highly associated and integrated in most Merkel cell carcinomas (5). However, several unanswered questions relating to persistence, transmission, and transformation remain.

Most veterinary diagnostic laboratories receive large numbers of raccoon (*Procyon lotor*) carcasses for diagnosis, yet tumors of any type are rarely reported (15–17). In northern California and southern Oregon, we diagnosed 10 cases of olfactory tract/frontal lobe brain tumors in free-ranging raccoons during March 2010–May 2012. During the same period, no other raccoon tumors were reported in diagnostic laboratories across the United States and Canada. The clustering of cases and association of the tumors with the olfactory pathways suggested an infectious cause with a distinct route of transmission and

tropism. We characterized from these tumors a candidate etiologic agent, raccoon polyomavirus (RacPyV).

Materials and Methods

Tissue Samples

All tissues were obtained by necropsy from the California Animal Health and Food Safety Laboratory, Veterinary Medical Teaching Hospital, at the School of Veterinary Medicine, University of California, Davis, California, or the Veterinary Diagnostic Library of Oregon State University, Corvallis, Oregon. DNA was extracted by using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions.

Consensus PCR

Reactions were performed by using Phusion polymerase (New England Biolabs, Ipswich, MA, USA). Consensus PCR primers were designed on the basis of conserved stretches of amino acid sequences in multiple sequence alignments (Vector NTI, Invitrogen, Carlsbad, CA, USA) of LT-Ags from multiple PyV species. Primer sequences are listed in the online Technical Appendix Table (wwwnc.cdc.gov/EID/pdfs/12-1078-Techapp.pdf).

PCR conditions were as follows: 98°C for 10 sec, followed by 30 cycles of 98°C for 10 sec, 44°C for 40 sec, 72°C for 30 sec; then 72°C for 10 min; and a 4°C hold. Amplification products were subjected to electrophoresis on a 1% agarose gel, and amplicons were purified by using a QIAquick Gel Extraction Kit (QIAGEN).

Sequencing Reactions

PCR products were Sanger sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). This process was conducted on an ABI 3770 Genetic Analyzer (Applied Biosystems).

Phylogenetic Analysis

Reference viral sequences from the following PyVs were obtained from GenBank: JCV (accession no. J02226.1), SV40 (J02400.1), MCPyV (JF813003.1), murine polyomavirus (J02288.1), gorilla polyomavirus (HQ385752.1), chimpanzee polyomavirus (2c/6413) (HQ385751.1), chimpanzee polyomavirus (Azzie) (FR692336.1), and orangutan polyomavirus (FN356901.1). Amino acid sequence alignments of the predicted RacPyV LT-Ag, small T-antigen (sT-Ag), viral protein (VP) 1, and VP2 were generated by using ClustalW, implemented in MEGA5 (www. megasoftware.net). Aligned sequences were used to generate phylogenetic trees in MEGA5 by using the neighbor-joining method with amino acid

p-distances and 1,000 bootstrap replicates. Intron splice sites in RacPyV LT-Ag were predicted by using the NetGene2 server (www.cbs.dtu.dk/services/NetGene2/).

Immunohistochemical Analysis

Tissue sections from the tumors and normal brain tissue from raccoons without tumors were sectioned at 4 μm thickness and placed on electromagnetically charged slides. Immunohistochemical analyses were performed by using the avidin-biotin peroxidase method as described (18). The primary antibodies used for this study included a mouse monoclonal antibody against a peptide from exon 2 of the MCPyV that specifically recognizes LT-Ag and 57-kDa isoforms but does not cross-react with sT-Ag (CM2B4; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a mouse monoclonal anti–wild-type p53 (Clone DO-7; Dako Laboratories, Carpinteria, CA, USA). Double labeling immunofluorescence was performed according to the protocol described above; however, secondary antibodies were tagged with Alexa-Fluor 488 for the LT-Ag primary and Alexa-Fluor 568 for the p53 primary (Invitrogen, Carlsbad, CA USA).

Southern Blot Hybridizations

Genomic DNA from tumor and normal brain tissue was extracted by using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's instructions. RacPyV_LT_Probe1, a 799-bp digoxygenin-labeled probe designed to hybridize to the RacPyV LT-Ag coding region, was PCR amplified from RacPyV DNA according to the manufacturer's instructions by using the PCR DIG Probe Synthesis Kit (Roche, Indianapolis, IN, USA) and verified (online Technical Appendix Figure 1). One microgram DNA undigested or digested with KpnI at 37°C for 16 h was separated on a 0.8% agarose gel and transferred to positively charged nylon membranes (Roche) overnight by capillary action in 20× SSC. DNA was UV cross-linked and then hybridized with RacPyV_LT_Probe1 (online Technical Appendix Figure 2). Hybridization occurred for 16 h at 48°C. Membranes were washed with washing buffer (0.1 mol/L maleic acid, 0.15 mol/L NaCl; pH 7.5; 0.3% [volume/volume] Tween 20) and developed with NBT/BCIP stock solution (Roche) for up to 2 h.

Rolling Circle Amplification

Rolling circle amplification (RCA) was conducted by using the illustra TempliPhi 100 Amplification Kit (GE Healthcare Life Sciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Briefly, 1 μL DNA extracted from tumor or normal brain tissue was added to 5 μL sample buffer and incubated for 5 min at 95°C. The sample was cooled on ice and then combined with 5 μL reaction buffer and 0.2 μL enzyme mix. The reaction was

then incubated at 30°C for 16 h, followed by incubation at 56°C for 10 min to deactivate the polymerase. RCA amplified products were *Kpn*I digested at 37°C for 16 h and separated on a 0.8% agarose gel.

Results

Clinical Findings and Tumor Pathology

During March 2010–May 2012, 52 raccoons were submitted to the California Animal Health and Food Safety Laboratory at the University of California, Davis, for full necropsy, with rabies screening performed as part of state diagnostic protocol. Ten (19%) raccoons had brain tumors within the cranial portion of their frontal lobe(s), most of which spanned the cribiform plate of the ethmoid bone and extended into, or from, the olfactory tract (Figure 1, panels A, B). Nine of the affected animals represented separate collection events from 3 adjacent counties in California, and 1 affected animal was shipped from southern Oregon.

In all instances, the raccoons were exhibiting neurologic signs, including wandering in the daylight, approaching humans, or exhibiting lack of consciousness. Overall, other tissues, including lymphoid and hematopoietic tissues such as bone marrow, thymus, lymph nodes, and spleen, were generally within normal limits.

In one of the affected raccoons (Rac7), a presumed early (grossly undetectable) tumor was exclusively localized within the olfactory tract and segments of the axonal bundles of olfactory nerves within the ethmoid turbinates, which makes this the likely site of tumor origin. Most of the tumors were histologically pleomorphic, and each of the 10 tumors have been alternately and preliminarily classified as MPNSTs or glioblastomas on the basis of criteria such as anatomic location, histopathology (Figure 1, panels C and D), and a panel of histochemical and immunohistochemical stains, including reticulin stain, glial fibrillary acidic protein, vimentin, pancytokeratin, neuron-specific nuclear protein, Olig2,
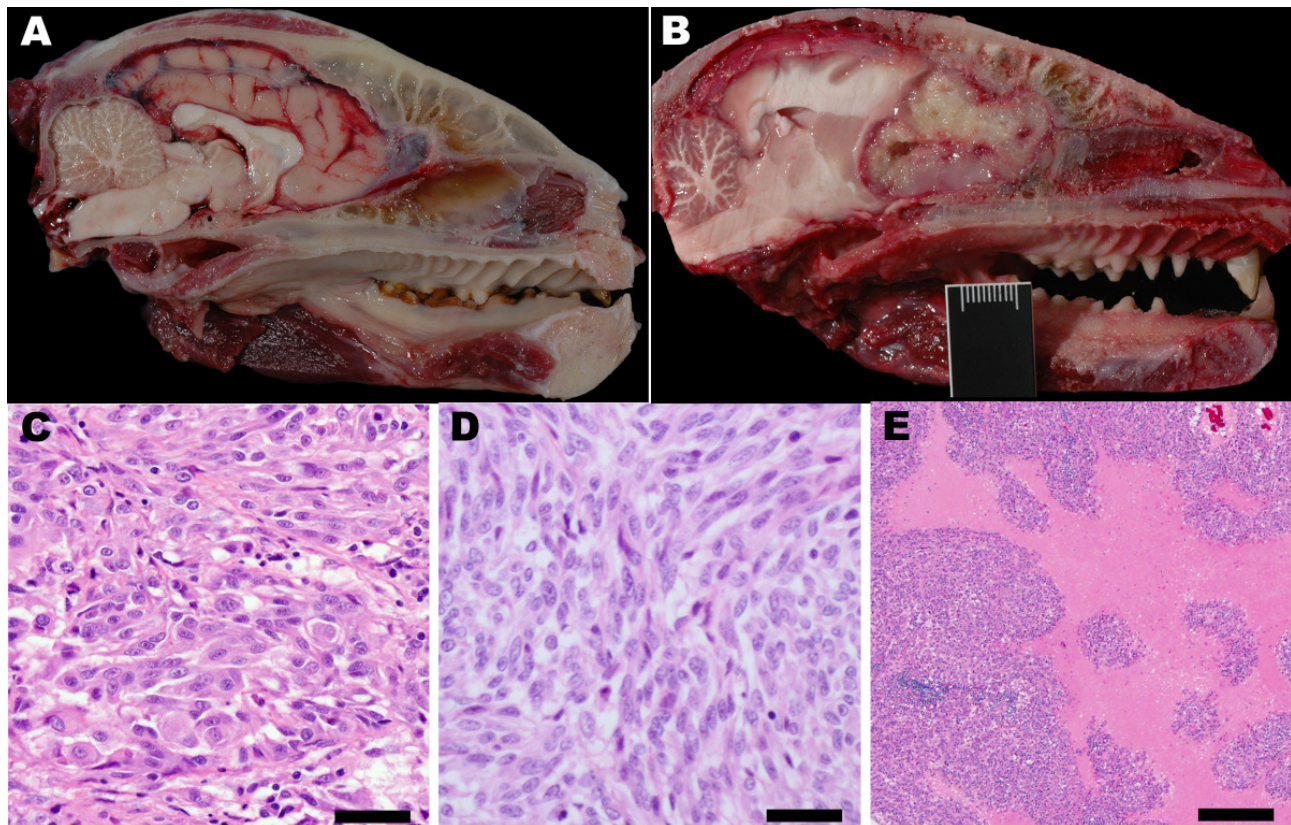
Figure 1. Pathology of raccoon polyomavirus–associated tumors. A) Normal anatomy, head of unaffected raccoon, midsagittal section. An intact cribriform plate separates the ethmoid turbinates from the olfactory tract. B) Gross pathology, head of raccoon no. 9 (Rac9), left parasagittal section. The tumor obliterates the left olfactory tract and extends into the left frontal lobe to the level of the midbrain. The tumor compresses the brain and distorts the cerebellum. The raccoon head in length (crown to nose) is 16 cm. C) Histopathology (hematoxylin and eosin staining) for Rac5. Marked anisokaryosis and anisocytosis are evident. Original magnification ×40. D) Histopathology (hematoxylin and eosin staining) for Rac10. Shown is a region that has streams of elongated cells. Original magnification ×40. Scale bar = 40 mm. E) Histopathology (hematoxylin and eosin staining) for Rac3. Cell-dense sheets of neoplastic cells are interrupted by vast regions of necrosis. Original magnification ×20. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/19/1/12-1078-F1.htm).

synaptophysin, and laminin. Among consistent findings in the glial tumors was expression of glial fibrillary acidic protein, variably sized regions of necrosis (Figure 1, panel E), and high mitotic activity. Tumors classified as MPNSTs also were positive for glial fibrillary acidic protein; however, neoplastic cells were individually surrounded by a reticulin-positive framework, which was immunoreactive with laminin. Viral inclusions were not detectable histologically, and no viral particles were visualized by transmission electron microscopy.

RacPyV Discovery by Consensus PCR

JCV is a human PyV that has been associated with brain tumors, including a broad range of glial-origin tumors. In experimental studies, transgenic mice expressing JCV LT-Ag under the control of the Mad-4 promoter develop MPNSTs and under the regulation of the CY (archetype) promoter develop medulloblastomas and glioblastomas (19). Because LT-Ag is a principal PyV protein that orchestrates oncogenesis (13,20–25), we chose it as the target for consensus PCR detection. Using an alignment of 21 PyVs, we designed 4 sets of degenerate primers to amplify conserved regions within LT-Ag (online Technical Appendix Figure 3). Amplification was successful by using primers PLMA_F3 and PLMA_R1, yielding a 270-bp product from tumor tissue of the 3 raccoons originally tested. Thus far, the virus has not been detected in brain tissue or multiple other tissues (kidney, spleen, feces, salivary gland, lung, tonsil, nasal swab) tested in 20 unaffected animals collected from the same geographic region. Full length viral genomes (6/10) or partial genomes (4/10, ranging from 2,998 bp to 4,667 bp) were generated by using a combination of primer walking and specific RacPyV primers.

RacPyV Genome Analysis

The novel virus was most closely related, phylogenetically, to a clade of viruses that includes MCPyV and was named raccoon polyomavirus (RacPyV). PyVs are small, circular, double-stranded DNA viruses, and analysis of the RacPyV genome showed characteristic organization for *Polyomaviridae* (Figure 2) (6). RacPyV encodes putative open reading frames (ORFs) with homology to PyV LT-Ag, sT-Ag, the structural VPs 1–3, and a single bidirectional noncoding regulatory region (NCRR). Because of sequence variation among the raccoon isolates, the designations 1–10 indicate the order of animal submission and which raccoon harbored which respective RacPyV; that is, RacPyV1 was found in Rac1 and was the first tumor and RacPyV discovered.

Phylogenetic trees of the deduced PyV proteins constructed by using the neighbor-joining method show that the putative early and late proteins of RacPyV are most

closely related to those of MCPyV and chimpanzee PyV (Figure 3). The 10 RacPyV genomes had a total of 30 single nucleotide polymorphisms (SNPs) (Figure 4). Most (29/30) of the SNPs were synonymous substitutions or in noncoding regions, as seen with strain variants of other PyVs. Half (15/30) of the SNPs were unique to the single Oregon case (Rac6), perhaps reflecting its geographic distance from the California raccoons. Three distinct nucleotide deletion events occurred across RacPyV5 and RacPyV10. RacPyV5 had a 26-bp deletion within the putative LT-Ag intron and a 12-bp deletion within the putative NCRR; RacPyV10 had a 1-bp deletion within the putative LT-Ag intron. The genome of RacPyV3 is deposited in GenBank (accession no. JQ178241).

The NCRR of RacPyV encodes multiple consensus pentanucleotide LT-Ag binding sites (GAGGC) on both strands as seen in other PyVs. RacPyV LT-Ag has 46% homology to MCPyV LT-Ag and has features conserved among PyV LT-Ags, including the LXCXE motif necessary for LT-Ag association with the retinoblastoma protein and the Walker A box GPXXXGKT, which suggests that RacPyV LT-Ag is an ATPase that binds to p53 (26). In experimental models, these sites are crucial components of virus-mediated transformation (20,22,23).
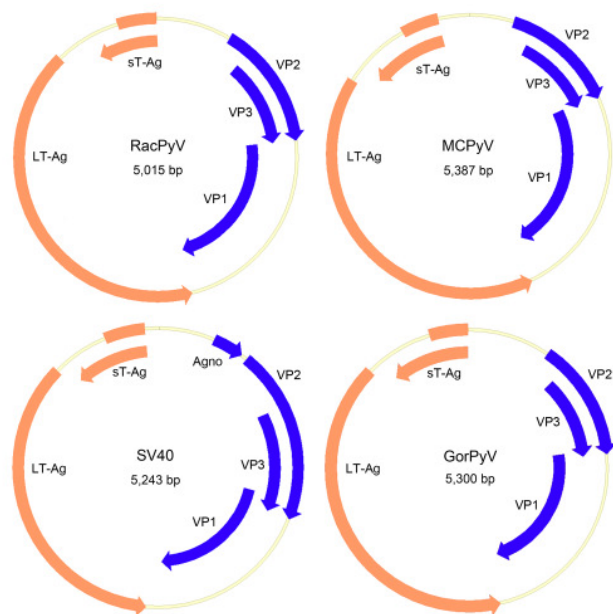
Figure 2. Genome organization of RacPyV. The entire dsDNA viral genome for RacPyV10 comprises 5,015 bp. The viral genome has a noncoding regulatory region and putative open reading frames for the late proteins VP1, VP2, and VP3 and the early proteins LT-Ag and sT-Ag. MCPyV and GorPyV, which are phylogenetic neighbors, and SV40 are presented for comparison. RacPyV, raccoon polyomavirus; LT-Ag, large T-antigen; sT-Ag, small T-antigen; VP, viral protein; MCPyV, Merkel cell polyomavirus; SV40, simian virus 40. GorPyV, gorilla polyomavirus.
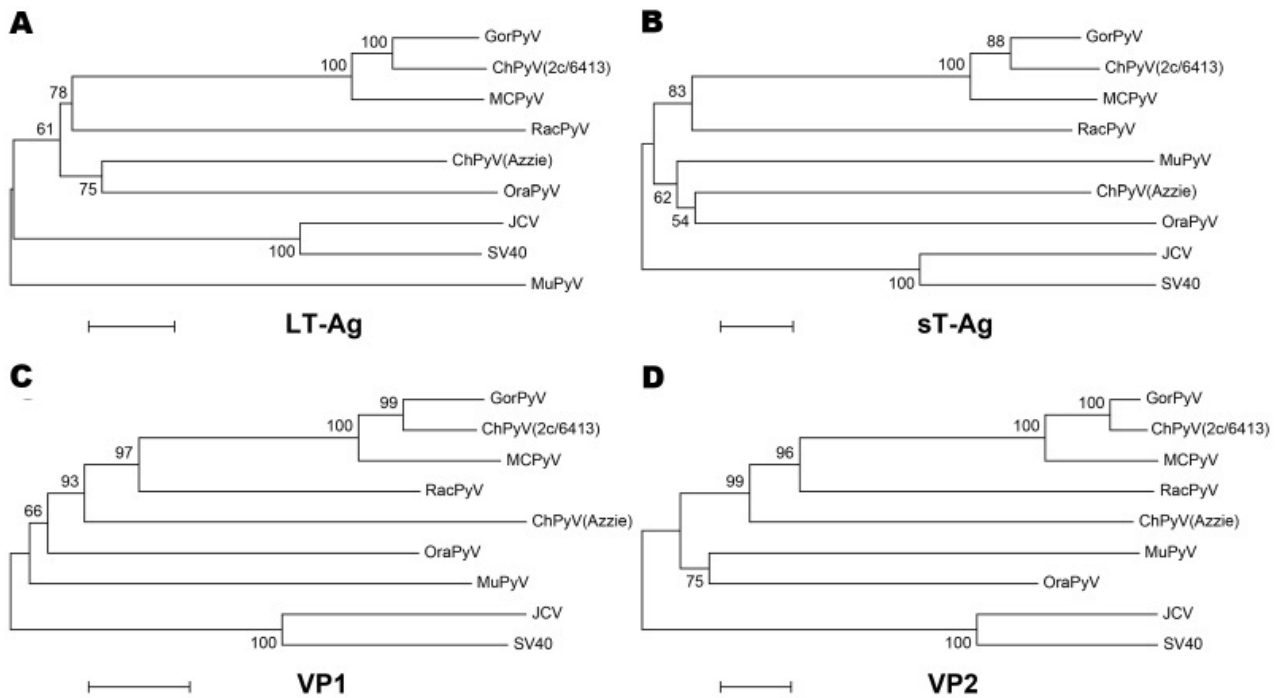
Figure 3. Phylogenetic relationship of RacPyV with representative polyomavirus species. Phylogenetic trees were individually generated on the basis of amino acid sequences of LT-Ag (A), sT-Ag (B), VP1, and VP2 (C, D) by using the neighbor-joining method with p-distance and 1,000 bootstrap replications. RacPyV, raccoon polyomavirus; LT-Ag, large T-antigen; sT-Ag, small T-antigen; VP, viral protein; GorPyV, gorilla polyomavirus; ChPyV, chimpanzee polymavirus; MCPyV, Merkel cell polyomavirus; OraPyV, orangutan polyomavirus; JCV, JC virus; SV40, simian virus 40; MuPyV, murine polyomavirus. The bar represents 5% estimated phylogenetic divergence.

The putative ORF encoding LT-Ag in RacPyV encodes putative splice sites surrounding a 387-bp intron. In other PyVs, sT-Ag, which shares a start site with LT-Ag, promotes cell transformation through negative regulation of the protein phosphatase 2A (PP2A) family of serine-threonine phosphatases (26,27). In transcription and expression studies, sT-Ag terminates at a stop codon within the LT-Ag intron. Aside from chimpanzee PyV (Azzie isolate) (28), all PyVs discovered thus far contain this canonical intronic stop codon that defines the ORF for sT-Ag. However, among the 7 RacPyVs for which the intron of LT-Ag was completely sequenced, only 2 (RacPyV5
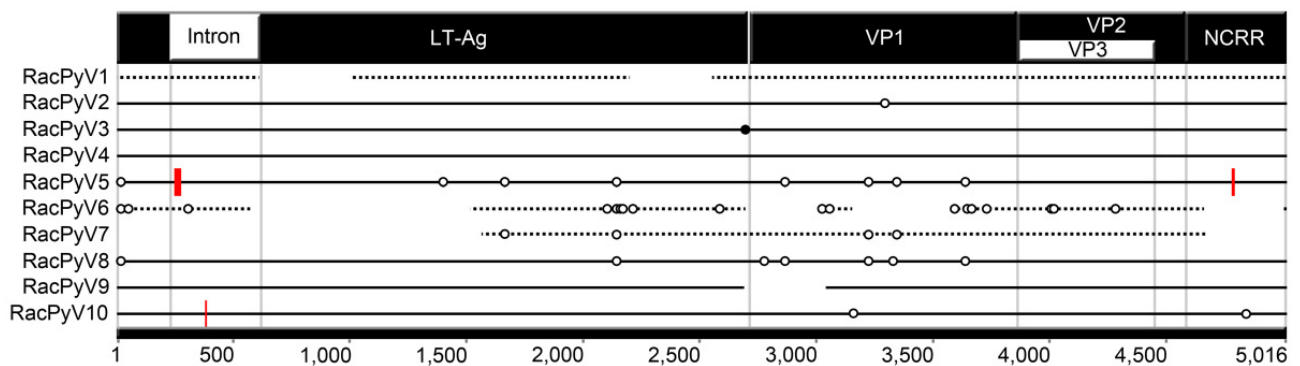
Figure 4. Partial genome sequences (ranging from 2,998 bp in raccoon polyomavirus 6 [RacPyV6] to 4,667 bp in RacPyV9) were obtained from 4 raccoons that had either undergone prolonged storage (RacPyV9) or were available only as formalin-fixed, paraffin-embedded tissue (dashed lines, RacPyVs 1, 6, and 7). Gaps in the sequences correspond to regions where amplification reactions failed. The genomes of RacPyVs 2, 3, 4, 5, 8, and 10 were sequenced in their entirety by using a primer walking method to complete the RacPyV circular genome. Open circles represent noncoding mutations; the closed circle represents a coding mutation. Horizontal bars indicate deletions. LT-Ag, large T-antigen; VP, viral protein; NCRR, noncoding regulatory region.
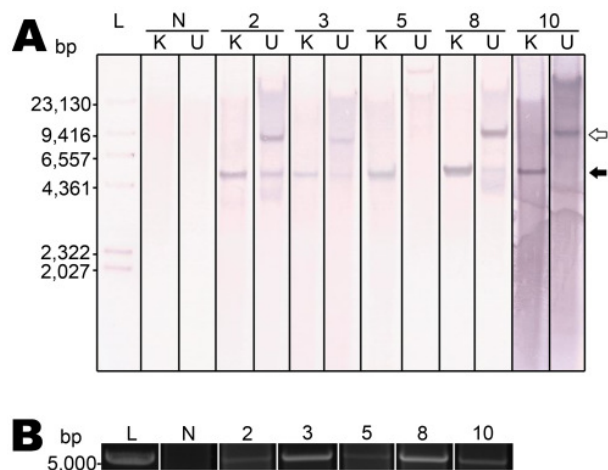
Figure 5. Raccoon polyomavirus (RacPyV) is episomal in raccoon brain tumors as detected by Southern blot hybridization and rolling circle amplification. A unique KpnI site in viral protein 1 (online Technical Appendix Figure 2, wwwnc.cdc.gov/EID/pdfs/12-1078-Techapp.pdf) conserved across the viral genomes was predicted to linearize circular RacPyV DNA. Genomic DNA was KpnI digested and probed with a 799-bp probe designed to hybridize to large T-antigen (online Technical Appendix Figure 3). A) Southern blot hybridization. DNA digested with KpnI (K) and undigested DNA (U) and hybridized with RacPyV_LT_Probe1 shows identical banding patterns in each tumor. In KpnI-digested samples, a single band appears at ≈5 kb (closed arrow), which is the expected position for linearized RacPyV genome. L, DNA ladder; N, DNA extracted from a raccoon that did not have a brain tumor (i.e., normal raccoon). B) RCA applied to RacPyV DNA by using random hexamers was digested with KpnI. Amplification of the circular RacPyV genome occurred in the same cohort of samples that was successful for the Southern blot hybridization. L, DNA ladder.

and RacPyV10) contain intronic in-frame stop codons that would define an independent sT-Ag. In the 5 other RacPyVs, the entire T-antigen coding region is in-frame as a putative single ORF. Although expression studies are needed, among possible explanations are that a single T-Ag contains both LT-Ag and sT-Ag domains, that sT-Ag could be expressed as an alternately spliced form of the T-Ag coding region, that sT-Ag is generated proteolytically, or that it is not expressed. Of the 2 viruses that have a stop codon in-frame within the LT-Ag intron, RacPyV10 has a stop codon that terminates sT-Ag after the PP2A domains, as is seen in other PyVs, whereas RacPyV5 encodes a stop codon before the PP2A domains. However, the sT-Ag of MCPyV constructs altered to lack a functional PP2A binding domain still initiates cell transformation (29).

Episomal RacPyV

Southern blot hybridizations and RCA were performed to ascertain whether RacPyV existed in episomal, integrated, or a combination of these forms. Viral DNA of sufficient quantity and quality was present in 5/10

tumors for these assays. In all cases, an ≈5-kb band for the KpnI-digested samples was absent or present faintly in undigested conditions (Figure 5, panel A, closed arrow). The band variably present at ≈9 kb is expected to be a circular nicked form of the genome (Figure 5, panel A, open arrow). RCA, by using random hexamers, was performed on DNA extracted from the same set of tumor tissues. RCA products of the tumor tissues produced the expected ≈5-kb band after KpnI digestion, confirming the Southern blot result that RacPyV is not detectably integrated in the host genome and demonstrating that it exists in circular form in tumor cells (Figure 5, panel B). As expected, Southern blot and RCA analyses performed on DNA extracted from normal, unaffected raccoon brain tissue produced no bands (Figure 5, panels A, B).

Expression of RacPyV LT-Ag in the Nuclei of Tumor Cells

To determine the expression of LT-Ag, we performed immunohistochemical analyses with a monoclonal antibody that recognizes a peptide within exon 2 of MCPyV LT-Ag. The LT-Ag was absent (Figure 6, Appendix, panel A, wwwnc.cdc.gov/EID/article/19/1/12-1078-F6.htm) in unaffected regions of the brain and absent in unaffected raccoon tissues but was detected within the nuclei of a subset of neoplastic cells in 4 tested raccoon tumors (Figure 6, Appendix, panels B, C). Immunohistochemical analyses for wild-type p53 also was positive in a subset of neoplastic cells (Figure 6, Appendix, panel D) but was negative in the adjacent normal brain. Furthermore, double-labeling immunofluorescence showed the co-localization of LT-Ag with wild-type p53 in the nuclei of neoplastic cells (Figure 6, Appendix, panels E–G).

Discussion

We consider this an outbreak of brain tumors because a retrospective case search and literature review of ≈700 necropsies from California and other regions of the United States and Canada showed only 2 primary brain tumors in raccoons, both classified as low-grade astrocytomas (30,31). Because of the consistent anatomic location of the tumors, the absolute association with RacPyV, and the expression of LT-Ag, we propose that RacPyV might play a role in tumorigenesis. In our sampling of tissues from affected and unaffected raccoons, viral genomic sequences were limited to the neoplastic tissue of the brain and olfactory tract. A more extensive set of samples might uncover RacPyV viremia and shedding sites, but it is also worth considering that the raccoon might be a dead-end host for viral replication and that viral persistence and shedding might occur in a different species. Raccoons in the affected geographic region have a nomadic suburban lifestyle and use sewer and water lines for travel and presumed daily

exposure to urine, garbage, environmental toxins, and environmental pathogens, including PyVs, which have been consistently detected in raw urban sewage samples worldwide (*32*).

Compared with other PyVs, RacPyV has several unique features. sT-Ag, for example, which in SV40 and other PyVs contributes to cell transformation (*27,29,33*), had a predicted termination site within the LT-Ag intron in only 2/7 RacPyVs analyzed. RacPyV has no predicted ORF or sequence homology for the Agno gene, which is found in many PyVs. RacPyV does not appear to be integrated into the host genome because both Southern blot hybridization and RCA analyses indicate that full-length genome exists in an episomal, circular state. Integration is one method of achieving replication incompetence of PyV and is considered by many researchers to precede cancer development (*34*). However, PyVs have alternate mechanisms of replication incompetence, and late gene suppression might occur in episomal virus. For example, Carbone et al. have implicated antisense RNA molecules produced in SV40 infection in late gene silencing through Dicer-mediated degradation (*35*). Natural and experimental infections with PyVs result in a spectrum of tumors that have some distinctions on the basis of route of infection, cofactors, and/or viral tropism (*7,8,10,11*). If RacPyV is driving oncogenesis, the transformation location has been remarkably consistent.

In humans, onset of viral-associated cancer typically occurs decades after initial infection with a transforming virus; however, free-ranging raccoons are short lived (≈2–3 years), so this naturally occurring transformation event must be relatively rapid. Natural models of viral-induced tumorigenesis provide an excellent opportunity to analyze mechanisms of transmission, transformation, viral persistence, and routes of shedding and infection. Raccoons are undeniably successful in the human suburban environment and have been proposed as a sentinel species for environmental contaminants and exposure to pathogens (*36*). New animal PyV species have been identified frequently during the last few years, but the strong association of RacPyV with a relatively sudden occurrence of brain tumors distinguishes it from other PyVs, and its discovery could help shed light on the etiologic role of PyVs in oncogenesis.

Acknowledgments

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Novel Framework for Assessing Epidemiologic Effects of Influenza Epidemics and Pandemics

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The effects of influenza on a population are attributable to the clinical severity of illness and the number of persons infected, which can vary greatly between seasons or pandemics. To create a systematic framework for assessing the public health effects of an emerging pandemic, we reviewed data from past influenza seasons and pandemics to characterize severity and transmissibility (based on ranges of these measures in the United States) and outlined a formal assessment of the potential effects of a novel virus. The assessment was divided into 2 periods. Because early in a pandemic, measurement of severity and transmissibility is uncertain, we used a broad dichotomous scale in the initial assessment to divide the range of historic values. In the refined assessment, as more data became available, we categorized those values more precisely. By organizing and prioritizing data collection, this approach may inform an evidence-based assessment of pandemic effects and guide decision making.

Pandemic influenza results from the emergence of a new influenza A virus to which the population possesses little or no immunity (*1*). Past pandemic influenza viruses have spread rapidly worldwide, affecting persons of all ages and causing substantial illness and death. Influenza can result in a wide spectrum of clinical outcomes in infected persons, including asymptomatic infection, medically and non–medically attended respiratory illness, hospitalization, or death. The likelihood of these outcomes is variable and depends on many factors, including the age of the patient, the presence of underlying medical conditions, and characteristics of the virus itself (*2*).

The overall number of illnesses and deaths from influenza in the population may be primarily attributable to a combination of both the clinical severity of illness in infected persons and the transmissibility of the infection in the population. Figure 1 shows the increasing expected number of deaths in the US population as both the cumulative incidence of influenza in the population and the case-fatality ratio (CFR) increase.

Because the risk for severe outcomes and differences in the rates of transmission of the virus can vary, the effects on the population observed during pandemics have ranged from those similar to severe seasonal influenza epidemics to those experienced during the 1918 influenza pandemic. Depending on the overall population effects, a pandemic could overwhelm the capacities of public health and health care systems or result in societal disruption because of
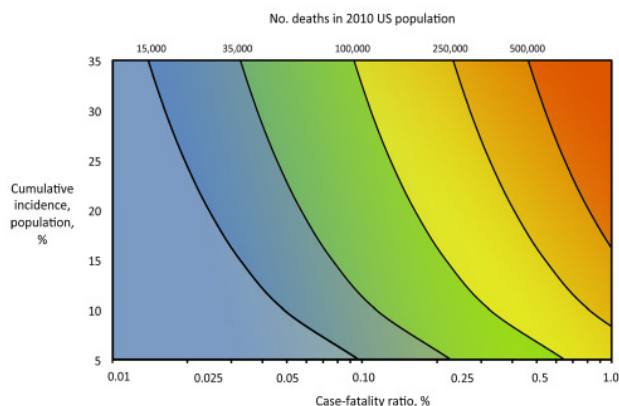
Figure 1. Estimates of influenza deaths in the 2010 United States population (308,745,538 persons) across varying values of case-fatality ratio and the cumulative incidence of infection in the population. Selected estimated numbers of deaths are indicated with a black line, across each relevant combination of case-fatality ratio and cumulative incidence. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/19/01/12-0124-F1.htm)

school or workplace absenteeism, which could affect critical infrastructure (*1,3*).

Historically, assessment of influenza pandemic effects has been characterized by using an estimate of the overall CFR (*4*). Although this approach provided guidance for planning and projections of the expected number of deaths from pandemic influenza in the population, using that ratio alone presents several challenges. First, deaths from influenza may occur weeks after illness begins and can also be subject to reporting bias, delaying the ability of public health and government leaders to quickly issue recommendations for evidence-based public health interventions if they lack an accurate estimate of CFR. Second, a single overall CFR does not fully account for the varying effects a seasonal epidemic or pandemic could have on vulnerable population subgroups, which could include children or the elderly, those with chronic conditions, or certain racial and ethnic minorities. Finally, CFR does not address other societal effects, such as absenteeism or the demand on health care services from excess outpatient visits and hospitalizations, that could result from increased transmission. Because of these limitations, relying on CFR as a single measure of the effects on a population may make an assessment difficult if such data are not yet available early in a pandemic or misleading if the available data are not well characterized and the biases are not well understood.

The ability to synthesize epidemiologic data collected early during a pandemic to characterize its anticipated public health effects is of vital importance to public health officials in the United States and worldwide. Here we provide a conceptual framework with which to characterize the expected effects of a pandemic in the context of past experience with influenza epidemics and pandemics in the United States. We examined published data from past influenza seasons and pandemics to determine the range of effects of influenza in the United States. The framework provides a basic structure by which to synthesize epidemiologic data and on which preparedness plans can be developed to guide and communicate the pandemic influenza response.

Methods

We developed the assessment framework using a 4-step process. The steps included were the following: 1) identify and evaluate available measures of influenza transmissibility and severity, 2) create a standard scale for selected measures, 3) summarize and scale available measures, and 4) provide historical context.

Step 1: Identify and Evaluate Measures of Transmissibility and Severity

We first identified epidemiologic measures that may be indicators of either the transmissibility of a novel influenza virus or the clinical severity in infected persons. The iden-

tification of relevant measures within these categories was based on an extensive review of historical seasonal and pandemic influenza literature, including published articles and reports of surveillance data collected from the 1918 pandemic forward. Three criteria were used to evaluate the identified measures: 1) the availability and quality of data related to the measures during the early stages of past influenza pandemics and seasonal influenza epidemics; 2) the presence of enough variation in the measure to produce a biologically plausible and measurable scale; and 3) the epidemiologic strengths and limitations of the measure (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0124-Techapp.pdf).

Step 2: Scaling Measures of Transmissibility and Severity

From the list of measures identified in step 1, we abstracted data from the literature review on the measures as reported during previous influenza seasons and pandemics. To create a comparable scale across the various measures of transmissibility and clinical severity, we first identified the range of values that had been observed historically for each measure. The data for each measure were then categorized into a uniform scale that was consistent across indicators of transmissibility and across indicators of clinical severity.

Because the availability and quality of epidemiologic information will increase throughout the course of a pandemic, we divided the assessment process into 2 assessment frameworks: 1) an "initial assessment" when data are sparse or very uncertain, and 2) a "refined assessment" when data are more available and more certain. A uniform scale of the transmissibility and clinical severity indicators was developed for each framework. When transmission of a novel influenza virus is identified, early epidemiologic measures provide a broad initial assessment, albeit with a high level of uncertainty, and were categorized by using a broad dichotomous scale. The assessment framework would become more refined as additional epidemiologic and clinical information are gathered and the biases in the earliest measures are better characterized. During this period, a similar general framework would incorporate a finer scale, allowing for more discrete separation of seasonal epidemics and pandemics.

Step 3: Summarize and Score Available Measures

During the initial assessment, a combination of the dichotomous scale for indicators of transmissibility and the dichotomous scale for indicators of severity results in a framework with 4 profiles (A, B, C, D) (Figure 2). An initial assessment can be made as soon as data on some measures become available and would continue to be reviewed and revised as the data warrant. As early data become available, issues of data quality are also essential to consider; we include a list of such considerations in

the online Technical Appendix. Once more robust data are available, the assessment could transition to the more detailed scale of the refined assessment framework, with scaled values of severity and transmissibility plotted along an x-axis and y-axis, respectively (Figure 3). Because the effects of an influenza pandemic may vary between age groups, the refined assessment could also be conducted with age-stratified data on indicators of transmissibility and clinical severity and then plotted by using the same scale and framework (Figure 4).

Step 4: Provide Historical Context

For the refined assessment, we scaled and plotted data from obtained from our literature riew for 4 pandemics (2009, 1968, 1957, 1918) and 3 nonpandemic influenza seasons that ranged in transmissibility and severity (1978–79, 2006–07, and 2007–08) (online Technical Appendix). When multiple measures for transmissibility or severity were present, we used the median score across all available measures. Age-stratified data from the 2009 influenza A (H1N1) pandemic were also similarly scaled and plotted by using the age categories <18 years, 18–64 years, and ≥65 years.

Results

Initial Assessment

Early in a pandemic, the spread of a novel virus is likely to be restricted to a particular geographic area, mostly in focal clusters of infections, and epidemiologic data are limited. To reflect the uncertainty in early data, we divided each measure of transmissibility and severity for the initial assessment framework into a dichotomous scale corresponding to the low-moderate and moderate-high ends of the range of values from the literature review. Scaled values for the initial assessment are shown in Table 1.

We recognized that early measures are likely to have substantial biases. Early measures of the transmissibility of the virus are likely to come from larger recognized outbreaks, which may lead to higher estimates than would eventually occur in the whole population. Likewise, early indicators of severity may be overestimated if severe illnesses are more likely to be recognized, as was seen worldwide early in the 2009 influenza A (H1N1) pandemic (5,6). For example, reports to the Centers for Disease Control and Prevention (Atlanta, GA, USA) of confirmed cases in the first few weeks of the 2009 pandemic indicated a crude CFR of 0.3% (7), »10-fold higher than it was estimated to be following adjustment for underdetection (5,8). To account for this bias in early measurements, we set the midpoint of the CFR in the initial assessment 10′ higher than the midpoint in the refined assessment.

Early measures of transmissibility were then scaled along a y-axis, and early measures of clinical severity were
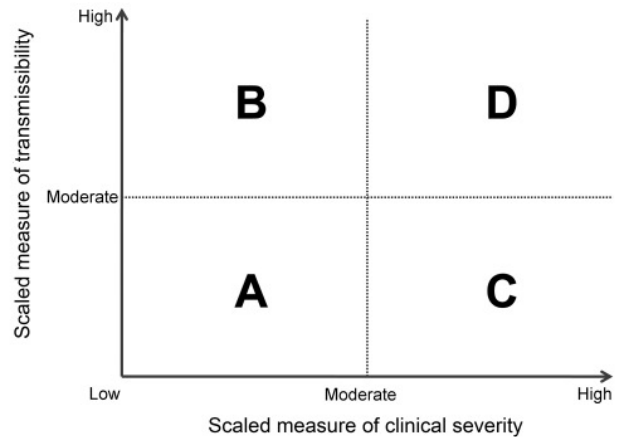
Figure 2. Framework for the initial assessment of the effects of an influenza pandemic.

scaled along an x-axis. From the combination of these 2 dichotomous scales, the initial framework results in 4 quadrants (Figure 2). In quadrant A, for example, available indicators appear similar to the range seen in annual seasonal epidemics. For quadrant B, although clinical severity is in the range of that seen in seasonal epidemics, the transmissibility is greater and thus overall rates of severe outcomes may be greater. Conversely, in quadrant C, transmissibility is similar to that of seasonal epidemics, but severity is expected to be higher, again leading to increased expected rates of severe outcomes, but for a different reason. Finally, in quadrant D, both indicators are greater than expected during annual seasonal epidemics. Consequently, recommended guidance and interventions during the pandemic response may be different between the quadrants.
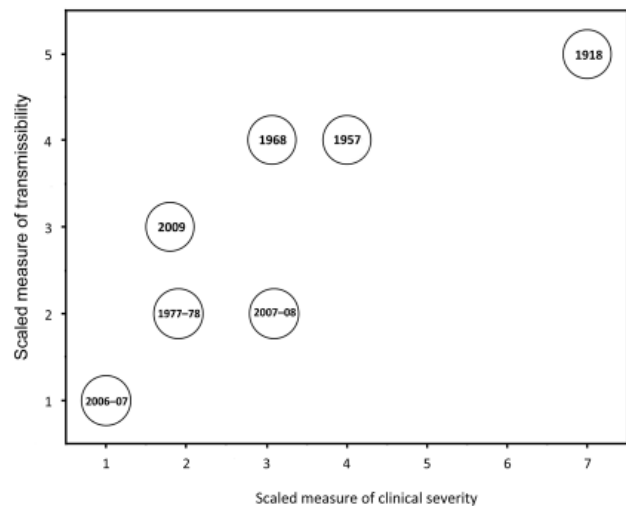
Figure 3. Framework for the refined assessment of the effects of an influenza pandemic, with scaled examples of past pandemics and past influenza seasons. A color version of this figure is available online (wwwnc.cdc.gov/eid/article/19/1/12-0124-F3.htm).
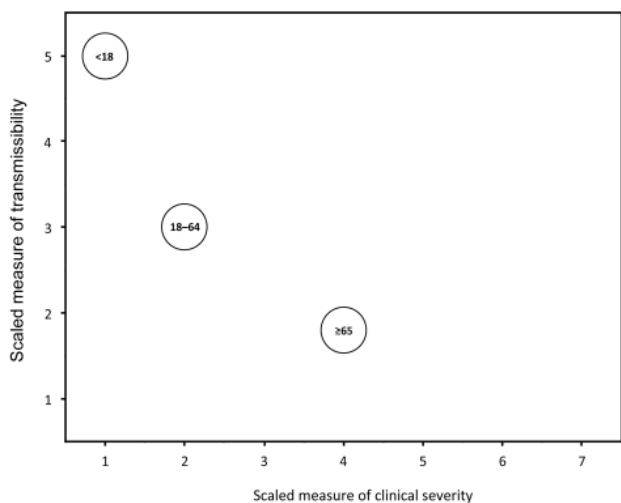
Figure 4. Framework for the refined assessment of the effects of an influenza pandemic, stratified by age group with scaled examples from the 2009 pandemic. A color version of this figure is available online (wwwnc.cdc.gov/eid/article/19/1/12-0124-F4.htm).

Refined Assessment

Although the assessment would be updated routinely as new data become available, an increase in the amount and quality of data will allow results to be presented in a more precise, refined assessment. For this framework, the range for each measure of transmissibility was divided into a 5-point scale while the range for each measure of clinical severity, which covered a broader range of values, was divided along a 7-point scale. To illustrate this assessment framework, we selected 5 measures of transmissibility and 3 measures of severity to scale on the basis of information obtained in our literature review. Detailed discussions of

the measures and their strengths and limitations are in the online Technical Appendix. Table 2 displays the ordinal scales for the measures of transmissibility and clinical severity that we developed for the refined assessment. For example, a cumulative symptomatic attack rate of 12% would be classified as a 2 on the scale, whereas a cumulative symptomatic attack rate of 28% would be a 5 on the scale. Likewise, a CFR of 0.01% would be a 1 on the clinical severity scale, whereas a CFR of 1.2% would be a 7. Each measure followed this approach with a scale of 1, representing the lowest observed values for that parameter, with values increasing as the scale increases.

Using available measures of transmissibility and clinical severity and the scale in Table 2, we plotted the coordinates for several sample years on the refined assessment framework. For example, using the 2009 pandemic (Table 3), available measures of clinical severity included the symptomatic CFR, the symptomatic case-hospitalization ratio, and the ratio of deaths to hospitalizations (5,8). Each of these measurements was a 2 on the ordinal scale of clinical severity. Available measures of transmissibility from 2009 included a household secondary attack rate (9–11), an estimated population clinical attack rate (12), an estimated R_0 (13), and a peak percent of visits for influenza-like illness from national surveillance (14). Each of these measurements was a 3 on the scale of transmissibility. This is illustrated at the coordinate (2,3) in Figure 3. We likewise characterized data abstracted from past pandemics and selected previous seasons and also plotted them as shown in Figure 3. Further details are included in the online Technical Appendix.

In addition, we abstracted and scaled data from the 2009 pandemic by age group. These values were plotted in Figure 4, with the dashed box representing the overall

Table 1. Scaled measures of transmissibility and clinical severity for the initial assessment of pandemic influenza effects		
	Scale	
Parameter no. and description	Low-moderate	Moderate-high
Transmissibility		
1. Secondary attack rate, household, %	≤20	>20
2. Attack rate, school or university, %	≤30	>30
3. Attack rate, workplace or community, %	≤20	>20
4. R_0: basic reproductive no.	1.0–1.7	≥1.8
5. Underlying population immunity	Some underlying population immunity present	No underlying population immunity present
6. Emergency department or other outpatient visits for influenza-like illness, %	<10	≥10
7. Virologic characterization	Genetic markers for transmissibility absent	Genetic markers for transmissibility present
8. Animal models—transmission studies	Less efficient or similar to seasonal influenza	More efficient than seasonal influenza
Clinical severity		
1. Upper boundary of case-fatality ratio, %	<1	≥1
2. Upper boundary of case-hospitalization ratio, %	<10	≥10
3. Ratio, deaths: hospitalizations, %	<10	≥10
4. Virologic characterization	Genetic markers for virulence absent	Genetic markers for virulence present
5. Animal models	Less virulent or similar to seasonal influenza	More virulent than seasonal influenza

Table 2. Scaled measures of transmissibility and clinical severity for the refined assessment of pandemic influenza effects

Parameter no. and description	Scale						
	1	2	3	4	5	6	7
Transmissibility							
1. Symptomatic attack rate, community, %	$\leq$10	11–15	16–20	21–24	$\geq$25		
2. Symptomatic attack rate, school, %	$\leq$20	21–25	26–30	31–35	$\geq$36		
3. Symptomatic attack rate, workplace, %	$\leq$10	11–15	16–20	21–24	$\geq$25		
4. Household secondary attack rate, symptomatic, %	$\leq$5	6–10	11–15	16–20	$\geq$21		
5. R_0: basic reproductive no.	$\leq$1.1	1.2–1.3	1.4–1.5	1.6–1.7	$\geq$1.8		
6. Peak % outpatient visits for influenza-like illness	1–3	4–6	7–9	10–12	$\geq$13		
Clinical severity							
1. Case-fatality ratio, %	<0.02	0.02–0.05	0.05–0.1	0.1–0.25	0.25–0.5	0.5–1	>1
2. Case-hospitalization ratio, %	<0.5	0.5–0.8	0.8–1.5	1.5–3	3–5	5–7	>7
3. Ratio, deaths: hospitalization, %	$\leq$3	4–6	7–9	10–12	13–15	16–18	>18

assessment of the 2009 pandemic. As shown, the available data indicated that persons <18 years of age had a high incidence of infection during the pandemic (an overall symptomatic attack rate of 26% [12], 5 on the transmissibility scale), but relatively few in that age group who became ill died (a CFR of 0.005% [5,8], 1 on the clinical severity scale). Those ≥65 years of age, however, had little illness (an overall symptomatic attack rate of 15% [12], 2 on the transmission scale), but more of those who became ill died (a CFR of 0.18% [5,8], 4 on the clinical severity scale). Persons 18–64 years of age had values that were similar to those of the overall assessment.

Discussion

A new framework to assess pandemic effects was developed to systematically assess the potential population effects of an influenza pandemic by characterizing data on both transmissibility and clinical severity and providing historical context from past pandemics and influenza seasons. We divided the framework into 2 periods. In the initial assessment, during the early stages of a pandemic, few epidemiologic data may be available and early indicators can be variable. These indications were thus categorized by using a broad dichotomous scale. In the refined assessment, as increased data become available later in a pandemic, the ranges of transmissibility and severity measures were more finely categorized.

Rather than rely only on a single measure, such as the CFR, to assess the potential effects of a pandemic, which may be misleading if those data are unavailable or not representative early in the pandemic, we incorporated several epidemiologic measures into the framework, although the CFR remains a valuable measure of clinical severity. With the creation of a standard scale that includes multiple epidemiologic measures, a variety of data may be incorporated to help synthesize these different measures into an overall indicator of transmissibility and clinical severity.

The visualization of epidemiologic data in the framework provides epidemiologists, public health officials, and policy makers with an evidence-based assessment of influenza transmissibility and clinical severity in the context of

previous influenza seasons and pandemics. Although the 3 selected influenza seasons are positioned in a cluster in the lower left of Figure 3, discernible differences exist between the seasons. During the 2006–07 season, subtype A/H1N1 viruses predominated (15), producing what has been generally regarded as a milder season in the United States; this season received the lowest score for both transmissibility and clinical severity. Conversely, during the 2007–08 season, subtype A/H3N2 viruses predominated (16) to produce what has been generally regarded as a more severe season. This season is positioned toward the center of the graph, which indicates greater transmissibility and clinical severity than was seen in 2006–07. The 3 modern pandemics (2009, 1968, and 1957) are clustered in the upper center of the graph, indicating that these pandemics had higher transmissibility but that overall clinical severity was either at or moderately above the level observed during some recent influenza seasons. In contrast, the 1918 pandemic was positioned at the upper right corner of the graph, indicating a very transmissible and clinically severe pandemic with extensive effects in the population.

An evidence-based assessment of pandemic effects is essential to inform decision makers early in a pandemic and enable them to develop and communicate preventive recommendations to reduce illness and death. The context

Table 3. Indicators of severity and transmissibility from the 2009 influenza (H1N1) pandemic and the corresponding assessment scale

Parameter	Value	Score
Clinical severity		
Symptomatic case-fatality ratio, %	0.02	2
Symptomatic case-hospitalization ratio, %	0.05	2
Ratio, deaths: hospitalization, %	4.7	2
Overall		2
Transmissibility		
Household secondary attack rate, symptomatic, %	13	3
Symptomatic attack rate, community, %	20	3
Peak % visits for influenza-like illness	7	3
R_0: basic reproductive no.	1.4	3
Overall		3

provided by the assessment of transmissibility and severity can inform the selection of pharmacologic and nonpharmacologic interventions that may be appropriate to mitigate the anticipated effects of a pandemic. For example, although the early initial assessment was categorized into only 4 quadrants, this broad early assessment can help organize available information to facilitate early decision-making that may need to be initiated when data are still limited. When clinical severity is high (quadrants C/D), measures may be initiated to provide early treatment to all who are ill and to reduce spread to limit severe disease outcomes and demand on health systems. If clinical severity appears to be similar to seasonal epidemics, but incidence is high (quadrant B), measures may be taken to reduce transmission and prepare for the possibility of disruption in schools and workplaces due to absenteeism. As more data are collected, the assessment transitions into a more detailed refined assessment, and a better characterization of the risks of transmissibility and clinical severity. Subsequently, recommendations and communications may be refined to better reflect the potential effects of the evolving pandemic. Work is ongoing at the Centers for Disease Control and Prevention to use the assessment framework to select different combinations of transmissibility and clinical severity and develop prepandemic guidance on the basis of the potential effects in the population.

Although this framework provided an assessment of the potential population effects from an influenza pandemic, it should not be used in isolation of other epidemiologic data. As this study illustrated, the assessment may be stratified to incorporate data on transmissibility and severity by age group or other risk factors to assess how the expected effects might vary in and across these groups. In addition, decision makers should consider the potential effects in relation to the time at which the pandemic emerges and the particular course of the epidemic in an area (i.e., early vs. approaching peak activity). For example, although the United States experienced a peak of pandemic activity in the late spring of 2009, for most of the country that wave ultimately accounted for only ≈5%–8% of the total estimated burden of influenza during the first year of the pandemic (5,12). Decision makers should also consider additional factors that are relevant to their individual communities, regions, and states when formulating guidance for interventions based on the epidemiologic impact assessment. These considerations include factors such as access to adequate health care and public health interventions among the affected population, the demographic make-up, the presence of vulnerable populations, or the population density.

Our assessment is subject to some limitations. We conducted a literature review of published data on measures of transmissibility and clinical severity from past influenza seasons and pandemics. Some data were sparse or contradictory, making it difficult to fully understand the variability within measures and the comparability between measures. However, building the framework around a standard scale provides flexibility to refine how measures are categorized as additional data become available and allows for other measures to also be incorporated into the scale. This lack of data underscores the need for ongoing study of the epidemiology of annual epidemics of influenza to improve our ability to accurately characterize the variability in the transmissibility and severity of influenza. An increased understanding of the effects of seasonal influenza will help the public health community prepare for the potential effects of a novel influenza virus.

In addition, there will be biases and limitations in the measurement or availability of epidemiologic data to incorporate in the framework. The online Technical Appendix describes an evaluation of several epidemiologic measures and available data sources. We attempted to account for some of the known biases by adjusting the scales used in the initial assessment on the basis of the most recent experience of the 2009 pandemic. However, changes in care-seeking behavior or testing practices may require readjusting the scale to more accurately reflect future trends. It is also possible that severity could be underestimated initially because of the delay from illness to death, which we did not directly account for (17). In the case of influenza, however, this underestimation may have less bearing than the substantial underrecognition of community transmission (6).

Continued refinement of the methods by which we collect and analyze data annually on influenza will improve our ability to have accurate and reliable data during a pandemic. A key challenge in assessing the effects of an influenza pandemic is that many cases of influenza are mild, even in the most severe pandemics, and not all persons will seek medical care or be tested for influenza. This leads to an underestimation of the incidence by missing persons who do not seek medical care and biases estimates of severity by disproportionately detecting more severe cases. Developing novel methods to better characterize the community effects of influenza will be vital to define a more accurate case denominator. In addition, strengthening systematic surveillance methods and better characterizing existing systems will also help address some of the biases in the detection of influenza and the estimation of key epidemiologic parameters.

Although we used data from the United States, the framework provides a basic structure to synthesize epidemiologic data that may be useful in other settings as well. The measures used to characterize epidemics and pandemics of influenza have both strengths and limitations; thus, we developed a the framework that is flexible and can be adapted over time to incorporate or refine measures as more data become available or better characterized. Further evaluation of the framework will be needed to determine

whether it will be used as a formal policy for pandemic planning and response. This standardized approach informs the assessment of pandemic impact by organizing available epidemiologic information using a set of key parameters to prioritize data collection and facilitate decision making.

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Seroepidemiologic Effects of Influenza A(H1N1)pdm09 in Australia, New Zealand, and Singapore

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To estimate population attack rates of influenza A(H1N1)pdm2009 in the Southern Hemisphere during June–August 2009, we conducted several serologic studies. We pooled individual-level data from studies using hemagglutination inhibition assays performed in Australia, New Zealand, and Singapore. We determined seropositive proportions (titer ≥40) for each study region by age-group and sex in pre- and postpandemic phases, as defined by jurisdictional notification data. After exclusions, the pooled database consisted of, 4,414 prepandemic assays and 7,715 postpandemic assays. In the prepandemic phase, older age groups showed greater seropositive proportions, with age-standardized, community-based proportions ranging from 3.5% in Singapore to 11.9% in New Zealand. In the postpandemic phase, seropositive proportions ranged from 17.5% in Singapore to 30.8% in New Zealand, with highest proportions seen in school-aged children. Pregnancy and residential care were associated with lower postpandemic seropositivity, whereas Aboriginal and Torres Strait Islander Australians and Pacific Peoples of New Zealand had greater postpandemic seropositivity.

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Australia, New Zealand (NZ), and Singapore all experience regular influenza seasons that coincide with winter in the Southern Hemisphere. After pandemic influenza A(H1N1) 2009 (A[H1N1]pdm09) emerged during spring in North America (1), influenza notifications and other markers of influenza activity peaked in Australia, NZ, and Singapore during July 2009 (2–4). The 3 countries continued to experience the circulation of an influenza strain closely related to the original virus until at least the following winter (5).

Most influenza surveillance systems are passive, laboratory-based systems that capture only symptomatic patients who seek medical advice and are then appropriately tested and case notifications sent. Therefore, these systems are likely to underestimate the true attack rate. Measurement of antibodies against A(H1N1)pdm09 can be used to assess the extent of population exposure to the virus (6). The emergence of a novel influenza virus provided a unique opportunity to study the behavior of influenza viruses to better understand their differential effects across various population groups.

Standardization of epidemiologic and serologic techniques across our region enabled more direct comparison of the effects of pandemic influenza on the different popula-

tions studied. Three of the countries in our region performed such studies, with publications originating from Australia (7–15), NZ (16), and Singapore (17). We pooled individual-level serologic data from studies that used the hemagglutination inhibition (HI) assay to describe the effects of the 2009 winter influenza pandemic in the Southern Hemisphere.

Methods

Identification of Studies

A working group for pandemic influenza serologic studies was formed with assistance from the Australian Seasonal Influenza Surveillance Strategy Working Group. The aims of this group included standardization of methods to facilitate analysis of pandemic serosurveillance research undertaken across Australia. The group convened its first teleconference on September 29, 2009, and continued to meet regularly as the studies were performed. Through this group and its contacts, 11 teams of researchers were identified who had performed serologic studies. After expressions of interest from researchers in Singapore (which lies just north of the equator but has a mid-year peak in influenza notifications) and NZ, 2 additional groups were identified.

Additionally, we searched Embase and PubMed for the period January 2009 to April 2011 using a combination of database-specific controlled vocabulary and general free text terms, including the following: "influenza A virus, H1N1 subtype," "seroepidemiologic studies," "influenza," "seroepidemiology," "serosurvey," and geographic terms for regions of the Southern Hemisphere. No further studies were identified by using these search strategies.

Inclusion Criteria

Studies were eligible for inclusion if they assessed serologic immunity against A(H1N1)pdm09 by HI assay across a population group in the Southern Hemisphere or Singapore. Studies were eligible if collected before vaccination programs against the virus commenced or if strategies were in place to allow for vaccine effect. Investigators from contributing studies provided HI assay titers, collection date, age, and geographic location at the individual level. The Figure shows the study profile.

Pandemic Phases

We defined the study region as NZ, Singapore, or Australian state or territory. Because the definition of pandemic phases varied between included studies, we defined pandemic phases using generally more stringent criteria than those used in contributing studies. Prepandemic specimens were defined as those collected before the first notified case in the corresponding region. Postpandemic phases were defined using notification data from NZ and the Australian Government Department of Health and Ageing by week and

region. For these countries, we defined postpandemic specimens as those collected at least 2 weeks after the date on which 90% of 2009 laboratory notifications had occurred for the region. In Singapore, continuing pandemic activity was noted through late 2009. Because the adult studies from Singapore were repeated collections from prospective cohorts, the latest collection was used for estimates of postpandemic immunity, generally from October 2009. The postpandemic collection from children in Singapore was from September 1, 2009, to June 2, 2010, and all of these specimens were included as postpandemic. Specimens that did not meet criteria for prepandemic or postpandemic were defined as intrapandemic and excluded from further analysis.

Statistical Analysis

Most studies were performed as cross-sectional or analysis of continuous prospective collections of available specimens collected for other purposes. Studies that used a purposive sampling technique were analyzed in the same way as
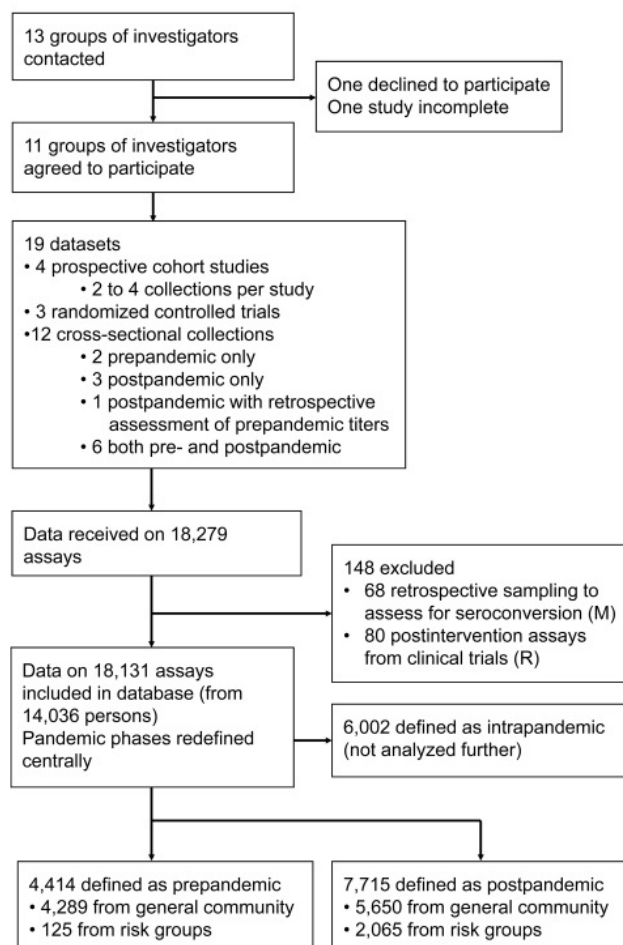
Figure. Flow chart showing profile of serologic studies to estimate attack rates of influenza A (H1N1) pandemic 2009 in the Southern Hemisphere during winter 2009.

those that used convenience collections. In the case of cohort collections and clinical trials, pre- and postpandemic assays from the same person were delinked and analyzed independently for consistency with other study techniques. For clinical trials, preintervention data from the intervention group and all data from the control group were included, whereas postintervention data from the treatment group were excluded. One study (M) used a postpandemic, cross-sectional design with retrospective assessment of prepandemic titers for those specimens found to be seropositive. For this study, only the postpandemic collection was included.

All studies used 2-fold serial dilutions from an initial dilution of 1:10 to determine titers. A titer of ≥40 was used to define seropositivity because all included studies used this cutoff value. Two studies (L, O) reported the geometric mean of 3 assays for each specimen, and for these studies, geometric mean titers of ≥40 were used to define seropositivity. Seropositive proportions are expressed as the proportion of reciprocal titers ≥40, with 95% CIs. Seropositive proportions are only reported for groups represented by ≥20 specimens. For comparability, age-standardized assessments of the proportion seropositive were calculated, weighted by 5-year age brackets to a reference population (Australian population on June 1, 2009) (18). Attack rates are calculated (for populations for which pre- and postpandemic seropositive proportions were available) as the difference in proportions of the immune population between pre- and postpandemic groups, age-standardized to the same reference population.

Using data from the 11 community-based studies, we performed multivariate logistic regression for the outcome of seropositivity in pre- and postpandemic phases. Exposure variables included in the model consisted of sex, age group, and study region because no other variables were consistently available across datasets.

To quantify the effect of study methods and the presence of potential risk factors on seropositivity, we compared pairs of studies performed in similar populations, using multivariate logistic regression, on the outcome of seropositivity. Data from the reference study were included along with data from a study of persons with the most similar characteristics. Exposure variables consisted of age, sex, and the binary variable of comparison group versus reference group. Analyses were restricted to specimens taken from patients during the same pandemic phase with comparable demographic characteristics (age, region, and population). Data management and statistical analysis were carried out with Stata 11.0 (StataCorp LP, College Station, TX, USA).

Results

Datasets were received from 11 groups of investigators, consisting of data from 10 published and 3 unpublished studies. Data were received from NZ, Singapore, and New South Wales (NSW), the Northern Territory (NT), Queensland, Tasmania, Victoria, and Western Australia in Australia. Datasets are listed by study design and population, with pandemic phases referring to investigators' definitions, which resulted in 19 datasets for analysis. Study designs consisted of 4 prospective cohorts (E–H), 3 randomized controlled trials (L, O, R), 2 prepandemic cross-sectional studies (A, D), 1 retrospective cohort study (M), and 6 unpaired pre-and postpandemic cross-sectional studies (I, J, K, N, P, S). Eleven datasets were community based, whereas 8 were from groups with potential risk factors.

Laboratory techniques common to all studies included HI assay, per inclusion criteria, and use of egg-grown, β-propiolactone–inactivated A/California/07/2009 reference virus as the antigen source. All studies provided titers and patient's age in years for each assay, and all Australian studies provided geographic data to at least state/territory level. Table 1 illustrates study characteristics that differed between included studies, with studies differing by design, enrolment criteria, specimen type, and specific HI method (a longer version can be found online; wwwnc.cdc.gov/EID/article/19/1/11-1643-T1.htm). All studies but 2 (I, K) provided data on patient's sex for all assays. All studies attempted to avoid contamination of specimens from vaccination effect, but approaches used to achieve this differed.

Received data consisted of 18,279 individual specimens, of which 18,131 assays (from 14,036 persons) were eligible for analysis, whereas 148 did not meet inclusion criteria. Samples were reclassified as prepandemic (4,414), intrapandemic (6,002), or postpandemic (7,715), according to the criteria described, with intrapandemic assays excluded from further analysis (Figure). The timing of the pandemic phases and the sample taking is summarized in the online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/11-1643-Techapp.pdf). Of the assays eligible for analysis in the pre- or postpandemic groups, 125 prepandemic specimens and 2,065 postpandemic specimens were from risk groups, while the remainder were from community-based datasets.

Tables 2 and 3 show seropositive proportions in the pre- and postpandemic periods. Overall, the age-standardized prepandemic seropositive proportion was 9.4%, with regional estimates of 10.6% in Australia, 11.9% in NZ, and 3.5% in Singapore. Higher levels of immunity were seen with increasing age, with only 1 of 5 studies (A) of children finding evidence of preexisting immunity in age group 0–4 years, whereas markedly higher seropositive proportions were seen in those ≥75 years of age.

In the postpandemic period, the age-standardized seropositive proportion was 24.3%, giving an attack rate of 14.9% (Table 3). Attack rates by country were 13.1% for Australia, 19.0% for NZ, and 14.0% for Singapore. For all regions in which children 5–14 years of age were assessed,

Table 1. Characteristics of selected collections included in database to estimate population attach rates of influenza A (H1N1) 2009 in the Southern Hemisphere, winter 2009*

Code (reference)	Study design	No. assays by redefined phase	Population	Age range, y†	Enrollment	Region	Monovalent pandemic vaccine effect
A (16)	Pre cross-section	524 pre	Outpatients	1–99	Opportunistic from stored specimens	NZ	Not applicable
B (16)	Post cross-section	1147 post	Primary care patients	1–89	Active recruitment of registered GP patients	NZ	Collection prior to vaccination program
C (16)	Post cross-section	532 post	HCWs‡	21–109	Active recruitment of hospital and clinic staff	NZ	Collection prior to vaccination program
D (7)	Pre cross-section	152 pre	Residents of aged-care facilities‡	59–100	Outbreak investigations of non-H1N1 viruses	NSW	Not applicable
E (17)	Prospective cohort (pre and post collections)	788 pre 671 intra 689 post	Community residents	21–74	Sub-cohort of existing cohort collection	Singapore	Collection prior to vaccination program
F (17)	Prospective cohort (pre and post collections)	1 pre 1,138 intra 391 post	HCWs‡	20–67	Email and word of mouth staff recruitment at hospital	Singapore	Collection prior to vaccination program
G (17)	Prospective cohort (pre and post collections)	300 intra 250 post	Staff and residents of long-term care facilities‡	19–109	Active recruitment by invitation	Singapore	Collection prior to vaccination program
H (17)	Prospective cohort (pre and post collections)	1915 intra 637 post	Military personnel‡	18–62	Active recruitment by invitation	Singapore	Collection prior to vaccination program
I (8)	Pre and post cross-sections	447 pre 221 intra 229 post	Community residents	0–19	Opportunistic from pathology laboratory	WA	Collection prior to vaccination program
J (8)	Pre and post cross-sections	201 pre 170 intra 116 post	Pregnant women‡	21–45	Opportunistic from pathology laboratory	WA	Collection prior to vaccination program
K (9)	Pre and post-cross-sections	474 pre 750 intra 497 post	Outpatients	0–100	Opportunistic from pathology laboratories	NSW	Collection prior to vaccination program
L (10)	RCT of pandemic vaccine (pre-vaccine collection)	166 intra	Healthy adults	.	Active recruitment of volunteers	Adelaide	Collection prior to vaccination program

*An expanded version of this table showing all collections in the database is available online (wwwnc.cdc.gov/EID/article/19/1/11-1643-T1.htm). NZ, New Zealand, HCWs, healthcare workers; NSW, New South Wales; WA, Western Australia; RCT, randomized controlled trial.
†Age range for specimens included in pre- or postpandemic phases.
‡Defined as risk groups for analysis.

this age group had the highest levels of postpandemic seropositivity, except for NSW, in which those 15–34 years of age showed the greatest seropositivity. Among risk groups, unweighted seropositive proportions were 28.4% in HIV-positive persons in NSW, 21.5% in hemodialysis patients in NSW, 26.7% in NZ health care workers, 9.5% in Singaporean health care workers, 33.9% in Singaporean military personnel, 6.8% in staff and residents of Singaporean residential care facilities, 14.7% in pregnant women in WA, 29.5% in indigenous residents of the NT, 34.3% in Maori in NZ, and 43.7% in Pacific Peoples in NZ.

Logistic regression performed in assays from postpandemic, community-based collections showed that the age groups 5–14 years and 15–34 years, as well as residence in NZ, were associated with increased seropositivity. Negative effects were seen for older age groups and those with residence in Singapore (Table 4).

In 2 instances, the same demographic group was assessed by 2 studies using different methods, allowing for assessment of the effect of study design. The 2 cross-sectional studies performed in adults in NSW in the postpandemic phase (performed in different laboratories) were

Table 2. Prepandemic seropositive proportions by country, region, and risk group of influenza A (H1N1) 2009 in the Southern Hemisphere, winter 2009*

Code	Pop.	Age groups, y						Sex		Overall	
		0–4	5–14	15–34	35–54	55–74	≥75	F	M	Raw	Age-stand.
A, E, I, N, P, R, S	Overall	1.4	2.7	12.0	4.8	11.5	47.4	7.8	8.5	8.4	9.4
I, N, P, R	AU	0	1.6	12.2	7.9	12.3	48.8	11.7	9.3	11.0	10.6
A	NZ	7.0	14.9	8.4	5.3	20.1	33.3	9.2	16.5	12.0	11.9
E & S	Sing	0	1.6	12.7	2.0	1.4		2.2	3.3	4.7	3.5
K	NSW	0	5.1	14.2	4.4	24.6	51.9	15.1	9.5	19.6	11.9
P	NT	0	0	4.4	8.7	8.2	26.7	6.5	8.5	7.4	6.9
N	QLD			12.3	11.3	14.4		15.0	10.6	12.6	
I, R	WA	0	0	15.7	0	7.7		12.5	4.2	4.7	9.8
					Risk group collection						
D	NSW res. care					28.0	50.5	55.8	23.7	46.0	

*Pop., population; age-stand., age-standardized; AU, Australia; NZ, New Zealand; Sing, Singapore; NSW, New South Wales; QLD, Queensland; NT, Northern Territory; WA, Western Australia; res., residential. Blank cells indicate no data. CIs are included in online version.

included in a regression model that examined the effect of using serum specimens collected to identify pathologic agents from outpatients by comparing them to results with plasma units of donated blood. The 2 collections obtained from WA adults in the postpandemic phase were analyzed similarly to examine the effect of using volunteers in a randomized-controlled trial by comparing those results to results using plasma units of donated blood. Neither the use of opportunistically collected blood specimens nor the use of adults enrolled in randomized controlled trials showed a significant difference on the outcome of seropositivity from results using plasma units of donated blood (Table 5).

We compared several other pairs of datasets by logistic regression to examine the effect of specific risk factors on the outcome of seropositivity. The odds ratio for the binary variable of study of origin (comparison study vs. reference study) is displayed as an estimate of the effect of the risk factor on the outcome. In NSW during the prepandemic phase, living in a residential care facility was associated with lower levels of preexisting seropositivity than was living in the general community. However, in the postpandemic phase, we found that persons with HIV infection or those who were undergoing hemodialysis were not significantly more likely to be seropositive than were community control subjects. In WA in the postpandemic phase, pregnancy was associated with lower levels of seropositivity. Health care workers in NZ had levels of postpandemic seropositivity similar to community controls, but those of health care workers in Singapore were lower. In Singapore, military personnel had similar levels of postpandemic seropositivity, while staff and residents of residential care facilities had lower levels compared to community controls. Aboriginal and Torres Strait Islander residents of the NT had higher levels of postpandemic immunity than other ethnic groups, as did Pacific Peoples of NZ (Table 5).

Discussion

We obtained estimates of the full epidemiologic effects of A(H1N1)pdm09 in the 2009 Southern Hemisphere winter by pooling data from several serologic studies performed across our region. We believe that population-based serologic studies give a more direct measure of community exposure to the virus than notification-based data, which are inherently limited by the proportion of cases of infection that are captured by the notification system. The individual-level data enabled us to apply consistent statistical methods across studies. This enabled estimates of seropositivity to be made across more directly comparable groups, as well as assessments of the effects of specific risk factors on seropositivity.

Our community-based, age-standardized estimates of prepandemic seropositive proportions ranged from 3.5% to 11.9%, with Singapore demonstrating a lower level of prepandemic immunity than Australia and NZ. The increased levels of prepandemic immunity in those ≥75 years of age are likely to be partially due to cross-reacting antibody responses to influenza A/South Carolina/1/1918 and related viruses that were circulating in the early 20th century (20). However, the steady increase in seropositivity with age across age groups suggests more recent circulation of influenza viruses with the potential to elicit cross-reacting antibody responses (21).

The finding of peak postpandemic seropositivity in the 5- to 14-year age group is consistent with greater social mixing of school-aged children, lower prepandemic immunity, and results from other population-wide studies (22,23). Despite the low level of prepandemic cross-reactive antibodies to the virus, Singapore remained the region with the lowest proportion of population-standardized seropositivity in the postpandemic phase (17.5%), whereas estimates from Australia and NZ ranged from 22.1% to 32.8%. The implication of lower postpandemic seropositivity in more tropical regions is consistent with estimates from India and Hong

Table 3. Postpandemic seropositive proportions by country, region, and risk group of influenza A (H1N1) 2009 in the Southern Hemisphere, winter 2009, with age-characterized AR*

Code	Pop.	Age groups, y						Sex		Overall		Age stand. AR
		0–4	5–14	15–34	35–54	55–74	≥75	F	M	Raw	Age stand.	
A, B, E, I, K, N, O, P, R, S	Overall	27.6	34.3	30.5	16.8	18.0	23.3	23.0	22.3	23.8	24.3	14.9
I, K, N, O, P, R	AU	24.0	32.2	29.8	17.8	18.8	17.0	23.3	21.2	23.1	23.7	13.1
B	NZ	37.2	46.3	38.1	22.3	20.1	35.8	30.5	30.1	30.3	30.8	19.0
E, S	Sing.	24.5	29.6	17.2	11.0	6.8		10.7	13.4	19.2	17.5	14.0
K, N	NSW	17.3	18.4	37.8	19.3	18.8	21.6	25.5	24.2	26.2	27.2	15.3
P	NT	16.7	37.2	22.0	18.1	16.3	14.3	20.5	18.3	19.5	21.8	15.0
N	QLD			29.6	9.3	14.8		19.2	18.0	18.5		
N	Tas			35.9	28.9	26.7		35.6	24.5	30.6		
N, O	Vic	36.1		30.8	12.5	21.4		31.3	13.6	21.5		
I, N, R	WA	24.0	39.5	31.6	18.2	34.3		27.4	27.5	31.4	30.3	20.5
Risk group collections												
M	NSW, HIV+				29.5	30.4			35.6	28.4		
Q	NSW, hemo.					21.7	25.0	20.8	22.0	21.5		
C	NZ, HCWs			31.3	23.7	27.6	33.3	26.3	28.2	26.7		
F	Sing. HCWs			11.0	6.8	11.1		10.1	6.3	9.5		
G	Sing. res. care			4.3	2.7	6.8	11.4	4.9	9.4	6.8		
H	Sing. military			35.7	3.4				34.5	33.9		
J	WA, preg. women			13.3	19.2			14.7		14.7		
P	NT, indig.		37.5	28.4	28.1	32.9		28.4	30.9	29.5	29.8	22.1
B	NZ, Maori			42.3	26.2	20.6		39.4	28.0	34.3		
B	NZ, Pacific People	56.0	55.6	53.1	39.5	24.3		43.6	45.1	43.7		
Overall attack rates, community-based studies												
A, B, E, I, K, N, O, P, R, S	Overall	26.2	31.6	18.5	12.1	6.4	–24.1	15.2	13.7	15.3	14.9	
Overall geometric mean titers, community-based studies												
A, E, I, N, P, R, S	Pre	6.03	5.86	8.63	6.97	8.57	24.13	15.2	13.7	15.3	14.9	
A, B, E, I, K, N, O, P, R, S	Post	15.42	16.87	16.09	10.10	11.29	14.74	15.2	13.7	15.3	14.9	

*AR, attack rate; Pop., population; Age-stand, age-standardized; AU, Australia; NZ, New Zealand; Sing., Singapore; NSW, New South Wales; NT, Northern Territory; QLD, Queensland; Tas, Tasmania; Vic, Victoria; WA, Western Australia; HCWs, health care workers; hemo., hemodialysis; res., residential; preg., pregnant; indig., indigenous; pre, prepandemic; post, postpandemic. Blank cells indicate no data or not applicable. CIs are included in online version.

Kong (23,24), as well as with the nonsignificant trend toward greater seropositivity in the more northerly Australian regions (NT and Queensland) on logistic regression. Although overall attack rates were similar for Australia and Singapore, this finding highlights the geographic heterogeneity of influenza spread and suggests that latitude may be a critical predictor of susceptibility to influenza, which might be explained by increased efficiency of transmission of influenza in cold temperatures (25) or population levels of vitamin D stores (26). The negative overall attack rate in those ≥75 years of age may have been an anomaly, because this age group had the smallest number of specimens and the prepandemic specimens were predominantly from NSW, while the postpandemic specimens were a combination of specimens from NSW, NZ, and NT. Other age groups had more similar compositions between pre- and postpandemic phases and so are likely to be more directly comparable. Alternative explanations include waning immunity in elderly persons in the months after seasonal influenza vaccination or limitations of study K, which observed the greatest decrease in titers in this age group.

Although several coexisting conditions have been found to be associated with severity of infection with A(H1N1) pdm09, most laboratory-confirmed cases across the Southern Hemisphere have occurred in persons without known risk factors (27). We found no increase in risk for postpandemic seropositivity among hemodialysis patients and a group of persons with generally well-controlled HIV infection. These

Table 4. Multivariate logistic regression models on outcome of pre- and postpandemic seropositivity in community-based studies of influenza A (H1N1) 2009 in the Southern Hemisphere with exposure variables of region, age group, and sex, winter 2009*

Exposure variable	Prepandemic phase, n = 4,289		Postpandemic phase, n = 5,650	
	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Region				
New South Wales†	1		1	
New Zealand	1.18 (0.77–1.80)	0.45	1.44 (1.17–1.79)	0.001
Northern Territory	0.62 (0.38–1.02)	0.06	0.82 (0.66–1.01)	0.06
Queensland	1.25 (0.79–1.98)	0.34	0.78 (0.53–1.14)	0.20
Singapore	0.40 (0.27–0.61)	<0.001	0.56 (0.43–0.74)	<0.001
Tasmania			1.50 (0.95–2.35)	0.08
Victoria			1.37 (0.94–2.01)	0.11
Western Australia	0.41 (0.25–0.67)	<0.001	1.04 (0.76–1.43)	0.79
Age group, y				
0–4†	1		1	
5–14	2.34 (0.92–5.92)	0.07	1.60 (1.24–2.06)	<0.001
15–34	13.70 (5.85–32.07)	<0.001	1.50 (1.18–1.91)	0.001
35–54	6.24 (2.50–15.57)	<0.001	0.75 (0.58–0.98)	0.04
55–74	14.60 (5.98–35.62)	<0.001	0.73 (0.56–0.95)	0.02
≥75	47.43 (18.58–121.08)	<0.001	0.95 (0.64–1.41)	0.80
Sex				
F†	1		1	
M	0.99 (0.73–1.34)	0.96	0.97 (0.85–1.24)	0.70
Unknown	2.44 (1.74–3.42)	<0.001	1.67 (1.25–2.24)	0.001

*Blank cells indicate no data. The 2 regression models are displayed vertically.
†Reference category.

results are consistent with the observation that HIV-infected patients admitted to a hospital for influenza have similar clinical outcomes as do non-HIV patients (*28*). Pregnant women represented ≈7%–9% of patients with laboratory-confirmed cases, severe infections, and admissions to intensive care units in the Southern Hemisphere (*27*). Although it has been postulated that this occurred because of the patients' younger ages and close contact with children, our results suggest that pregnancy is associated with a lower likelihood of infection, possibly because pregnant women actively avoid infection. Therefore, pregnant women appear more susceptible to severe illness with A(H1N1)pdm09 infection, which may relate to lower levels of immunoglobulin G_2 (*29*). By contrast, our results suggest that the 6- to 7-fold higher rates of hospitalization seen in indigenous persons in our region are likely to be partially attributable to a higher attack rate in Australia (*30*). We did not find evidence for a higher attack rate among health care workers or military personnel, with levels of seropositivity comparable to those of the general community. A study in a Finnish garrison found that 22.3% of personnel had titers of ≥40, a level lower than the 33.9% observed in Singaporean military personnel postpandemic, even though this study was performed in response to a recent outbreak (*31*). Levels of seropositivity in those living in residential care appeared lower than in community-living persons of comparable age, both in a prepandemic comparison in NSW and in a postpandemic comparison in Singapore.

The unavoidable limitation to our comparisons is that they included data from multiple studies that used differing methods. Studies differed by epidemiologic approach, specimen type, and laboratory methods, and the jurisdictions studied exhibited different public health responses.

We excluded from analysis data we considered to have been obtained with methods that were unlikely to give a population-wide estimate of serologic immunity, for example, 1 retrospective prepandemic collection from persons with postpandemic seropositivity (M) and the postintervention assessments from clinical trials (L, O, R, S). Several studies used convenience collections of specimens taken for clinical indications before routine discarding. These studies enabled population-based estimates but were subject to selection bias, given that conditions predisposing to influenza might increase the chance of being tested. By contrast, the use of blood donor specimens may select for a healthier sample. Cohort studies (E–H) were analyzed in the same manner as for cross-sectional surveys, although samples included in these datasets were determined by selection biases relating to original enrollment in the cohort as well as to enrollees dropping out. Previous evidence indicates that this is a valid approach to estimating population-wide immunity (*32*). Moreover, our analysis found no effect from differing study methods when comparing 2 pairs of studies performed in the same populations. Therefore, while the differences seen between the risk groups could have been caused by differences in study method, we found no evidence of this from the data available.

Whether the epidemiologic differences are due to differences in transmission in differing populations or because of the effectiveness of public health responses is difficult to gauge. In Australia, most jurisdictions moved from the Delay to the Contain phase on May 22 and from the Contain to the Protect phase on June 22. Only Victoria, which contributed 234 specimens to this pooled analysis, differed in the timing of its response phases (*33*). Although there were

Table 5. Multivariate logistic regression models comparing specific collections on outcome of seropositivity, with exposures of region, age group, and sex, in community-based studies of influenza A (H1N1) 2009 in the Southern Hemisphere, winter 2009*

Collections compared		No. assays included	Characteristics of model		ORs 95% CI for exposure variables		
Comp.	Ref.		Restrictions to inclusion	Rationale	Male sex	Age†	Comp. group/study compared with ref. group/study
K	N	493	Residence in NSW; post; age 16–78 y	Stored pathology specimens survey vs. survey of blood donors (NSW)	0.98 (0.65–1.49); p = 0.93	0.74 (0.66–0.84); p<0.001	1.37 (0.89–2.09); p = 0.15
R	N	204	Residence in WA; post	Patients voluntarily enrolled in RCT vs. blood donors (WA)	1.05 (0.56–1.98); p = 0.88	1.06 (0.86–1.31); p = 0.56	1.48 (0.79–2.79); p = 0.22
D	K	278	Pre; age ≥58 y	Persons in res,care vs. community control group (NSW)	0.49 (0.31–0.79); p = 0.003	2.79 (2.01–3.86); p<0.001	0.34 (0.15–0.79); p = 0.01
M	K	278	Post; age 19–77 y	Persons with HIV infection vs. community control group (NSW)	1.43 (0.80–2.57); p = 0.23	0.74 (0.61–0.90); p = 0.003	1.26 (0.66–2.41); p = 0.48
Q	K	192	Post; age 43–88 y	Hemo. patients vs. community control group (NSW)	0.90 (0.42–1.95); p = 0.79	0.91 (0.68–1.21); p = 0.50	1.65 (0.75–3.63); p = 0.21
J	N, R	316	Res. in WA; post ; age 21–45 y	Preg. women vs. community control group (WA)		0.72 (0.48–1.06); p = 0.10	0.44 (0.24–0.81); p = 0.008
C	B	1,316	Post; age ≥21	HCWs vs. community control group (NZ)	0.92 (0.70–1.22); p = 0.56	0.95 (0.88–1.03); p = 0.26	1.09 (0.83–1.42); p = 0.54
F	E	1,080	Post	HCWs vs. community control group (Singapore)	1.12 (0.74–1.71); p = 0.59	0.78 (0.66–0.93); p = 0.006	0.65 (0.41–1.01); p = 0.06
H	E	996	Post; age 21–62 y	Military personnel vs. community control group (Singapore)	1.19 (0.75–1.88); p = 0.45	0.71 (0.58 – 0.85); p<0.001	0.97 (0.58–1.60); p = 0.89
G	E	858	Post	Res. care group vs. community control group (Singapore)	1.38 (0.89–2.16); p = 0.15	0.81 (0.68–0.96); p= 0.02	0.44 (0.22–0.90); p = 0.03
P	P	1,689	Post	Aboriginal and Torres Strait Islanders vs. nonindig. people (NT)	0.95 (0.74–1.22); p = 0.68	0.88 (0.82–0.94); p<0.001	2.67 (2.08–3.42); p<0.001
B	B	1,147	Post	Maori vs nonindig. people (NZ)	0.95 (0.73–1.22); p = 0.66	0.86 (0.82–0.91); p <0.001	1.17 (0.83–1.64); p = 0.38
B	B	966	Post	Pacific Peoples vs. nonindig. people (NZ)	1.04 (0.78–1.37); p = 0.80	0.87 (0.82–0.92); p<0.001	1.99 (1.41–2.82); p<0.001

*ORs, odds ratios; comp., comparison; ref., referent; NSW, New South Wales; post, postpandemic phase; WA, Western Australia; RCT, randomized controlled trial; pre, prepandemic phase; res., residence/residential; hemo., hemodialysis; preg., pregnant; HCWs, health care workers; NZ, New Zealand; NT, Northern Territory; nonindig., nonindigenous.
†Age is considered as a continuous variable with OR for each decade of increasing age.

notable differences between public health management of the response to the outbreak in NZ and Singapore, the timing of the transition to containment was broadly similar, with NZ focusing primarily on containment from April 25 to June 21 (*34*), while Singapore began its transition to the Mitigation phase on June 29 (*35*).

Protocols for the HI assay may differ between laboratories in terms of specimen source and preparation (serum or plasma, erythrocyte adsorption), reagents (erythrocyte species, antigen preparation), procedure (incubation conditions), and controls. Furthermore, use of fresh erythrocytes for HI assays means inherent within-laboratory variability must be managed. To minimize variability between labora-

tory method and erythrocyte batches, control panels of serum samples were shared and results were standardized. A common source of virus antigen was also shared. Such comparative experiments were performed early in the pandemic between 3 of the 4 laboratories described in this analysis, with minimal variation seen. These 3 laboratories used a common source of A(H1N1)pdm09 antigen for at least 15 of the 19 datasets included. International standards were also available in 2009 for standardization of serologic assays around the world. Notably, the source of erythrocytes to detect influenza virus may vary, depending on the binding specificity of the hemagglutinin protein for each virus. A(H1N1)pdm09 virus recognized human, turkey, and guinea pig erythrocytes. This

enabled laboratories to use cells that were available and that they were experienced in handling.

Although all studies used a titer of ≥40 as the cutoff for seropositivity, this is an over-simplification of the complex immune response to influenza infection, which includes both cellular and humoral components (*36*). Although a titer of 40 was achieved in 80%–90% of persons with PCR-confirmed infection with A(H1N1)pdm09 (*37*), in unpaired analysis, no single cutoff reliably determines past infection and subsequent immunity. Serologic studies that incorporated interviewing participants about symptoms of influenza-like illness suggest that as many as half of those with serum titers of ≥40 in the postpandemic phase do not have a history of a compatible illness (*16,31*). This finding partly reflects the fact that a proportion of those patients who were seropositive in the postpandemic phase were already seropositive in the prepandemic phase, but also suggests that some persons who seroconverted did not experience or report symptoms. Despite this, pre- and postpandemic cross-sectional serologic surveys are the most convenient and inclusive method for assessing population-wide serologic immunity.

Our results provide a broad picture of the effects of A(H1N1)pdm09 in the Southern Hemisphere during the winter of 2009. The absence of clear differences between estimates with different study methods suggests that pooling of data is likely to be useful in estimating the effects of the virus across population groups. We found greater levels of prepandemic seropositivity as patient's age increased, particularly in those ≥75 years of age. By contrast, in the postpandemic period, school-aged children showed the greatest levels of immunity. Health care workers, military personnel, persons with HIV infection, and hemodialysis patients had levels of postpandemic seropositivity similar to those of the general community. Pregnancy and residential care appeared protective from infection, suggesting more severe disease in those infected. Despite recording the lowest prepandemic levels of immunity, Singapore retained comparatively lower levels of seropositivity after the pandemic.

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Sheep-to-Human Transmission of Orf Virus during Eid al-Adha Religious Practices, France

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Five persons in France were infected with Orf virus after skin wounds were exposed to infected sheep tissues during Eid al-Adha, the Muslim Feast of Sacrifice. Infections were confirmed by electron microscopy, PCR, and sequence analysis. Prevention and control of this underdiagnosed disease can be achieved by educating physicians, slaughterhouse workers, and persons participating in Eid al-Adha.

Orf virus (genus *Parapoxvirus*, family *Poxviridae*) is endemic to most countries. The virus primarily causes contagious ecthyma in wild and domestic ruminants, mostly sheep and goats (*1*). Human infections caused by occupational and household exposures have been described (*2–5*); they most commonly cause lesions on the hands (*1,3,6*). We report Orf virus infection in 5 humans who had household exposure to the virus.

The Cases

Case-patient 1, a 51-year-old woman, was examined on November 28, 2011, by a surgeon (CF) for an 8-mm lesion without local complications on her left thumb. Case-patient 2, the 33-year-old niece of case-patient 1, was hospitalized on November 29 for surgical excision of a phleg-

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monous lesion on the fifth finger of her left hand; she also had a fever and a lesion without local complications on the second finger of the same hand. The surgery was performed by CF, the same surgeon who examined case-patient 1. By chance, case-patient 3, the 38-year-old brother of case-patient 2, met CF on November 29 while visiting his sister in the hospital; he asked CF if she would examine lesions on his left hand. Clinical examination revealed 2 lesions (1 each on the thumb and third finger) without local complications (Figure 1, panels A and B). The man reported having fever and malaise 1 day before the examination.

While examining case-patient 3, CF became aware that case-patients 1–3 were members of the same family and that they had butchered or handled several lambs on November 6, 2011, in preparation for Eid al-Adha (also called Eid al-Kabir), the Muslim Feast of Sacrifice. Using a smartphone, CF photographed the lesions on case-patient 3 and transmitted the photographs to 2 infectious disease specialists. The specialists indicated that the lesions appeared to be typical of parapoxvirus infection. CF interviewed case-patients 1–3 again, and they reported having knife wounds after preparing lambs for the religious feast and seeing lesions on the gums and tongue of 1 lamb. Swab (Virocult; Medical Wire and Equipment Co. Ltd., Corsham, United Kingdom) specimens were obtained from lesions on case-patients 1 and 3 and sent, along with a surgical skin biopsy specimen from case-patient 2, to the virology laboratory at Public Assistance–Hospitals of Marseille, Marseille, France.

Case-patient 4, a 64-year-old woman, sought medical care on December 9 for an ulcerovegetative lesion on the third finger of her left hand; the lesion was on the internal face of the interphalangeal joint, and phlegmon and cellulitis were present. The patient reported that she had injured herself with a kitchen knife on November 6 while butchering lamb meat for Eid al-Adha. Ten days later, she noticed vesicular lesions at the injury site; a pustule complicated by superinfection subsequently developed. The lesion was surgically excised on December 12, and skin biopsy samples were sent to the virology laboratory at Public Assistance–Hospitals of Marseille.

Case-patient 5, a 42-year-old woman, sought medical care on December 14 for a painful 2-cm papulonodular lesion on her right wrist (Figure 1, panel C). She recalled being injured on November 6 with a knife used to cut off the head of a lamb that was being prepared for Eid al-Adha. The lesion was surgically excised, and a sample was sent to the virology laboratory at Public Assistance–Hospitals of Marseille.

Specimens from case-patients 1–3 were received at the laboratory on December 2. Negative-stain electron microscopy (EM) was immediately performed: images revealed

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Figure 1. Orf virus infection in 5 persons who butchered or prepared lambs as part of a religious practice for Eid al-Adha (the Muslim Feast of Sacrifice), Marseille, France, 2011. Cutaneous lesions on hands of case-patient 3 (A, B) and case-patient 5 (C) are shown. Negative-staining electron microscopy of samples from case-patient 3 (D) and case-patient 5 (E, F) show ovoid particles (≈250 nm long, 150 nm wide) with a crisscross appearance; the size and appearance of these particles are highly suggestive of parapoxvirus virions.

typical poxvirus-like particles in the specimen from case-patient 3 (Figure 1, panel D). To detect the presence of poxviruses, we subjected the samples to 2 broad-range PCRs with high-GC and low-GC primers (7). A 627-bp product was amplified with the high-GC primers from samples from case-patients 1 and 3 (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/pdfs/12-0421-Techapp. pdf). The products were directly sequenced, and results were subjected to BLAST analysis, which confirmed Orf virus infection (8). Skin-biopsy specimens from case-patients 4 and 5 were received at the laboratory on December 13 and 15, respectively, and processed as described above. EM revealed pox-like particles in both samples (Figure 1, panel E–F), and both were positive for Orf virus by PCR (online Technical Appendix Figure 1). Figure 2 shows the time from lesion onset to laboratory diagnosis of Orf virus infection for case-patients 1–5.

Immediately after Orf virus infection was confirmed, we attempted virus isolation by cell culture, using Vero cells (the only cells available at the time) in 12.5-cm^2 flasks; none of the samples yielded infectious virus. We later attempted virus isolation again, using fetal bovine esophagus cells, and isolated Orf viruses from samples from case-patients 3 and 5.

The 4 partial sequences obtained from samples from case-patients 1 and 3–5 were identical (GenBank accession no. JQ596637). We used ClustalX (9) to align the sequences for comparison with other homologous Orf virus sequences and other high-GC poxviruses. We performed phylogenetic analysis by using the neighbor-joining method (jukes-cantor algorithm) in MEGA5.0 software (10) (online Technical Appendix Figure 2).

County veterinary services traced the origins of the sheep considered to be responsible for these human cases of Orf virus infection. The first 3 cases were linked to an illegal slaughterhouse within the county where sheep from France and Spain had been housed for 1 month. Case 4 was linked to sheep carcasses that were purchased from 2 legally operating butchers. Case 5 was linked to a certified temporary slaughterhouse that had sheep from regional counties, Spain, and Romania.

Conclusions

Although human Orf virus infections have typically been associated with occupational animal contact (1–3,6), they also have been linked to Muslim religious practices and, more globally, to household meat processing or animal slaughter (4,11–15). Our findings show that clinical
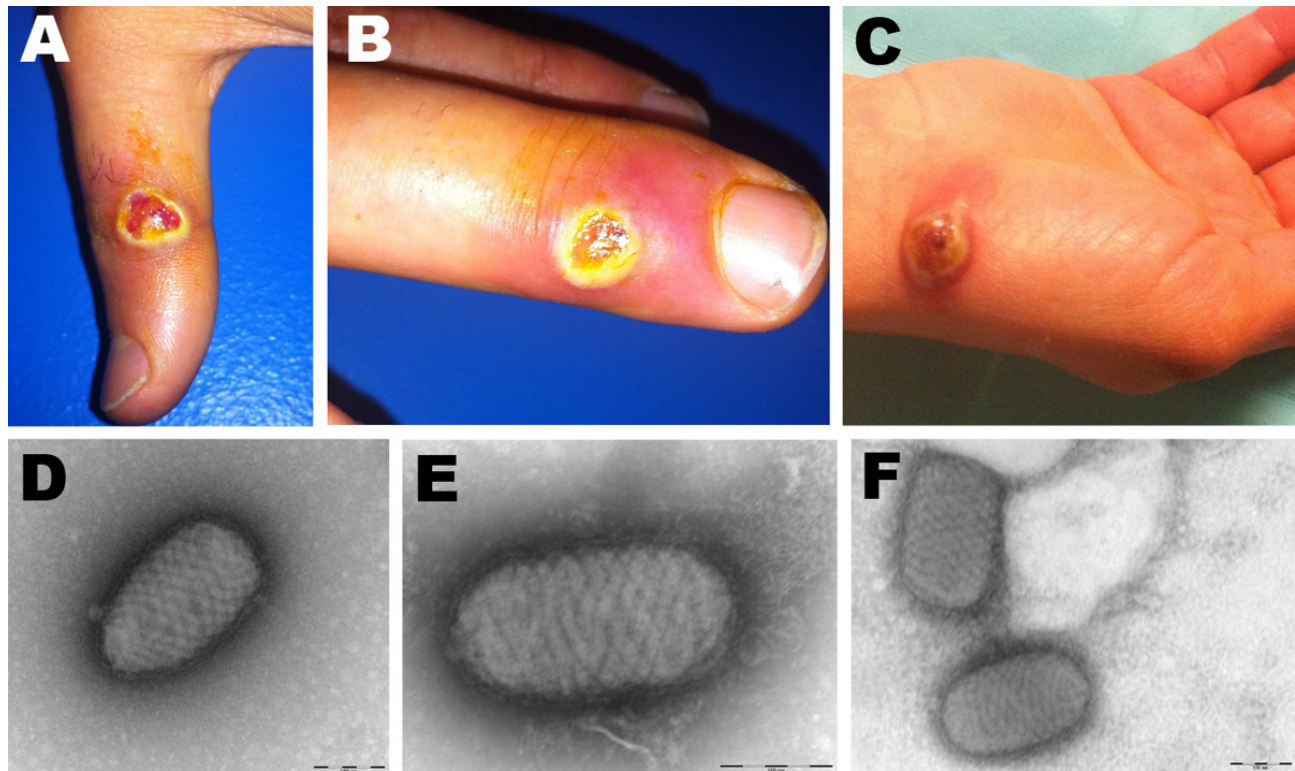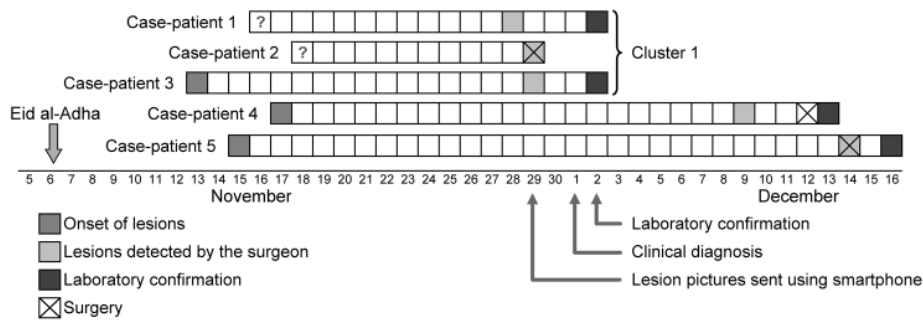
Figure 2. Natural history of Orf virus infection and diagnosis in 5 persons who butchered or prepared lambs as part of a religious practice for Eid al-Adha (the Muslim Feast of Sacrifice), Marseille, France, 2011. Arrows indicate events for the first cluster of cases among 3 related persons (a brother and sister and their aunt).

microbiology laboratories (other than national reference centers) can accurately detect and identify poxviruses by using EM and broad-spectrum PCR, such as that described by Li et al. (*7*). Our results also suggest that PCR is highly sensitive for detection of poxviruses, and they show that samples obtained by Virocult swab are well-suited for detection of Orf virus by EM or PCR and could replace more invasive methods (e.g., skin biopsy).

The finest, unblemished animals (e.g., cows, goats, sheep) were initially reserved for the ritual sacrifice during Eid al-Adha. Today, however, Muslims in developed countries (especially in cities) mostly buy lambs, which are cheaper and more plentiful but also highly susceptible to Orf virus infections (*1*). This change in buying practices has created a large market for possibly infected animals and an associated potential health risk for persons who butcher and prepare the animals.

The cases reported here stress the need for using appropriate measures to prevent animal-to-human transmission of pathogens. Public health officials should educate persons with occupational or household exposure to animals about the possibility for disease transmission and ways to avoid infection. Persons at increased risk for exposure to Orf virus include livestock owners, slaughterhouse workers, and persons who prepare animals at home for religious practices. Persons who handle animals should wear nonpermeable gloves, avoid exposure of open wounds, and meticulously wash skin wounds with soap and water after handling animals (*4*). In addition, slaughterhouses should verify that all animals to be sold or butchered are in good health; animals with Orf virus lesions should be disposed of in a safe manner. Physicians, including dermatologists, should be informed of the potential for Orf virus infection, a heretofore underdiagnosed disease (*15*), and suspected infections should be confirmed by microbiology laboratories. The first 3 cases presented here were rapidly diagnosed, and emergency department physicians were promptly advised of the cases, enabling rapid detection and confirmation of the subsequent cases.

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etymologia

Orf
[orf]

Origins of the term are unclear, but some sources (the Oxford English Dictionary, Webster's) derive it from Old Norse *hrūfa* ("crust on a wound, scab"). Another source (Stedman's) derives it from the Old English *orfcwealm* ("murrain, any infectious disease of livestock"), from *orf* ("cattle") + *cwealm* ("destruction"). Paradoxically, although "orf" may trace its origin to a word meaning "cattle, orf does not naturally infect cattle.

Orf (contagious ecthyma) is an exanthematous infectious disease caused by a parapoxvirus that occurs primarily in sheep and goats but can be spread to humans through contact with an infected animal or fomites. Although most human cases are associated with occupational exposure to sheep or goats, cases have recently been reported in persons who were infected through household meat preparation or ritual sacrifice.

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Schmallenberg Virus in *Culicoides* spp. Biting Midges, the Netherlands, 2011

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To determine which species of *Culicoides* biting midges carry Schmallenberg virus (SBV), we assayed midges collected in the Netherlands during autumn 2011. SBV RNA was found in *C. scoticus*, *C. obsoletus sensu stricto*, and *C. chiopterus*. The high proportion of infected midges might explain the rapid spread of SBV throughout Europe.

During early summer 2011, Schmallenberg virus (SBV), a novel orthobunyavirus of the Simbu serogroup, spread across much of northern Europe, infecting ruminant livestock. The Simbu serogroup (family *Bunyaviridae*, genus *Bunyavirus*) includes Shamonda virus, Akabane virus, Sathuperi virus, and Aino virus. These viruses cause teratologic effects in ruminants and are arthropod-borne, and most have been isolated in the Old World from mosquitoes and *Culicoides* spp. biting midges (*1*). Recent preliminary studies indicate that ≥1 species of *Culicoides* midges act as field vectors for SBV in Europe (*2*). To determine which *Culicoides* midge species harbor SBV, we analyzed midges collected from 3 livestock holdings in eastern and northeastern parts of the Netherlands.

The Study

Throughout September and early October 2011, *Culicoides* spp. biting midges were trapped almost daily at a dairy in the municipality of Ermelo (eastern Netherlands) by various methods, including the standard Onderstepoort-type blacklight trap. In addition, during several days in August and September 2011, *Culicoides* spp. biting midges were trapped near sheep flocks in the municipalities of Bilthoven (central Netherlands) and Midden-Drenthe (northeastern Netherlands) by using the Onderstepoort-

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type trap and a drop-tent cage. Captured midges were stored in 70% ethanol.

Female midges were categorized as nulliparous, parous, gravid, or freshly blood fed (engorged) (*3*); only midges belonging to the first 3 categories were assayed. The 6,100 selected midges were divided into 610 species-specific pools, 10 midges per pool. Under a dissecting microscope, the heads were separated from abdomens by use of a scalpel; 10 heads were then pooled and assayed for SBV, whereas the corresponding abdomens (also pooled) were stored in 70% ethanol.

All midges were identified morphologically, but because female *C. obsoletus* sensu stricto midges cannot be separated with confidence from *C. scoticus* midges, they were pooled and are referred to jointly as the *C. obsoletus* complex. The number of pools assayed for each species was as follows: *C. obsoletus* complex (230), *C. chiopterus* (144), *C. dewulfi* (130), *C. punctatus* (105), and *C. pulicaris* (1). After assays were conducted, the species identity of each SBV-positive midge pool was established by using molecular techniques.

Only when a pool of 10 heads was found SBV positive was the corresponding pool of dissected abdomens retrieved and assayed. In this instance, the 10 abdomens were assayed singly, so that the individual abdomen that was SBV-positive could be identified molecularly, to establish exactly which of the 2 species of the *C. obsoletus* complex was involved and to confirm or refute the morphologic identifications that had been made for the remaining *Culicoides* species.

RNA extraction was performed according to a protocol developed by CODA-CERVA (Centrum voor Onderzoek in Diergeneeskunde en Agrochemie, Centre d'Étude et de Recherches Vétérinaires et Agrochimiques), Brussels, Belgium; whereas, reverse transcription PCR (RT-PCR) was performed according to a method recently developed to detect the small segment of SBV (*4*). The RT-PCR cutoff value for the pooled heads was set at a cycle threshold (C_t) value of 35. Pools with C_t >35 were retested and considered positive when confirmed. Reported C_t values for blood samples from infected cattle in Germany, tested by using the same RT-PCR, were 24–35 (*5*) and were used as the guide for our choice of cutoff value. If a specific pool of midge heads tested positive, individual abdomens from the corresponding stored pool were tested separately by RT-PCR. For molecular identification of the SBV-positive midges, the 18S internal transcribed spacer 1 (ITS1) 5.8S region was amplified by using the PanCulF and PanCulR primer set, adapted from Cêtre-Sossah et al. (*6*). The ITS1 sequences obtained from the SBV-positive abdomens were used to develop a *Culicoides* spp. phylogeny (Figure), which includes GenBank sequences representing all 5 species of the subgenus *Avaritia* (including *C. imicola*) known
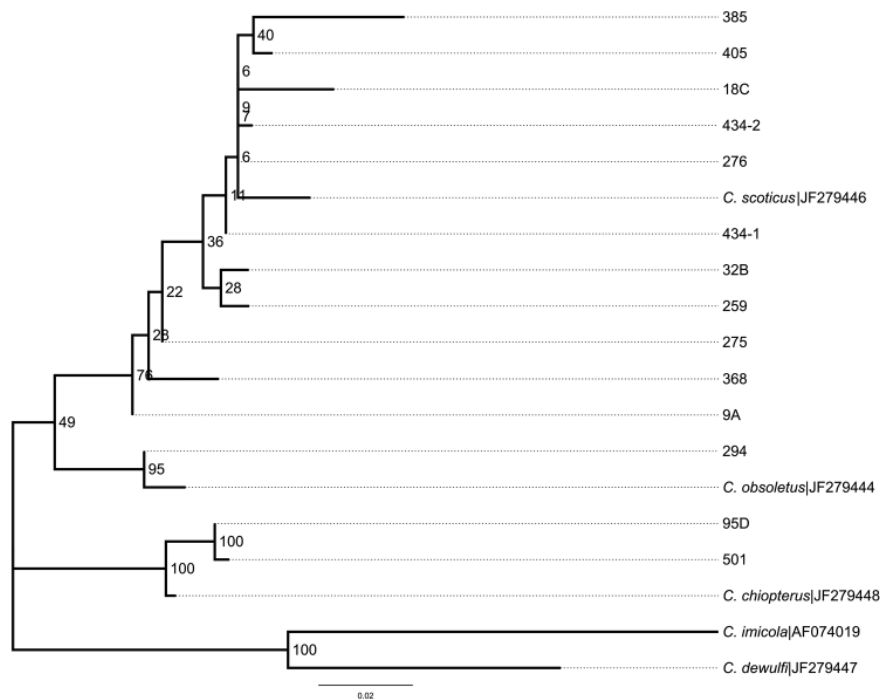
Figure. Phylogenetic tree comparing Schmallenberg virus–positive *Culicoides* spp. biting midge abdomens isolated in different regions in the Netherlands, 2011, with reference sequences from Deblauwe et al. (*7*). *C. imicola* was used as an outgroup. Bootstrap values are indicated at the significant nodes. Scale bar indicates nucleotide substitutions per site.

to be involved in the transmission of arboviruses in western Europe.

Of the 610 *Culicoides* midge head pools, 14 (2.3%) were SBV positive according to RT-PCR (Table 1): 11 *C. scoticus*, 1 *C. obsoletus* s.s., and 2 *C. chiopterus*. Of the 14 pools, 13 comprised midges from the dairy in Ermelo; midges in the remaining *C. chiopterus* pool came from Midden-Drenthe. C_t values for 12 of the 14 pools ranged from 19.6 to 30.44; C_t values for the remaining 2 pools were 34.98 and 36.78 (Table 2). C_t values for 13 of the individual midge abdomens linked to each pool of SBV-positive heads were lower (meaning a higher viral load) than those obtained for their corresponding heads. In 1 pool of *C. obsoletus* complex midges, 2 of 10 abdomens were positive for SBV, 1 strongly and 1 weakly. RT-PCRs for SBV were negative for all 130 pools of *C. dewulfi*, 105 pools of *C. punctatus*, and the 1 pool of *C. pulicaris* midges.

The species of all but 1 midge abdomen could be molecularly identified on the basis of ITS1 (Table 2). Not only did the molecular results confirm most of the morphologic identifications, but they also showed that *C. scoticus* seems

to have played a more prominent role than *C. obsoletus* s.s. in transmission of SBV. The ITS1 sequences obtained from samples 95-D and 501 were almost identical to those published for *C. chiopterus*; the same applies to sample 294, which represented *C. obsoletus* s.s. (Table 2) (*7*). Although sequence polymorphism in *C. scoticus* was diverse, we were able to unambiguously assign each of the 11 SBV-positive abdomens to this species (Figure).

Prevalence of SBV among the *Culicoides* spp. midges was 0.25% (15/6,100 midges tested). More specifically, the prevalence of SBV in the 2 species that comprised the *C. obsoletus* complex was 0.56% (13/2,300 tested). This prevalence is similar to that obtained for Akabane virus in *C. brevitarsis* midges from Australia (*8–11*) but about 10× higher than that reported for bluetongue virus (*12*). For *C. chiopterus* midges, prevalence of SBV was 0.14% (2/1,440 tested), ≈5× higher than prevalence of bluetongue virus (*13*).

Conclusions

Our results demonstrate that SBV was harbored in 3 species of field-collected *Culicoides* biting midges: *C.*

Table 1. Schmallenberg virus RNA in *Culicoides* spp. biting midges collected August–September 2011, the Netherlands

Municipality (Province)	*C. obsoletus* complex	*C. dewulfi*	*C. chiopterus*	*C. punctatus*	*C. pulicaris*	Total
		Pools, no. positive/no. tested*				
Bilthoven (Utrecht)	0/10	0	0	0	0	0/10
Midden-Drenthe (Drenthe)	0/5	0	1/39	0	0	1/44
Ermelo (Gelderland)	12/215	0/130	1/105	0/105	0/1	13/556
Total	12/230	0/130	2/144	0/105	0/1	14/610

*Tested by reverse transcription PCR.

Table 2. C$_t$ values of Schmallenberg virus–positive *Culicoides* spp. biting midges collected August–September 2011, the Netherlands*

	Pooled heads			Individual abdomens		
	Species identification by	C$_t$ value		Species identification by	C$_t$ value	
Pool no.	morphologic examination	First test	Second test	DNA sequencing	First test	Second test
95-D	*C. chiopterus*	27.88	NA	*C. chiopterus*	24.59	NA
501	*C. chiopterus*	35.36	34.98	*C. chiopterus*	36.45	35.07
9-A	*C. obsoletus* complex	30.44	NA	*C. scoticus*	24.75	NA
18-C	*C. obsoletus* complex	28.24	NA	*C. scoticus*	24.95	NA
32-B	*C. obsoletus* complex	21.84	NA	*C. scoticus*	18.32	NA
259	*C. obsoletus* complex	19.60	NA	*C. scoticus*	18.16	NA
275	*C. obsoletus* complex	20.72	NA	*C. scoticus*	20.39	NA
276	*C. obsoletus* complex	36.02	36.78	*C. scoticus*	36.68	NA
293	*C. obsoletus* complex	20.43	NA	No reliable sequence	19.95	NA
294	*C. obsoletus* complex	24.60	NA	*C. obsoletus* sensu stricto	20.06	NA
368	*C. obsoletus* complex	25.21	NA	*C. scoticus*	21.80	NA
385	*C. obsoletus* complex	20.67	NA	*C. scoticus*	20.25	NA
405	*C. obsoletus* complex	23.38	NA	*C. scoticus*	21.64	NA
434†	*C. obsoletus* complex	23.68	NA	*C. scoticus*	23.10	NA

*C$_t$ values determined by reverse transcription PCR. All midges were collected from cattle in the Ermelo municipality except no. 95-D, which was collected from sheep in the Midden-Drente municipality. C$_t$, cycle threshold; NA, not applicable.
†Two abdomens from this pool were positive; C$_t$ values for the second abdomen were 35.75 and 35.37.

scoticus, C. chiopterus, and *C. obsoletus* s.s. These species were among the more abundant of the 15 species found at the livestock holdings sampled. The holdings were situated in the center of the epidemic area, and of the ≈100 animals at the dairy in Ermelo, >96% had seroconverted to SBV. The low C$_t$ values indicate that concentrations of the virus in most SBV-positive *Culicoides* midges were high. The fact that the C$_t$ values for the heads of midges matched closely with those from the associated abdomens renders it certain that SBV had replicated to transmissible levels in these midges and supports the contention that 2 species of the *C. obsoletus* complex, along with *C. chiopterus,* act as natural vectors for SBV. Despite the relatively large numbers of SBV-negative pools, our findings should not be interpreted to exclude the involvement of other species, such as *C. dewulfi* or *C. punctatus*, in field transmission of SBV. We conclude that the high proportion of SBV-positive *Culicoides* spp. midges and the multiple vector species could help explain the rapid spread of SBV throughout much of Europe during 2011.

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Hepatitis E Virus Genotype 4 Outbreak, Italy, 2011

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During 2011, 5 persons in the area of Lazio, Italy were infected with a monophyletic strain of hepatitis E virus that showed high sequence homology with isolates from swine in China. Detection of this genotype in Italy parallels findings in other countries in Europe, signaling the possible spread of strains new to Western countries.

Hepatitis E virus (HEV) represents the major etiologic agent of enterically transmitted, non-A, non-B hepatitis. One third of the world population is estimated to have been infected with HEV, although the global extent of infection is unknown (http://apps.who.int/gb/ebwha/pdf_files/A62/A62_22-en.pdf). Recent evidence indicates that the family *Hepeviridae* may contain several genera and that viruses from some genera can be transmitted from animals to humans and vice versa (1). Gene sequence analysis demonstrates that HEV isolates are divided into 4 genotypes and ≥24 subgenotypes (2). Increasing evidence shows that genotypes 3 and 4 are zoonotic, with domestic pigs a likely reservoir of infection; these genotypes have also been found in boars and deer (3).

HEV genotype 4 is endemic among humans in China, Japan, India, and Indonesia (4) and was detected during 2008 from swine fecal samples in Belgium (5). Human infections with imported strains of this genotype that later became endemic have subsequently been described in Germany (6) and northern France (7). Recently, multiple cases of HEV infection have been described in southern France (8,9).

In different areas of Italy, HEV seroprevalence estimates range from 1% to 6% (10); prevalence is 2.9%

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in the Lazio region and 2.5% in the province of Rome (11). However, the number of acute hepatitis cases caused by HEV reported in Italy is relatively low compared with surrounding European countries and is probably underestimated; most cases are travel-related and caused by genotype 1, but sporadic cases spread within Italy have been caused by genotype 3 (12). We report an outbreak of HEV genotype 4 infection among persons living in Lazio, an administrative region of Italy that encompasses Rome.

The Study

During March and April 2011, diagnoses of acute HEV infection were made for diagnosed for 5 patients admitted to 3 hospitals in Lazio: 2 in Rome and 1 in Latina. Diagnosis was made on the basis of clinical and laboratory signs of acute hepatitis and detection of IgG and IgM against HEV by immunoassay (Radim S.P.A., Rome, Italy); Other causes of acute liver injury, including drug toxicity, autoimmune hepatitis, and infection by hepatitis A, B, and C viruses, as well as by cytomegalovirus and Epstein-Barr virus, were excluded (Table 1). All patients recovered rapidly with supportive care.

The 5 case-patients lived in the same area (maximum distance apart: 27 km) (Figure 1). The cases were reported to the health authorities, who conducted a structured interview to determine travel history and other risk factors (Table 2). None of the case-patients reported recent travel to disease-endemic areas, and relationships among them or exposure to a common source were not identified. It was not possible to conduct the complete interview for 1 patient (isolate no. E2107).

Serum samples were tested for RNA by reverse transcription-nested PCR by using primers designed within open reading frame (ORF) 1 and ORF2 (12,13). The sequence data from these genetic regions identified a monophyletic strain belonging to genotype 4, subgenotype d. ORF1 nucleotide sequences (172 bp) from the outbreak showed high similarity among patients (99.2%) and 96% and 95% identity with HEV4d swine hb-3 and human T1 isolates from China, respectively (GenBank accession nos. GU361882 and AJ272108). Sequences identified in the ORF2 region (411 bp) in samples from the 5 case-patients showed 100% similarity. The sequences also were closely related to the strains KMsw-3 (nucleotide similarity 98.5%; accession no. HQ008864) and KMsw-1 (98.3%; accession no. HQ008863) isolated from swine and to the strain GS-NJ-13 isolated from humans (97.8%; accession no. JF309220.1), all of which originated in China. Figure 2 shows phylogenetic trees for ORF1 and ORF2 partial gene sequences.

The similarity of the strain from this outbreak to strains from recent autochthonous HEV genotype 4 infections in Europe was relatively low: 73.5% in the

Table 1. Clinical and demographic characteristics of 5 case-patients involved in outbreak of HEV infection, Lazio, Italy, March–April 2011*

Characteristic	Case-patient 1	Case-patient 2	Case-patient 3	Case-patient 4	Case-patient 5
HEV isolate no.	E2104	E2105	E2107	E2111	E2115
Age, y/sex	60/M	38/M	66/M	57/M	74/M
Date of symptom onset	Mar 10	Mar 12	Mar 14	Apr 1	Apr 2
Date of first HEV Ab detection	Mar 19	Mar 23	Mar 25	Apr 11	Apr 14
Clinical signs, symptoms	Fatigue, jaundice, dark urine	General malaise, fatigue	Fatigue, dark urine	Jaundice	Fatigue, vomiting
Duration of hospitalization, d	18	25	14	13	16
Alanine aminotransferase, IU/L†	4,017	2,648	2,648	5,163	3,001
Total bilirubin, mg/dL‡	6.5	20.0	15.6	14.7	8.0
Hepatitis A virus IgG	Positive	Negative	Negative	Positive	Positive
HBV surface antigen	Negative	Negative	Negative	Negative	Negative
HBV core Ab	Negative	Negative	Negative	Positive	Negative
Hepatitis C virus Ab	Negative	Negative	Negative	Negative	Negative
HIV Ab	Negative	Negative	Negative	Negative	Negative
Cytomegalovius IgM	Negative	Negative	Negative	Negative	Negative
EBV EA IgG	Negative	Negative	Negative	Negative	Negative
EBV VCA IgM	Negative	Negative	Negative	Negative	Negative

*HEV, hepatitis E virus Ab, antibody; HBV, hepatitis B virus; EBV, Epstein–Barr virus; EA, early antigen; VCA, viral capsid antigen.
†Reference range 7–34 IU/L.
‡Reference range 0.3–1.2 mg/dL.

overlapping 37–172-nt ORF1 region of the isolate from Germany (GenBank accession no. EU879120) (6), 85.7% in the overlapping 31–373-nt ORF2 region of the isolate from northern France (accession no. GU982294) (7), and 84.7%–84.8% in the 329-nt overlapping ORF2 region of 5 isolates from southern France (9). Consistent with these data, 85.1% and 85.3% identity was observed in the 537-nt ORF2 region of 2 of the isolates from southern France (accession nos. JF900631 and JF900632) (8), (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0983-Techapp.pdf).

Conclusions

An outbreak of HEV infection caused by genotype 4 in Italy involved 5 case-patients who lived in the same area and did not travel to disease-endemic areas. The high genetic similarity in ORF1 and ORF2 among the 5 HEV isolates supports a point-source outbreak and not sustained
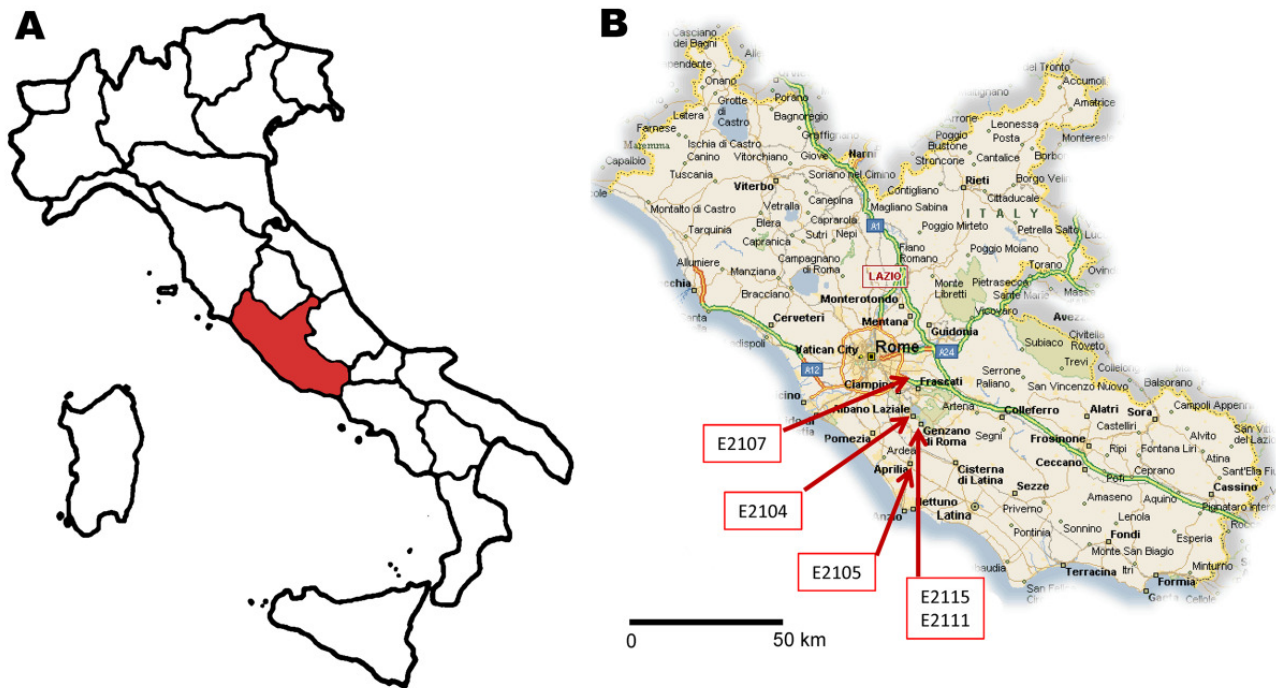
Figure 1. A) Lazio region of Italy (black). B) Location of 5 cases of hepatitis E virus (HEV) subtype 4d infection involved in an autochthonous outbreak, March–April 2011. (Map source: CartineGeografiche, Catania, Sicily, Italy; www.cartinegeografiche.eu).

Table 2. Exposure and potential risk factors for 4 patients infected with hepatitis E virus during outbreak in Lazio, Italy, March–April 2011*

Factor	Case-patient 1	Case-patient 2	Case-patient 4	Case-patient 5
Underlying disease	IHD	None	IHD	IHD
Farm residence or employment	No	No	No	No
Manure used for fruits/vegetables grown in backyard	No	No	No	No
Professional or amateur hunter	No	No	Yes	No
Occupational exposure to animals	No	No	No	No
Household pet or domestic animal ownership	Dogs	No	Dogs, chicken	No
Risk behavior during previous 2 months				
Received blood transfusion	No	No	No	No
Traveled abroad	No	London	No	No
Had close contact with a recent traveler	Yes†	No	No	No
Visited a farm or petting zoo	No	No	No	No
Drank water from a well	No	No	No	No
Had contact with surface water	No	No	No	No
Had contact with waste water	Yes	No	No	No
Food products consumed during previous 2 months				
Mussels	Yes	No	Yes	No
Shellfish	Yes	Yes	Yes	No
Poultry, undercooked	No	No	No	No
Pork products, raw or undercooked	No	Yes	Yes	Yes
Pork meat, undercooked	No	No	No	No
Horse meat	No	No	No	No
Beef, raw or undercooked	No	No	Yes	Yes
Wild boar, cooked or undercooked	No	No	Yes	No
Wild-animal meat (other than wild boar)	No	No	No	No
Cattle liver	No	No	No	Yes
Pig liver	No	No	No	Yes
Other (e.g., kidney, gut)	No	No	No	No

*Complete information was not available for case-patient 3 (isolate no. E2107). IHD, ischemic heart disease.
†Contact with a person returning from Poland and Romania.

local circulation of this strain. Epidemiologic information did not identify the transmission route; available data ruled out direct transmission among patients, and parenteral transmission is unlikely because none of the patients had received blood transfusions, tattoos, or drug injections.

Three patients had IgG against hepatitis A virus (HAV); no information on HAV vaccination status was available. However, the presence of these antibodies does not necessarily imply higher than average levels of exposure to enterically transmitted viruses; a HAV seroprevalence >60% has been reported in persons in the birth cohorts of the 5 case-patients in central Italy (14).

Consumption of contaminated food (i.e., pork or wild animal meat, bivalve mollusks, or shellfish) is considered the most likely source of infection with HEV genotype 3 in Europe (8,12). For genotype 4, uncooked deer meat was indicated as a source of human infection in Japan (15), and undercooked pork meat was the probable source of infection in southern France (8,9). The isolates from this study had the highest genetic similarity to subgenotype 4d strains of swine origin from China (Figure 2), which suggests a possible zoonotic origin through either direct (e.g., ingestion of raw or undercooked pork products) or indirect (e.g., by water contaminated with animal excreta) transmission.

Possible sources of infection with this HEV strain that cannot be ruled out include contaminated food from abroad and direct introduction through infected immigrants from China or other countries in Asia. However, the proportion of immigrants from Asia in this area of Italy, 0.77%, is lower than the national average (1.26%; www.comuni-italiani.it/statistiche/stranieri.html). Available data do not support correlations between immigration from China and spread of this HEV genotype in Lazio.

Strong sequence similarity (>96%) was observed between HEV isolates from human cases in northern and southern France and the strain isolated from swine in Belgium (5), classified as subgenotype 4b. Human infection with HEV genotype 4 reported in Germany in 2008 (6) was attributed to a different subgenotype (4f). The strain involved in the outbreak in Italy showed relatively poor genetic resemblance with any of these strains, which indicates that different HEV genotype 4 strains have been recently introduced in Europe.

In summary, this outbreak of HEV genotype 4 infection in Italy was not linked to infection by imported foods or persons traveling from endemic areas, which suggests the possibility that newly imported strains might spread this virus to new areas. Molecular characterization of HEV outbreaks in Europe is needed to implement epidemiologic mapping of infection with introduced strains of HEV and subsequent circulation.
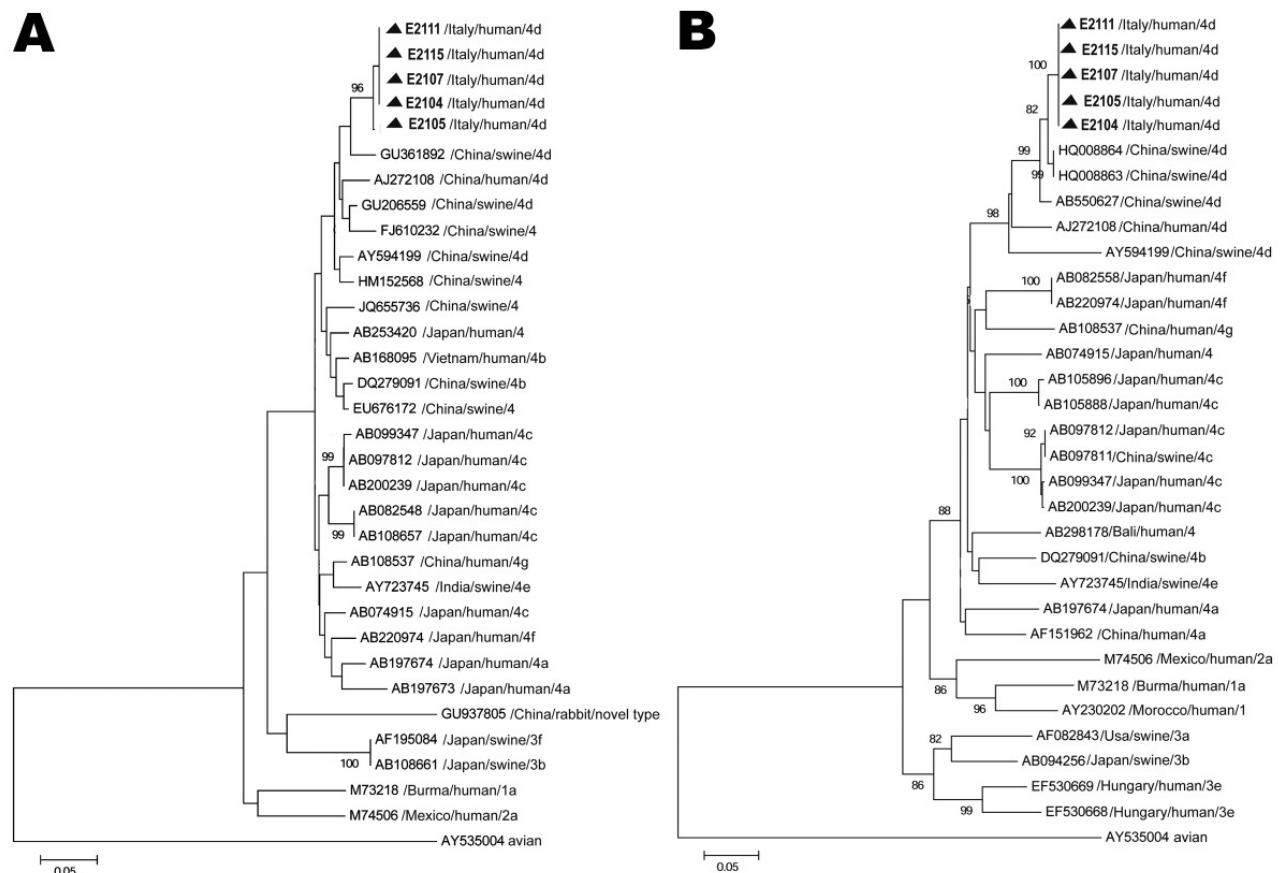
Figure 2. Phylogenetic trees based on partial open reading frame (ORF) sequences of the hepatitis E virus monophyletic strain involved in an outbreak in Lazio, Italy, March–April 2011. A) ORF1, 172 nt. Sequences from the outbreak in Italy could not be submitted to GenBank, being <200 nt long; they are available on request from the authors. B) ORF2, 411 nt. The ORF 2 sequence (identical in all 5 patients) described in this panel was submitted to GenBank (accession no. JX401928). Neighbor-joining trees were built by using MEGA5.1 software (www.megasoftware.net), applying the Jukes-Cantor p-distance model of nucleotide substitution. Bootstrap values were determined on 1,000 resamplings of the data set; bootstrap values >80 are shown. Reference strains from GenBank are also included in the trees. Reference viral strains are identified by GenBank accession number, source, country of origin, and respective genotype and subtype. The avian strain AY535004 was used as outgroup. Triangles indicate sequences recovered during the outbreak in Italy. Scale bars represent nucleotide substitutions per site.

Acknowledgment

Dr Garbuglia is a senior scientist at the virology laboratory of the Lazzaro Spallanzani National Institute for Infectious Diseases in Rome. Her primary research interests include virus evolution analysis and host–pathogen interaction.

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Characterization of Full Genome of Rat Hepatitis E Virus Strain from Vietnam

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We amplified the complete genome of the rat hepatitis E virus (HEV) Vietnam strain (V-105) and analyzed the nucleotide and amino acid sequences. The entire genome of V-105 shared only 76.8%–76.9% nucleotide sequence identities with rat HEV strains from Germany, which suggests that V-105 is a new genotype of rat HEV.

Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus (1), classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (2). Hepatitis E, caused by HEV infection, is a serious public health concern in developing countries and is recognized as sporadic and endemic acute hepatitis (3). To date, at least 4 genotypes of HEV have been isolated from humans (4). In addition, HEV has been isolated from other mammals, including pigs, wild boars, wild deer, rabbits, ferrets, bats, chickens, and wild rats (5–9). Much direct evidence indicates that HEV is transmitted from pigs or wild boars to humans, and therefore hepatitis E caused by genotypes 3 and 4 is recognized as a zoonotic disease (6,8,10).

Rat HEV was first isolated from Norway rats in Germany (7,11). Since then, rat HEV strains have been isolated from wild rats in other areas of Germany and detected in wild rats in the United States and Vietnam (12–14). Those results suggest that rat HEV infection is not restricted to Germany but is broadly distributed in wild rats throughout the world. The nucleotide sequences of the rat HEV isolated in Germany and the United States are similar; however, the partial sequences of the Vietnam rat HEV strain (V-105, JN040433) have been found to have 78.18%–79.43% identities with isolates from Germany, R63 and R68 (14). To confirm whether new genotypes of rat

HEV exist, we amplified the entire genome of the rat HEV V-105 strain and analyzed the sequences. We confirmed that the rat HEV strain isolated in Vietnam belongs to a new genotype of rat HEV.

The Study

The rat HEV used in this study was isolated from a 10% lung homogenate of a wild rat from Vietnam, which was positive for rat HEV RNA by reverse transcription PCR (RT-PCR) (14). Because of the limited availability of rat specimens that are positive for HEV RNA, we first transmitted the rat HEV to a laboratory rat (Wistar) to produce a large amount of virus for RNA extraction and genome amplification. After intravenous inoculation of the rat, fecal specimens positive for HEV RNA were collected, and a 10% suspension was prepared as described (15). RT-PCR was performed by using Superscript II RNase H⁻ (Invitrogen, Carlsbad, CA, USA) and primer TX30SXN (14). The full-length genome of the V-105 strain was amplified by RT-PCR with primers based on the nucleotide sequences of GU345042 and JN040433 (Table 1). All PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and cloned into TA cloning vector pCR2.1 (Invitrogen). The nucleotide sequencing was carried out by using an ABI 3130 Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA).

Because 901 nt of V-105, corresponding to nt 4108–5008 of the R63 genome, were already known (14), primers F13 and open reading frame (ORF) 1–R12 were designed. An ≈2,100-nt fragment of the C-terminus of the rat HEV V-105, nt 4923-poly (A) tail, was amplified with a pair of primers, F13 and TX30SXN, by the first RT-PCR. The ORF1 region was amplified with primers ORF1-F1 and ORF1-R12. Two fragments, 440 nt (nt 11–450) and 1,182 nt (nt 2990–4171), were amplified by nested PCR with 2 sets of primers, ORF1-F2/ORF1-R1 and ORF1-F7/ORF1-R12, respectively. On the basis of the nucleotide sequences of those amplified fragments, ORF1-F9, ORF1-F16, ORF1-R16, ORF1-F18, and ORF2-R21 were designed, and 3 fragments, 1,830 nt (nt 388–2217), 996 nt (nt 2080–3075), and 1,110 nt (nt 3991–5100), were amplified with 3 sets of primers, ORF1-F9/ORF1-R10, ORF1F16/ORF1-R16, and ORF1-F18/ORF2-R21, respectively.

To amplify the N-terminus nonstructural region of V-105, we synthesized cDNA with primer ORF1-R14, and a DNA anchor (P-CACGAATTCACTATCGATTCTGG AACCTTCAGAGG-NH₃) was linked to the N-terminus of the cDNA by T4 RNA Ligase I (BioLabs, Tokyo, Japan). By using this anchor-cDNA as the template, the first and the nested PCRs were carried out with 2 sets of primers, anchor-1/ORF1-R14 and anchor-2/ORF1-R13, respectively.

Table 1. Oligonucleotides used in amplifying the complete genome of the rat HEV Vietnam strain, V-105*

Primers	Product length, bp
Forward ORF1-F1 (1-21)† 5'-GCAACCCCCGATGGAGACCCA-3'‡	
Reverse ORF1-R12 (4149-4171) 5'-GGCGGCCTCGAACTTCTCCTGAA-3'	§
Forward ORF1-F2 (11-30) 5'-ATGGAGACCCATCAGTATGT-3'†	
Reverse ORF1-R1 (431-450) 5'-GTGCAAAAGGAAAGATCAGT-3'	440
Forward ORF1-F9 (388-408) 5'-AGCTAACAACATCCGCCGTTG-3'	
Reverse ORF1-R10 (2197-2217) 5'-TGGGTTCGGTCGAAGGCCTCT-3'†	1,830
Forward ORF1-F16 (2080-2100) 5'-TGCAGCCGTTTATGAGGGAGA-3'	
Reverse ORF1-R16 (3055-3075) 5'-CGCCATTCTGTGGGTTCTAGA-3'	996
Forward ORF1-F7 (2990-3009) 5'-GACCCAAGGCAGATCCCTGC-3'†	
Reverse ORF1-R12 (4149-4171) 5'-GGCGGCCTCGAACTTCTCCTGAA-3'	1,182
Forward ORF1-F18 (3991-4011) 5'-ATTCACCACAGACGAGCCAGT-3'	
Reverse ORF2-R21 (5079-5100) 5'-GGTGATAGCCAATTGGTAAGCT-3'	1,110
Forward F13 (4896-4915) 5'-AATAACACTCTGGGCTGTAG-3'	
ReverseTX30SXN 5'-GACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3'	2,092
Forward primer Anchor-1: 5'-CCTCTGAAGGTTCCAGAATCGATAG-3'	
Reverse primer ORF1-R14 (276-296) 5'-TAGACCTAGGGTGCGCACCGA-3'	§
Forward primer Anchor-2: 5'-GAATCGATAGTGAATTCGTG-3'	
Reverse primer ORF1-R13 (200-220) 5'-AACACGCTGTACCGGATGCGA-3'	240

*HEV, hepatitis E virus; ORF, open reading frame.
†Numbers in a parentheses show the positions of primers corresponding to the entire genome of rat HEV V-105.
‡Primer designed based on rat HEV (GU345042).
§The PCR product was not detected (reverse).

The V-105 genome consisted of 6,927 nt plus a poly (A) tail of a still-undetermined length (GenBank accession no. JX120573). The genomic structure of V-105 was, from the N-terminus toward the C-terminus, the N 5'–untranslated region (UTR) at nt 1–10, ORF1 at nt 11–4900, ORF3 at nt 4917–5225, ORF2 at nt 4928–6862, the 3'-UTR at nt 6863–6927, and the poly (A) tail starting at nt 6928. ORF2 and ORF3 encode 644 aa and 102 aa, respectively, as do R63 and R68. However, ORF1 of V-105 encodes 1,629 aa, which is 7 aa shorter than either R63 or R68. The V-105 genome possessed 2 aa insertions (Ser-Pro) between the aa residues 591 and 592 and 9 aa deletions (Ser-Pro-Pro-Gly-Pro-Pro-Pro-Ala-Gly) between aa residues 852 and 853, corresponding to those of R63. The 3'-UTR was 65 nt as were R63 and R68. Unlike R63 and R68, only 1 additional putative ORF, corresponding to ORF4 (nt residues 27–578), was found in V-105, suggesting that other putative ORFs, ORF5 and ORF6 found in R63 and R68, are not common in rat HEV.

When the V-105 genome was compared with reported HEV genomes, the V-105 genome shared identities of only 50.5% with avian HEV, 53.6% with rabbit HEV, 53.7%–54.0% with wild boar HEV, and 53.1%–53.5% with HEV genotypes 1–4. In contrast, V-105 shared relatively high nucleotide sequence identities (76.8%–76.9%) with rat HEV strains (R63 and R68) (Table 2). The nucleotide and amino acid sequences of ORF1, ORF2, and ORF3 of V-105 were compared with those of other HEV genotypes, and the identities among them are shown in Table 2. Together, these results suggest that V-105 is more similar to rat HEV than to other HEV genotypes.

Phylogenetic trees were generated on the basis of the nucleotide sequences derived from the entire genome and ORF3 of the genotypes 1–4, wild boar, rabbit, chicken, and rat HEV isolates. These trees demonstrated that V-105 does not belong to any known genotype and should probably be classified into a new genotype (Figure).

Table 2. Nucleotide and deduced amino acid sequence identities between human, wild boar, rabbit, rat, and avian HEV strains, compared with Vietnam rat HEV V-105 strain*

HEV strain (GenBank accession no.)		Vietnam rat HEV strain					
		Nucleotides, %			Amino acids, %		
	Entire genome	ORF1	ORF2	ORF3	ORF1	ORF2	ORF3
Genotype 1 (NC_001434)	53.5	50.7	60.8	51.0	54.1	55.5	33.3
Genotype 2 (M74506)	53.3	51.2	59.1	51.4	53.1	54.6	30.6
Genotype 3 (AF060668)	53.3	50.8	59.2	53.8	51.6	57.1	26.5
Genotype 4 (AJ272108)	53.1	50.9	58.9	52.6	50.7	55.4	24.5
Wild boar HEV (AB573435)	53.7	51.5	59.6	53.4	50.3	56.3	28.2
Wild boar HEV (AB602441)	54.0	51.5	59.6	53.4	50.3	56.3	28.2
Rabbit HEV (FJ906895)	53.6	51.3	59.3	51.5	52.1	56.2	26.2
Rat HEV (GU345042)/R63	76.9	75.7	79.6	80.6	87.0	91.6	66.7
Rat HEV (GU345043)/R68	76.8	75.5	79.8	80.9	86.4	92.1	66.7
Avian (chicken) HEV (AY535004)	50.5	49.7	54.2	47.0	44.7	47.4	33.9

*HEV, hepatitis E virus; ORF, open reading frame.
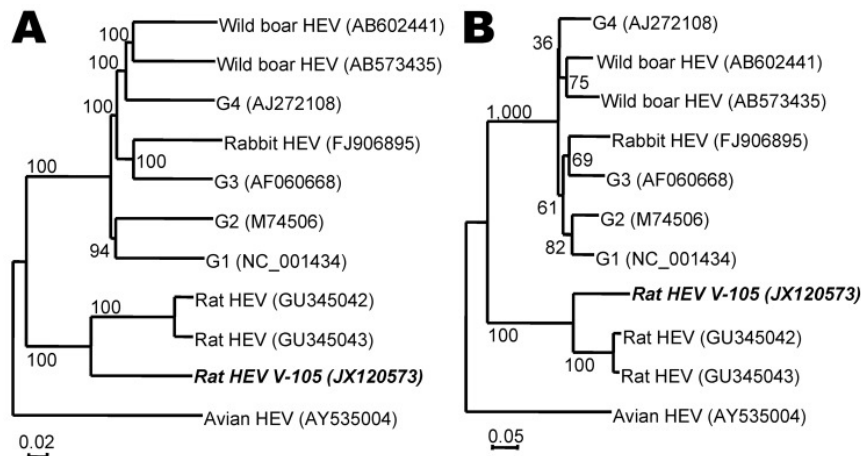
Figure. Phylogenetic relationships among genotypes 1–4, wild boar, rabbit, avian (bird), and rat hepatitis E virus (HEV) isolates. The nucleic acid sequence alignment was performed by using ClustalX 1.81 (www.clustal.org). The genetic distance was calculated by the Kimura 2-parameter method. A phylogenetic tree with 1,000 bootstrap replicates was generated by the neighbor-joining method, based on the entire genome (A) and open reading frame 3 (B) of the genotypes 1–4, wild boar, rabbit, chicken (avian), and rat HEV isolates. Scale bar indicates nucleotide substitutions per site. **Boldface** indicates isolate used in this study.

Conclusions

In this study we successfully amplified the entire genome of an HEV strain isolated from a wild rat in Vietnam. Phylogenetic analyses and nucleotide and amino acid sequence comparisons demonstrated that the complete rat HEV genome sequences were consistently well separated from those of mammalian genotypes 1–4, wild boar, rabbit, and chicken HEV and close to those of the rat HEV strains. Although the entire genome of V-105 shared nucleotide sequence identities of only 76.8%–76.9% with the isolates from Germany (R63 and R68), the ORF1 and ORF3 amino acid identities between V-105 and these isolates were 86.4%–87.0% and 66.7%, respectively, which suggests that V-105 can be classified into a new genotype of rat HEV. However, ORF2 has relatively high amino acid identities with R63 and R68 (91.6%–92.1%), indicating that the V-105 and rat HEV isolates from Germany share similar antigenicity. In fact, rat HEV–like particles derived from R63 are cross-reactive to serum from V-105–infected wild rats (*14*).

In conclusion, we isolated and identified rat HEV strain V-105 from a wild rat in Vietnam, and this strain was highly divergent from known rat HEV isolates. We propose that the strain from Vietnam, V-105, is a new member of the rat HEV genotype.

Dr Li is a senior researcher at the National Institute of Infectious Diseases, Tokyo. His research focuses on epidemiology, expression of viral proteins, and the 3-dimensional structure of HEV.

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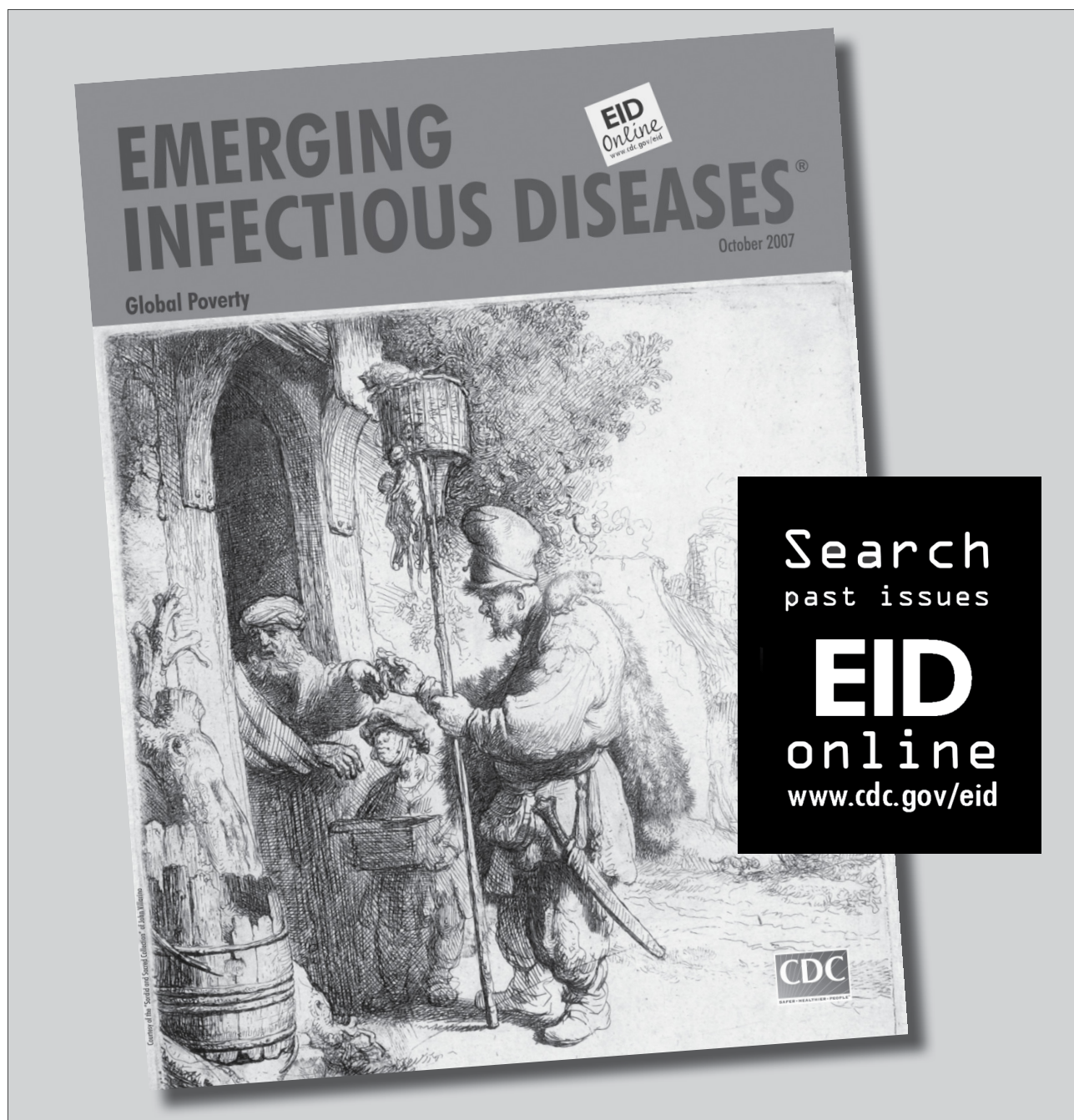
Adenovirus Serotype 14 Infection, New Brunswick, Canada, 2011

Gabriel Girouard, Richard Garceau,
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Nathalie Bastien, and Yan Li

We describe 3 culture-proven cases of adenovirus serotype 14 infection in New Brunswick, Canada, during the summer of 2011. Strains isolated from severely ill patients were closely related to strains of a genomic variant, adenovirus 14p1, circulating in the United States and Ireland. Physicians in Canada should be aware of this emerging adenovirus.

Originally discovered in 1955, human adenovirus serotype 14 (HAdV-14) had rarely been reported in medical literature for over 30 years. This archetype strain, known as "agent de Wit," seemed to have almost vanished from Earth and had not been identified in the Western Hemisphere (1). Since 2005, however, the number of reports of a newly emerging HAdV-14 strain has increased, mainly across the United States (2), and in 2009, cases were identified in Ireland (3). This strain, currently designated as HAdV-14p1, was confirmed by enzymatic restrictions profiles as a new genomic variant, and it has a unique signature 6-nt deletion in the knob region of the fiber gene (4). Outbreaks among communities and military training camps have been described, all showing high rates of infection and increased risk for hospitalization and death (5,6). It is not known whether this new circulating variant is truly more virulent or whether current reports represent only the severe side of the natural clinical spectrum of HAdV-14 in immunologically naive populations (7).

The Study

In July 2011, a 74-year-old aboriginal (Micmac) woman from eastern New Brunswick sought medical care at our hospital for an influenza-like illness, including a dry cough and acute diarrhea, of 3 days' duration. Her medical history

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revealed heavy smoking but was otherwise unremarkable. Chest radiographs obtained at admission revealed bilateral alveolar infiltrates. Paraclinical data for the patient during hospitalization are shown in the Table. Broad-spectrum antimicrobial drug treatment for community-acquired bacterial pneumonia was started, but the patient's respiratory condition deteriorated rapidly. On day 1, she was transferred to the intensive care unit and intubated. Severe acute respiratory distress syndrome developed in the patient, and she died 6 days after admission.

Results for PCR of nasopharyngeal and tracheal aspiration samples were negative for influenza. Results were also negative for urinary antigen detection for pneumococcus and *Legionella* spp., hantavirus IgM, and tracheal and blood cultures. Gram staining of the respiratory specimen was notable for its abundance of leukocytes and absence of bacteria. Respiratory viral cultures revealed a cytopathic effect after 6 days of incubation, and adenovirus was confirmed by indirect immunofluorescence (Light Diagnostics Respiratory Panel I Viral Screening and Identification IFA; Millipore, Billerica, MA, USA).

Given the severity of this case, the virus isolate from the patient was sent to the National Microbiology Laboratory in Winnipeg, Manitoba, Canada, to determine its serotype by partial hexon gene sequencing, as described (8); the infecting strain was found to be HAdV-14. Postmortem examination of the patient revealed dark purple, heavy lungs with marked consolidation and patchy hemorrhagic foci; the right lung weighed 1,593 g and the left 1,268 g (reference range 350–450 g). Examination by microscopy showed diffuse alveolar damage, fibrinohemorrhagic changes, areas of necrosis, and cellular debris (Figure 1). Immunohistochemistry (Anti-Adenovirus Antibody, clone 20/11 | MAB8052; Millipore) for adenovirus showed few epithelial cells which had cytoplasmic granular and nuclear positivity.

Once HAdV-14 had been isolated, we conducted a retrospective study of all adenovirus-positive viral cultures performed at the Provincial Laboratory for Viral Illnesses, Centre hospitalier universitaire Dr-Georges-L.-Dumont (Moncton, New Brunswick, Canada), to search for other HAdV-14 cases that might have occurred during March 21, 2011–August 31, 2011. All 14 single-patient, adenovirus-positive isolates found during the survey were submitted for partial hexon gene sequencing; HAdV-14 was found in 2 of the isolates. Of the 12 non–HAdV-14 isolates, 1 was HAdV serotype 1, 8 were HAdV serotype 2, and 3 were HAdV serotype 3. The clinical data for the 2 HAdV-14 cases was limited. The first was a case of upper respiratory tract infection and acute bilateral otitis media in a 1-year-old boy who was hospitalized in July 2011. The second was a case of severe pneumonia in a 45-year-old man who was hospitalized in August 2011. Both patients survived.

Table. Laboratory test results for a patient with a fatal case of human adenovirus-14–associated pneumonia, New Brunswick, Canada, 2011*

Laboratory test, value	Hospitalization day				Reference range
	Admission	3	5	7	
Cell count, $\times 10^9$/L					
Leukocytes	6.5	5.8	5.5	6.7	4.0–11.0
Polymorphonuclear cells	5.6	3.8	4.3	5.0	1.8–7.7
Lymphocytes	0.7	0.6	0.9	0.3	1.0–4.8
Platelets, $\times 10^9$/L	111	104	125	134	130–400
Hematocrit	0.368	0.336	0.236	0.256	0.370–0.470
PaO_2/FiO_2	ND	146	60	34	ND
Natremia, mmol/L	137	141	133	137	136–145
Creatinine, μmol/L	224	115	241	ND	46–92
Aspartate aminotransferase, U/L	132	63	104	55	14–36
Lactate dehydrogenase, U/L	885	1,138	1,398	1,020	313–618

*PaO_2, arterial oxygen tension; FiO_2, fraction of inspired oxygen; ND, no data available.

The 3 cases of HAdV-14 infection were severe, requiring hospitalization of the patients. Pneumonia developed in 2 of the patients, 1 of whom died. The cases occurred within a brief 43-day period, and the young boy and man

Figure 1. Hematoxylin and eosin–stained lung tissue (original magnification ×10) from a patient in New Brunswick, Canada, with a fatal case of pneumonia caused by adenovirus 14. A) Arrow shows a patchy area of hemorrhagic consolidation. B) Arrow shows an area of necrosis.

lived within a 35-km radius of the female patient who died. The clinical outcome for these cases is consistent with previously reported clinical data on HAdV-14 infection and the apparently enhanced virulence of this strain.

We performed nucleotide sequencing of the E1A, fiber, and hexon genes of the HAdV-14 isolates from Canada and compared the sequences with those for published adenovirus subspecies B2 (type 11, 14, 34, and 35) (Figure 2). We found no differences in the nucleic acid sequences of the 3 isolates from Canada, which were also identical to HAdV-14p1 strains from the United States and Ireland. The fiber gene sequence of the isolates from Canada contained the 6-nt deletion identified in HAdV-14p1 strain from the United States and Ireland. Phylogenetic analysis of the E1A, fiber, and hexon genes revealed that isolates from Canada, the United States, and Ireland clustered together. Restriction enzyme analysis was not performed on the 3 isolates from Canada; however, phylogenetic analysis and the presence of the 6-nt deletion suggested that these isolates corresponded to the genomic variant HAdV-14p1.

Conclusions

Our findings confirm the presence of HAdV-14 in Canada since the emergence of this serotype in North America was first described. In the case we describe, the patient sought medical care at our hospital after being ill for 3 days. She received a diagnosis of community-acquired pneumonia, was hospitalized, and died 6 days later. The patient maintained a normal level of total leukocytes during her illness but was lymphocytopenic. It has been reported that lymphocytopenia could be more common among severely ill patients with adenovirus infection (5). Epidemiologic data obtained from the patient's family showed that she had not traveled outside New Brunswick in the 14 days preceding hospitalization, and she frequently participated in social gatherings. Transmission appeared to occur through close, person-to-person contact and among members of tight social networks, similar to transmission during an outbreak of HAdV-14 in Alaska (1). Family mem-

bers also reported that in the weeks following the patient's illness, many aboriginal members of the community had influenza-like symptoms, which could represent other unconfirmed cases of HAdV-14 infection.
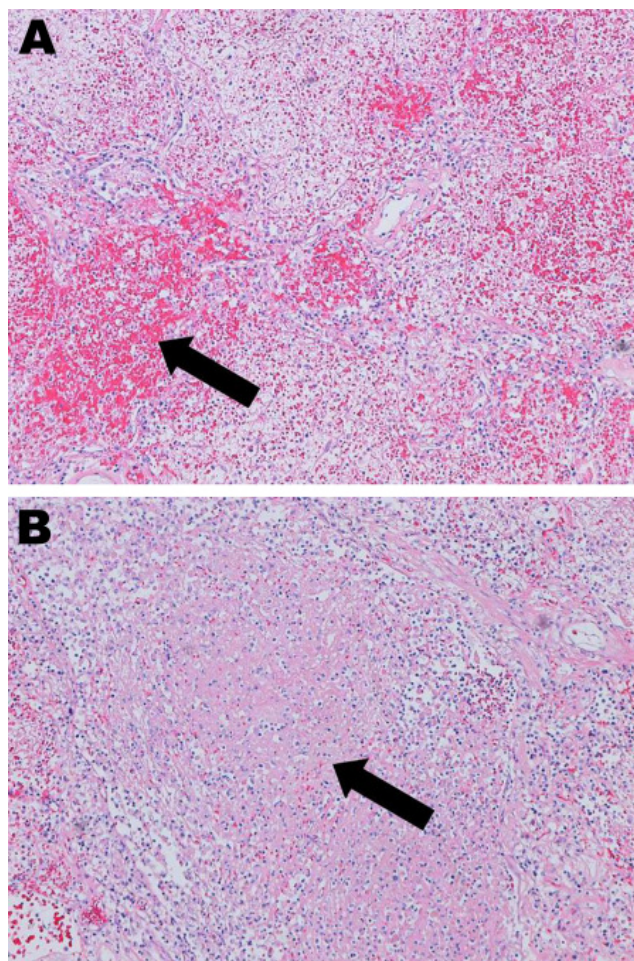
Figure 2. Phylogenetic analysis of human adenovirus 14 (HAdV-14) isolates from patients in New Brunswick, Canada, 2011. Nucleotide sequences were determined for the fiber (A), E1A (B), and hexon (C) genes. The corresponding gene sequences from previously reported HAdV-14 isolates are also included. Phylogenetic analysis was performed by using the neighbor-joining method of the MEGA2 program (9). Scale bars indicate nucleotide substitutions per site. Numbers on branches and at nodes indicate bootstrap proportions. The GenBank accession numbers for the fiber, E1A, and hexon genes for HAdV-14 (Canada) RV1360 are JQ815083, JQ815080, JQ815086, respectively; those for RV1368 are JQ815084, JQ815081, JQ815087, respectively; and those for RV1370 are JQ815085, JQ815082, JQ815088, respectively.

Epidemiologic data is lacking for the HAdV-14 variant emerging in Canada. Because clinicians do not always perform diagnostic tests for respiratory illnesses, cases of HAdV-14 infection might be diagnosed simply as acute respiratory illnesses or as unspecified sporadic viral pneumonias. During a 2008–2009 outbreak of adenovirus type 3 in New Brunswick, the same province in which the current study patient lived, we serotyped 17 strains and found no evidence of HAdV-14 (10). Moreover, a large 2007 Canadian study in the Toronto region failed to find any HAdV-14 strains among the 200 strains that were submitted; circulating HAdV strains were largely subtypes 1, 2, and 3 (11).

After finding these 3 cases, we notified public health authorities and the provincial Centre for Disease Control, who, in turn, informed physicians of the presence of HAdV-14 in New Brunswick. No other cases have been reported.

Acknowledgment

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Staphylococcus aureus Causing Tropical Pyomyositis, Amazon Basin, Peru

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and Jan Jacobs

We studied 12 *Staphylococcus aureus* isolates causing tropical pyomyositis in the Amazon Basin of Peru. All isolates were methicillin-susceptible; 11 carried Panton-Valentine leukocidin–encoding genes, and 5 belonged to multilocus sequence type 25 and possessed an extensive set of enterotoxins. Our findings suggest sequence type 25 is circulating in tropical areas of South America.

Pyomyositis is an acute bacterial infection characterized by suppuration within large skeletal muscles manifesting as single or multiple abscesses. Its exact pathogenesis is unknown but is thought to occur through bacteremic seeding (*1*). The infection is often seen in tropical countries, hence the name tropical pyomyositis, and usually occurs in young, otherwise healthy persons. In addition, the infection is increasingly reported from temperate regions in patients receiving immunosuppressive therapy or with HIV infection (*1,2*). The most common bacterial causes of tropical pyomyositis are *Staphylococcus aureus* (90% in tropical areas, 75% in temperate zones) and group A streptococcus (1%–5%); less common causes are group B, C, and G streptococcus, pneumococcus, *Haemophilus* spp., and gram-negative bacilli (*2*). Furthermore, the increasing incidence of pyomyositis in temperate regions has been correlated with the emergence and spread of community-associated (CA)–methicillin-resistant *S. aureus* (MRSA) clones (*3*). These CA-MRSA clones usually produce Panton-Valentine leukocidin (PVL), a pore-forming toxin encoded by 2 genes, *lukF-PV* and *lukS-PV* (*4*).

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Few studies have been done on the genetic characteristics of *S. aureus* that cause tropical pyomyositis. To help fill this void, we determined the molecular characteristics of *S. aureus* isolates causing tropical pyomyositis in the Amazon Basin of Peru.

The Study

We analyzed 12 *S. aureus* isolates from patients with tropical pyomyositis. Of the 12 isolates, 10 were obtained from patients hospitalized in a 120-bed public hospital located in Iquitos, the largest city in the Amazon Basin of Peru (160,000 inhabitants). A retrospective chart review showed that 38 patients were hospitalized with tropical pyomyositis during 2009–2010; these patients represented 0.9% of 4,445 hospital admissions. The 10 isolates from hospitalized patients came from these patients; the 2 other isolates were obtained in 2005 from 2 persons hospitalized in Yurimaguas, a city 388 km southeast of Iquitos (63,000 inhabitants). We obtained clinical data by reviewing patients' charts.

Isolates were analyzed at the Instituto de Medicina Tropical Alexander von Humboldt in Lima, Peru. Colonies were identified as *S. aureus* by Gram staining and by positive reactions for catalase, DNAse, and tube coagulase tests. We screened for oxacillin resistance by using the cefoxitin (30 μg) disk diffusion test, and we used disk diffusion to assess susceptibilities to clindamycin, erythromycin, gentamicin, ciprofloxacin, rifampin, and trimethoprim-sulphamethoxazole (*5*).

Detection of 16S rRNA, *mec*A, and *nuc* genes by multiplex PCR was performed at the Centre National de Référence *S. aureus*, Erasme Hospital, Brussels, Belgium (*6*). We used PCR to detect the presence of the PVL- and toxic shock syndrome toxin 1–encoding genes (*lukS-lukF* PV and *tst*, respectively), exfoliatin A– and B–encoding genes (*eta* and *etb*, respectively) (*7*), and enterotoxin genes (*sea, seb, sec, sed, see, seg, seh, sei, sej, sek, sem, sen, seo, sep, seq, ser*) as described by Omoe et al. (*8*). We determined *spa* type as described (*9*), and we performed multilocus sequence typing on 1 randomly selected isolate of each *spa* type (*10*).

Demographic characteristics of the patients are shown in the Table. Of the 12 patients, 9 (75%) were <5 years of age, similar to age distributions recorded in other studies (*2*). No patient revealed a history of chronic disease. Two groups of muscles were involved in 2 patients, and 10 patients had fever. All patients had local edema and pain, needed surgical drainage, and had received antimicrobial drugs (oxacillin and/or clindamycin) before samples were obtained.

Of the 12 *S. aureus* isolates we studied, 11 (92%) carried PVL genes; PVL genes were also reported in 12% of clinical MSSA isolates recovered in hospitals in Lima (*11*). In the United States and France, PVL has been detected in

Table. Characteristics of patients with tropical pyomyositis caused by methicillin-susceptible *Staphylococcus aureus*, Amazon Basin, Peru*

Patient age	Year patient hospitalized	Body site involved	*spa* type	Multilocus ST†	*mecA*	*eta*	*etb*	*lukS-lukF*	*tst*	Enterotoxin genes
12 mo	2005	Lower limb	t6465	**ST121**	–	–	–	+	–	*sei, seg*
3 y	2005	Lower limb	t6465	ST121	–	–	–	+	–	*sei, seg*
6 mo	2009	Upper limb	t701	**ST6**	–	–	–	+	–	*sea*
12 y	2009	Thorax, lower limb	t078	**ST25**	–	–	–	+	–	*seb, sed, sei, sej, seg, sem, sen, seo, ser*
24 mo	2009	Lower limb	t078	ST25	–	–	–	+	–	*seb, sed, sei, sej, seg, sem, sen, seo, ser*
45 y	2009	Thorax	t1778	**ST1**	–	–	–	+	–	*seh*
3 y	2009	Abdomen, upper limb	t701	ST6	–	–	–	+	–	*sea*
28 d	2010	Lumbar	t164	**ST20**	–	–	–	-	–	*seg, sei, sem, sen, seo*
3 y	2010	Lower limb	t078	ST25	–	–	–	+	–	*seb, sed, sei, sej, seg, sem, sen, seo, ser*
26 y	2010	Abdomen	t078	ST25	–	–	–	+	–	*seb, sed, sei, sej, seg, sem, sen, seo, ser*
4 y	2010	ND	t1778	ST1	–	–	–	+	–	*seh*
18 mo	2010	Thorax	t078	ST25	–	–	–	+	–	*seb, sed, sei, sej, seg, sem, sen, seo, ser*

The spanning header over *mecA, eta, etb, lukS-lukF, tst* is "Gene"; the group over *spa* type through Enterotoxin genes is "*S. aureus* isolate".

*ST, sequence type; –, negative; +, positive; ND, no data.
†**Boldface** indicates isolates that were randomly selected by *spa* type for multilocus sequence typing (MLST). The other isolates were not analyzed by MLST. Results for those isolates were extrapolated from results for the analyzed isolates of the same *spa* type.

20% and 10%, respectively, of CA-MSSA soft tissue infections (*3,12*). *S. aureus* carrying PVL has received more attention by public health officials since the emergence, in the United States and other countries, of skin and soft tissue infections caused by the PVL-positive CA-MRSA clone USA300. A recent publication from the United States noted that 69% of MRSA isolates causing skin and soft tissue infections carried PVL (*3*).

None of the isolates described in this study carried genes associated with the toxic shock syndrome toxin (*tst*) or the staphylococcal scalded skin syndrome (*eta, etb*), but all possessed several enterotoxin genes. The presence of toxin genes was strongly linked to *spa* type (Table). In particular, isolates belonging to *spa* type t078 (sequence type [ST] 25) harbored the *egc* operon (coding genes *seg, sei, sem, sen, seo*), coding genes *sed, sej* and *ser* (probably carried on the same plasmid) (*13*), and *seb*. Along with toxic shock syndrome toxin 1, *seb* is one of the most powerful staphylococcal superantigens.

Of particular interest in our study is the high proportion of isolates belonging to ST25. This ST has been detected in bovine and human isolates in the United Kingdom (*14*), and an ST25 PVL–positive MSSA was detected in a patient in Brazil with life-threatening sepsis with pneumonia and myositis (*15*). ST25 PVL–positive isolates are thought to be rare in Peru: in 2008–2009, only 6 (3.6%) of 169 MSSA blood isolates from hospitalized patients in Lima (non-tropical area of Peru) harbored *spa* type t078 or related *spa* types (C. Garcia, unpub. data). Our findings raise the hypothesis that ST25 is circulating in the tropical areas of South America.

This study has several limitations. First, tropical pyomyositis cases from the referring hospitals were defined by the patients' physician, and isolates from all patients were not assessed. Second, because this was a retrospective study, many variables were not recorded, including history of blunt trauma and duration of antimicrobial drug therapy. However, given that studies of the genetic characteristics of *S. aureus* that cause tropical pyomyositis are lacking and given the striking presence of PVL genes among the isolates in our study, our findings add to the insights about the pathogenesis of this acute suppurative infection.

Conclusions

This study describes a high rate of PVL encoding genes among *S. aureus* causing tropical pyomyositis in the Amazon Basin of Peru. Further investigation in areas geographically different from the Amazon Basin should be done to confirm the association of PVL and other toxins in the pathogenesis of tropical pyomyositis.

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Puumala Virus Infections Associated with Cardiovascular Causes of Death

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We studied the causes of death of patients in Sweden with diagnoses of hemorrhagic fever with renal syndrome (HFRS) during 1997–2009. Cardiovascular disorders were a common cause of death during acute-phase HFRS and were the cause of death for >50% of those who died during the first year after HFRS.

Hantaviruses cause 2 acute diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). HFRS is caused by the prototypic hantavirus Hantaan and by Dobrava virus, Puumala virus (PUUV), and Seoul virus in Eurasia; HCPS is caused by Andes virus, Sin Nombre virus and related hantaviruses in the Americas. Case-fatality rates differ: ≤10% for HFRS and ≤40% for HCPS (1). PUUV causes HFRS in Europe; >225,000 cases of HFRS have been reported (2). One of the largest PUUV outbreaks recorded occurred in northern Sweden; an incidence of 313 cases per 100,000 persons was reported (3). The case-fatality rate for HFRS is 0.4% overall in Sweden and reaches 6% among elderly persons (4).

HFRS can cause pulmonary complications and HCPS can cause renal signs and symptoms, suggesting that these 2 diseases might have more in common than previously believed (5,6). However, as indicated by their respective names, HFRS is mainly considered a hemorrhagic fever with affected renal functions, and HCPS is characterized by severe cardiac and respiratory signs and symptoms (1). The primary causes of death during HCPS are known to be associated with cardiopulmonary failure (7). However, less is known regarding causes of death during the acute phase of HFRS and those that occur after HFRS related to possible sequelae of the illness. To explore patterns of death among persons who died during and after HFRS, we reviewed all

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causes of death of persons infected with PUUV in Sweden during 1997–2009.

The Study

PUUV infection is a notifiable disease in Sweden, according to the Swedish Communicable Disease Act. Diagnosed cases are reported with each patient's unique personal identity number to the Swedish Institute for Communicable Disease Control. These notifications are stored in a database; we obtained a permit from the Regional Ethical Review Board, Stockholm, to further analyze these patients. During the study period, HFRS associated with PUUV was diagnosed in 5,903 persons in Sweden. Of those, 59 persons lacked personal identity numbers, resulting in a database of 5,844 records (Table) that were compared to the Swedish cause of death registry administered by the Swedish National Board of Health and Welfare. The resulting study group, the Cause of Death (COD) cohort, comprised 238 deceased persons in whom HFRS had been diagnosed during 1997–2009 (median age at death 75 years, range 18–100, interquartile range [IQR] 66–82); 163 were male (median age at death 74 years, IQR 65–82), and 75 were female (median age at death 76 years, IQR 66–84).

Of the COD cohort, 24 (10%) patients (9 female, 15 male) died during the acute phase of HFRS (deaths within 90 d of HFRS diagnosis). The main cause of death for patients in the acute phase was categorized by using International Classification of Disease, 10th Revision (ICD-10), codes as specified by the World Health Organization: HFRS (A94, A98.5, N15.0); cardiovascular diseases (CVD) (I00–I99); renal diseases (N00–N39, excluding N15.0); pulmonary diseases (J00–J99, R06.8); neoplasms (C00–D48); gastrointestinal diseases (K55–K63); infectious diseases (A00–B99, excluding A94.0 and A98.5); endocrine diseases (E00–E99); and central nervous system (CNS) diseases (F00–F99, G00–G99). Unexpectedly, we found at least as many deaths caused by CVDs as by HFRS and related symptoms during the acute phase of HFRS (Figure 1). Causes of death for remaining persons in the cohort were neoplasms, infectious diseases, endocrine diseases, and CNS diseases (Figure 1). The mean ± SD time from diagnosis to death for persons in the COD cohort by disease was as follows: HFRS, 10.8 ± 5.3 d (n = 6); CVDs, 31.2 ± 14.4 d (n = 7); pulmonary, 15 d (n = 1); renal, 17 d (n = 1); neoplasms, 34.3 ± 20.1 d (n = 3); gastrointestinal, 44 ± 39.5 d (n = 2); infections, 77.5 ± 16.3 d (n = 2); endocrine, 22 d (n = 1); and CNS, 88 d (n = 1).

The prominence of CVD as a main cause of death for acute HFRS prompted us to explore patterns of cardiovascular-related deaths in the remaining decedents (n = 214, 90% of the COD cohort). We compared the percentage of cardiovascular-associated deaths by 12-month intervals according to when, after HFRS diagnosis, the patients died

Table. Characteristics of patients with diagnoses of HFRS, Sweden, 1997–2009*

| Cohort | No. patients | No. (%) patients by sex | | No. (%) patients by age group, y | | |
		F	M	Median age†	Age range	Interquartile range
HFRS	5,844	2,349 (40.2)	3,495 (59.8)	52	2–95	39–62
COD	238	75 (31.5)	163 (68.5)	71‡	18–94	62–78
HFRS, no PIN	59§	21 (37.5)	35 (62.5)	45‡¶	10–75	28–54

HFRS, hemorrhagic fever with renal syndrome; COD, cause of death; PIN, personal identification number. Data from the HFRS database (Swedish Institute for Infectious Disease Control) were merged with data from the Cause of Death Register, National Board of Health and Welfare, identifying persons by PINs culminating in the cause of death (COD) cohort.
†Information regarding the age of 6 patients was missing.
‡Information regarding the sex of 3 patients was missing.
§The age in years of the patients at HFRS diagnosis according to the HFRS database.
¶There was a significant difference in ages of the persons in the COD cohort and entire HFRS cohort, and between those patients lacking PIN and the entire HFRS cohort (p<0.001 and p = 0.001, respectively, independent samples, Mann-Whitney U-test).
#The ages of persons in the "HFRS, no PIN" group were significantly younger than those in the COD cohort (p<0.001, independent samples, Mann-Whitney U-test).

(Figure 2). During year 1 (months 3–14 after HFRS diagnosis), CVD deaths/total n = 10/19, median age at death 72 years (IQR 59–79); during year 2 (months 15–26), n = 16/39, 72 years (62–81); and during year 3 (months 27–38), n = 9/27, 73 years (66–81); and year 4 (≥39 months), n = 46/129, 77 years (68–84). A disproportionate percentage of deaths were caused by CVDs for those who died during the first 12 months after acute HFRS compared with later and with deaths among the general population of Sweden (Figure 2). The proportion of CVD as cause of death during the second 12-month interval and later after diagnosis of acute HFRS was similar to that for the general Swedish population (Figure 2), indicating that HFRS might cause a transient elevated risk for death caused by CVDs. We found a significant difference (p <0.001) between the ages of the deceased persons and those of all persons with HFRS (Table), suggesting that age could have a confounding influence on the results of this study.

Conclusions

Our finding indicate that CVD is a common cause of death from acute HFRS and might also be overrepresented as cause of death in the year after the acute phase of HFRS. PUUV infection is most likely a trigger of cardiovascular events that eventually lead to death rather than directly causing CVD, which can take 1–2 decades to develop. Other infections have been shown to trigger acute cardiovascular events (8) (e.g., compared with noninfected persons, persons with respiratory viral infections have 2–3× increased risk for acute coronary syndromes soon after infection) (9). The elevated risk is more pronounced during the acute phase of respiratory viral infection but can still be increased 3 months afterwards (9). Abnormal electrocardiograms (10,11) and myocarditis (12) have been observed during the acute phase of PUUV infection, indicating an association between PUUV infection and cardiovascular disorders. Furthermore, PUUV antigen has been found in cardiac tissue (6), and other studies have documented the essential role of coagulopathy in HFRS disease severity

(13–15). Moreover, >25% of HFRS patients in Sweden had evidence of disseminated intravascular coagulation, which correlated with HFRS disease severity (14). Taken together, data from these studies (6,10–15) and the present data indicate a possible association between PUUV infection and precipitation of CVD events that could eventually lead to death.

A weakness of this study is the small sample number. Furthermore, this study is based entirely on data from public health registries, which poses the limitation of the inability to validate the cause of death data, including the risk for coding errors, which could affect the overall interpretations of our results.

Our data suggest it would be prudent for health care providers to monitor elderly patients for affected cardiovascular functions during the first year after a diagnosis
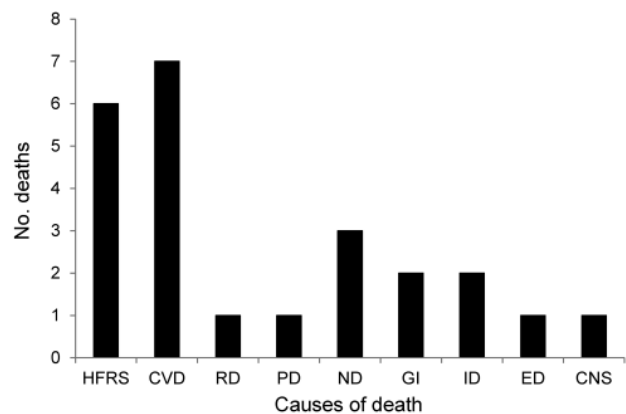
Figure 1. Main causes of death for patients in the acute phase of hemorrhagic fever with renal syndrome (HFRS), Sweden 1997–2009. Acute phase includes any death within 90 days of HFRS diagnosis. Data from the HFRS database, Swedish Institute for Communicable Disease Control, Cause of Death Register, National Board of Health and Welfare. HFRS, hemorrhagic fever with renal syndrome; CVD, cardiovascular disease; RD, renal disease; PD, pulmonary disease; ND, neoplastic disease; GI, gastrointestinal disease; ID, infectious disease; ED, endocrine disease; CNS, central nervous system disease.
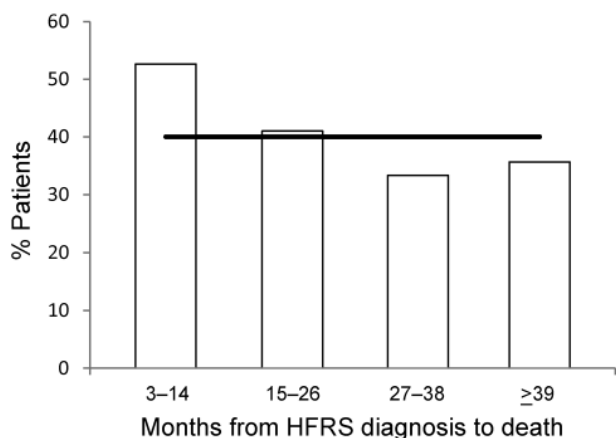
Figure 2. Main cardiovascular causes of death after the acute phase of hemorrhagic fever with renal syndrome (HFRS), Sweden 1997–2009. Bars indicate percentage of patients with cardiovascular diseases during 12-month intervals after HFRS as a main cause of death is displayed; horizontal line indicates the background rate of cardiovascular disease (40%) as main cause of death for the general population in Sweden. Data from the HFRS database, Swedish Institute for Communicable Disease Control, Cause of Death Register, National Board of Health and Welfare.

of HFRS. This potential association between CVDs and HFRS deserves further consideration in other cohorts of persons in whom HFRS was diagnosed, particularly in countries where more severe hantaviruses, such as Hantaan and Dobrava viruses, exist and in countries with large numbers of HFRS cases.

Dr Connolly-Andersen is a postdoctorate fellow in the Division of Infectious Diseases, Department of Clinical Microbiology, Umeå University. Her primary research interest is the pathogenesis of viral hemorrhagic fevers, including Crimean-Congo hemorrhagic fever and HFRS.

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Linezolid Dependence in *Staphylococcus epidermidis* Bloodstream Isolates

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We document linezolid dependence among 5 highly linezolid-resistant (LRSE) *Staphylococcus epidermidis* bloodstream isolates that grew substantially faster at 32 μg/mL linezolid presence. These isolates carried the mutations T2504A and C2534T in multiple 23S rRNA copies and 2 mutations leading to relevant amino acid substitutions in L3 protein. Linezolid dependence could account for increasing LRSE emergence.

Linezolid is highly effective against *Staphylococcus epidermidis* (*1*). Linezolid-resistant *S. epidermidis* (LRSE) isolates are limited worldwide (*2*), and few LRSE outbreaks have occurred (*3,4*). Linezolid resistance in *S. epidermidis* has been attributed to specific 23S rRNA mutations (G2576U, G2447U, U2504A, C2534U, and G2631U) (*5,6*), *cfr* gene (*7*), or mutations in ribosomal proteins L3, L4, and L22 (*7*).

Dependence on linezolid for bacterial growth has not been reported but has been described for other antimicrobial drugs (*8–10*). We report the characteristics of partially linezolid-dependent LRSE causing bloodstream infections (BSIs).

The Study

Twenty-seven LRSE isolates were randomly selected for study among the 46 single-patient LRSE isolates recovered from BSIs in Tzaneio General Hospital (Piraeus, Greece) during 2008–2010. Isolates were

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identified by Vitek 2 (bioMérieux, Marcy l'Etoile, France). Chloramphenicol and clindamycin MIC was determined by E-test (bioMerieux) and linezolid MIC by using broth microdilution (*11*).

The 27 LRSE isolates were tested by pulsed-field gel electrophoresis (PFGE) as described (*12*) and screened for *cfr* gene (*7*). Mutations in the peptidyl-transferase center were identified for each separate 23S rRNA copy as described (*13*).

In 8 LRSE isolates representing all PFGE types, genes encoding the L3, L4, and L22 ribosomal proteins that factor in ribosome assembly were sequenced to identify mutations conferring linezolid resistance (*6*). Nucleotide and amino acid sequences were analyzed by using Lasergene software (DNASTAR, Madison, WI, USA) and compared with those of the linezolid-susceptible *S. epidermidis* (LSSE) strain ATCC 12228 (GenBank accession no. AE015929).

Growth curves were conducted in the presence and absence of linezolid for the above 8 LRSE isolates, 1 clinical LSSE isolate (A1521, linezolid MIC 2 μg/mL), and the ATCC 29213 *S. aureus* strain (linezolid MIC 0.5 μg/mL) as controls. Linezolid concentrations tested were half-MIC for controls and 3 LRSE isolates with low MIC (16–32 μg/mL) and 8, 16, 32, 64, and 128 μg/mL for 5 LRSE isolates with MIC >256 μg/mL. Growth curves were performed in triplicate by diluting 20 μL Mueller-Hinton broth culture in 2 mL broth, followed by incubation at 37°C under constant shaking; turbidity of cultures (McFarland scale) was measured every 6 h for 36 h. We statistically compared isolate growth at each time point using the paired *t* test and Minitab software version 13.31 (www.minitab.com); p≤0.05 indicated statistical significance.

We retrospectively examined medical records (anonymized demographic data, clinical characteristics, comorbidities, prior linezolid treatment for ≥3 days, and in-hospital deaths) of the 27 patients harboring LRSE to ascertain factors influencing resistance acquisition and outbreak persistence. Each of the 27 patients yielding LRSE had prolonged hospitalization and carried a central venous catheter. Twenty-one were mechanically ventilated, and 25 received linezolid treatment (Table 1).

Linezolid MICs were >256 μg/mL for 23 LRSE isolates and 8–32 μg/mL for 4 LRSE isolates. All isolates were co-resistant to clindamycin and chloramphenicol, but the *cfr* gene was not detected by PCR in any isolate (*7*). Three PFGE types were identified. PFGE type I comprised the 23 highly LRSE isolates, which all carried mutations T2504A and C2534T; 3 LRSE isolates were related to each other (type II) and carried the mutations G2576T and C2534T; and 1 LRSE isolate was unique (type III) and carried G2576T along with novel mutations C2356T or T2334C in different 23S rRNA copies each. All isolates

Table 1. Demographic and clinical characteristics of 27 patients with bloodstream infections who yielded linezolid-resistant *Staphylococcus epidermidis*, Greece, 2008–2010

Patient characteristics	Finding
Mean age, y, ± SD	46.9 ± 21.7
Male sex, no. (%)	16 (59.3)
Comorbidities >2, no. (%)	8 (29.6)
Mean hospital stay, d ± SD	27.1 ± 9.8
Use of mechanical ventilation	
Isolates recovered during ventilation, no. (%)	21 (77.8)
Mean duration, d ± SD	23.4 ± 9.7
Presence of central venous catheter	
No. (%) patients	27 (100)
Mean duration, d ± SD	27.1 ± 9.8
Presence of foreign material, no. (%)	11 (40.7)
Admission from other hospital, no. (%)	6 (22.2)
Prior hospitalization, no. (%)	10 (37.0)
Linezolid administration	
No. (%) patients	25 (92.6)
Mean duration, d ± SD	12.9 ± 7.4
In-hospital deaths, %	18.5

had mutations in 3–6 copies of 23S rRNA. The *cfr* gene was not detected in any isolate.

Characteristics of the 8 LRSE isolates tested by growth analysis are shown in Table 2; curves of the 5 highly LRSE isolates at 0, 32, and 128 µg/mL linezolid and of the 3 low-level LRSE and controls at half-MIC linezolid are shown in Figures 1 and 2. The growth of all 8 LRSE isolates was significantly slower than for the *S. aureus* control (p<0.05 at 24 h and at 36 h incubation for all isolates). Exposure to 8 µg/mL linezolid did not affect growth of the 5 highly LRSE isolates (p>0.05 for all isolates; data not shown). The 3 low-level LRSE isolates and the LSSE control showed moderately slower growth (p>0.05 at 24 h and 36 h) and the *S. aureus* control showed significantly slower growth (p<0.05 at 24 h and 36 h) at half-MIC linezolid than without linezolid. However, exposure of the 5 highly LRSE isolates to 32 and 128 µg/mL linezolid resulted in significantly faster growth compared with linezolid absence (p<0.05 at 24 and 36 h with 32 µg/mL linezolid and p<0.01

at 24 and 36 h with 128 µg/mL linezolid for all 5 isolates), suggesting partial linezolid dependence. Remarkably, all 5 linezolid-dependent LRSE isolates grew significantly faster with 128 µg/mL linezolid than did the 3 low-level LRSE isolates and the LSSE control with half-MIC and without linezolid (p<0.05 at 24 h and 36 h). Furthermore, 3 linezolid-dependent LRSE isolates (A2864, A2562[1], 217) grew significantly faster with 128 µg/mL linezolid than did the *S. aureus* control without linezolid (p<0.05 at 24 h and 36 h).

The 5 linezolid-dependent LRSE isolates had 2 potentially relevant amino acid substitutions, G152D (shift from a small amino acid to a negative hydrophilic) and D159Y (shift of hydrophilic to hydrophobic amino acid), and a less significant one (L101V) in L3 protein. No amino acid changes were observed in the remaining 3 isolates tested for proteins L3, L4, and L22 or in proteins L4 and L22 for any isolate tested.

Conclusions

All study isolates were recovered from patients with BSIs, indicating relatively high infectivity. Most of the LRSE isolates were clonally related, but 3 distinct PFGE types were detected, implying that linezolid resistance emerged in at least 3 different strains, which subsequently spread between patients. However, all linezolid-dependent isolates were clonal, implying that dependence possibly emerged once or on few occasions.

Antimicrobial drug resistance associated with dependence has been described for streptomycin and vancomycin (8–10). While investigating linezolid resistance in 8 LRSE isolates, we observed slower growth without linezolid than in controls, possibly resulting from mutations conferring resistance-exerted fitness cost. Surprisingly, linezolid concentrations at ≥32 µg/mL caused impressive growth acceleration in all 5 highly LRSE isolates, rendering

Table 2. Characteristics of 8 linezolid-resistant *Staphylococcus epidermidis* isolates tested for growth in the presence and absence of linezolid, Greece, 2008–2010*

Isolate designation	PFGE type	Mutations in each allele of the 23S rRNA						MIC, µg/mL		
		rrlA	rrlB	rrlC	rrlD	rrlE	rrlF	Linezolid	Chloramphenicol	Clindamycin
A2562(1)	I	T2504A	–	T2504A	T2504A	T2504A	T2504A	>256	>256	>256
		C2534T	–	C2534T	C2534T	C2534T	C2534T			
A2570	II	–	–	–	C2534T	C2534T	C2534T	16	>256	>256
		–	–	–	G2576T	G2576T	G2576T			
E371	I	T2504A	–	T2504A	T2504A	T2504A	T2504A	>256	>256	>256
		C2534T	–	C2534T	C2534T	C2534T	C2534T			
A2864	I	T2504A	–	T2504A	T2504A	T2504A	T2504A	>256	>256	>256
		C2534T	–	–	C2534T	C2534T	C2534T			
217	I	C2534T	T2504A	–	T2504A	T2504A	T2504A	>256	>256	>256
		–	C2534T	–	C2534T	–	C2534T			
605-2	I	T2504A	–	–	T2504A	T2504A	T2504A	>256	>256	>256
		–	–	–	C2534T	C2534T	C2534T			
A1702	II	G2576T	G2576T	G2576T	C2534T	G2576T	C2534T	32	64	>256
		–	–	–	G2576T	–	G2576T			
A2490	III	C2356T	T2334C	–	C2356T	C2356T	C2356T	32	>256	>256
		G2576T	G2576T	–	G2576T	G2576T	G2576T			

*PFGE, pulsed-field gel electrophoresis; –, absence of mutated position in the respective 23S rRNA copy.

Figure 1. LRSE isolated from patients with bloodstream infections, Greece, 2008–2010. Effect of growth under exposure to linezolid at 128 µg/mL is shown for the 5 highly LRSE: A) A2562[1], B) E371, C) A2864, D) 217, and E) 605–2. LRSE, linezolid-resistant *Staphylococcus epidermidis*.

significantly faster growth than without linezolid. Linezolid dependence is evident starting from relatively low linezolid concentrations, against which LRSE may be exposed in vivo during linezolid treatment. In fact, most of these 27 patients, including all 5 harboring linezolid-dependent LRSE, had prolonged linezolid treatment before yielding LRSE. This exposure also may have fostered the transition from resistance to dependence as suggested previously in vancomycin-dependent enterococci (8). Therefore, the high intrahospital linezolid consumption may favor not only LRSE selection but also their competitive survival. Should linezolid dependence prove common in highly LRSE isolates, it could explain their increasing clinical occurrence and the emergence of LRSE outbreaks (3,4,13). To support this hypothesis, growth with and without linezolid needs to be tested on larger collections of LRSE

isolates. Growth characteristics of LRSE isolates reported previously should also be studied.

The underlying mechanism by which linezolid binding to the mutated ribosomal subunits enhances growth may be complex. All 5 linezolid-dependent isolates harbored mutations T2504A combined with C2534T, whereas the linezolid-nondependent isolates harbored other mutations in 23S rRNA genes (Table 2). Also, only the linezolid-dependent isolates carried mutations in the ribosomal protein L3, known to stimulate ribosome assembly. The coupling of rRNA synthesis from precursor RNA molecules and ribosome assembly possibly affects the overall rate of protein synthesis in vivo (14). Linezolid may interfere in this interaction, thus affecting the ribosomal assembly and enabling interactions with precursor forms of the 50S subunit, as demonstrated for erythromycin (15). We speculate that
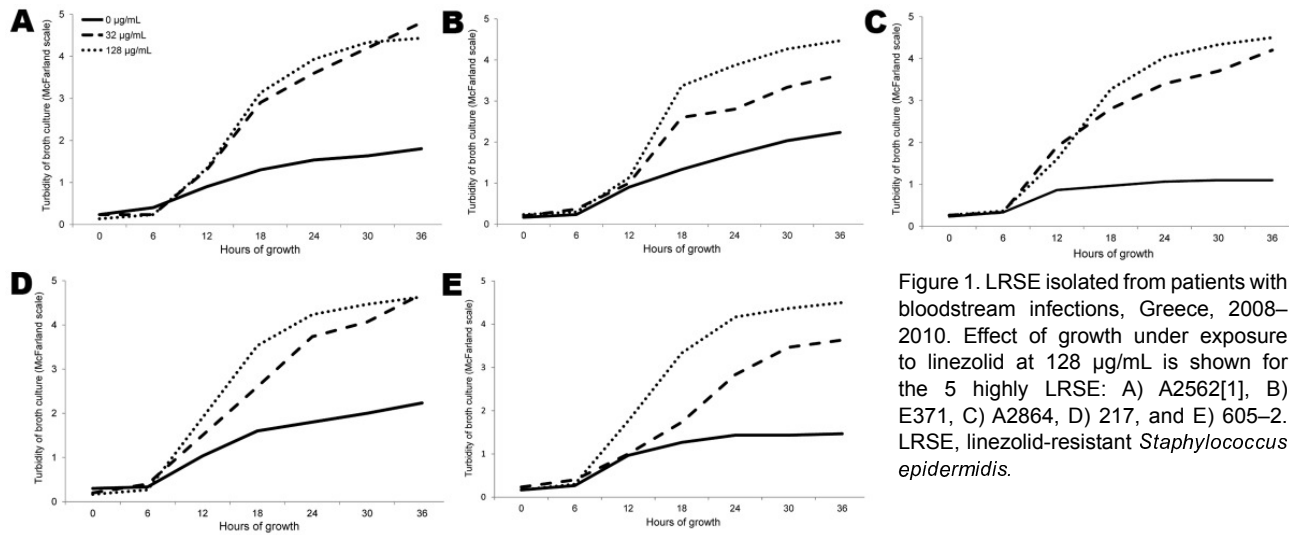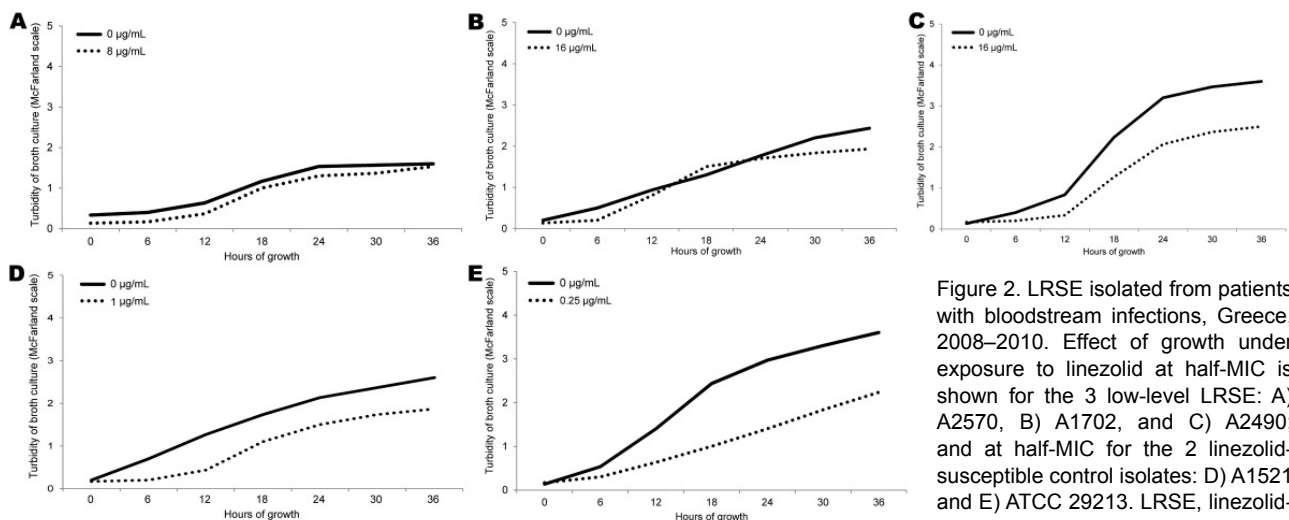

Figure 2. LRSE isolated from patients with bloodstream infections, Greece, 2008–2010. Effect of growth under exposure to linezolid at half-MIC is shown for the 3 low-level LRSE: A) A2570, B) A1702, and C) A2490; and at half-MIC for the 2 linezolid-susceptible control isolates: D) A1521 and E) ATCC 29213. LRSE, linezolid-resistant *Staphylococcus epidermidis*.

linezolid-dependent cells may possess linezolid-dependent ribosomal precursor particles exhibiting different structural conformation, which favors a faster rate of the overall protein synthesis recovery. This feature might explain the linezolid-dependent growth of the isolated strains. Further functional ribosomal characterization is required to elucidate linezolid dependence.

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Klebsiella pneumoniae Antimicrobial Drug Resistance, United States, 1998–2010

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We studied antimicrobial-resistant *Klebsiella pneumoniae* for 1998–2010 by using data from The Surveillance Network. Susceptibility results (n = 3,132,354) demonstrated significant increases in resistance to all antimicrobial drugs studied, except tetracycline. Cross-resistance among carbapenem-resistant *K. pneumoniae* was lower for tetracycline and amikacin.

*K*lebsiella spp. are among the most common pathogens isolated in intensive care units (ICUs), and *K. pneumoniae* is the most frequently encountered carbapenemase-producing *Enterobacteriaceae* (*1*). Increasing antimicrobial drug resistance, including carbapenem-resistant *K. pneumoniae* (CRKP), accounts for substantial increases in illness and death (*1*). Few antimicrobial therapy options exist for infections caused by CRKP (*2*).

The emergence of *K. pneumoniae* resistance to carbapenems is well documented (*3*). However, few studies have analyzed the trends and prevalence of in vitro *K. pneumoniae* antimicrobial drug resistance since carbapenem resistance emerged in the United States during the late 1990s (*4*). Furthermore, few investigations have examined antimicrobial drug resistance with regard to specimen source or cross-resistance patterns among CRKP.

We examined the prevalence of *K. pneumoniae* antimicrobial drug resistance in US inpatients using a large national surveillance system. Our objectives were to analyze *K. pneumoniae* antimicrobial drug resistance among US inpatients, resistance patterns by specimen source, and cross-resistance among imipenem-resistant *K. pneumoniae* isolates.

The Study

We examined inpatients' antimicrobial susceptibility test results from The Surveillance Network (TSN) Database-USA (Eurofins Medinet, Chantilly, VA, USA) for 1998–2010. TSN is a nationally representative repository of antimicrobial susceptibility results from ≈200 community, government, and university health care institutions in the United States and has been used in investigations of trends and prevalences of antimicrobial drug resistance (*5*). Susceptibility testing of isolates is conducted onsite by using Food and Drug Administration (FDA)–approved testing methods and interpreted by using Clinical Laboratory Standards Institute breakpoint criteria for all agents except tigecycline, for which FDA breakpoints were used. Details of quality control in TSN Database-USA have been described (*6*). No institutional review board approval was needed for this research because no personal identifying information was collected.

K. pneumoniae antimicrobial susceptibility results were stratified by specimen source (blood, sputum, urine, and wounds). Imipenem-resistant *K. pneumoniae* isolates from 2010 were examined for cross-resistance to other antimicrobial agents and prevalence in ICU versus non-ICU settings. We used χ^2 testing to determine whether changes in *K. pneumoniae* antimicrobial drug resistance were statistically significant from 2000 to 2010 and whether 2010 antimicrobial drug resistance differed by specimen source. The α level was set at 0.05. Analyses were performed by using R version 2.11.0 (www.r-project.org).

We analyzed a total of 3,132,354 *K. pneumoniae* antimicrobial susceptibility results for 1998–2010 (Table 1). Statistically significant increases in antimicrobial drug resistance to all agents (p<0.0001) except tetracycline (p = 0.0745) (Figure 1) were observed. Resistance to imipenem first appeared in TSN Database-USA in 2004 and rose gradually to 4.3% by the end of our study period. In 2010, *K. pneumoniae* resistance to tigecycline was 2.6% (data not shown). The largest increases in antimicrobial drug resistance from 1998 to 2010 were observed for aztreonam (7.7% to 22.2%), ceftazidime (5.5% to 17.2%), and ciprofloxacin (5.5% to 16.8%). Changes in resistance were smaller for tetracycline (14.2% to 16.7%) and amikacin (0.7% to 4.5%).

In 2010, isolates from the lower respiratory tract showed higher levels of resistance than did isolates from urine for all antimicrobial agents (p<0.0001) except tetracycline (p = 0.54) (Table 2). CRKP was more prevalent in ICU settings than in non-ICU settings (6.3% vs. 3.8%, respectively) (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0310-Techapp.pdf).

Table 1. *Klebsiella pneumoniae* antimicrobial drug resistance among inpatients, by year, United States, 1998–2010*

Antimicrobial drug	No.	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	Total change†
TET	80,862	14.2	14.7	14.6	13.9	14.2	13	15.6	14.8	15.6	15.2	15.3	16.4	16.7	2.5
AMK	232,933	0.7	0.9	0.8	0.9	1	1.1	2.1	3.7	4.9	4.6	4.9	4.6	4.5	3.8
IPM	259,589	0	0	0	0	0	0	0.3	0.6	0.6	1.5	3.3	3.8	4.3	4.3
GEN	344,597	4.9	4.6	4.6	5.2	5.5	5.6	6.9	8.3	8.4	7.8	8.7	9.1	9.2	4.3
CPM	211,134	2.1	2	2.7	2.3	2.3	2.5	5.1	5.3	5.5	5.8	7.7	7.3	7.7	5.6
TMP/SXT	344,522	10.9	11	10.9	10.4	10.4	10.9	13	15	16.2	16.5	19.4	18.9	19.3	8.4
TZP	250,554	4	5.5	5.4	5.3	5.3	5.6	7.7	8.8	8.4	7.8	10.5	12	12.7	8.7
TOB	289,287	4.6	4.5	4.4	4.8	5.2	5.5	8	9.8	11.9	10.4	13.2	13.2	13.8	9.2
CRO	287,091	1.8	2.7	3.2	2.9	2.6	3.1	5.7	6.9	8.1	8.7	11.1	11.2	12.1	10.3
CIP	306,348	5.5	6.1	6.5	7	7.6	7.9	9.9	11.7	14.4	14.6	17	16.8	16.8	11.3
CAZ	248,004	5.5	6.8	7.9	7.9	7.7	7.8	11.3	13.3	12.3	12.3	15.6	15.2	17.2	11.7
ATM	184,981	7.7	7.7	7.7	7.5	8	7.3	11.2	12.9	12.8	12.8	16.6	16.1	22.2	14.5

*TET, tetracycline; AMK, amikacin; IPM, imipenem; GEN, gentamicin; CPM, cefepime; TMP/SXT, trimethoprim/sulfamethoxazole; TZP, piperacillin/tazobactam; TOB, tobramycin; CRO, ceftriaxone; CIP, ciprofloxacin; CAZ, ceftazidime; ATM, aztreonam.
†Change from 1998 to 2010. p<0.0001 for all antimicrobial agents except tetracycline (p = 0.0745).

Imipenem-resistant isolates of *K. pneumoniae* showed the lowest resistance to tetracycline (19.9%) and amikacin (36.8%). High prevalence of cross-resistance was observed for ciprofloxacin (96.4%) (Figure 2).

Conclusions

In our study, the proportion of *K. pneumoniae* isolates resistant to carbapenems was lower than those previously reported (*7,8*). In 2010, we observed a resistance rate of 4.3% for imipenem. The Centers for Disease Control and Prevention (CDC) reported that, among health care–associated infections, 8% of *Klebsiella* spp. isolates were carbapenem resistant in 2007 compared with <1% in 2000 (*9*). Most studies of *K. pneumoniae* antimicrobial drug resistance have focused on patient populations with higher exposures to antimicrobial agents, such as those in critical care and academic hospital settings. In contrast, the lower

Figure 1. *Klebsiella pneumoniae* antimicrobial drug resistance, United States, 1998–2010. ATM, aztreonam; SXT, trimethoprim/sulfamethoxazole; CAZ, ceftazidime; CIP, ciprofloxacin; TET, tetracycline; TOB, tobramycin; TZP, piperacillin/tazobactam; CPM, cefepime; AMK, amikacin; IPM, imipenem. Ceftriaxone and gentamicin were not included for better data presentation.

prevalence of CRKP in our study might have resulted from a wider variety of institution types and inclusion of isolates from hospital patients outside of the critical care setting. Furthermore, within our study, a high percentage of isolates were from urine and showed lower levels of resistance than did isolates from respiratory samples. Interpretive breakpoint criteria for the antimicrobial agents included did not change during the study period.

The low cross-resistance to tetracycline among CRKP and stable resistance rate of *K. pneumoniae* to this agent during the study period are noteworthy. In our analysis of cross-resistance among imipenem-resistant *K. pneumoniae*, tetracycline had the greatest antimicrobial activity against CRKP. Although resistance of *K. pneumoniae* increased for all antimicrobial agents studied, resistance to tetracycline increased only slightly from 1998 to 2010. Later-generation tetracyclines may prove useful in the treatment of CRKP-related infections because of their improved tissue penetration, antimicrobial activity, and decreased propensity to develop antimicrobial drug resistance compared with their older counterparts (*10*). Tigecycline, a glycylcycline antimicrobial agent that is structurally similar to tetracycline, has been used to treat CRKP-related infections and is often active against carbapenemase–producing *K. pneumoniae* (*11,12*). Data for tigecycline that used FDA interpretive breakpoints showed *K. pneumoniae* antimicrobial drug resistance was 2.6% in 2010. Tigecycline data were included only for 2010 because the drug was not FDA approved until 2005 and an insufficient number of results were available before 2010.

The widespread transmission of carbapenemase-producing *K. pneumoniae* has become the most common cause of carbapenem resistance among *Enterobacteriaceae* in the United States (*13*) and probably accounts for most of the imipenem resistance shown in this study. The spread of carbapenemase-producing organisms threatens to extend carbapenem resistance to the community (*14*). The
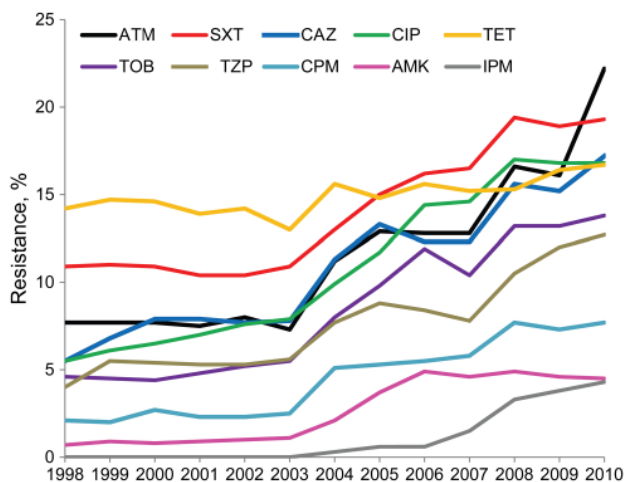
Table 2. *Klebsiella pneumoniae* antimicrobial drug resistance rates, United States, 2010*

Source, no. samples	AMK	GEN	TOB	TZP	ATM	IPM	CAZ	CRO	CPM	TMP/SXT	CIP	TET
All, 187,359	4.5	9.2	13.8	12.7	22.2	4.3	17.2	12.1	7.7	19.3	16.8	16.7
Blood, 20,185	4.2	10.6	14.9	15	23.6	5.9	19.2	13.4	7.1	21.3	18.9	17.1
Urine, 112,567	4.1	7.8	12.4	10.6	21	3.8	15.1	10.9	7.1	18.3	15.1	17.3
Wound, 22,225	4.5	10.1	14.4	14.7	20.9	5	17.5	12.2	8.3	19.5	16.8	16.4
Respiratory, 32,382	5.8	11.1	15.8	16.4	24.5	4.7	21.3	15.5	9.9	20.2	20	15.5

*AMK, amikacin; GEN, gentamicin; TOB, tobramycin; TZP, piperacillin/tazobactam; ATM, aztreonam; IPM, imipenem; CAZ, ceftazidime; CRO, ceftriaxone; CPM, cefepime; TMP/SXT, trimethoprim/sulfamethoxazole; CIP, ciprofloxacin; TET, tetracycline.

increasing antimicrobial drug resistance to *K. pneumoniae* in our study, a concurrent lack of novel antimicrobial agent development (*15*), and limited therapeutic options available for treating CRKP-related infections add further urgency to improve prevention efforts and treatment strategies.

Our study data have strengths and limitations. The strengths are the wide variety of antimicrobial agents included, the number of laboratories reporting data, the nationally representative geographic distribution of these institutions, and the large number of isolates. Geography is a critical consideration with surveillance of this organism because distribution of *K. pneumoniae* antimicrobial drug resistance varies within the United States (*13*). The limitations of these data include a lack of central laboratory testing and the variety of test methods used. Because of a lack of Clinical Laboratory Standards Institute or FDA interpretive breakpoints for *K. pneumoniae* and colistin or fosfomycin, these data were not collected by TSN Database-USA and were not included in this study. Resistance to carbapenems might have been underreported at the beginning of our study period because of a lower frequency of susceptibility testing of these agents and the inability of antimicrobial susceptibility test methods to detect low-level carbapenem resistance.

Our study shows that *K. pneumoniae* antimicrobial drug resistance increased for every antimicrobial class studied except tetracyclines. Cross-resistance among imipenem-resistant *K. pneumoniae* was high for ciprofloxacin but

lower for tetracycline and amikacin. This emerging problem presents a major threat to public health and warrants due diligence in future surveillance efforts.

Acknowledgments

Mr Sanchez is studying medicine and epidemiology as a physician assistant student at The George Washington University. His research interests are infectious disease epidemiology and emerging antimicrobial drug resistance.

Figure 2. Prevalence of antimicrobial cross-resistance among imipenem-resistant *Klebsiella pneumoniae* isolates, United States, 2010. TET, tetracycline; AMK, amikacin; GEN, gentamicin; CPM, cefepime; SXT, trimethoprim/sulfamethoxazole; CRO, ceftriaxone; TOB, tobramycin; CIP, ciprofloxacin; TZP, piperacillin/tazobactam; CAZ, ceftazidime; ATM, aztreonam.

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West Nile Virus Infection among Humans, Texas, USA, 2002–2011

Melissa S. Nolan, Jim Schuermann, and Kristy O. Murray

We conducted an epidemiologic analysis to document West Nile virus infections among humans in Texas, USA, during 2002–2011. West Nile virus has become endemic to Texas; the number of reported cases increased every 3 years. Risk for infection was greatest in rural northwestern Texas, where *Culex tarsalis* mosquitoes are the predominant mosquito species.

The first documented case of West Nile virus (WNV) infection in North America occurred during an outbreak of encephalitis in 1999 in New York, New York, USA (*1*). Within a few years, WNV had rapidly spread across the United States and was being transmitted throughout most of the country (*2*). In Texas, the first cases of human infection with WNV were reported in 2002, and cases have been reported every year since. Our objective was to epidemiologically describe WNV infections among humans over the first decade of virus transmission in Texas.

The Study

We analyzed deidentified surveillance data for all cases reported to the Texas Department of State Health Services (TxDSHS) during 2002–2011. Reporting of West Nile neuroinvasive disease (WNND) to the TxDSHS was made mandatory in 2000, and West Nile fever (WNF) was added to the list of reportable conditions in 2005. Onset dates were categorized according to work week for each year and were used to create an epidemic curve and yearly incidence graph. Attack rates (no. cases/100,000 population) were stratified by demographics, and a 2-tailed t test was used to detect differences between the overall attack rate for the state and each demographic variable. Stratified calculations were based on the number of variable-specific reported cases over the variable-specific population. We estimated the seroprevalence by using published ratios (no. infected: no. WNND cases) and state population estimates for 2011 (*3–5*). By using the number of reported WNND

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cases, defining WNF as 26% of seroprevalence estimates, and using previously published cost estimates for WNND and WNF cases, we calculated the total economic cost of infections over the past decade (*6,7*).

Relative risks (RRs) for each county were calculated for each year. Expected number of cases per county (e) were calculated by using a validated equation $e = (r \times n)(c)$, where r is the state incidence rate, n is the state population, and c is the county population (*5,8*). To assess potential associations between counties and increased RRs and to examine urban–rural characteristics of counties and increased RRs, we performed Kruskal-Wallis 1-way analysis of variance by ranks. All calculations were run by using Stata version 12.0 software (StataCorp, College Station, TX, USA).

During 2002–2011, a total of 2,274 cases were reported to TxDSHS. Most (n = 735) cases were reported in 2003, and additional peaks occurred in 2006 (n = 354) and 2009 (n = 115). These peaks occurred every 3 years, starting in 2003, with a higher number of cases reported in those peak years than in the years before and after (Figure 1, panels A, B; Figure 1, panel C, Appendix, wwwnc. cdc.gov/EID/article/19/1/12-1135-F1.htm). Transmission season in Texas was April–December; the epidemic curve peaked in August.

Of those cases reported, 749 (33%) were WNF and 1,525 (67%) were WNND. Most cases were in non-Hispanic white persons (1,224 [54%] cases), followed by Hispanic (550 [24%]), other/unknown (350 [15%]), non-Hispanic black (141 [6%]), and Asian (9 [1%]) persons. The median age of case-patients was 54 years (range 21 days through 99 years), and most (1,335 [59%]) were male. Case-fatality rate was 6.3% (143 deaths/2,274 cases); all deaths were attributed to WNND.

We calculated the attack rates for WNV for Texas, stratified by demographic variables (Table). The overall attack rate was 7.1 WNV cases per 100,000 population. Attack rates were higher among male than among female persons (10.2 cases and 6.7 cases/100,000 population, respectively), and attack rates among non-Hispanic white persons were slightly higher than those among persons of other races (11 and 8 cases/100,000 population, respectively). No statistical differences were found between the state overall attack rate and attack rates by sex or race/ethnicity. Our findings contradict those of previous studies that found higher attack rates for persons who were male or members of a minority group (*3,9,10*). Analysis of these variables on a smaller geographic level might provide more insight into the influence of demographics and socioeconomic status on infection rates.

Attack rates for WNV increased progressively with age; the rate among persons >65 years of age was 23×higher than that among children 5–15 years of age. These findings
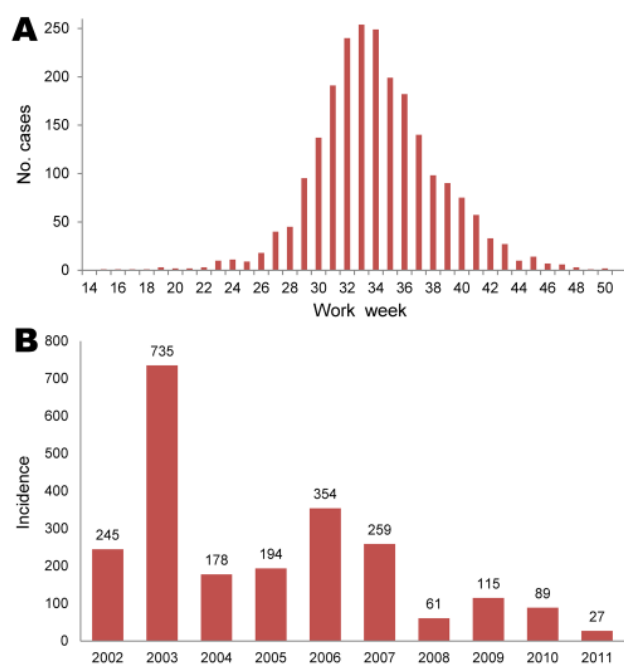
Figure 1. Reported cases of West Nile virus infection among humans, Texas, USA, 2002–2011. A) Epidemic curve. B) Incidence (no. cases/100,000 population).

are consistent with those of studies that found that WNV disease severity increases with age, resulting in a higher risk for disease among elderly persons (3,4,11).

By using published seroprevalence estimates of the ratio of asymptomatic blood donors to reported WNND cases, we were able to estimate that ≈534,000 persons in Texas have been infected with WNV since 2002 (Table). Using these numbers, we estimated an overall WNV seroprevalence of 2.5% and an estimated total economic cost of acute WNV infection in Texas of »$112 million. WNF cases cost $42 million ($302/case), and WNND cases cost $70 million ($46,530/case).

For the 10-year period, attack rates were calculated per county. Rates (no. cases/100,000 population) were highest for predominately rural counties within the northwest area of the state: Castro (34.2), King (27.9), Crosby (21.3), Swisher (18.7), and Parmer (16.4) Counties. Attack rates were low for the most densely populated counties; rates for the 5 metropolitan counties were as follows: Dallas (1.1), Tarrant (1.0), Harris (1.0), Travis (0.5), and Bexar (0.2) Counties.

Over the 10 years, average RR was greater than expected (RR>1) for 79 (31%) of the 254 counties. The 10-year average RR was mapped by county (Figure 2). After mapping the RRs, we noticed spatial clustering and examined RR by region as defined by the Texas Association of Regional Councils (12). Analysis of variance indicated a significant difference of RR (p<0.001) by region. Odds of WNV infection in humans were significantly higher for 3 regions (ORs 23.6, 37.48, 2.7) in the northwestern part of the state than for other regions.

To further investigate RRs and environmental factors, we examined the percentage of urban versus rural land use in each county. Increased RR was positively associated (p = 0.01) with rural environments; median percentage of rural land use was 53% for rural counties with RR<3, compared with 85% for rural counties with RR≥3.

We propose that predominant mosquito species combined with urban versus rural factors might have influenced disease transmission in Texas. Population densities varied; urban areas were generally in the eastern part of the state and rural areas in the western part (5). Culex quinquefasciatus mosquitoes are the predominant species in eastern Texas and are associated with urban areas; C. tarsalis mosquitoes are predominant in western Texas and are associated with rural areas (13–15). Unfortunately, reporting of WNV-positive mosquito species to TxDSHS is voluntary and was incomplete because of inadequate resources for surveillance in most Texas counties. This lack of mosquito

Table. Sex- and age-stratified ratio of infected population by reported cases of WNND, Texas, USA, 2002–2011*

Patient age, y	Texas population†	Reported WNND cases‡	WNND attack rate/100,000 population	Ratio of estimated no. infected for each reported WNND case§	Estimated WNV-infected population	Estimated seroprevalence of WNV-infected population, %
Children, 5–15	3,754,316	40	1.1	4,167	166,680	4.44
Adult men						
16–24	1,502,524	48	3.2	719	34,512	2.30
25–44	3,204,788	167	5.2	356	59,452	1.86
45–64	2,839,796	310	10.9	248	76,880	2.71
≥65	1,098,089	358	32.6	50	17,900	1.63
Adult women						
16–24	1,442,664	37	2.6	1,231	45,547	3.16
25–44	3,066,278	140	4.6	330	46,200	1.51
45–64	2,920,842	190	6.5	387	73,530	2.52
≥65	1,413,691	224	15.8	61	13,664	0.97
Overall	21,242,988	1,514	7.1	353	534,442	2.52

*WNND, West Nile neuroinvasive disease; WNV, West Nile virus.
†Eestimates from State Data Center (www.idserportal.utsa.edu/sdc/projections).
‡Reported to the Texas Department of State Health Services.
§Ratio from references (3,4).
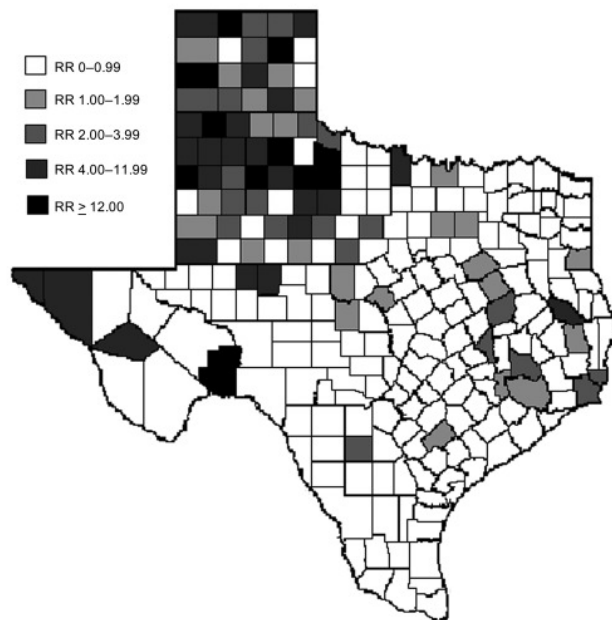
Figure 2. Average relative risk (RR) for human infection with West Nile virus, by county, Texas, USA, 2002–2011. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/19/1/12-1135-F2.htm).

data prohibited us from statistically evaluating the influence of *C. tarsalis* mosquitoes on WNV transmission to humans. According to the literature and our preliminary data, we believe *C. tarsalis* mosquitoes might be a factor associated with increased RR in western compared with eastern Texas, where *C. quinquefasciatis* mosquitoes are the more established species.

Conclusions

During the 10 years of WNV emergence and human infection in Texas, a total of 2,274 cases were reported (67% WNND, 33% WNF); case-fatality rate was 6%. The total economic toll of human disease in Texas was $112 million. Although attack rates and RRs were higher for persons in rural counties, specifically in northwestern Texas, the highest numbers of cases were reported from metropolitan cities.

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Seropositivity for Influenza A(H1N1) pdm09 Virus among Frontline Health Care Personnel

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Seroprevalence of antibodies to influenza A(H1N1) pdm09 virus among 193 emergency department health care personnel was similar among 147 non–health care personnel (odds ratio 1.4, 95% CI 0.8–2.4). Working in an acute care setting did not substantially increase risk for virus infection above risk conferred by community-based exposures.

Transmission of infectious disease from acutely ill patients to health care personnel (HCP) is a critical concern during disease outbreaks. During the initial months after the emergence of influenza A(H1N1)pdm09 virus, comparisons to prior pandemic viruses (1) and reports of increased illness and death in younger adults (2,3) heightened concerns about the safety of frontline HCP caring for patients with A(H1N1)pdm09 and the ability of the health care system to meet demands for health care services if infected HCP had to stay home from work.

New York, New York, was one of the first densely populated areas in the United States to experience outbreaks of A(H1N1)pdm09. These early outbreaks and the concomitant surge in patient volumes in our emergency

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department (ED) provided the opportunity to evaluate and compare risk for A(H1N1)pdm09 virus infection among frontline HCP and non-HCP from the same community in a virus-naive population before availability of the A(H1N1) pdm09 monovalent vaccine.

The Study

Written informed consent was obtained and the study approved by the Human Subjects Review Board of the Feinstein Institute for Medical Research of the North Shore–Long Island Jewish Health System. Long Island Jewish Medical Center and the adjoining Cohens Children's Hospital are tertiary care teaching hospitals in Queens, New York. During April 24–June 11, 2009, the volume of all-cause ED visits to these 2 institutions increased by 62% compared with the same period during 2008. There were 5,100 visits with influenza-like illness (ILI) as the primary manifestation, which coincided with a surge of ILI visits to EDs throughout New York, New York (4).

HCP who worked in an acute care or specially designated influenza area during April 24–June 11, 2009, were asked to participate in our study during October 28–December 16, 2009, by completing a survey and submitting a blood sample. During the same time, we enrolled a convenience sample of non-HCP adults >18 years of age residing in the same region as HCP. None of the participants received the A(H1N1)pdm09 monovalent vaccine before enrollment. Assuming a 20% seroprevalence of antibodies to A(H1N1)pdm09 among the general population and a type I error probability of 5% and type II error probability of 20% (power 80%), a sample size of 140 HCP and 140 non-HCP would be sufficient to show a 15% difference in seroprevalence between HCP and non-HCP.

Serum samples were tested by using hemagglutination inhibition and microneutralization assays with A/Mexico/4108/2009, an A/California/07/2009 (H1N1)–like virus (5). Participants with a single serum sample with a microneutralization titer $\geq$40 and a hemagglutination inhibition titer $\geq$20 were considered seropositive for antibodies to A(H1N1)pdm09 virus. This combination of antibody titers in single convalescent-phase serum samples was shown to provide 90% sensitivity and 96% specificity for detection of A(H1N1)pdm09 infection in persons <60 years of age and 92% specificity in persons 60–79 years of age (5).

Separate analyses comparing seropositive and seronegative persons were performed for HCP and non-HCP by using either a χ^2 statistic, Fisher exact test, or Mann-Whitney test. In multivariable logistic regression models, factors associated with seropositivity in univariate analysis (p<0.10) or hypothesized to be exposure risk factors were included. Analyses were performed by using SAS version 9.2 software (SAS Institute Inc., Cary, NC, USA).

We enrolled 193 HCP and 147 non-HCP in the study. Non-HCP were older (median 47 years, range 18–80 years) than HCP (median 40 years, range 21–65 years) and less likely to recall symptoms of an ILI (Table 1). A similar proportion of HCP and non-HCP reported contact with a household member who had confirmed or suspected A(H1N1)pdm09 and living with children <18 years of age.

Among 193 HCP, 41 (21.2%) were seropositive for antibodies to A(H1N1)pdm09 virus; of these, 12 (29.3%) reported no influenza-like symptoms during the study period. Age, sex, and HCP role were not associated with seropositivity. However, a higher proportion of attending physicians who took care of children were seropositive than those who took care of adults (30.8% vs. 8.7%; p<0.07). Seropositive HCP did not work more ED shifts than seronegative HCP (mean 20 vs. 22 shifts; p = 1.0) or temporary influenza treatment center shifts (mean 8 vs. 5 shifts; p = 0.5) during April 24–June 11, 2009. The proportion of seropositive HCP who reported contact with a patient with suspected or confirmed A(H1N1)pdm09 was similar (76.3% vs. 73.2%; p = 0.9).

Among 147 non-HCP, 24 (16.3%) were seropositive for antibodies to A(H1N1)pdm09 virus. A higher proportion of persons living with children <18 years of age were seropositive (54.2% vs. 34.2%; p = 0.06) than those not living with children <18 years of age. However, this finding did not reach statistical significance.

Among the 340 study participants, 65 (19%) were seropositive for antibodies to A(H1N1)pdm09 virus. HCP were no more likely to be seropositive than were non-HCP (21.2% vs. 16.3%; p = 0.30). In a multivariate model that included age, sex, receipt of seasonal influenza vaccine, having children <18 years of age in the household, and occupation, only living in a household with children <18 years of age was associated with being seropositive (Table 2).

Conclusions

We found that 21% of frontline HCP working in the ED or specially designated influenza areas during the first wave of A(H1N1)pdm09 virus circulation in New York, New York, were seropositive for antibodies to A(H1N1) pdm09 virus, similar to non-HCP. Overall, our estimated seroprevalence among HCP and non-HCP was 19%, which was similar to estimates after the first wave of A(H1N1) pdm09 virus circulation from other studies (6,7). Living with children <18 years of age was the only identified risk indicator for seropositivity.

Among HCP, the reported seroprevalence of antibodies to A(H1N1)pdm09 virus in other countries ranges from 7%

Table 1. Baseline characteristics of 340 health care personnel tested for seropositivity to influenza A(H1N1)pdm09 virus*

Characteristic	No. (%) health care personnel, n = 193	No. (%) non–health care personnel	p value
Sex			
M	70 (36.3)	68 (46.3)	0.07
F	123 (63.7)	79 (53.7)	NA
Age, y			
<30	35 (18.1)	21 (14.3)	0.01
30–40	58 (30.1)	27 (18.4)	NA
41–50	48 (24.9)	33 (22.5)	NA
51–60	43 (22.3)	49 (33.3)	NA
>60	9 (4.7)	17 (11.6)	NA
Age, y (dichotomized)			
<60	184 (95.3)	130 (88.4)	0.02
≥60	9 (4.7)	17 (11.6)	NA
Children <18 y of age in home	85 (44.0)	55 (37.4)	0.22
Received seasonal influenza vaccine during 2008 or 2009	190 (98.5)	123 (83.7)	<0.01
Contact with household member with confirmed or suspected A(H1N1)pdm09	39 (20.2)	23 (15.7)	0.26
Clinical contact with confirmed or suspected A(H1N1)pdm09	146 (75.7)	NA	NA
Health care worker role			
Attending physician	47 (24.4)	NA	NA
Resident physician	30 (15.5)	NA	NA
Registered nurse	58 (30.1)	NA	NA
Other†	58 (30.1)	NA	NA
Cared for primarily adults	126 (65.3)	NA	NA
Cared for primarily children	67 (34.7)	NA	NA
Influenza-like symptoms‡ during spring–summer 2009	81 (42.0)	53 (36.1)	0.27
ILI§ during spring–summer 2009	27 (14.0)	7 (4.8)	0.01
ARI¶ during spring–summer 2009	48 (24.9)	30 (20.4)	0.33

*NA, not applicable; ILI, influenza-like illness; ARI, acute respiratory illness.
†Patient care technicians, registrars, and other emergency department staff.
‡Any of the following: fever, cough, body aches, chills, headache, fatigue, runny nose, sore throat, diarrhea, nausea, and vomiting.
§Fever AND either cough or sore throat.
¶Two or more of the following: fever, cough, runny nose, sore throat.

Table 2. Univariate and multivariate analysis of risk indicators for seropositivity for influenza A(H1N1)pdm09 virus among 340 study participants*

Characteristic	No. (%) seropositive, n= 65	No. (%) seronegative, n = 257	Crude OR (95% CI)	Adjusted OR (95% CI)
Sex				
M	22 (33.9)	116 (42.2)	Referent	NA
F	43 (66.2)	159 (57.8)	1.41 (0.80–2.49)	NA
Age, y				
<30	15 (23.1)	41 (14.9)	2.01 (0.60–6.81)	NA
30–40	20 (30.8)	65 (23.6)	1.57 (0.48–5.15)	NA
41–50	14 (21.5)	67 (24.4)	1.15 (0.34–3.86)	NA
51–60	12 (18.5)	79 (28.7)	0.84 (0.25–2.85)	NA
>60	4 (6.2)	22 (8.0)	Referent	NA
Age, y (dichotomized)				
<60	61 (93.9)	253 (92.0)	1.32 (0.44–3.96)	NA
>60	4 (6.2)	22 (8.0)	Referent	NA
Received seasonal influenza vaccine during 2008 or 2009	61 (93.9)	252 (91.6)	1.30 (0.43–3.91)	NA
Contact with person with suspected A(H1N1)pdm09 virus infection	13 (20.0)	49 (17.8)	1.17 (0.59–2.31)	NA
Children <18 y of age at home	36 (55.4)	104 (37.8)	1.96 (1.13–3.40)	1.96 (1.13–3.40)
Occupation				
Non-HCP	24 (36.9)	123 (44.7)	Referent	NA
HCP	41 (63.1)	152 (55.3)	1.35 (0.77–2.36)	NA
ILI† during spring–summer 2009	15 (23.1)	19 (6.9)	3.97 (1.85–8.49)	NA
ARI‡ during spring–summer 2009	25 (38.5)	53 (19.3)	2.55 (1.41–4.60)	NA

*OR, odds ratio; NA, not applicable; HCP, health care personnel; ILI, influenza-like illness; ARI, acute respiratory illness. ILI and ARI were not included in the adjusted logistic regression model because they did not reflect risk for exposure.
†Fever AND either cough or sore throat.
‡Two or more of the following: fever, cough, runny nose, sore throat.

to 30% (8–12). Consistent with findings from our study, seroprevalence among HCP in most studies comparing HCP and non-HCP was similar to that for non-HCP (10–12). However, some studies identified differences in seroprevalence associated with HCP role and between first-line and second-line HCP (8,11). Although our study did not have adequate power to detect such differences, we observed a trend toward higher seropositivity among physician providers caring for children.

Several studies documented reduction in spread of influenza by facemask use and handwashing (13,14). Measures taken to limit the spread of infection during A(H1N1)pdm09 in our hospitals included isolation of patients with ILI upon hospital arrival; HCP use of N95 protective masks, gloves, and gowns; and standard precautions such as handwashing. We did not evaluate the effect of these precautions on risk for A(H1N1) pdm09 virus infection because we did not measure HCP adherence to prevention measures. However, seropositivity for A(H1N1)pdm09 virus might have been higher among HCP in our study if these preventions measures were not in place.

Our study had several limitations. Our control group may not have been representative of the general community. We did not assess for use of influenza antiviral medications after potential exposures among participants. On the basis of our sample size, we only had adequate power to detect a ≥5% difference in seropositivity between HCP and non-HCP. We did not

have prepandemic serum samples from study participants to evaluate for pre-existing cross-reactive antibodies to A(H1N1)pdm09 virus. However, we found that that our criteria for seropositivity were highly specific for detection of A(H1N1)pdm09 virus infection.

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Human Gastroenteritis Outbreak Associated with *Escherichia albertii*, Japan

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Although *Escherichia albertii* is an emerging intestinal pathogen, it has been associated only with sporadic human infections. In this study, we determined that a human gastroenteritis outbreak at a restaurant in Japan had *E. albertii* as the major causative agent.

*E*scherichia albertii* is an emerging human and bird pathogen that belongs to the attaching and effacing group of pathogens. This group of pathogens forms lesions on intestinal epithelial cell surfaces by the combined action of intimin, an *eae* gene–encoded outer membrane protein, and type III secretion system effectors (*1–4*).

Recently, we found that *E. albertii* represents a substantial proportion of the strains that had previously been identified as *eae*-positive *Escherichia coli*, enteropathogenic *E. coli* or enterohemorrhagic *E. coli*; 26 of the 179 *eae*-positive strains analyzed were found to be *E. albertii* (*5*). Furthermore, *E. albertii* is also a potential Shiga toxin 2f (Stx2f)–producing bacterial species (*5*). However, no *E. albertii*–associated gastroenteritis outbreak has been reported, which generates doubts regarding the clinical role of this microorganism. In this study, we revisited an outbreak of gastroenteritis that was presumed to have been caused by *eae*-positive atypical *E. coli* OUT:HNM (*6*) to determine if it was actually caused by *E. albertii*.

The Study

An outbreak of gastroenteritis occurred at the end of May 2011 in Kumamoto, Japan, among persons who attended 1 of 2 parties held in a Japanese restaurant on May 29. We

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reviewed case records for the 94 persons who attended the parties. A total of 48 persons became ill; 43 of them attended the first party (a total of 86 attended), and 5 attended the second party (a total of 8 attended). The ill participants had not eaten any food in common except for the meals served at the restaurant. The main symptoms of the patients were diarrhea (83%), abdominal pain (69%), fever (44%; mean temperature 37.2°C), and nausea (29%). The mean incubation period was 19 h.

A routine protocol to identify bacteria and viruses (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0646-Techapp.pdf) was used by our laboratory to examine 54 fecal specimens from 44 party participants and 10 members of the restaurant kitchen staff (7 party participants and all of the kitchen staff were asymptomatic). Atypical *E. coli* (lactose negative; OUT:HNM) strains harboring the *eae* gene and *E. coli* OUT:H18 strains harboring the *stx2d* and *astA* (but not *eae*) genes were isolated from 24 and 3 specimen, respectively; 7 specimens yielded both strains (Table 1). The *stx2*-positive/*eae*-negative *E. coli* strains were found to be serotype O183 (a recently described O serotype) by agglutination testing with O183-specific antiserum (S. Iyoda, M. Ohnishi, unpub. data).

All atypical *E. coli* strains showed identical or nearly identical *Xba*I-digested DNA banding patterns by pulsed-field gel electrophoresis, and the 10 *E. coli* O183:H18 strains also exhibited identical patterns (Figure). The source of the infection was most likely the meals served in the restaurant, but a bacteriological examination of the meal or of the ingredients used to prepare the meal was not possible because none of the food was preserved for analysis.

The lactose-negative/*eae*-positive features of the OUT:HNM strains suggested that these strains might be *E. albertii*. We examined additional biochemical properties of these strains and found that they exhibited the *E. albertii*–specific features described (*4,5*). These features include nonmotility, inability to ferment xylose and lactose, and inability to produce β-D-glucuronidase. The *E. coli* O183:H18 strains demonstrated common phenotypic and biochemical properties of *E. coli* (*7*).

To determine whether the *E. albertii*–like OUT:HNM strains were *E. albertii*, we randomly selected 6 strains and determined their phylogeny by multilocus sequence analysis as described (*5*) (online Technical Appendix Table). Results indicated that although the *E. coli* O183:H18 strain analyzed in parallel belongs to *E. coli sensu stricto*, the *E. albertii*–like OUT:HNM strains belong to the *E. albertii* lineage; all 6 strains showed identical sequences (online Technical Appendix Figure).

We further examined the intimin subtype by sequencing the *eae* gene, the chromosome integration site of the locus of enterocyte effacement encoding the *eae* gene and a

Table 1. Isolates from fecal specimens of party participants during outbreak of gastroenteritis associated with *Escherichia albertii*, Japan*

	Origin of isolates				
	Participants, n = 44			Kitchen staff, n = 10	
Isolate	Symptomatic	Asymptomatic	No information	Symptomatic	Asymptomatic
E. albertii†	21	1	0	0	2
E. albertii† and *E. coli* O183:H18‡	7	0	0	0	0
E. coli O183:H18‡	3	0	0	0	0
None	6	5	1	0	8

*None, negative for both pathogens.
†Initially identified as atypical (lactose negative) *E. coli* OUT:HNM harboring the intimin (*eae*) gene.
‡Initially identified as *eae* negative *E. coli* OUT:H18 harboring the Shiga toxin 2d and enteroaggregative *E.coli* heat-stable toxin genes.

set of type III secretion system genes, and the presence and subtype of the *cdtB* gene as described (*5*). Results showed that the *E. albertii* strains had intimin σ, which is rarely identified in enteropathogenic *E. coli* or enterohemorrhagic *E. coli*; the locus of enterocyte effacement was integrated into the *pheU* tRNA gene; and the *cdtB* gene of the II/III/V subtype group was present. These features are consistent with recently described genetic features of *E. albertii* (*5*).

We divided the party participants into 4 groups according to strain isolation patterns and statistically assessed the association of strain isolation patterns with incidence of clinical symptoms (Table 2). The results indicated that persons infected with only *E. albertii* or persons infected with *E. albertii* and *E. coli* O183:H18 had diarrhea and abdominal pain more frequently than did uninfected persons (p<0.05) and that the incidence of asymptomatic carriers was lower among persons infected only with *E. albertii*.

Nucleotide sequences obtained in this study have been deposited in the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank database. Accession numbers and other information on sequence analyses are shown in the online Technical Appendix.

Conclusions

In this gastroenteritis outbreak, *E. albertii* or *stx2*-positive *E. coli* O183:H18 was isolated from 24 ill patients; both strains were isolated from 7 patients. Thus, although the responsible meal or food was not identified, it was most likely contaminated with these 2 microorganisms. The contribution or involvement of *E. coli* O183:H18 in this outbreak is unknown because there were 3 patients from whom only *E. coli* O183:H18 was isolated and because there were no differences in clinical symptoms between persons infected with *E. coli* O183:H18 and persons not infected (Tables 1, 2). In contrast, *E. albertii* was isolated
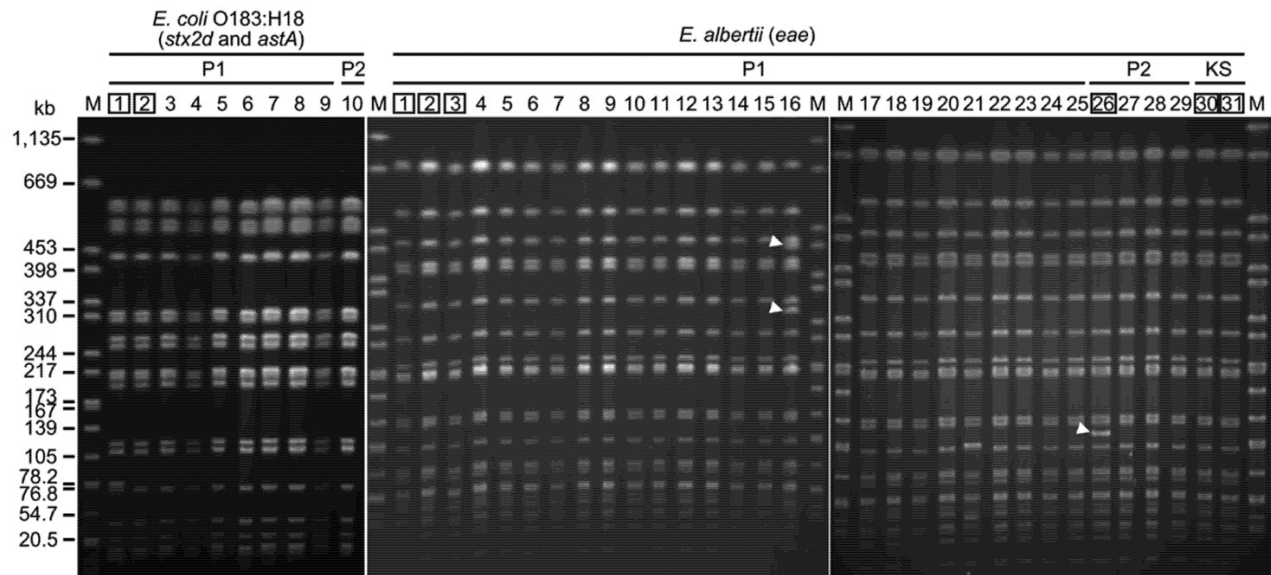
Figure. *Xba*I-digested pulsed-field gel electrophoresis profiles of isolates from fecal specimens collected from patients during an outbreak of human gastroenteritis associated with *Escherichia albertii*, Japan. Extra bands observed in 2 *E. albertii* isolates are indicated by arrowheads (only 1 or 2 band differences). The 2 *E. coli* O183:H18 and 6 *E. albertii* isolates indicated by numbers in boxes were subjected to multilocus sequence analysis (see text and online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0646-Techapp.pdf). *stx2d*, Shiga toxin 2d gene; *astA*, enteroaggregative *E. coli* heat-stable toxin gene; *eae*, intimin gene. Lane M, *Salmonella enterica* serovar Braenderup strain H9812 (used as a DNA size standard); lanes P1, Party 1; lanes P2, Party 2; lanes KS, kitchen staff.

Table 2. Clinical symptoms of party participants during outbreak of gastroenteritis associated with *Escherichia albertii*, by pathogen identified, Japan*

Symptom	*E. albertii*, n = 21†	*E. albertii* and *E. coli* O183:H18, n = 7	*E. coli* O183:H18, n = 3	None,‡ n =11§
Diarrhea	17 (81)¶	7 (100)§	1 (33)	4 (36)
Abdominal pain	16 (76)¶	6 (86)§	2 (67)	3 (27)
Nausea	5 (24)	5 (71)§	0	1 (9)
Fever	8 (38)	4 (57)	2 (67)	4 (36)
None	1 (5)¶	0	0	5 (45)

*Values are no. (%). None, negative for both pathogens.
†One symptomatic person was excluded because no clinical record was available.
‡Negative for both pathogens.
§One person was excluded because no clinical record was available.
¶A 2-tailed Fisher exact test (p<0.05) showed significant differences between the groups from which *E. albertii* or *E. coli* O183:H18 was isolated and the groups from which they were not isolated.

from a larger number of patients, and many fecal specimens yielded only *E. albertii* (Table 1).

The proportion of persons who had clinical symptoms was also higher for *E. albertii*–positive party participants than for uninfected persons (Table 2). Therefore, it is plausible that *E. albertii* was the major causative pathogen of this outbreak. This information indicates that *E. albertii* can cause gastroenteritis outbreaks among humans (5).

More attention should be given to sporadic cases and outbreak cases caused by this emerging pathogen. It may also be informative to revisit past outbreak cases caused by *eae*-positive atypical *E. coli* if pathogens were recorded as being nonmotile, unable to ferment lactose and xylose, and unable to produce β-D-glucuronidase.

Acknowledgments

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Novel Epidemic Clones of *Listeria monocytogenes,* United States, 2011

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We identified a novel serotype 1/2a outbreak strain and 2 novel epidemic clones of *Listeria monocytogenes* while investigating a foodborne outbreak of listeriosis associated with consumption of cantaloupe during 2011 in the United States. Comparative analyses of strains worldwide are essential to identification of novel outbreak strains and epidemic clones.

In September 2011, the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, was notified of an increase of listeriosis cases linked to eating cantaloupe (*1*). The outbreak isolates were categorized into 4 pulsed-field gel electrophoresis (PFGE) profiles and serotypes 1/2a and 1/2b, the latter being seldom associated with large outbreaks (*1,2*). During August 2012, a fifth outbreak-associated subtype responsible for 1 case was detected, and CDC reported a final total of 147 cases from 28 US states, causing 33 deaths and 1 miscarriage (www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/index.html). The Food and Drug Administration (FDA) inspected the involved farm; outbreak strains matching 3 of the PFGE profiles from clinical samples were isolated from washed cantaloupes and various environmental surfaces within the facility (www.fda.gov/Food/FoodSafety/CORENetwork/ucm272372.htm#report).

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Epidemic clones (ECs) of *Listeria monocytogenes* are defined as isolates of a presumably common ancestor that are genetically related and involved in different temporally and geographically unrelated outbreaks (*2*). Previously, multivirulence locus sequence typing (MVLST) accurately identified the 5 known ECs of *L. monocytogenes*, ECI–V (*3,4*). Also, *comK* prophage junction fragment (JF) sequences were demonstrated to be unique to EC strains of *L. monocytogenes* in individual facilities that processed ready-to-eat meat and poultry or in multiple plants manufacturing similar ready-to-eat products (*5*). The *comK* prophage may represent a rapid adaptation island that enables *L. monocytogenes* to rapidly adapt to and form biofilms in specific environmental niches (*5*).

Nine foodborne outbreak-associated isolates related to cantaloupe, representing the 4 outbreak strains initially identified, were selected for multilocus sequence typing (MLST) (*6*), MVLST (*3*), and *comK* prophage JF sequencing (*5*) to determine if they represented previously identified outbreak strains or known/novel ECs of *L. monocytogenes* (*2–4*). Isolates from cantaloupe samples were also compared with 29 US Department of Agriculture (USDA) isolates of *L. monocytogenes* retrieved from 2 US chicken processing plants (*7,8*).

The Study

CDC confirmed identification of *L. monocytogenes* using the AccuProbe LISTERIA MONOCYTOGENES Culture Identification Test (Gen-Probe, San Diego, CA, USA) and by FDA according to the FDA Bacteriological Analytical Manual (www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm). Isolates were serotyped by using commercial antisera (Denka Seiken, Tokyo, Japan) and analyzed by PFGE (*9*) (Table; Figure 1). The online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/12-1167-Techapp.pdf) shows the relative distribution of the 4 PFGE profiles among clinical, food, or environmental samples.

Isolates were grown overnight in tryptic soy broth with yeast extract at 37°C, and DNA was extracted by using the Ultra Clean Microbial DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA, USA) for isolates from CDC and USDA and the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) for isolates from FDA. Sequence types (STs) identified by using MLST were assigned as described (*6*) on the basis of whole genome sequence data (C. Tarr, Y. Chen, unpub. data) and compared with those publicly available (www.pasteur.fr/mlst). MVLST data were obtained as described (*3*) or extracted from whole genome sequences (Y. Chen, unpub. data). Sequences were compared with those on the MVLST database available in the laboratory of S.K. (*3,4*) and analyzed by using MEGA5.0 (*10*). New virulence

Table. Characteristics of *Listeria monocytogenes* isolates representing 1 novel outbreak strain and 2 newly defined epidemic clones, ECVI and ECVII, United States, 2011*

Isolate†	Agency	Outbreak year, location, source (type of source)	Serotype	MLST ST (CC)	MVLST VT (EC)	UP PT	DOWN PT
PFGE profile1‡							
L2624	CDC	2011, US, cantaloupe (C)	1/2b	5 (5)	63 (VI)	–	–
LIS0075	FDA	2011, US, cantaloupe (F)	1/2b	5 (5)	63 (VI)	–	–
LIS0078	FDA	2011, US, cantaloupe (E)	1/2b	5 (5)	63 (VI)	–	–
233	USDA	2002, US, chicken plant A (F)	1/2b	5 (5)	63 (VI)	–	–
466	USDA	2006, US, chicken plant B (F)	1/2b	5 (5)	63 (VI)	–	11
10–0810	NML	1996, Canada, imitation crabmeat (C)	1/2b	5 (5)	63 (VI)	11	11
10–0811	NML	1996, Canada, imitation crabmeat (F)	1/2b	5 (5)	63 (VI)	11	11
PFGE profile 2							
L2625	CDC	2011, US, cantaloupe (C)	1/2a	29 (29)	74	–	–
PFGE profile 3							
L2626	CDC	2011, US cantaloupe (C)	1/2a	561 (7)§	56 (VII)	–	–
LIS0077	FDA	2011 US, cantaloupe (E)	1/2a	561 (7)	56 (VII)	–	–
PFGE profile 4							
L2676	CDC	2011, US, cantaloupe (C)	1/2a	7 (7)	56 (VII)	18	13
LIS0072	FDA	2011, US, cantaloupe (F)	1/2a	7 (7)	56 (VII)	18	13
LIS0087	FDA	2011, US, cantaloupe (E)	1/2a	7 (7)	56 (VII)	18	13
261	USDA	2002, US, chicken plant A (E)	1/2a	7 (7)	56 (VII)	18	13
498	USDA	2006, US, chicken plant B (E)	1/2a	7 (7)	56 (VII)	–	10
10–813	NML	2000 Canada, whipping cream (C)	1/2a	7 (7)	56 (VII)	–	13
10–812	NML	2000 Canada, whipping cream (F)	1/2a	7 (7)	56 (VII)	–	13

*For comparison, additional molecular subtype data from unrelated foodborne outbreaks in Canada were obtained (*4*). PFGE, pulsed-field gel electrophoresis; CDC, Centers for Disease Control and Prevention; FDA, Food and Drug Administration; USDA, US Department of Agriculture; NML, National Microbiology Laboratory of Canada, Division of the Public Health Agency of Canada; MLST ST (CC), multilocus sequence typing, sequence type (clonal complex); MVLST VT (EC), multivirulence locus sequence typing, virulence type (epidemic clone); UP PT, upstream *comK* prophage type; DOWN PT, downstream *comK* prophage type; –, no PCR amplification of junction fragment.
†The numbering of outbreak strain used differs from the FDA's final update on the outbreak (www.fda.gov/Food/FoodSafety/CORENetwork/ucm272372.htm#report).
‡PFGE profiles based on CDC and FDA analysis of isolates from cantaloupe-associated outbreak.
§ST561 differs from ST7 by 1 nonsynonymous single nucleotide polymorphism (G/A) in *lhkA*.

types (VTs) were assigned to USDA isolates: VT60 (isolates 239, 441, 442, 458, 541, 565, 577); VT68 (350, 470); VT69 (247); VT70 (502); VT71 (450); VT72 (342), and VT73 (267). *comK* prophage JFs were sequenced as described (*5*). Prophage types (PTs) were assigned by comparing JF sequences with those available from previous reports (*4,5*). *comK* prophage JF sequences were submitted to GenBank for isolate L2676 (accession nos. JQ407079 and JQ407080) and 3 USDA isolates (accession nos. JQ750615–JQ750618).

Isolates L2624, LIS0075, and LIS0078 (PFGE profile 1) belonged to the globally disseminated ST5 (*6*) and had the same VT (VT63) as 5 other 1/2b isolates in the database: isolates 10-0810 and 10-0811, from an imitation crabmeat–borne outbreak in Canada during 1996 (*4,11*); and isolates 98-0041, 233, and 466 (Table; Figure 2). Because VT63 isolates were associated with multiple outbreaks, they should be considered part of a novel EC (ECVI). ECVI isolates from cantaloupe and USDA isolate 233 showed no amplification of *comK*

Isolates	PFGE profile	AscI pattern	ApaI pattern
L2624, LIS0075, LIS0078	1	GX6A16.0019	GX6A12.0227
L2625	2	GX6A16.0029	GX6A12.0069
L2626, LIS0077	3	GX6A16.0099	GX6A12.0001
L2676, LIS0072, LIS0087	4	GX6A16.0001	GX6A12.0001
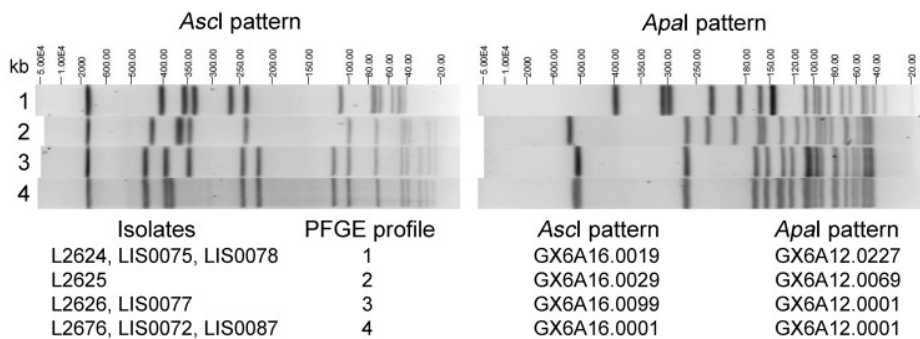
Figure 1. Four *Asc*I / 4 *Apa*I pulsed-field gel electrophoresis (PFGE) profiles (identified at the time the research was performed) displayed by *Listeria monocytogenes* clinical isolates (L2624, L2625, L2626, and L2676) and isolates from food or environmental samples (LIS0072, LIS0075, LIS0077, LIS0078, and LIS0087) associated with the 2011 listeriosis outbreak traced to cantaloupe. PFGE profiles 3 and 4 differ by ≈40-kb shift in 1 band in the AscI pattern, likely related to the loss or acquisition of the comK prophage, because the size of this prophage was ≈40 kb as calculated by using the whole genome sequencing data (not shown).

prophage JFs (Table). PT11/11 was identified during the 1996 imitation crabmeat–associated outbreak in Canada (*4*) and in USDA isolate 466 (Table). Further research is needed to determine why *comK* PTs were identical during different years and in different geographic locations and food processing plants.

Isolate L2625 (VT74, PFGE profile 2) from cantaloupe differed by 1 single nucleotide polymorphism in *inlC* from 3 other serotype 1/2a VT61 isolates (10-4758, 10-4754, and 06-6956) associated with the 2002 cheese-associated listeriosis outbreak in Canada (*4,12*) (Table; Figure 2). L2625 was assigned to ST29, an infrequent sequence type in the Institut Pasteur MLST database that differs from the ST (ST405) assigned to the isolates from cheese in the 2002 outbreak in Canada. No amplification of *comK* prophage JFs was observed, consistent with the PTs in the 2002 cheese-associated outbreak in Canada (*4*). Given this evidence, isolate L2625 does not represent a novel EC but should be considered a novel outbreak strain.

Isolates L2626 and LIS0077 (PFGE profile 3, ST7) and L2676, LIS0072, and LIS0087 (PFGE profile 4, ST561) from cantaloupe samples shared the same VT (VT56) as isolates 10-0813 and 10-0812 associated with a listeriosis outbreak related to whipping cream during 2000 in Canada (*4,12*) and isolates 06-6909, BL0047, 261, and 498 (Table; Figure 2). These *Listeria* isolates from cantaloupe displayed 2 highly similar PFGE profiles and STs, and the same serotype, *Apa*I PFGE pattern, and VT (Table; Figure 1). Isolates L2626 and LIS0077 showed no amplification of *comK* prophage JFs, which was also consistent with the upstream PT in the outbreak associated with whipping cream in Canada (Table). The JF sequences in isolates L2676, LIS0072, and LIS0087 were identical to those in USDA isolate 261 (Table). These isolates matched those from the whipping cream–associated outbreak in Canada in terms of VT56 and downstream PT (PT13) (Table). However, the upstream JF could not be amplified in the strain identified in whipping cream (*4*), possibly because of extensive recombination within the *comK* prophage (*13*), especially in the upstream JF (*5*). These STs and VTs were also found in clinical isolates over extended periods (*6*). Therefore, by definition (*2,3*), these isolates also represent a novel EC (ECVII).

Conclusions

Different clones, particularly ECVI and ECVII, might have cocolonized niches or harborage sites within the cantaloupe processing facility, possibly explaining the multiple strains associated with this outbreak. Serotype 4b *L. monocytogenes* strains, of the same genetic lineage as serotype 1/2b strains, reportedly survived and grew substantially better in mixed-serotype biofilms containing a specific strain of serotype 1/2a (*14*). Although a biofilm was not detected in the cantaloupe facility, because
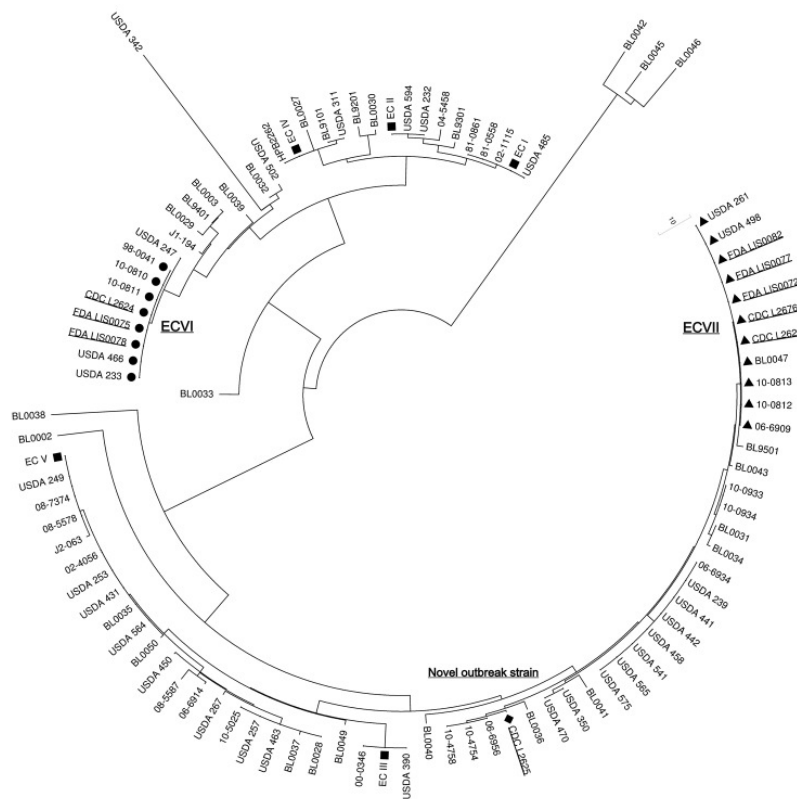
Figure 2. Unrooted neighbor-joining tree computed in MEGA 5.0 (*10*) for multivirulence locus sequence typing) data based on sequencing of 6 virulence genes, *prfA, inlB, inlC, dal, clpP,* and *lisR* (*3*) obtained for the 93 *Listeria monocytogenes* isolates compared in this study. Nine cantaloupe-associated outbreak isolates were from the Centers for Disease Control and Prevention (CDC) (GenBank accession nos. JQ407055–JQ407078) and Food and Drug Administration (FDA) (JX141237–JX141275), 23 isolates from Knabel et al., 2012 (*4*), 29 isolates from Chen et al., 2007 (*3*), 29 from Berrang et al., 2005 and 2010 (*7,8*) (JQ946653–JQ946836), and 3 other isolates for which sequence data were available in GenBank. Squares indicate reference strains representing the 5 currently known *L. monocytogenes* epidemic clones. Underlined text indicates CDC and FDA isolates associated with the isolates from the 2011 cantaloupe-associated outbreak. Circles indicate strains classified as epidemic clone (EC)VI. Triangles indicate strains classified as ECVII. Diamond indicates the novel outbreak strain.

the facility had already been extensively cleaned and sanitized before FDA sampling, further research is needed to determine the potential for these strains to cocolonize with biofilms.

Six of the 7 currently identified ECs were found at some point in 1 or both of the US chicken processing plants included in the study (Figure 2). Listeriosis cases and outbreaks have been associated with consumption of undercooked raw chicken and ready-to-eat poultry products (2,4). Additional research is needed to determine whether poultry or poultry processing plants could be responsible for the global dissemination of ECs of *L. monocytogenes*.

The molecular epidemiology of *L. monocytogenes* strains involved in the 2011 multistate cantaloupe-associated outbreak was greatly enhanced by the use of subtyping markers with different levels of epidemiologic resolution. Particularly, MVLST enabled the detection of 1 novel 1/2a outbreak strain and 2 novel ECs of *L. monocytogenes*. In contrast to focusing on isolates from a single outbreak (15), our findings demonstrate that to detect new ECs it is important to analyze isolates from many sources around the world.

Acknowledgments

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Rinderpest Virus Sequestration and Use in Posteradication Era

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After the 2011 declaration of rinderpest disease eradication, we surveyed 150 countries about rinderpest virus stocks. Forty-four laboratories in 35 countries held laboratory-attenuated strains, field strains, or diagnostic samples. Vaccine and reagent production and laboratory experiments continued. Rigorous standards are necessary to ensure that stocks are kept under safe conditions.

During 2011, a major milestone in global infectious disease control was achieved. Rinderpest was declared eradicated by the Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE). Rinderpest is a disease caused by rinderpest virus, a paramyxovirus of the genus *Morbillivirus*; the disease has had a devastating effect on livestock health, productivity, and welfare. It became the second disease to be eradicated, 30 years after smallpox eradication. The last reported outbreak of rinderpest occurred during 2001 in Kenya (*1*).

Unintentional or deliberate virus release remains a serious concern as long as virus-containing material remains in the possession of vaccine manufacturers or research and diagnostic laboratories. An example to substantiate this concern is the last reported case of smallpox in a human, which originated from a laboratory (*2*). To manage the risk for rinderpest reintroduction, identifying these potential virus sources is essential. We conducted a questionnaire survey to assess the location and number of rinderpest virus stocks, their uses, and their storage conditions.

The Study

We developed 2 password-protected online questionnaires, 1 for national veterinary authorities and 1 for labo-

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ratory staff. Questionnaires were available in the 6 official languages of the United Nations: English, French, Spanish, Russian, Arabic, and Chinese. A snowball sampling approach was adopted. Questionnaires were first sent in May 2011 to 192 national veterinary authorities and to 126 laboratories for which contact details were known. National veterinary authorities were asked to forward the laboratory questionnaire to all government, university, and private laboratories that might be involved in cattle virus diagnostics, research, or vaccines within their countries. Laboratory personnel were also asked to forward the survey to other laboratories. Questionnaire responses were inconsistent for 4 survey participants, who were then contacted for clarification. Survey data were treated confidentially.

This survey captured data from 100 countries (81 national veterinary authorities and 100 laboratories). It was complemented with the results of a previous questionnaire survey undertaken during 2010 by the FAO and OIE to identify countries holding rinderpest virus stocks and with information obtained from unpublished, or "grey," literature and direct discussions with laboratory staff. As a result, information was available for 150 (76%) of the 198 OIE or FAO member and associate member countries (Figure).

Thirty-five countries held rinderpest virus–containing material: 37% in Asia, 29% in Africa, and 26% in Europe. Extrapolating these results for countries for which information was not available, we estimated a global tally of 41 (95% credible interval 37–47) countries that held rinderpest virus (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0967-Techapp.pdf).

A susceptible host infected by a laboratory-attenuated strain cannot become infectious unless mutations facilitating virus reversion to transmissibility occur during infection. Laboratory-attenuated strains were the most commonly held virus type, kept in 34 countries, mainly as live vaccines or virus seed for vaccine production. Live vaccine stocks could be quantified for 11 countries, 4 of which had 100,000–4,000,000 doses. Field strains, which included mild to virulent strains that are transmissible between hosts, were held in 11 countries. We also received incomplete information; for example, several laboratory personnel reported holding virus in the past but did not specify whether their laboratories still held it. When we accounted for this incomplete information, we determined that 14 countries could be holding field strains (Figure). Diagnostic samples, which included blood and tissues from animals infected or suspected to be infected, were held in 10 countries.

Rinderpest virus manipulations were being performed by some countries. A total of 115,000 live vaccine doses were produced in 1 country in 2011, and diagnostic reagent tests have been produced by using live virus in 3 countries since 2010. Basic research was still being conducted in 3 countries, of which 1 was conducting in vivo experiments
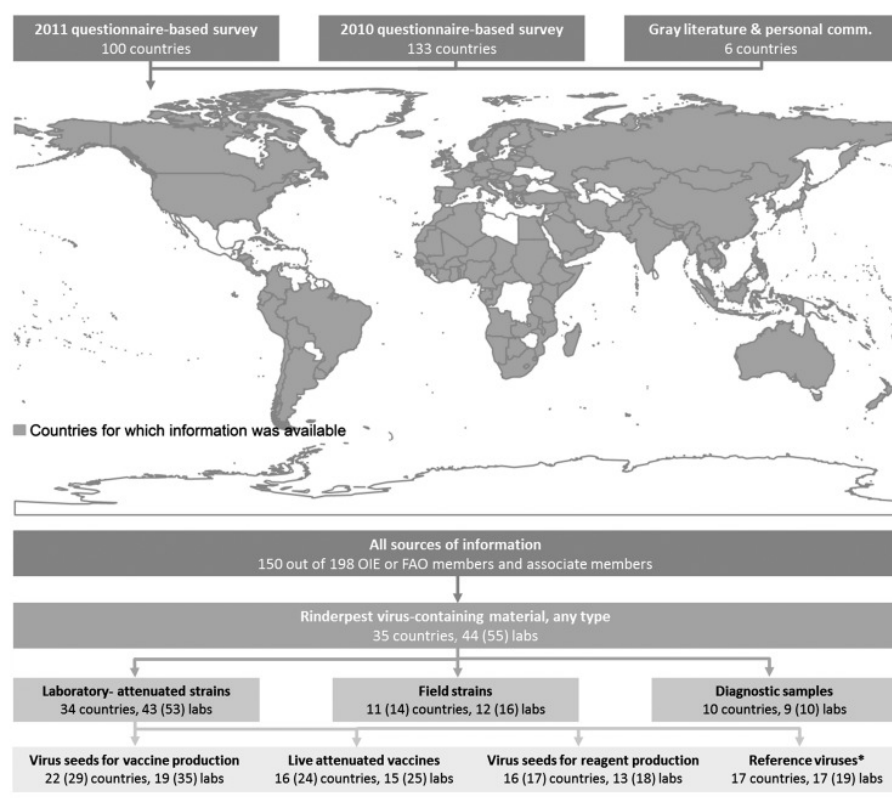
Figure. Countries and laboratories holding material containing rinderpest virus, June 2011. Numbers in parentheses indicate the potential number of laboratories or countries holding a given type of virus when accounting for incomplete information. *Reference virus or specimens as part of a collection for research or teaching. FAO, Food and Agriculture Organisation of the United Nations; OIE, World Organisation for Animal Health.

on rabbits using laboratory-attenuated strains. Laboratories in some countries stated that they planned to develop marker and recombinant vaccines and to maintain and upgrade diagnostic reagent stocks. The sequencing of field strains was also being considered.

We identified 44 laboratories holding viruses. Among the 36 laboratories for which biosafety level (BSL) was known, 11 (31%) of 36 laboratories holding attenuated strains were classified as BSL-2 or lower, as were 2 (17%) of 12 laboratories holding field strains and 3 (38%) of 8 laboratories holding diagnostic samples. Nine (39%) of 23 laboratories reported providing veterinary authorities with an inventory of the material containing rinderpest virus that were held, and 3 (13%) laboratories reported that authorization from veterinary authorities was required before they could manipulate rinderpest viruses.

Fourteen national veterinary authorities reported holding a complete and up-to-date inventory of all rinderpest virus for all laboratories within the country. However, 43% of them did not report all of the virus types that were reported as being held by the laboratories themselves.

Veterinary authorities of the 66 countries where rinderpest vaccines were used during the past 30 years were invited to forward a separate online questionnaire to field veterinarians, to assess their awareness of rinderpest and vaccine use. Of 70 respondents from 21 countries, none reported any use of rinderpest vaccines after 2003; however,

36% of respondents from 9 countries estimated that the likelihood of using rinderpest vaccines for routine vaccination against rinderpest or peste des petits ruminants, another morbillivirus disease, in their region within the next 5 years was "possible" to "very likely."

Conclusions

Because data were not available for all countries or all laboratories within countries, the number of virus stocks was probably underestimated. Some laboratory staff could retain material containing rinderpest virus without having ever conducted research on rinderpest or reported their stocks to their national authority. These laboratories might have been missed by our sampling approach. The number of respondents might have been greater if we had made questionnaires available in other languages. However, despite the sampling limitations, we can conclude that a large number of countries hold material containing rinderpest virus.

Some veterinary authorities did not report all the virus types held in their countries, despite most of them claiming to maintain an inventory. Unless all virus stocks in all countries can be assessed, virus sources could be missed by future relocation plans.

The reasons given for conducting basic research on rinderpest included that rinderpest viruses were a good model for studying aspects of morbilliviruses, such as mechanisms

of replication, host pathogenicity, and interactions between host cell factors and viruses. Although such research might still be relevant and the production and management of vaccine and diagnostic reagent stocks could be key components of contingency plans, modifications to laboratory activities and protocols are needed to meet compliance standards recently recommended by FAO and OIE (*3*). Under these new standards, all laboratories holding any virus stocks must meet BSL-3 criteria, and manipulations involving rinderpest virus must be approved by the national veterinary authority, as well as by FAO and OIE. Adhering to the new standards and subjecting activities to approval also applies to the potential reconstruction of rinderpest virus de novo, given that the technical capacity and genome sequences are available.

Some field veterinarians reported believing that rinderpest vaccines might be used in their region in the near future. We did not investigate the reasons behind these opinions, but awareness clearly needs to be raised in the veterinary sector about risks for rinderpest reintroduction despite eradication and, more specifically, about the restricted use of rinderpest vaccines solely for the management of confirmed rinderpest outbreaks (*3*).

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Schmallenberg Virus in Central Nervous System of Ruminants

To the Editor: In 2011, a new virus spread throughout ruminant populations in the Netherlands, Germany, and other European countries (*1*). Infected dairy cattle exhibited fever, reduced milk yield, loss of appetite, and diarrhea. Weeks after these signs appeared, epidemic abortions; births of malformed or stillborn animals; and perinatal deaths of calves, lambs, and goat kids were reported. Metagenomic analysis identified a novel orthobunyavirus (family *Bunyaviridae*), termed Schmallenberg virus (SBV), which is closely related to Akabane and Shamonda viruses (1). These arthropod-borne viruses are well-recognized ruminant pathogens in Africa, Asia, and Oceania; fetal infection is associated with an arthrogryposis–hydranencephaly syndrome.

Previous reports about SBV have addressed clinical signs (*2*), serologic findings (*3*), pathologic findings (*4*), and SBV RNA distribution in organs of malformed calves (*5*) detected by real-time quantitative reverse transcription PCR (qRT-PCR). However, pathogenesis and viral cell tropism are largely unknown. Therefore, our aims were to establish an in situ hybridization method (ISH) to detect SBV mRNA, to evaluate the usefulness of this method as a complementary diagnostic tool, and to further analyze SBV pathogenesis.

The in situ probe was generated according to established protocols (*6*). The SBV qRT-PCR product and primers used for diagnostics (*5*) were used to amplify an 88-bp segment of the nucleoprotein, encoded by the small segment of the SBV genome. The amplicon was cloned into a pCR4-TOPO vector (Invitrogen, Darmstadt, Germany) and sequenced. For generation of a digoxigenin-labeled antisense probe to detect viral mRNA, we used M13 reverse and SBV-S-469R prim-

ers (*5*). Specificity of the probe was ensured by distinct ISH signals in the brains of SBV-positive (as determined by qRT-PCR) animals (*6*). No reaction was detected in SBV-negative (as determined by qRT-PCR) goats, sheep, and calves; in ruminants with various non–SBV-associated nervous system lesions; or in the brain of a mouse that was experimentally infected with Akabane virus.

Thereafter, we investigated SBV mRNA distribution in the central nervous system (CNS) of 82 naturally infected ruminants (46 lambs, 2 goat kids, and 34 calves), all of which had previously been found by qRT-PCR to be positive for SBV. In addition, ISH was used to examine the following tissues from 10 of these animals (4 lambs, 1 goat kid, and 5 calves): placenta, muscle, eye, heart, aorta, lung, trachea, liver, kidney, spleen, small and large intestine, mesenteric and pulmonary lymph nodes, thymus, adrenal gland, testis, and uterus.

SBV mRNA was found in varying amounts, predominantly in neurons (Figure) of the cerebrum, cerebellum, brain stem, medulla oblongata, and spinal cord of 7 lambs, 1 goat kid, and 2 calves. Randomly distributed clusters of SBV-positive neurons were frequently found in small ruminants, whereas only single positive cells were found in both calves. SBV mRNA was not found in any peripheral organ. The online Technical Appendix (wwwnc. cdc.gov/EID/pdfs/12-0764-Techapp. pdf) provides an overview of ISH results in relation to malformations and inflammatory CNS lesions. Histologic examination identified encephalitis, characterized by lymphohistiocytic, perivascular cuffs, in 11 (9 lambs, 1 goat kid, and 1 calf) of the 82 animals. Among animals that were positive according to ISH, all small ruminants showed inflammation, whereas both calves lacked inflammatory changes.

The most frequent macroscopic lesions or conditions in animals that were SBV positive by qRT-PCR,

especially calves, were arthrogryposis, brachygnathia inferior, torticollis, kyphosis, lordosis, scoliosis, and muscle hypoplasia (especially in calves) (*4*). The predominant CNS lesions or conditions were cerebellar and cerebral hypoplasia, hydranencephaly, porencephaly, hydrocephalus, and micromyelia. Among the 8 small ruminants that were SBV positive by ISH, hydrocephalus was found in 5, cerebellar hypoplasia in 5, hydranencephaly in 1, and arthrogryposis in 6. For the 2 calves that were SBV positive, micromyelia and arthrogryposis were found in both and cerebellar hypoplasia in 1.

This study indicates that neurons are the predominant target in SBV-infected neonates. Surprisingly, only 10 of 82 ruminants were positive for SBV by qRT-PCR and ISH. This discrepancy could result from low SBV mRNA copy numbers per cell, which are detectable by qRT-PCR but not ISH, and/or from reduced viral load in individual cells because of the long time between assumed infection in early gestation and time of examination after birth (≈17 weeks in sheep and 27 weeks in calves). The latter explanation is indicated by the high incidence of malformations (*7*) typical for teratogenic insults and might especially apply to calves. Detection of CNS inflammation in all small ruminants that were SBV positive according to ISH indicates a cause-and-effect association between high SBV mRNA copy numbers per cell and lymphohistiocytic immune response. Furthermore, low numbers of infected cells and/or reduced replication as assumed for animals that were positive by qRT-PCR but negative by ISH might explain the absence of inflammation in the calf brains. The 3 tested animals in which inflammation was found but that were SBV negative by ISH could represent an advanced resolving lesion after viral clearance or reduction. In situ detection of SBV mRNA represents a suitable way to study SBV pathogenesis, especially in the active phase of infection, and
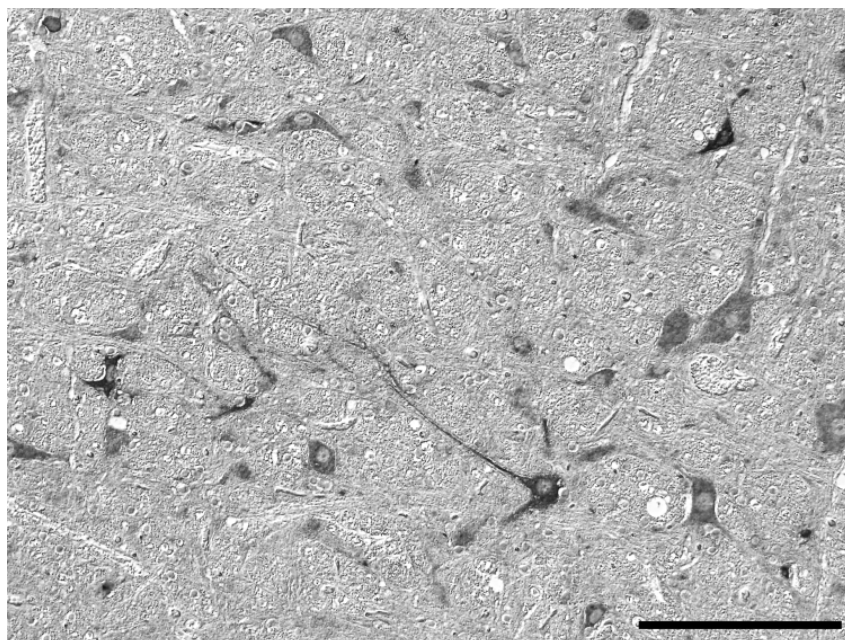
Figure. In situ detection by using Nomarski differential interference contrast microscopy of Schmallenberg virus mRNA in neurons in the medulla of a neonatal sheep for which Schmallenberg virus infection was confirmed by real-time quantitative reverse transcription PCR. Scale bar = 100 μm.

might enable identification of SBV as the causative agent in cases of CNS inflammation of unknown etiology.

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Polyomavirus in Saliva of HIV-infected Children, Brazil

To the Editor: Human polyomaviruses (HPyVs) are members of the family *Polyomaviridae*. Nine distinct PyVs can infect humans: BKPyV, JCPyV, WUPyV, KIPyV, MCPyV, TSPyV, HPyV6, HPyV7, and HPyV9 (*1*). Primary infections generally occur early in life, are typically subclinical, and are followed by persistence of the virus in the person. Reactivation of infection has been associated with disease in immunocompromised persons (*2–6*). We detected the excretion of HPyV in the saliva of HIV-infected children and compared this finding with its prevalence in healthy control children to evaluate the possible association between viral infection and the stage of immunodeficiency.

Samples were collected during August 2009–June 2011 from patients attending the School of Dentistry of the Federal University of Rio de Janeiro, Brazil. Saliva samples were obtained from 60 HIV-infected children (27 (44.9%) boys, 33 (55.1%) girls), 6–13 years of age (median 9.5 years),

Table. HPyVs detected in saliva from HIV-infected and healthy control children, Brazil*

Virus	No. (%) HIV-infected children, n = 60	No. (%) control children, n = 60
BKPyV	3 (5.0)	0
JCPyV	2 (3.3)	4 (6.6)
WUPyV	1 (1.7)	1 (1.7)
KIPyV	8 (13.3)	0
BKPyV+KIPyV	1 (1.7)	1 (1.7)
JCPyV+KIPyV	2 (3.3)	0
Total	17 (28.3)	6 (10.0)

*HPyV, human polyomavirus.

and 60 healthy children (47.9% male, 52.1% female), 7–12 years of age (median 9.04 years). The study protocol was approved by the Ethics Committee of the Hospital Universitário Clementino Fraga Filho/University of Rio de Janeiro. The parents of all children involved in the study gave informed consent.

Virus DNA was extracted by using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Specimens were tested for HPyV by real-time PCR (7,8). For BKPyV, we used primer pair BKV-F: 5′-GGT-GTAGATCAGAGGGAAAGT-3′ and BKV-R: 5′-TTGCCAGTG AT-GAAGAAG C-3′. Statistical significance was assessed by p<0.05.

HPyVs were detected in 17 (28.3%) and 6 (10%) of HIV-infected and control children, respectively (Table). A higher frequency of viral infection was observed in the HIV-infected group (p = 0.01). Frequency of KIPyV infection was significantly higher among immunocompromised children (p = 0.02). No difference was observed for BKPyV, JCPyV, or WUPyV. The virus loads were similar in both groups (data not shown).

HIV-infected persons were classified into 3 immunologic categories: no evidence of immune suppression (CD4+ >500 cells/µL; n = 38), moderate suppression (CD4+ 200–499 cells/µL; n = 13), and severe suppression (CD4+ <200 cells/µL; n = 9). HPyV was more frequently detected among children with severe immunosuppression (n = 7; p<0.001). However, no significant correlation was observed between the frequency of

HPyV DNA detection and the use of highly active antiretroviral therapy (HAART) (p = 0.156).

Because the immunosuppressed population is increasing around the world, the role of HPyVs as opportunistic pathogens in these persons has become a great concern (2–6). In this study, we found that the frequency of HPyV infections was higher among HIV-infected children than among the general pediatric population, although infection was not associated with the person's CD4+ cell count. The viral loads were similar in both groups, suggesting that efficiency of viral replication is not related to the person's immune status. None of the HPyV-positive children, including those with severe immunosuppression (data not shown), showed any symptoms of illness associated with these viruses, such as urinary tract, neurologic, or respiratory tract infection.

Previous studies analyzed the occurrence of HPyV infections in immunosuppressed persons with AIDS. Sharp et al. investigated the presence of WUPyV, KIPyV, and MCPyV in lymphoid tissue samples from persons with AIDS and healthy controls and found a much higher frequency of infection in the immunosuppressed group (9). Babakir-Mina et al. investigated the frequency KIPyV and WUPyV in blood of HIV-1–infected patients compared with blood donors and demonstrated that WUPyV infection was more frequent in HIV-infected patients but the frequency of infection for KIPyV was similar in both groups; they also found no as-

sociation between CD4+ cells count and HPyV infection (2). Machado et al. investigated the urinary excretion of BKPyV and JCPyV among HIV-1–infected children and adolescents and healthy controls and demonstrated a significantly higher BKPyV viruria in HIV-infected patients. No difference was observed for JCPyV excretion, however, and no association was found between CD4+ values and viral shedding (3). Jeffers et al. assessed the salivary shedding of BKPyV on a cohort of healthy and HIV-immunosuppressed persons and found that BKPyV DNA levels in the saliva were significantly higher in HIV-infected patients. They also demonstrated the ability of a BKPyV to replicate in vitro in salivary gland cells and suggested that salivary glands may constitute a reservoir for BKPyV (10). Jeffers and Webster-Cyriaque, while investigating the contribution of viral infection to the pathogenesis of salivary gland diseases, detected BKPyV shedding in the saliva of HIV-positive patients with salivary gland diseases more often than in healthy controls and suggested that it played a possible role in the disease (4). In contrast, other studies did not detect BKPyV or JCPyV in saliva of either HIV-infected or healthy controls (6,7).

In this study, we detected DNA of BKPyV, JCPyV, WUPyV, and KIPyV in saliva samples of both HIV-positive and healthy control children, although the frequency of infection was significantly higher among the HIV-infected children. These findings suggest that saliva may be a route of HPyV transmission and that the oral cavity could be a site of virus replication and persistence.

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Carbapenem-hydrolyzing Oxacillinase-48 and Oxacillinase-181 in Canada, 2011

To the Editor: In 2001, a *Klebsiella pneumoniae* isolate from a patient in Turkey was found to harbor a novel class D carbapenem-hydrolyzing oxacillinase, OXA-48 (1). Although this enzyme hydrolyzes carbapenems at a low level and shows weak activity against expanded-spectrum cephalosporins, it is often associated with other β-lactamases and is multidrug resistant. Reports of *Enterobacteriaceae* harboring OXA-48 have been described across Europe, the Mediterranean area, and the Middle East (2). In addition, OXA-181, which differs from OXA-48 by 4 aa substitutions, has been described in India (2). We describe the emergence of OXA-48 and OXA-181 in Canada.

Hospital and provincial public health laboratories in Canada voluntarily submitted *Enterobacteriaceae* isolates to the National Microbiology Laboratory. Isolates submitted by the laboratories were not susceptible to carbapenems and were to be tested by PCR for carbapenemase genes (KPC, NDM, IMP, VIM, OXA-48, and GES) (3). During April–November 2011, a total of 4 isolates (3 *K. pneumoniae*, 1 *Escherichia coli*) tested positive for the *bla*OXA-48–type gene. Sequencing, using the primers preOXA-48A and -48B (4), revealed that 3 of the isolates (*K. pneumoniae* 11-882 and 11-2720 and *E. coli* 11-1498) possessed the *bla*OXA-48 gene, and the other isolate (*K. pneumoniae* 11-2568) possessed the *bla*OXA-181 gene. We conducted additional β-lactamase PCR and sequencing as described (3) (Table). The Modified Hodge test (using a 10-μg disk of ertapenem and meropenem) showed that all isolates were strongly positive for carbapenemase production.

Table. Patient data and antimicrobial drug susceptibility profiles for isolates of *Klebsiella pneumoniae* and *Escherichia coli* harboring OXA-type carbapenemases, Canada, 2011*

| | Bacterial isolate, strain no., OXA-type | | | | | | |
| | *K. pneumoniae* | | | *E. coli* | Plasmid | | |
Variable	11-882, OXA-48	11-2568, OXA-181	11-2720, OXA-48	11-1498, OXA-48	p48-11-882	p181-11-2568	*E. coli* DH10B†
Patient data					NA	NA	NA
Recent travel	NK	India	Lebanon	Dubai	NA	NA	NA
Site of bacterial isolation	Urine	Perirectal	Urine	Skin swab	NA	NA	NA
Infection/colonization	NK	Colonization	Infection	Colonization	NA	NA	NA
Hospitalization	NK	General ward	Outpatient	ICU	NA	NA	NA
Bacterial isolate data							
β-lactamase	TEM-1, SHV-11	TEM-1, SHV-11, CTX-M-15, OXA-1	SHV-11, OXA-1	TEM-1, CTX-M-24	TEM-1	Neg	NA
Sequence type	395	147	831	38	NA	NA	NA
Antimicrobial drug susceptibility test, drug‡							
Vitek2,§ MIC µg/mL							
Amikacin	<2.0	4.0	<2.0	<2.0	<2.0	<2.0	<2.0
Aztreonam	<1.0	>64.0	<1.0	32.0	<1.0	<1.0	<1.0
Cefazolin	32.0	>64.0	16.0	>64.0	>64.0	32.0	<4.0
Cefepime	<1.0	>64.0	<1.0	<1.0	<1.0	<1.0	<1.0
Ceftriaxone	<1.0	>64.0	<1.0	>64.0	<1.0	<1.0	<1.0
Ciprofloxacin	>4.0	>4.0	<0.25	<0.25	<0.25	<0.25	<0.25
Ertapenem	>8.0	>8.0	4.0	4.0	>8.0	4.0	<0.5
Gentamicin	<1.0	>16.0	<1.0	>16.0	<1.0	<1.0	<1.0
Imipenem	4.0	>16.0	<1.0	<1.0	8.0	2.0	<1.0
Meropenem	1.0	>16.0	<0.25	0.5	8.0	<0.25	<0.25
Tigecycline¶	1.0	2.0	<0.5	<0.5	<0.5	<0.5	<0.5
TMP/SXT	>320.0	>320.0	<20.0	>320.0	>320	<20	<20.0
Etest,§ MIC µg/mL							
Imipenem	1.0	24.0	0.38	0.38	0.5	0.5	0.19
Meropenem	0.5	24.0	0.19	0.38	0.125	0.19	0.032
Ertapenem	2.0	>32.0	0.5	2.0	0.38	0.75	0.008
Colistin#	0.38	0.5	0.5	0.5	0.125	0.125	ND
Disk diffusion,** zone diameter, mm							
Imipenem	20	8	23	22	24	25	33
Meropenem	20	7	23	22	22	28	35
Ertapenem	16	6	19	16	23	22	35

*NK, not known; NA, not applicable; ICU, intensive care unit; Neg, negative; TMP/SXT, trimethoprim/sulfamethoxazole; ND, not done.
†OXA-48–type plasmids were electroporated into *E. coli* DH10B cells.
‡Additional antimicrobial drugs on panel: ampicillin, ampicillin/sulbactam, nitrofurantoin, piperacillin/tazobactam, tobramycin.
§Test from bioMérieux Canada Inc., St. Laurent, Quebec, Canada.
¶MIC breakpoints for tigecycline were based on the US Food and Drug Administration criteria for *Enterobacteriaceae*: susceptible, <2 µg/mL; intermediate, 4 µg/mL; resistant, >8 µg/mL.
#MIC breakpoints for colistin followed Clinical Laboratory Standards Institute breakpoint criteria for *Acinetobacter* spp.: susceptible, MIC <2 µg/mL; resistant, MIC >4 µg/mL.
**Test from Clinical Laboratory Standards Institute (www.clsi.org/source/orders/free/m100-s22.pdf).

The Table shows clinical data and antimicrobial drug susceptibility profiles for the *K. pneumoniae* and *E. coli* harboring OXA-type carbapenemases. Half of the isolates were from men >65 years of age. Three of the case-patients had a history of travel to regions where OXA-48–type carbapenemases are endemic (Lebanon, India, and Dubai), and while still abroad (shortly before being hospitalized in Canada), 2 case-patients had sought medical attention.

Pulsed-field gel electrophoresis of 3 *K. pneumoniae* isolates digested with *Xba*I showed unique fingerprint patterns (data not shown). Multilocus sequence typing (www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) of *K. pneumoniae* 11-2568, 11-882, and 11-2720 showed that they belonged to sequence type (ST) 147, ST395, and ST831, respectively. ST395 has been identified in *K. pneumoniae* from Morocco, Amsterdam, and France harboring blaOXA-48 (2). E. coli 11-1498 was shown to belong to sequence type 38, previously described in a nosocomial outbreak and in isolated cases of

OXA-48 in France; travel to Morocco and Egypt was linked to those cases (5,6). Phylogenetic grouping of the *E. coli* isolate revealed that it belonged to group D, which is associated with virulent extraintestinal strains (7).

Antimicrobial drug susceptibilities were determined by using Vitek 2 (GN-25) (bioMérieux, Canada Inc., St Laurent, Quebec, Canada) and interpreted by using the 2012 guidelines (M100-S22) of the Clinical Laboratory Standards Institute (www.clsi.org/source/orders/free/m100-s22.pdf) (Table). All but 1 isolate (*K. pneumoniae*

11-2720) was multidrug resistant (defined as resistant to >3 antimicrobial drug classes). All isolates were resistant to ampicillin, cefazolin, and pipericillin/tazobactam. Two isolates were resistant to broad-range cephalosporins, both of which contained the CTX-M–type extended-spectrum β-lactamase. All isolates were also resistant to ertapenem, 2 of 4 were resistant to imipenem, and 1 was resistant to meropenem. Results were confirmed by Etest (bioMérieux, Canada Inc.) and Clinical Laboratory Standards Institute disk diffusion, with the exception of results for *K. pneumoniae* 11-882, which showed susceptibility to meropenem and imipenem by Etest and intermediate susceptibility by disk diffusion (Table). All isolates were susceptible to tigecycline and colistin.

PCR mapping, using previously described primers (8), identified the *bla*OXA-48 gene located on the transposon Tn1999. The *bla*OXA-181 gene was found downstream of IS-Ecp1, as described (4). We isolated plasmid DNA by using QIAGEN Plasmid Mini Kits (QIAGEN, Mississauga, ON, Canada) and attempted to transfer the plasmid harboring the *bla*OXA-48–type gene, using electroporation into *E. coli* DH10B, as described (3). Plasmid transfer of *bla*OXA-48–type genes was successful only for *K. pneumoniae* 11-882 and 11-2568. PCR revealed the transfer of *bla*TEM-1 and *bla*OXA-48 along with a plasmid of ≈114 Kb (p48-11-882). In addition, *bla*OXA-181 was transferred along with a plasmid of »4 Kb (p181-11-2568) with no additional β-lactamases present.

Replicon typing (9,10), using primers for incompatibility group IncR (IncRrv 5′-GTGTGCTGTGGT-TATGCCTCA-3′), showed that p48-11-882 belonged to IncR and to the OXA-48–associated IncL/M. Plasmid p181-11-2568 was negative for all replicons tested.

Transformants were resistant to ampicillin, cefazolin, and pipericillin/

tazobactam but susceptible to third- and fourth-generation cephalosporins. This finding is not surprising because OXA-48 and OXA-181 show weak activity against expanded-spectrum cephalosporins (1,4). Resistance to third- and fourth-generation cephalosporins in clinical isolates is likely caused by a combination of additional mechanisms, such as porin mutations, or additional β-lactamases, such as CTX-M–type, extended-spectrum β-lactamases (Table).

We report the emergence of OXA-48 and OXA-181 in North America. The emergence of carbapenem-resistant *Enterobacteriaceae* in Canada is of concern because they are difficult to detect in the laboratory, and treatment options are lacking. The history of travel by the patients in our study to regions where carbapenem-resistant *Enterobacteriaceae* are endemic highlights the necessity for understanding the potential risk factors associated with acquiring these multidrug-resistant pathogens.

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Characterization of Peste des Petits Ruminants Virus, Eritrea, 2002–2011

To the Editor: Peste des petits rumniants (PPR) is a highly contagious viral disease causing high rates of mortality among domestic and wild small ruminants in Africa and Asia. The disease causes economic losses in the countries where it is present. Peste des petits ruminants virus (PPRV) is a negative-sense, single-stranded RNA virus of the genus *Morbillivirus*, in the family *Paramyxoviridae*. Analysis of a small sequence of the PPRV nucleoprotein (NP) gene permits classification of the strains of the unique serotype of circulating PPRV into 4 genetically distinct lineages (1–3). The geographic distribution of lineages I and II is restricted mainly to western and central Africa and that of lineage III mainly to eastern Africa. Lineage IV is more widely distributed in Southeast Asia, the Arabian Peninsula, and the Middle East. Lineage IV is also currently circulating across northern and central Africa (1).

Although PPR is endemic in Eritrea, there are no data on the molecular characterization of the circulating viruses. As part of a program of cooperation between the National Animal and Plant Health Laboratory of Asmara and the Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise "G. Caporale" in Teramo, we analyzed 41 sheep and goat tissue samples that were collected by the Eritrean veterinary service during several outbreaks of PPR. Samples were collected in the following villages: 5 samples in Gahitelay (Northern Red Sea region) in 2003; 4 in Gulee and Weki (Maekel region), 3 in Hukum (Anseba region), and 6 in Torat and Keih Adi (Debub region) in 2005; and 6 in May Harish (Debub region) in 2011. For 17 samples, the region was not recorded; instead, they were identified as "Eritrea," followed by the year of collection. Of the 41 samples, 22 were from goats and 6 from sheep; the source was not recorded for the other 13 samples. Nineteen samples were collected from lymph nodes, 10 from spleen, 9 from lung, and 1 each from tonsil, liver, and trachea. Samples were analyzed in the biosafety level 3 laboratory at the Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise.

Homogenates were prepared by using a mortar and sterile quartz to grind the tissue samples. The homogenates were then diluted (10% wt/vol) in phosphate-buffered saline, and tissue debris was removed by low-speed centrifugation. For lineage determination, total RNA was extracted by using the High Pure Viral Nucleic Acid Kit (Roche Diagnostic, Mannheim, Germany), RNA was amplified by reverse transcription PCR using the primer set NP3/NP4 (4) and the QIAGEN One-Step RT-PCR Kit (QIAGEN, Hilden, Germany). After agarose gel electrophoresis, 34 samples showed specific amplification of the 351-nt fragment of NP gene. PCR products were then purified by using the QIAquick PCR Purification Kit (QIAGEN) and sequenced by using the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Nucleotide sequences were obtained for 24 (59%) samples. The geographic distribution of animals in Eritrea with tissue samples from which we obtained viral sequences is shown in the online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/12-1072-Techapp.pdf). Sequence editing, assembly, and alignment were performed by using BioEdit version 7.0.5.3 (5). BLAST (www.ncbi.nlm. nih.gov) was used to find homologous hits in the sequence databases. Phylogenetic analysis (neighbor-joining) with bootstrap (1,000 replicates) was performed using MEGA4 (6). We performed phylogenetic analysis of a 255-nt sequence of PPRV NP gene, using a selection of reference strains: 7 new sequences from this study (GenBank accession nos. JX398126, JX398127, JX398128, JX398129, JX398130, JX398131, JX398132) and 34 sequences representing the 4 lineages of PPRV for which sequences are available (Figure). Phylogenetic analysis was repeated by using the distance (neighbor-joining) method in PHYLIP version 3.67 (http://evolution.gs.washington.edu/phylip.html). The results of the phylogenetic analyses showed that the PPRV we isolated from ruminants in Eritrea belongs to the Afro-Asian lineage 4 and could be further distinguished into 2 clusters.

The first cluster consisted of 5 PPRV strains (Eritrea_2002, Eritrea_Gahitelay_2003, Eritrea_Hukum_2005, Eritrea_Torat_2005, and Eritrea_2011), which are related to PPRV strains from Sudan (AlAzaza_BNSUD00) and Cameroon (Cameroon_97). Thus, the data suggest that these 5 isolates from Eritrea are related to viruses that originated in Sudan and Cameroon and spread across central Africa, probably by free ranging wild or susceptible domestic animals.

The second cluster consisted of 2 PPRV strains (Eritrea_Gulee_2005 and Eritrea_May Harish_2011); this
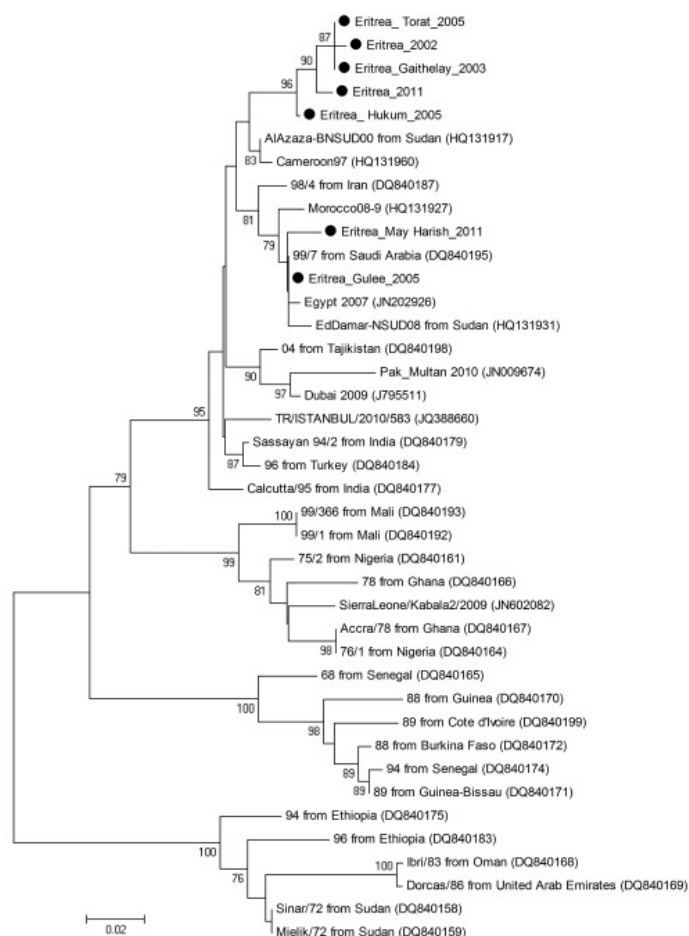
Figure. Phylogenetic tree showing the genetic relationships between isolates of peste des petits ruminants virus (PPRV). The tree was constructed on the basis of 255-nt sequences of the PPRV nucleoprotein gene. Black dots indicate sequences obtained in this study. Lineages are indicated on the right, and GenBank accession numbers are shown in parentheses. Analysis was performed by using the MEGA4 software (6) and neighbor-joining (maximum composite likelihood) methods. Bootstrap support values >70 are shown at nodes (1,000 replicates). The scale bar indicates nucleotide substitutions per site.

cluster also includes strains from Saudi Arabia (Saudi Arabia 99_7), Sudan (NSUD08), and Egypt (Egypt_2011). Genetic sequences are highly conserved in this group, particularly for Eritrea_Gulee_2005, which shares 100.0% nucleotide identity with Saudi Arabia 99_7, 99.6% identity with Egypt_2011, and 99.2% identity with NSUD08. The group is closely related to viruses collected in Morocco during outbreaks in 2008. The data suggest a clonal origin of the viruses belonging to this group, supporting the hypothesis that Eritrea could have been a gateway for the Saudi Arabia 99_7 strain to spread throughout Africa.

Our findings show that PPRV lineage IV is the dominant lineage circulating among ruminants in Eritrea. This information is crucial for further research aimed at defining strategies for the efficient prevention and control of PPR in this country.

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A Case of Endemic Syphilis, Iran

To the Editor: Endemic syphilis, also known as bejel, is a nonvenereal treponematosis with onset in early childhood; the disease is caused by the bacterium *Treponema pallidum* subsp. *endemicum*. Until the 1970s, the disease was endemic to many parts of the world, including the Middle East; aggressive treatment programs abated its prevalence, but such programs have since ceased. Transmission occurs through contact with infectious lesions on the skin and mucous membranes and with contaminated drinking vessels (*1*). We report a case of bejel in a young boy in Iran, manifested by gummatous ulcerating lesions of the face.

In November 2010, a 14-year-old Iranian boy was brought by his grandfather to our private infectious diseases clinic in Tehran, Iran, because of cutaneous lesions on his face, which had increased progressively over 9 months. This adolescent had spent his childhood in Izeh, in the southwest region of Iran. He had 5 healthy siblings, and the family medical history was unremarkable. He reported experiencing a rash in childhood without mucous membrane involvement but had no history of joint or bone pain.

Examination revealed disfiguring gummatous lesions infiltrating the skin of the nose, glabella, and forehead, with clustered nodules in the left interciliary region (Figure). No other abnormality was found. He denied any sexual contacts, and there were no stigmata of congenital syphilis. Skin biopsy was refused. Tuberculin skin test results were negative. Full blood count, erythrocyte sedimentation rate, and C-reactive protein level were within reference ranges. The Venereal Disease Research Laboratory test result was positive (titer ≥640), and a fluorescent treponemal antibody absorption test result was strongly

Figure. Disfiguring infiltration of the nose, glabella, and forehead with clustered nodules in left interciliary region of boy with endemic syphilis, Iran, 2010. Photograph by Alireza Abdolrasouli.

reactive. Because of the positive serologic test results and a preliminary diagnosis of benign tertiary syphilis, the patient was treated with 2.4 million units of benzathine penicillin G, by intramuscular injection, once per week for 3 weeks. The ulcerations completely resolved, and an atropic scar and peripheral hyperpigmentation developed over the 3-week period. The patient did not return for follow-up examination.

Serologic tests cannot distinguish between bejel and venereal syphilis. For this patient, lack of history of primary chancre, absence of cardiovascular and neurologic complications in the chronic stage of the infection, absence of history of any sexual activity, and socioeconomic background are suggestive of the nonvenereal subspecies. Because the boy had no syphilitic stigmata and his siblings were healthy, congenital syphilis is unlikely.

By the early 1970s, the global prevalence of endemic treponematoses (pinta, bejel, and yaws) had been reduced from 50 million to 2.5 million cases because of widespread use of long-acting, injectable penicillin in the 1950s and 1960s, led by the World Health Organization (WHO) and the

United Nations Children's Fund (*2*). However, penicillin mass treatment campaigns were not maintained and, as a result, the disease has reemerged. In 1995, WHO estimated the total number of treponematoses cases (infectious, latent, and late-stage) to be 2.6 million worldwide, including 460,000 infectious cases (*3*). Most of these were cases of yaws in Africa and Southeast Asia.

Bejel predominantly affects children <15 years of age. Poor personal hygiene and overcrowding facilitate transmission of infection (*1,4*). Manifestation as primary lesions is rare; secondary lesions or rashes are common and are usually succeeded by a period of latency. Angular stomatitis, papules, mucous patches, and macules on the moist areas of the body are the most typical manifestations. Condylomata lata, similar to those seen in venereal syphilis and yaws, can occur. If late-stage disease develops, it usually affects the skin, the long bones of the legs, and the cartilage. Cartilage damage may result in severe destruction of the nose and palate (gangosa). Whether bejel is transmitted congenitally is unknown (*1,4*).

Bejel was known to be endemic to the Middle East and was prevalent in Iraq and in the Bedouin population in Saudi Arabia until the 1980s (*5,6*). In 1995, it was diagnosed in 3 children and their father in southeastern Turkey, an area where no cases of bejel had been reported for >30 years (*7*). In 1954, 1 epidemiologic study of bejel in Iran reported a prevalence of 23%–34% in 4 remote villages of Khousistan (*8*), near the home of the case-patient in the current study. Since 1954, no cases in Iran have been reported to WHO.

This case report shows that bejel continues to be transmitted among isolated, poor rural communities in Iran. Our patient was living in a remote, rural district of low socioeconomic status, and his community had almost no access to medical facilities. WHO recently convened a meeting to discuss a new initiative for the eradication of yaws, after it was demonstrated that a single oral dose of azithromycin was as effective as injected penicillin in the treatment of this disease (*9,10*). Bejel should be equally susceptible to eradication, but only if health services are made available to poor rural communities in areas where the disease is endemic. We recommend that countries in which this disease was formerly declared endemic initiate surveillance programs with the goal of eradication if new cases are found.

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Antiretroviral Therapy–associated Coccidioidal Meningitis

To the Editor: Coccidioidomycosis, a reemerging fungal infection in the United States, comprises ≈150,000 cases annually (*1,2*). We report a case of postmortem examination–proven antiretroviral therapy (ART)–associated coccidioidomycosis manifesting as atypical lymphocytic meningitis, which we believe represents a rare presentation of immune reconstitution inflammatory syndrome (IRIS).

In September 2011, a 59-year-old man sought care in Atlanta, Georgia, USA, with new-onset headache, photophobia, and neck stiffness. He also reported fevers, chills, weight loss, dyspnea, and cough with scant hemoptysis. Two months earlier, he had sought care for epididymitis; HIV infection was diagnosed at that time (CD4+ T-cell count of 45 cells/μL [7%] and plasma HIV RNA level of 420,720 copies/mL [reference not detectable]). He was started on an ART regimen, and 1 week before his September 2011 illness, HIV RNA level had decreased to 790 copies/mL and CD4+ count had risen to 163 cells/μL (13%).

The patient was a thin African-American man who reported marked discomfort, with nuchal rigidity. Laboratory results were unremarkable except for serum sodium of 128 mEq/L (reference 132–144 mEq/L) and creatinine of 1.7 mg/dL (reference 0.7–1.2 mg/dL). Chest imaging showed a diffuse infiltrate in a miliary pattern. Noncontrast computed tomography scan of the head was normal. Cerebrospinal fluid (CSF) examination revealed an opening pressure of 31 cm H_2O, 365 leukocytes/μL (reference <11/mL), (93% lymphocytes, 80% described as atypical), glucose 13 mg/dL (reference 40–70 mg/dL), and protein 171 mg/dL (reference 15–45 mg/dL).

Closer examination of the atypical CSF lymphocytes showed mostly CD3+ T cells, markedly variable in size and morphology, containing substantially irregular nuclear membranes, coarsened chromatin with multilobulated flower-shaped nuclei, and increased cytoplasm (Figure, panel A). India ink stain, acid-fast bacillus stain, and Gram stain were all negative. CSF Venereal Disease Research Laboratory and cryptococcal antigen test results also were negative. Flow cytometry on the CSF was not performed. No abnormal-appearing lymphocytes were noted in the blood.

Empirically, the patient was placed on 4-drug therapy plus dexamethasone for tuberculous meningitis, along with bacterial and viral meningitis coverage. Results of CSF cultures and viral PCR studies were negative. Magnetic resonance imaging of the brain showed 2 areas consistent with cavernous malformations but no abnormal meningeal enhancement. On day 5 of admission, blurred vision and confusion developed and rapidly progressed to obtundation. A repeat head computed tomography scan showed marked edema and transtentorial herniation, and a ventricular drain was placed. Full-strength voriconazole

was added empirically for coverage of common fungal organisms. However, the patient's clinical status worsened, and he died 2 days later.

On postmortem examination, brain pathology showed areas of necrosis, along with massive hemorrhage. Special staining of the necrotic tissue revealed marked inflammation surrounding multiple characteristic *Coccidioides* spp. spherules, 1 of which had ruptured and was spilling endospores (Figure, panel B). Examination of the lungs showed multiple granulomas that also contained coccidioidal spherules.

Pathogenic *Coccidioides* spp. is not indigenous to the Atlanta area; however, the patient was homeless and could have traveled to *Coccidioides* spp.–endemic areas. The need for increased suspicion for coccidioidomycosis in areas to which it is not endemic was highlighted further by a recent report identifying a case in Rome, Italy (*3*).

Coccidioidal meningitis is the most severe complication of coccidioidomycosis and results from lymphohematogenous spread from the lung, manifesting as fever, headache, changes in sensorium, malaise, and meningismus. CSF studies typically

show 100–500 leukocytes/µL (predominantly lymphocytes), low glucose, and protein >150 mg/dL. Culture of *Coccidioides* spp. from the CSF is diagnostic but is much less sensitive than detection of anticoccidioidal antibodies in the CSF (*4*).

The most intriguing aspect of this case is the atypical pleocytosis, which initially suggested lymphomatous or tuberculous meningitis and obscured the true diagnosis of coccidioidal disease. Atypical reactive CSF lymphocytes were described in a lymphoma patient in whom coexistent cryptococcal meningitis was diagnosed (*5*). Those cells were initially confused for CNS lymphoma and caused a similar diagnostic and therapeutic dilemma.

Haddow et al. recently proposed case definitions for such clinical phenomena involving cryptococcosis, offering that newly defined cryptococcal disease identified after ART initiation be termed ART-associated cryptococcosis, with a more virulent subset of disease attributed to the unmasking of cryptococcal IRIS (*6*). We propose that the case described here parallels that described by Haddow et al. and illustrates ART-associated coccidioidomycosis. Furthermore, because of the significant inflammatory process, granulomatous pathology, and exaggerated clinical deterioration in the setting of rapid ART-induced CD4+ T-cell recovery, we suggest that this case meets the proposed criteria for the unmasking form of IRIS (*6,7*).

The high percentage of atypical lymphocytes described here is unusual for an IRIS response and might reflect an unusual variation of the diverse immune mechanisms used during an IRIS phenomenon (*8*). Another unusual case of coccidioidal IRIS manifested as superior vena cava syndrome (*9*). Additionally, the use of high-dose corticosteroids in the absence of antifungal therapy might have contributed to more aggressive disease progression. We suggest that the discovery of atypical lymphocytic meningitis in
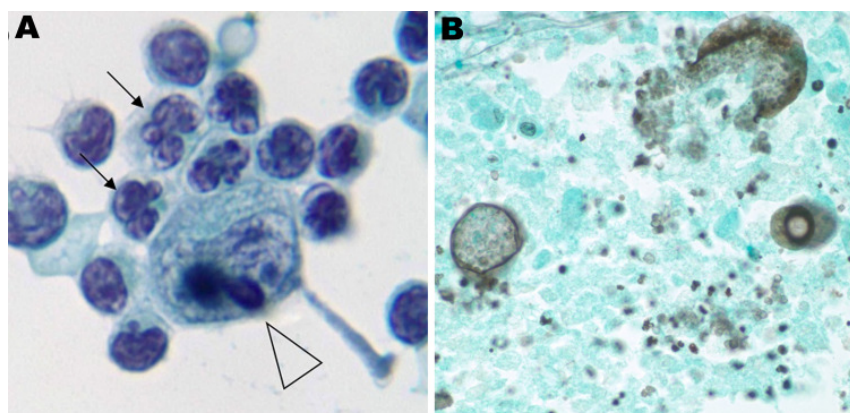
Figure. Test results of cerebrospinal fluid and brain tissue in HIV-positive man with signs and symptoms of meningitis, Atlanta, Georgia, USA, 2011. A) Atypical lymphocytes containing flower-shaped nuclei (arrows) and coarse chromocenters (arrowhead). Cerebrospinal fluid processed by Cytospin centrifuge (Thermo Electron Corp., Waltham, MA, USA) and prepared by Papanicolaou staining. Original magnification ×100. B) Gomori methenamine silver stain of sampled brain tissue showing multiple spherules and endospores of *Coccidioides* spp. Original magnification ×40.

a patient shortly after ART-associated immune recovery should alert the clinician to the possibility of coccidioidal meningitis.

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Concurrent Tuberculosis and Influenza, South Korea

To the Editor: The concurrence of active pulmonary tuberculosis (TB) and influenza in immunocompetent hosts is rarely reported. Such concurrence could distract clinicians from diagnosing TB during an influenza epidemic. We describe 7 cases of concurrent active pulmonary TB and influenza A(H1N1)pdm09 virus infection in South Korea.

At 2 teaching hospitals in Seoul, medical records were reviewed retrospectively. Among the 12,196 patients for whom A(H1N1)pdm09 infection was confirmed by real-time reverse transcription PCR from May 2009 through May 2011, a total of 7 (0.06%) were co-infected with newly diagnosed active pulmonary TB (Table). Patients who had a history of TB diagnosis were excluded.

Among the 7 co-infected patients, 6 (85.7%) were <30 years of age. All but 1 patient, who had colon cancer, had been previously healthy. No patients had diabetes mellitus or HIV infection. One patient was a current smoker. For 5 patients, pulmonary TB was diagnosed within 1 week from the date of influenza diagnosis; initial chest radiographic findings were suggestive of active TB or pneumonia. Another 2 patients, for whom radiographic examination was not performed at the first visit, experienced worsening cough and blood-tinged sputum after improvement of influenza; laboratory tests for TB were performed, and pulmonary TB was diagnosed 17 days after the date of influenza diagnosis. For 4 patients, computed tomography of the chest was performed, and multiple nodular lesions, cavities, and tree-in-bud appearance were found. Lymphopenia at initial visit was detected in 2 patients. All *Mycobacterium tuberculosis* isolates were sensitive to anti-TB drugs, and clinical outcomes were good for all patients.

For persons infected with *M. tuberculosis*, lifetime risk for development of active TB is 5%–10%; this risk increases for those with immunocompromising conditions (*1*). One study reported that pulmonary TB was a risk factor for A(H1N1)pdm09 infection (*2*). However, the concurrence of influenza and pulmonary TB has been reported only a few times, and the findings have been mostly descriptive and somewhat contradictory. An old report, from 1919, describes TB diagnoses for patients who were not recovered completely from influenza pneumonia (*3*). During 1957–1958, Löfgren and Callans (*4*) observed 46 patients with newly detected TB that had been diagnosed shortly after Asian influenza; among them, 4 had a history of typical influenza.

In South Africa, among 72 patients who died of A(H1N1)pdm09 infection, 7 also had active TB (*5*).

In Taiwan, TB and A(H1N1)pdm09 infection in a lung cancer patient was reported (6). In Japan, a fatal case of influenza pneumonia combined with *Streptococcus pneumoniae* and *M. tuberculosis* infection in a patient with diabetes mellitus was reported (7). Although the 2 patients from Taiwan and Japan had concurrent illnesses, 6 of the 7 patients in our study had been healthy (6,7). Radiographic abnormalities for the patients reported here were similar to those reported for other patients, but more cavitary lesions were found for the patients reported here.

Although it is not clear whether influenza accelerates emergence of TB, some animal studies suggest that influenza-associated TB is possible. In mouse studies, simultaneous injection of tubercle bacilli into the peritoneum and intranasal inoculation with influenza A virus (PR8) resulted in more rapid and extensive development of pulmonary tuberculous lesions than did infection with tubercle bacilli only (8). In a mouse model of chronic infection with *M. bovis* BCG, acute infection with influenza virus moderately increased the load of acid-fast bacilli in the liver, although this change was not significant (9).

It is possible that temporary suppression of T-cell immunity by A(H1N1)pdm09 virus might alter the course of *M. tuberculosis* infection. Among influenza patients, $CD4^+$ T cells were depleted and a subset of Th17 cells were preferentially lost at an early stage of infection; Th17 cells that produce proinflammatory cytokine interleukin-17 are associated with a protective immune response (10). Among 4 patients for whom laboratory examination was conducted at initial visit, 2 were lymphopenic. However, individual lymphocyte subsets were not checked, and a functional assay of lymphocytes was not conducted. Further studies of serial quantification and functional assay of lymphocytes at the acute stage of influenza and its effect on host susceptibility to TB in animals and humans are needed.

The concurrence of TB and influenza could be a simple overlap. In 2009, the case notification rate of pulmonary TB in South Korea was 58.2 cases per 100,000 population; in 2010, it was 56.5. However, if influenza actually amplifies TB, TB might be underestimated and missed in influenza patients. Thus, large-scale observational epidemiologic studies on the changing incidence of TB during the influenza postpandemic era are needed. Especially in TB-endemic areas, physicians should consider a concurrent pulmonary TB diagnosis for influenza patients with radiologic abnormalities consistent with TB or with prolonged respiratory symptoms.

Table. Case summary of concurrent active pulmonary TB and influenza A(H1N1)pdm09 infection*

Patient age, y/sex	Date of influenza diagnosis	Underlying disease	Days from influenza to TB diagnosis	Lymphocyte count at initial visit, cells/µL (%)	Specimen/ auramine-rhodamine stain result	Specimen/ TB PCR result	MTB source	Radiographic findings	Days hospitalized
17/F	2009 Sep	None	17	NA	BAL/+	BAL/+	BAL	Patchy consolidation in LUL	0
15/M	2009 Sep	None	2	380 (7.3)	Sputum/+	Sputum/+	Sputum	Pneumonic infiltration in both upper lobes, cavitary lesion in LUL	7
15/F	2009 Oct	None	17	NA	Sputum/+	Sputum/−	Sputum	Nodular infiltration with cavity in LUL	10
26/M	2009 Nov	None	1	1,406 (18.5)	Sputum/+	Sputum/+	Sputum	Multiple nodules and cavities in RUL and RML	6
29/M†	2010 Feb	None	2	NA	Sputum/−, BAL/−	BAL/+	Sputum, BAL	Pneumonic infiltration in RUL	0
68/M	2010 Feb	Colon cancer	1	230 (4.9)	Sputum/+	NA	Sputum	Lobar pneumonia in RLL	10
24/F	2011 Feb	Previous wedge resection to treat pneumothorax	7	1,433 (37.7)	Sputum/−, BAL/+	Sputum/−, BAL/+	BAL	Patchy opacity in left middle lung zone	0

*All patients survived. TB, tuberculosis; MTB, *Mycobacterium tuberculosis*; NA, not available; BAL, bronchoalveolar lavage; LUL, left upper lobe; RUL, right upper lobe; RML, right middle lobe; RLL, right lower lobe.
†This patient had a history of 7.5 pack-years of smoking.

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Cronobacter Infections Not from Infant Formula, Taiwan

To the Editor: Species of the genus *Cronobacter* are relatively heterogeneous at the phenotypic and molecular levels (*1*). In 2012, the following 7 *Cronobacter* species had been defined: *C. sakazakii, C. malonaticus, C. turicensis, C. dublinensis, C. muytjensii, C. condimenti,* and *C. universalis* (*2*). These opportunistic pathogens cause bacteremia and meningitis in neonates and are associated with necrotizing enterocolitis (*3*); ≈30% of infants with *Cronobacter* bacteremia or meningitis have died (*4*). *Cronobacter* spp. primarily infect infants, but infections among immunocompromised patients, particularly elderly patients, have been reported (*5*). Although these organisms are ubiquitous in the environment and have been isolated from a variety of foods, *Cronobacter* spp. infections in infants have been epidemiologically associated with ingestion of contaminated powdered infant formula (*6*). Few reports of *C. sakazakii* infections in adults have been published.

During 2002–2011, a total of 5 *C. sakazakii* isolates, 1 from each of 5 patients, were identified at the National Taiwan University Hospital in northern Taiwan (Table). These isolates were identified as belonging to the *C. sakazakii* group by use of 2 systems: PMIC/ID-30 (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) and the Vitek 2 System GN card (bioMérieux Inc., La Balme les Grottes, France). The phenotypic profiles that use 14 biochemical characteristics to differentiate 7 species and 3 subspecies of *C. dublinensis* within *Cronobacter* gen. nov. (*C. sakazakii* group) have been described (*2*). Although we did not apply the 14 biochemical tests to differentiate the 5 isolates (*2*), the isolates' lack of indole production and dulcitol utilization, obtained by use of Enterotube II (Becton Dickinson Diagnostic Systems), was compatible with identification of the following 3 species or subspecies: *C. sakazakii, C. malonaticus,* or *C. dublinensis* subsp. *lausannensis* (*2*). Results of partial 16S rRNA gene sequence analysis with primers 8FPL and 1492RPL indicated that the isolates were probably *C. sakazakii* (*7*), and results of a 2-step *rpoB*-based PCR that used 2 sets of primer pairs (*Csakf/Csakr* and *Cmalf/Cmalr*) confirmed that the isolates were *C. sakazakii* (*8*).

Serogroups of the 5 *C. sakazakii* isolates were determined by using 5 primer pairs specific to the *wehC, wehI,* and *wzx* genes (*9*). Of these 5 isolates, 3 were serogroup O1, and 2 were not typeable (not serogroups O1, O2, or O3).

Table. Characteristics of 5 patients with *Cronobacter sakazakii* infections, National Taiwan University Hospital, Taiwan, 2002–2011*

Patient age/sex	Year of diagnosis	Underlying medical conditions	Clinical presentation	Infection type	Outcome	Isolation site	Isolate GenBank accession no.	Isolate serogroup
77 y/M	2002	Laryngeal cancer, diabetes mellitus, pulmonary tuberculosis	Cardiac arrest at arrival at emergency department	Primary bacteremia	Died	Blood	FJ947061.1	O1
72 y/M	2002	Gastric cancer	Fever	Primary bacteremia	Survived	Blood	JF330153.1	NT
2 mo/M	2005	Congenital heart disease	Fever	Pneumonia	Survived	Sputum	F330133.1	O1
37 y/M	2008	None	Abdominal pain	Acute cholecystitis	Survived	Bile	JF330153.1	NT
64 y/F	2011	Breast cancer	Hemoptysis	Pneumonia	Survived	Sputum	GU727684.1	NT

*All isolates were identified as *C. sakazakii* I (99% identity) by 16S sequencing. NT, not typeable (not serogroups O1, O2, or O3).

Results of disk-diffusion susceptibility testing showed that all isolates were susceptible to cefotaxime, cefepime, piperacillin–tazobactam, ertapenem, imipenem, meropenem, ciprofloxacin, gentamicin, and amikacin and that all were resistant to cefazolin. The random amplified polymorphic DNA patterns generated by arbitrarily primed PCR that used 2 random oligonucleotide primers (M13 and ERIC1) differed among the 5 isolates, indicating that these 5 *C. sakazakii* strains were not clonally related (*10*).

The clinical and microbiological characteristics of the 5 patients (4 adult, 4 male) with *C. sakazakii* infection are summarized in the Table. Primary bacteremia was found in 2 patients, pneumonia in 2 (predominant growth of *C. sakazakii* from purulent sputum samples), and acute cholecystitis in 1.

The nonadult patient was a 2-month-old boy with congenital heart disease. Because of apnea and cyanosis, he was sent to an emergency department and later received assisted ventilation and supportive care in an intensive care unit. He was extubated on day 11 of hospitalization; however, fever and increased purulent sputum were noted on day 18. Bacterial culture of the suctioned sputum specimen yielded *C. sakazakii*. Before being hospitalized, the boy had been fed reconstituted powdered infant formula (Nestlé H.A.1, Gold; Nestlé Taiwan Ltd., Taipei, Taiwan) by mouth without other supplemental nutrition. During hospitalization, he received infant formula made by the hospital nutritional department through nasogastric tube. Although the powdered infant formula was not tested for *C. sakazakii*, initial sputum culture disclosed viridans group streptococci, and *C. sakazakii* was isolated from sputum obtained on day 18 of hospitalization. Thus, *C. sakazakii* from this infant might not have been associated with contaminated powdered infant formula.

Among the 4 adult patients, 3 had underlying solid organ malignancy and had received immunosuppressive drugs., and the other had bacteremia and died of cardiac arrest at arrival at the emergency department. The sources of *C. sakazakii* infection in the 1 nonimmunocompromised adult and the infant remain unclear; further research is needed to identify the source of *C. sakazakii* infections in Taiwan.

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Methicillin-Resistant *Staphylococcus pseudintermedius* in Rats

To the Editor: *Staphylococcus pseudintermedius* is a coagulase-positive species in the *S. intermedius* group. Previously misidentified as *S. intermedius*, *S. pseudintermedius* is now recognized as a leading cause of opportunistic infection in dogs (*1*) and a cause of sporadic infections in other species, including humans (*1,2*). Additionally, evidence of zoonotic transmission of *S. pseudintermedius* from dogs to humans has been reported (*3,4*). Although information regarding the pathogenic process of *S. pseudintermedius* is limited, the bacterium is known to possess virulence factors similar to those found in *S. aureus*, including a leukotoxin comparable to the Panton-Valentine leukocidase as-

sociated with community-acquired *S. aureus* infection (*1*).

Of concern is the emergence and widespread international recognition of methicillin-resistant *S. pseudintermedius* (MRSP) (*1*). One veterinary laboratory noted a 272% increase in MRSP cases from 2007–2008 through 2010–2011 (*5*). As with methicillin-resistant *S. aureus*, MRSP resistance is conferred by the *mecA* gene, making MRSP resistant to all β-lactam antimicrobial drugs and some other antimicrobial drug classes (*1*). Compared with methicillin-susceptible strains, MRSP seems better able to colonize humans (*3*).

The potential for zoonotic transmission and concerns that MRSP could be mistaken for other methicillin-resistant staphylococci (*1,2*) suggest the need for further investigation into the epidemiology of this pathogen. One question yet to be addressed is whether commensal pests, particularly rats (*Rattus* spp.), could serve as a source of MRSP because of their pervasiveness, their propensity toward close contact with humans, and the fact that they are the source of several other zoonotic diseases (*6*). We report MRSP carriage in wild Norway rats (*R. norvegicus*) in Vancouver, British Columbia, Canada.

During September–November 2011, Norway rats were trapped in a random sample of alleys in Vancouver's Downtown Eastside, an impoverished neighborhood with high levels of homelessness, intravenous drug use, and HIV infection. Immediately after the rats were euthanized, a sterile swab was used to sample the oropharynx and nares of each rat.

Swabs were placed in 2 mL of enrichment broth containing 10 g/L tryptone T, 75 g/L sodium chloride, 10 g/L mannitol, and 2.5 g/L yeast extract and incubated for 24 h at 35°C. Aliquots of 100 μL were streaked onto mannitol salt agar with 2 μg/mL oxacillin and incubated at 35°C for 48 h. Suspected staphylococcal isolates were subcul-

tured onto Columbia blood agar and identified according to colony morphologic appearance, Gram staining, and catalase reaction. Tube coagulase-positive isolates were speciated by using a multiplex PCR specific for the thermonuclease (*nuc*) gene (*7*). Methicillin resistance was confirmed by demonstrating penicillin-binding protein 2a antigen with the latex-agglutination test (Oxoid Ltd., Basingstoke, UK). Isolates were typed by sequencing of the *mec*-associated direct repeat unit (*dru* typing) (*8*). Antimicrobial drug susceptibility was evaluated by broth microdilution (Sensititre; Trek Diagnostics, Cleveland, OH, USA), according to Clinical and Laboratory Standards Institute guidelines (www.clsi.org/). The study was approved by the University of British Columbia Animal Care Committee.

MRSP was isolated from 5 (2.1%) of 237 rats trapped. However, lack of standardized screening methods for MRSP could have resulted in underestimation of MRSP prevalence. Of the 5 isolates, 3 were *dru* type dt11a, a strain commonly found in dogs (*8*), and the other 2 were a novel *dru* type (assigned dt7ac). All isolates tested demonstrated resistance to multiple antimicrobial drug classes (Table).

Carriage of MRSP has not been identified in wild rats; therefore, the epidemiologic and public health implications of these findings are difficult to determine. However, the isolation of a common dog-associated *dru* type from rats suggests that MRSP might be transmissible between dogs and rats. This possibility is not surprising given the potential for direct and indirect contact between these species. Indeed, rat-to-dog transmission of other bacterial pathogens has been recognized (*9*). Detection of a *dru* type not previously detected in methicillin-resistant staphylococci suggests that these isolates might have evolved independently of methicillin-resistant staphylococci in other animal species.

Table. Sensitivity profiles for methicillin-resistant *Staphylococcus pseudintermedius* isolated from wild Norway rats, Vancouver, British Columbia, Canada*

Drug	Isolate no. (strain type)†			
	1 (dt11a)	2 (dt11a)	3 (dt11a)	4 (dt7ac)
β-lactams	R	R	R	R
Clindamycin	R	R	R	R
Erythromycin	R	R	R	R
Daptomycin	S	S	S	S
Linezolid	S	S	S	S
Gentamicin	R	S	S	S
Tetracycline	S	S	S	R
Tigecycline	S	S	S	S
Trimethoprim–sulfamethoxazole	S	S	S	S
Quinupristin–dalfopristin	R	S	S	S
Rifampin	R	S	S	S
Chloramphenicol	S	S	S	S
Nitrofurantoin	S	S	S	S
Ciprofloxacin	R	S	S	S
Levofloxacin	I	S	S	S
Moxifloxacin	I	S	S	S
Vancomycin	S	S	S	S

*Of 5 isolates, 1 was not available for antimicrobial susceptibility testing. R, resistant; S, sensitive; I, intermediate.
†Strain types identified through direct repeat unit typing.

Rat carriage of MRSP does not prove that rats are capable of transmitting the bacterium to humans; however, rats are a source of other zoonotic pathogens (*6*). These pathogens are most commonly transmitted from rats to humans indirectly, through contamination of the environment or foodstuffs (*6*). Of note, MRSP has been shown to survive for extended periods in the environment (*4*), suggesting a mechanism through which the bacterium could be passed from rats to humans and dogs or vice versa.

In 2011, MRSA was detected in bedbugs from Vancouver's Downtown Eastside (*10*). We have identified another urban pest in this area as a potential source of a multidrug-resistant pathogen. As with bedbugs, rat infestations are most common in impoverished, inner-city neighborhoods, and residents of these neighborhoods are at greatest risk for rat-to-human pathogen transmission. These findings suggest that inner-city urban rats warrant further investigation as a potential source of MRSP and other contemporary zoonotic pathogens, including multidrug-resistant bacteria.

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Subcutaneous Infection with *Dirofilaria immitis* Nematode in Human, France

To the Editor: In March 2012, a 48-year-old woman was hospitalized with a subcutaneous nodule on her right thigh that was present for 4 weeks. She was living in Martigues near Marseille in southeastern France, owned cats and dogs, and never traveled out of France. Ultrasonography examination showed a diffuse subcutaneous edema without abscess. Results of initial blood count; ionogram; and tests for urea, creatinine, and liver enzyme levels were within reference ranges, but slight hypereosinophilia $(0.7 \times 10^9 \text{ cells/L})$ was noted 1 month later. Serologic results for toxocariasis, schistosomiasis, trichinosis, distomatosis, cysticercosis, and microfilaremia (3 assays) were negative. However, the result of an ELISA for *Dipetalonema viteae* nematodes was positive.

After surgical removal of the nodule, histopathologic analysis showed a female nematode surrounded by an aspecific inflammatory reaction. Transverse sections showed a parasite with a diameter of 300 μm and a layered cuticle <20 μm thick. The surface of the cuticle had numerous external, cuticular, longitudinal ridges. The nematode had 2 uterine tubules without microfilaremia, 1 intestinal tube, and a polymyarian-type musculature interrupted by 2 lateral chords. This worm showed similarities to an immature *Dirofilaria repens* female.

To identify the worm, we performed PCR amplification of a 12S rRNA gene fragment (*1*). PCR products (International Nucleotide Sequence Database Collaboration accession no. JX502021) were compared with sequences deposited in GenBank. Analysis showed 92% similarity and 89% coverage with the 12S rRNA gene of the *D. repens* nematode reference sequence (GenBank accession no. GQ292761.1) (*2*) but 100% similarity and 100% coverage with the 12S rRNA gene of the *Dirofilaria. immitis* nematode reference sequence (GenBank accession no. EU169125.1) (*3*).

To confirm this result, we amplified cytochrome c oxidase 1 (*cox*-1) and internal transcribed spacer 2 (ITS-2) genes by using a duplex real-time PCR as described (*4*). Results were positive for *D. immitis* (International Nucleotide Sequence Database Collaboration accession no. HE979797 [100% similarity and 100% coverage with the cox-1 gene of the *D. immitis* reference sequence] and GenBank accession no. JF461464.1) (*5*) and negative for the *D. repens* ITS-2 gene. These results confirmed an unexpected *D. immitis* subcutaneous infection that would have been misdiagnosed without molecular analysis.

D. repens and *D. immitis* nematodes are the most common species causing dirofilariasis in temperate and tropical areas. Dogs and cats are usual hosts, and main vectors are *Aedes*, *Culex*, and *Anopheles* spp. mosquitoes (*6*). In dogs and cats, *D. immitis* nematodes cause severe infections that affect lung vessels and heart cavities. In humans, this nematode is mainly responsible for benign asymptomatic pulmonary nodules, but *D. repens* nematodes usually induce periocular or subcutaneous lesions, as in our patient (*6*).

Until 2001, three areas in Europe (Iberian Peninsula, southern France, and Italy) were greatly affected by dirofilariasis. Its incidence has since increased in animals, and epidemiologic surveys showed spread of both nematode species to areas previously free of *Dirofilaria* nematodes, such as Germany and eastern Europe (Romania, Croatia, Serbia, Bulgaria, Czech Republic, and Rostov region in Russia) (*7*). Increased transport of microfilaremic pets throughout Europe, building construction and other human activities in new areas, emergence of new competent vectors (such as highly adaptable *Ae. albopictus* mosquitoes), and climate changes affected this spread (*6*). Climate changes indirectly influenced abundance of mosquitoes in specific areas, their period of activity, and development of *Dirofilaria* nematodes in vectors (*6*). Thus, incidence of human dirofilariasis is also expected to increase, although many asymptomatic infections are not diagnosed.

Fewer than 30 cases of human *D. immitis* infections have been reported in Europe since 1981, including only 4 in France. Moreover, 16 of these cases were questioned by Pampiglione et al. (*8*) because of unreliable diagnostic tools (serologic testing without a negative control) or atypical histologic criteria. Although other cases attributed to *D. immitis* nematodes have been reported, we found only 1 case in Europe attributed to *D. immitis* nematodes for which the diagnosis was confirmed by PCR (*9*).

Definitive diagnosis of dirofilariasis remains a difficult challenge. Noninvasive tests (mainly dirofilarial serologic assays) lack sensitivity, specificity, and standards to be considered reliable methods (*6*). Until recently, the standard test was histologic identification on the basis of diameter of the nematode, thickness of the cuticle, number and distribution of the fibers in the muscular layer, and study of external cuticular ridges (*8*). However, diagnosis by histopathologic analysis may be unreliable if worms are immature or subjected to necropsy, and *D. immitis* nematodes may be misidentified as *D. repens* nematodes.

Our case indicates the difficulties with histopathologic analysis (e.g., the nematode showed similarity with *D. repens* nematodes because of external cuticular ridges). However, using validated molecular techniques (*4,5*), we showed that infection with *D. immitis* nematodes would have been

erroneously identified as infection with *D. repens* nematodes. Thus, we believe that PCR-based identification should be considered as a new diagnostic method for dirofilariasis.

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Neurocysticercosis on the Arabian Peninsula, 2003–2011

To the Editor: Neurocysticercosis occurs when humans become intermediate hosts of the tapeworm *Taenia solium* by ingesting its eggs after contact with a *Taenia* spp. carrier. This parasitic disease is endemic to most of the developing world, where it represents a leading cause of acquired epilepsy (*1*). In conjunction with an increasing number of immigrants from disease-endemic areas, there has been a recent increase in the number of patients with a diagnosis of neurocysticercosis in industrialized countries.

On the basis of the incorrect assumption that human neurocysticercosis does not occur in countries in which religious laws prohibit swine breeding and consumption of pork, the disease has been considered nonexistent in Muslim countries of the Arab world. However, sporadic cases were reported during the last 2 decades of the 20th century, mainly in immigrants from India, and several case series have suggested that the prevalence of neurocysticercosis in the Arab world has been increasing over the past few years.

A Medline and manual search of the literature identified 7 reports of 39 patients with neurocysticercosis on the Arabian Peninsula during 2003–2011 (*2–8*). Of these patients, 30 were from Kuwait, 5 from Saudi Arabia, and 4 from Qatar. Mean ± SD age of these patients was 16.9 ± 13.4 years (age range 2–44 years), and 25 (64%) were women. Twenty-four patients (62%) were <18 years of age. Seizures were the primary manifestation of neurocysticercosis in 35 (90%) patients. Two of the remaining patients had focal neurologic deficits, 1 had cognitive disease, and 1 had disease that was fortuitously discovered.

Neuroimaging studies showed parenchymal brain cysticercosis in the 39 patients that appeared as 1 or 2 enhancing lesions in 34 patients (colloidal cysts) and as vesicular cysts in 5 patients. Enzyme-linked immunoelectrotransfer blotting of serum detected antibodies against cysticerci in 12 (91%) of 23 patients tested. Twelve patients received cysticidal drug therapy and 7 biopsy specimens of brain lesions were obtained from 7 patients (Table). According to currently accepted diagnostic criteria, 32 patients had definitive neurocysticercosis and 7 had probable neurocysticercosis (*9*). Results of testing for *Taenia* spp. eggs in fecal samples from 3 patients were negative. In contrast, fecal examinations of household contacts

Table. Characteristics of 39 patients with neurocysticercosis reported from countries on the Arabian Peninsula, 2003–2011*

Characteristic	Value
Mean age ± SD, y	16.9 ± 13.4
Sex, %	
M	36
F	64
Country	
Kuwait	30 (77)
Saudi Arabia	5 (13)
Qatar	4 (10)
Citzenship status	
Arabian Peninsula country	29 (74)
Immigrant from disease-endemic area	8 (21)
International traveler	1 (3)
Clinical manifestations	
Seizures	35 (90)
Focal neurologic deficit	2 (5)
Cognitive decrease	1 (3)
Asymptomatic	1 (3)
Form of disease	
1 or 2 parenchymal brain cysticercus granulomas	34 (87)
Vesicular parenchymal brain cysts	5 (13)

*Values are no. (%) except as indicated.

of the 3 patients identified 1 carrier of *Taenia* spp.

The 4 patients from Qatar were citizens of Qatar, and 4 of 5 patients from Saudi Arabia were citizens of this country; the other patient was an immigrant from India. One Qatari citizen had a history of traveling to disease-endemic areas, and 1 Saudi Arabian citizen had an immigrant housekeeper from a disease-endemic area. Of the 30 patients reported from Kuwait, 23 were Kuwaiti citizens and 7 were immigrants from disease-endemic areas (mainly India). Sixteen of the 23 Kuwaiti citizens had immigrants from disease-endemic countries working at their homes. Cases of neurocysticercosis in family members were reported by 7 persons.

This review suggests that the number of patients with neurocysticercosis on the Arabian Peninsula is increasing. Most cases were autochthonous, and many occurred in wealthy families who employed babysitters and housekeepers from disease-endemic areas. Although *Taenia* spp. eggs were not identified in most of these persons, it is likely that some were *Taenia* spp. carriers who infected persons for whom they worked through nonhygienic handling of food products or directly by the fecal–oral route.

Neurocysticercosis is a disease most often acquired from a human infected with *T. solium* tapeworms, and infected swine can perpetuate the infection (*1*). Although swine husbandry is not allowed on the Arabian Peninsula, *Taenia* spp. carriers who enter countries in this region every year might infect native persons and increase the prevalence of neurocysticercosis without infected swine.

The pattern of disease expression of neurocysticercosis in countries in this region is similar to that observed in patients from India, i.e., parenchymal brain cysticerci in the acute encephalitic phase (*10*). This benign form of the disease is characterized by development of 1 or 2 parenchymal brain cysts in the coloidal stage and occurs most often in persons who do not eat pork and who do not have contact with infected swine. Differential diagnosis with other infections of the nervous system is a problem with these patients. Proper interpretation of diagnostic criteria for neurocysticercosis will enable a correct diagnosis in most cases,

obviating the practice of unnecessary surgical procedures (*9*).

The prevalence and incidence of neurocysticercosis on the Arabian Peninsula is unknown, and many cases might not have been reported. Compulsory reporting of cases will help determine the incidence and prevalence of parasitic disease. Also, identification of *Taenia* spp. carriers among household contacts of neurocysticercosis patients will enable detection of potential sources of infection and reduce spread of this disease.

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Sapovirus Gastroenteritis in Preschool Center, Puerto Rico, 2011

To the Editor: Human sapoviruses belong to a group of viruses within the family *Caliciviridae*, which also includes noroviruses, that cause acute gastroenteritis (*1*). Evidence of worldwide distribution of sapovirus has been documented on the basis of detection of virus and antibody prevalence against sapovirus in different populations (*2*). However, no evidence of sapovirus infection or outbreaks in Latin America and the Caribbean Islands has been reported.

In this study, we describe a sapovirus-associated outbreak of gastroenteritis in a preschool center during February–March 2011 in Canóvanas, Puerto Rico. The center had 60 children 4–5 years of age enrolled who were divided in 3 groups of 20 students per classroom. Each classroom had 2 teachers. The children had lunch in their respective classrooms.

A study was conducted at the center and included all children and workers who met the case definition for gastroenteritis (vomiting or diarrhea accompanied by ≥1 other symptom, such as nausea, stomach ache, or fever, during February 15–March 15, 2011). Nine persons (8 students and 1 teacher) met the case definition. They were interviewed by using a standardized questionnaire. Major symptoms were vomiting (100%), nausea (71.4%), fever (62.5%), stomach ache (57.1%), and diarrhea (25%) (Table). Data for fever were based on subjective reports of case-patients.

The earliest date of illness onset identified in the outbreak was February 23 in a child whose symptoms began abruptly with a vomiting event in the classroom. The child had a second vomiting event in the bathroom before the child was sent home. An initial cleaning was made with an absorbent powder, and a chlorine bleach solution was used for disinfection. That child was absent from school on February 24 and 25 (Thursday and Friday) and returned to school on Monday, February 28, supposedly recovered. The next reported illnesses began on March 2.

Fecal specimens were collected from 6 ill persons who met the case definition. The specimens were collected 2–11 days after onset of illness. All specimens were negative for enteric bacteria. Three specimens were sent to the Centers for Disease Control and Prevention (Atlanta, GA, USA) for virologic analysis. All 3 specimens were negative for norovirus and positive for sapovirus by real-time quantitative reverse transcription PCR (Table).

An environmental inspection and evaluation was conducted at the preschool center and showed no deficiencies. Neither of the 2 food handlers associated with the school reported symptoms of gastroenteritis. Fecal specimens collected from both food handlers were negative for enteric bacteria but were not tested for viral pathogens. Sapovirus transmission from asymptomatic food handlers in foodborne outbreaks has been reported (*3*). However, sapoviruses are much less frequently associated with foodborne outbreaks than are noroviruses (*4*).

Transmission during this outbreak most likely occurred person-to-person directly through fecal–oral contact or by indirect exposure through contaminated objects or surfaces

Table. Clinical symptoms and laboratory results for 9 patients with acute gastroenteritis in preschool center, Puerto Rico, 2011*

Patient	Age, y/sex	Date of illness onset	Date of specimen collection	Norovirus RT-PCR result	Sapovirus RT-PCR result	Vomiting	Nausea	Fever	Stomach ache	Diarrhea
1	5/M	Feb 23	NS	NS	NS	+	+	–	–	–
2	5/F	Mar 2	NS	NS	NS	+	U	+	U	–
3	4/F	Mar 2	Mar 11	–	+	U	U	+	U	+
4	5/F	Mar 3	Mar 14	–	+	+	+	+	–	–
5	5/F	Mar 3	Mar 14	–	+	+	+	+	–	–
6	4/F	Mar 3	NS	NS	NS	+	–	+	+	–
7	4/F	Mar 7	Mar 9	NT	NT	+	–	+	+	–
8	53/F	Mar 8	Mar 11	NT	NT	+	+	–	+	+
9	4/M	Mar 8	Mar 9	NT	NT	U	+	–	+	+

*RT-PCR, reverse transcription PCR; NS, no sample; +, positive; –, negative; U, unknown (missing information); NT, not tested.

because 7 (78%) of the 9 ill persons were from the same classroom (attack rate 32% [7 of 22 students and teachers]). The other 2 ill persons were a child in a different classroom who was a cousin of 1 of the ill children in the affected classroom and a teacher from the other classroom that shared the bathroom with the affected classroom.

This investigation highlights the need for clinical diagnostics of viral pathogens in evaluation of persons with acute gastroenteritis. A recent study in the United States demonstrated that viruses were the leading cause of acute gastroenteritis among persons of all ages seeking medical care (5). Better understanding of the relative role of specific causes of acute gastroenteritis is needed to help guide clinical management and ultimately to develop more appropriate prevention strategies. Limited laboratory-based data are available on the role of viral agents in causing acute gastroenteritis for sporadic cases and outbreaks in Puerto Rico. On the basis of this investigation, sapoviruses appear to be circulating in Puerto Rico and should be considered a potential cause of gastroenteritis in children and adults. We recommend expanded use of sapovirus diagnostics in other Latin American countries and Caribbean Islands to better elucidate their role in cases of viral gastroenteritis.

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Cronobacter sakazakii ST4 Strains and Neonatal Meningitis, United States

To the Editor: To overcome various limitations of phenotyping and 16S rDNA sequence analysis of *Cronobacter* bacteria, we have established a comprehensive multilocus sequence typing (MLST) scheme as an open access database resource (www.pubMLST.org/cronobacter) (*1*). The scheme is based on 7 housekeeping genes (*atpD*, *fusA*, *glnS, gltB, gyrB, infB, ppsA*; 3,036 nt concatenated length) and has been used to study the diversity of the *Cronobacter* genus and new *Cronobacter* species (*2–4*). Previously, we compared the sequence type profile to severity of infection by compiling patient details, isolation site, and clinical signs and symptoms for strains isolated from around the world during 1953–2008 (*5*). This study revealed that most serious meningitis clinical cases caused by *Cronobacter* spp. in neonates during the previous 30 years in 6 countries were caused by a single sequence type (ST): *C. sakazakii* ST4. We were therefore interested in applying the MLST method to the *Cronobacter* strains associated with the highly publicized cases in the United States during December 2011 (*6*).

The Centers for Disease Control and Prevention (CDC) sent us the *Cronobacter* isolates they collected during 2011 for MLST analysis (Table). Ten specimens were clinical isolates from neonates or infants. These included 2 specimens (1577, 1579) associated with *Cronobacter* infections in Missouri and Illinois (*6*). Four specimens were from opened tins of powdered infant formula (PIF), and 1 was from PIF reconstitution water. DNA sequences for all specimens are available for download and independent analysis through the open access database.

Most (14/15) specimens were *C. sakazakii*; 1 was *C. malonaticus*. This predominance of *C. sakazakii* isolates matches reports of cases and outbreak studies (*7*). The *C. sakazakii* isolates were in 6 of 55 STs defined for *C. sakazakii* (*4*). However, there was an uneven distribution according to clinical records: all 5 cerebrospinal fluid (CSF) isolates were either ST4 or within the ST4 complex (clonal group where strains are identical in 4 or more loci). This group included strains from cases during December in Illinois (specimen 1577) and in Lebanon, Missouri (specimen 1579).

Specimen 1577 (ST110), isolated from CSF, is a triple-loci variant of ST4, distinguished by 5/3036 nt: *atpD*

(1/390nt), *gltB* (2/507nt), and *gyrB* (2/402nt). Specimen 1578 (ST111), isolated from the PIF reconstitution water associated with the case reported in Illinois, is distinguishable from ST4 in 4/7 loci: *fusA* (5/438nt), *glnS* (1/363), *infB* (4/441), and *ppsA* (19/495). The 2 Illinois strains, 1577 and 1578 (ST110 and ST111), differed from each other at all loci, in total, 35/3,036 nt difference.

Such sequence-based relationship analysis of isolates is not possible by using pulsed-field gel electrophoresis (PFGE). PFGE and MLST analyze the bacterial DNA content differently, and there are no *Xba*I sites (the endonuclease most commonly used with PFGE of Enterobacteriaceae) within the 7 MLST loci. *C. sakazakii* ST4 strains were also found in feces (specimen 1567), opened PIF (specimen 1571), and tracheal samples (specimen 1576) (Figure, Appendix,

wwwnc.cdc.gov/EID/article/19/1/12-0649-F1.htm). In addition, 2 single-loci ST4 variants were found; CSF specimen 1565 differed from the ST4 profile in the *fusA* loci by 6/438 nt, and specimen1572 from an opened tin of PIF differed in the *fusA* loci by 5/438 nt. These 2 strains differ from each other minimally, by 1 nt of 3,036 (concatenated length) in the *fusA* loci position 378 (A:T).

Several non-ST4 *C. sakazakii* strains were received by CDC in 2011. *C. sakazakii* ST8 was isolated from an opened powdered infant formula tin (specimen 1573) and 2 associated fecal samples from an infant who had diarrhea (specimens 1574 and 1575). One blood isolate (specimen 1569) was *C. malonaticus* ST112, found in an infant <1 month of age with meningitis who did not survive the infection. This finding is highly noteworthy because it has been proposed that *C. malonaticus*

predominates in adult infections (*5*), and no fatal meningitis cases have been attributed to this species.

This MLST analysis of 15 strains received by the CDC in 2011 reinforces the conclusion that CSF isolates are not evenly spread across the 7 *Cronobacter* species and are instead predominantly in the *C. sakazakii* ST4 clonal complex. Such infections in neonates are of high concern because of the risk for associated severe brain damage. As previously stated, whether this association is caused by greater neonatal exposure as a result of environmental factors or particular virulence capabilities remains uncertain (*5*).

Acknowledgments

Table. Multilocus sequence typing of *Cronobacter* isolates received by the CDC during 2011*

Cronobacter species	NTU strain ID no.	CDC patient ID no.	ST	Location	Isolation source	Comment
C. sakazakii	1579	2012-05-05	4	Missouri	CSF of <1 mo male, term infant; exposed to PIF	Patient died
	1566	2011-12-02	4	Ohio	CSF of 1 mo male infant; exposed to PIF	From twin of patient 2011-12-03
	1567	2011-12-03	4	Ohio	Feces of 1 mo male infant; exposed to PIF	From twin of patient 2011-12-02; asymptomatic
	1568	2011-12-04	4	Ohio	Opened PIF	Formula associated with 2011-12-02 and -03
	1570	2011-21-01	4	Minnesota	CSF of <1 mo male, term infant; exposed to PIF	Brain infarction
	1571	2011-21-03-01	4	Minnesota	Opened PIF	Formula associated with 2011-21-01
	1576	2193-02	4	Michigan	Tracheal secretion of <1 mo male, pre-term infant (30-wk EGA); not exposed to PIF	Symptoms were not caused by *Cronobacter* infection. Fortified breast milk fed only after culture was obtained
	1565	2011-12-01	107	Michigan	CSF of <1 mo male, term infant; exposed to PIF	Brain abscess; outcome unknown. Single locus variant of ST4
	1572	2011-21-03-02	108	Minnesota	Opened PIF	Single locus variant of ST4
	1577	2193-03	110	Illinois	CSF of 1 mo female, term infant; exposed to PIF	Triple locus variant of ST4
	1578	2193-08-01	111	Illinois	PIF reconstitution water	Bottled water associated with 2193-03 case
	1573	2011-18-05-02	8	Ohio	Opened PIF	Formula associated with 2011-18-01 and 2011-18-07
	1574	2011-18-01	8	Ohio	Feces of 4 mo female, term infant; exposed to PIF	Diarrheal symptoms
	1575	2011-18-07	8	Ohio	Feces of ≈5 mo female, term infant; exposed to PIF	Ongoing diarrhea; same patient as 2011-18-01
C. malonaticus	1569	2193-01	112	Wisconsin	Blood of <1 mo male pre-term infant (32 week EGA); exposed to PIF	Clinical meningitis; patient died

*NTU, Nottingham Trent University; ID, identification; CDC, Centers for Disease Control and Prevention; ST, sequence type; CSF, cerebrospinal fluid; PIF, powdered infant formula; EGA, estimated gestational age.

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Seroprevalence of Crimean-Congo Hemorrhagic Fever Virus, Bulgaria

To the Editor: Crimean-Congo hemorrhagic fever (CCHF) is endemic in southern Russia, southeastern Europe, Africa, the Middle East, and southwestern Asia (*1*). The incidence and spread of the disease have increased in recent years. In Bulgaria, located on the Balkan Peninsula, CCHF is endemic. The disease was first described in the country in 1952 (*2*). Since then, a mandatory reporting system has been introduced. Most of Bulgaria is an ecologically favorable environment for CCHF virus (CCHFV) circulation in nature. In the 1970s, numerous virologic and serologic studies were performed by Vasilenko et al., who showed that the most affected age group was 21–50 years and that most of those with CCHF were male (65%) (cited in [*3*]). A genetic study showed that CCHFV strains in Bulgaria cluster together with strains from other Balkan countries and Russia (*2*). A vaccine consisting of chloroform-inactivated CCHFV was developed in 1974, and the currently used vaccine strain, isolated from a Bulgarian patient, was characterized genetically (*4*).

In the last 10 years, <10 CCHF cases have been registered annually in Bulgaria. Although the number of cases is lower than previously, the disease has spread into new areas (southeast, northeast, south-central provinces). In 2008, a cluster of cases was observed in southwestern Bulgaria (Blagoevgrad district), a low-risk CCHF area (*5*). Since then, a substantial number of cases have been reported in this district. During the past 4 years (2008–2011), 30 CCHF cases have been registered in Bulgaria, 12 from Blagoevgrad district, 8 from Burgas district, 4 each from Haskovo and Sliven districts, and 1 each from Kardjali and Shumen districts.

To estimate the current situation on CCHFV seroprevalence in both disease-endemic and -nonendemic areas in Bulgaria, we tested serum samples for CCHFV IgG antibodies using a commercially available ELISA kit (Vector Best, Novosibirsk, Russia). The serum samples were collected prospectively during 2011 from 1,018 healthy persons (50.2% male) from 13 districts: Sofia (n = 116), Blagoevgrad (n = 100), Pazardjik (n = 52), Stara Zagora (n = 36), Smolyan (n = 46), Yambol (n = 60), Haskovo (n = 108), Kardjali (n = 50), Sliven (n = 50), Burgas (n = 200), Shumen (n = 50), Ruse (n = 100), and Pleven (n = 50); they were then tested for CCHFV IgG antibodies with a commercially available ELISA kit (Vector Best). The median age of participants was 48 years (range 2–89 years). Persons previously vaccinated against CCHFV were excluded from the study.

Twenty-eight persons (2.8%) had IgG antibodies to CCHFV. The highest seroprevalence was observed in Burgas (7.6%), followed by Kardjali (6%), Pazardjik (5.8%), and Haskovo (4.6%) districts (Figure, Appendix, wwwnc.cdc.gov/EID/article/19/1/12-0299-F1.htm). Low seroprevalence levels were detected in Sliven (2%), Blagoevgrad (1%), and Ruse (1%) districts. Generally, these results are consistent with the number of reported cases in different districts. Notably, Kardjali and Pazardjik districts showed high CCHFV seroprevalence but single reported cases in the last

Table. Univariate and multivariate regression analysis of CCHFV seropositivity in human population, Bulgaria*

Variable	No. (%) IgG positive, n = 28	No. (%) IgG negative, n = 990	Univariate analysis		Multivariate analysis	
			OR (95% CI)	p value	OR (95% CI)	p value
Age, y			1.00 (0.99–1.03)	0.494		
Median	46	48				
Range	20–83	2–89				
Sex				0.456		
M	16 (3.1)	495 (96.9)	1.33 (0.62–2.85)			
F	12 (2.4)	495 (97.9)	Ref			
Tick bite				<0.001		<0.001
Yes	15 (9.3)	147 (90.7)	6.62 (3.09–14.19)		5.40 (2.47–11.84)	
No	13 (1.5)	843 (98.5)	Ref			
Animal contact				0.253		
Yes	11 (3.7)	290 (96.3)	1.56 (0.72–3.38)			
No	17 (2.4)	700 (97.6)	Ref			
Farming				0.001		0.012
Yes	13 (6.7)	182 (93.3)	3.85 (1.80–8.23)		2.76 (1.25–6.08)	
No	15 (1.8)	808 (98.2)	Ref			

*CCHFV, Congo-Crimean hemorrhagic fever; OR, odds ratio; Ref, reference.

years. However, these regions were among the main endemic foci in the past. In contrast, the low seroprevalence rate found in district of Blagoevgrad conflicts with the high number of diagnosed CCHF cases, but this district has been at low risk for many years.

Multivariate analysis showed that having a former tick bite and farming were significant risk factors, while age and sex were not related to seropositivity (Table). Although no significant difference was seen among age groups, none of the samples from persons 0–19 years of age were seropositive, whereas seroprevalence levels were increasing in those 20–59 years (2.65%) and 60–89 years (3.37%). This increase would be expected because the probability of contacting the virus increases with age. The main risk factor for the 20–29 year age group was the tick bite, and farming and contact with animals were incriminated in the older age groups.

A similar study conducted in Greece, a neighboring country, showed an overall seroprevalence of 4.2%; slaughtering and agricultural activities were significant risk factors for CCHFV seropositivity (6). Notably, the seroprevalence levels in the Greek districts Rodopi and Evros (4.95% and 4.49%, respectively) were similar to those in neighboring Bul-

garian districts Kardjali and Haskovo (6% and 4.6%, respectively).

We found that the risk for seropositivity was increased 5.4-fold in persons bitten by ticks. Increased tick aggressiveness in years that have favorable climatic conditions results in high rates of attacks on humans and an increased number of tick-borne diseases (7). A recent survey for CCHFV in ticks in Haskovo, Kardzhali, and Stara Zagora districts showed that 4.83%, 2.09%, and 1.46%, respectively, were infected by CCHFV, and that the most infected tick was *Hyalomma marginatum* (8). These results coincide with those of the current study because Kardzhali and Haskovo were among the districts with the highest seropositivity.

Because of the increasing spread of CCHFV in new foci, public health awareness of this problem is essential. Studies giving information about the spread and ecology of the virus can provide the necessary data for risk assessment analysis and even for prediction of epidemics.

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Primary Multidrug-Resistant Leprosy, United States

To the Editor: Since the initiation of multidrug therapy for leprosy (Hansen disease) in the 1980s by using rifampin, dapsone, and clofazimine, resistance to rifampin and dapsone has been observed worldwide and is still prevalent (*1,2*). Because few alternative effective antileprosy drugs exist, resistance to these first-line drugs could seriously affect leprosy control programs. We report a documented case of primary multidrug-resistant (MDR) leprosy in the United States.

A man from American Samoa migrated to Hawaii at age 25 years and, at age 41 years, first sought care for generalized erythematous papules and plaques. A skin biopsy showed borderline lepromatous (BL) leprosy (Figure, panel A). He had no prior history of leprosy and no prior treatment. He was treated for 44 months with a daily regimen of dapsone (100 mg), clofazimine (100 mg), and rifampin (600 mg). He appeared to comply with this regimen, and the lesions slowly resolved. He remained free of any new lesions until 4 years after completing treatment, when multiple brown hyperpigmented patches appeared on his lower legs. A skin biopsy showed only hemosiderin deposition but no organisms.

At 51 years of age, 6 years after completing treatment, the man again sought care for a 2-week history of multiple generalized erythematous papules and plaques on his face, trunk, and extremities. Some lesions were pruritic but nontender. A skin biopsy showed chronic inflammatory infiltrates with numerous acid-fast bacilli (Figure, panel B). Clinically considered to have relapsed BL leprosy, he was again treated daily with dapsone (100 mg), clofazimine (50 mg), and rifampin (600 mg). After 1 month of this regimen, no clinical improvement was observed.

Real-time PCR using the *Mycobacterium leprae*–specific repetitive element assay (*3*) confirmed the presence of *M. leprae* in biopsy specimens taken at the initial diagnosis and at relapse. Molecular genotyping of these samples with a panel of single-nucleotide polymorphism (SNP) and variable number of tandem repeat (VNTR) markers (*4*) showed that both biopsy specimens harbored *M. leprae* with the identical SNP subtype 3I and VNTR profile. PCR/DNA sequencing of the drug resistance–determining regions of *M. leprae* from these samples showed mutations within codon 53 of the *folP1* gene (ACC→GCC) and in codon 425 of the *rpoB* gene (TCG→TTG). These mutations have been characterized to induce high-level resistance to dapsone and rifampin, respectively (*5,6*). Careful evaluation of electropherograms of these drug resistance–determining regions showed only the resistant alleles in both strains.

These data indicated that this patient had been infected with MDR *M. leprae* before his initial treatment for leprosy. Therefore, when he was initially treated with leprosy multidrug therapy, he was essentially given clofazimine monotherapy. This treatment appears to have resulted in a slow, temporary clinical improvement. After relapse, he was placed on a daily regimen of clofazimine (100 mg), clarithromycin XL (500 mg), and minocycline (100 mg). The lesions clinically improved within 2 weeks, and the patient no longer noted any pruritus or tenderness in the lesions.

This report documents a case of primary MDR leprosy in the United States. In evaluating several previous biopsy samples from other patients in Hawaii, we have not seen any rifampin-resistant or MDR isolates. Health officials in American Samoa, the patient's country of origin, indicated that they were not aware of drug-resistant *M. leprae* among their patients (D. Scollard, pers. comm.). The patient reported no family history of leprosy, and no other contact could be identified. The origin of the MDR *M. leprae* in this case cannot be definitively determined.

Drug-resistant leprosy, including dapsone- and rifampin-resistant and MDR leprosy, has been reported in other parts of the world, usually in association with relapse after insufficient therapy (*1,2*). Relapses in leprosy are not usually seen until many years after completion of treatment (*7,8*). In the United States, among patients treated for 2 years with a multidrug protocol involving daily rifampin, no relapses were observed after 10–15 years' follow-up (*9*). Most new or worsening skin lesions clinically suspected to be relapses are actually leprosy reactions (*10*),
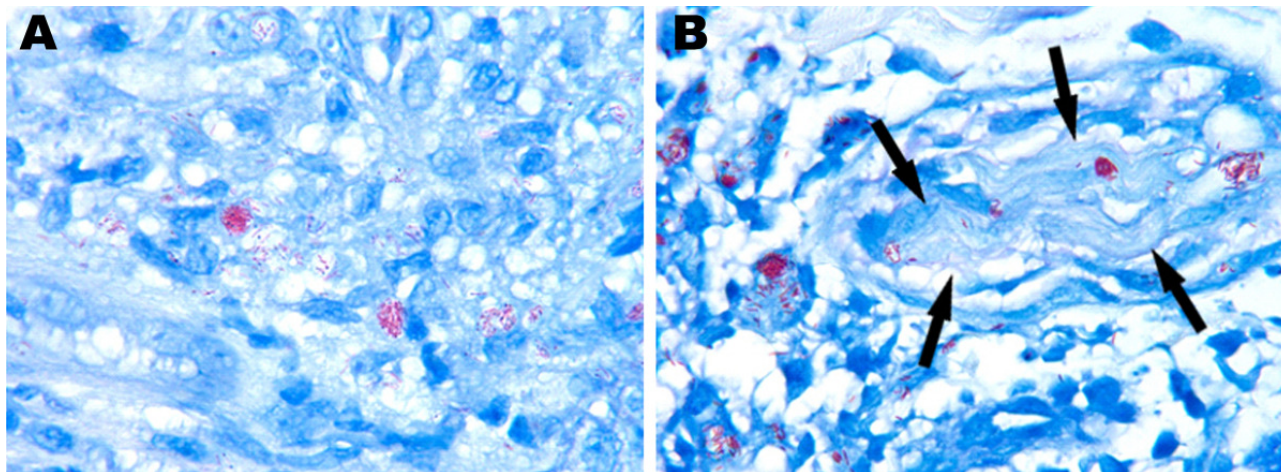
Figure. Acid-fast organisms from biopsy specimens of a man with leprosy, United States. Fite-stained sections show numerous acid-fast bacilli in the initial skin biopsy specimen (A) and in the biopsy specimen taken at relapse, 6 years after completion of treatment (B). Both specimens demonstrate the clumps of *Mycobacterium leprae* referred to as globi. In panel B, bacilli can be seen within a cutaneous nerve (arrows), a finding that is pathognomonic of *M. leprae*. Original specimen magnification ×1,000.

which affect 30%–50% of patients. In the patient reported here, leprosy relapsed with MDR *M. leprae* 6 years after completion of treatment.

The emergence of drug resistance poses a serious threat to leprosy control programs that rely on a secondary intervention, such as chemotherapy, because a leprosy vaccine is not available. Clinicians should be aware that persons who have acquired leprosy in other countries could have infection resulting from drug-resistant *M. leprae*. When resistance is suspected, biopsy samples should be analyzed by using molecular assays that enable rapid identification of mutations associated with drug resistance directly from paraffin-embedded biopsy specimens. For patients in the United States, this analysis is available through the National Hansen's Disease (Leprosy) Program (www. hrsa.gov/hansensdisease/diagnosis/index.html), and for US patients, the program provides the 3-drug regimen for leprosy free of charge. When needed, minocycline, clarithromycin, and ofloxacin are provided as alternatives to treat leprosy.

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RETRACTION

Triple Reassortant Swine Influenza A (H3N2) Virus in Waterfowl

To the Editor: We would like to retract the letter entitled "Triple Reassortant Swine Influenza A (H3N2) Virus in Waterfowl," which was published the April 2010 issue of Emerging Infectious Diseases (1). The nucleoprotein gene sequences from the viruses reported in that letter are very closely related to those from the earliest detected triple reassortant swine influenza viruses [CY095676 A/sw/Texas/4199–2/1998(H3N2)]. Although these viruses could have acquired a swine-origin segment, the branch lengths are quite short for 9 years of evolution. Therefore, we have withdrawn these 4 isolates from GenBank and subsequently retract this letter.

Sagar M. Goyal

Author affiliation: College of Veterinary Medicine– University of Minnesota, St. Paul, Minnesota, USA

Reference

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Pieter Cornelisz van Rijck (1558–1628) *Still Life with Two Figures* **(1622) Oil on canvas (123.8 cm × 148.6 cm)** North Carolina Museum of Art, Raleigh, purchased with funds from the State of North Carolina

One Rotten Apple Infects All in the Basket

Polyxeni Potter

Food was a precious commodity during the Dutch Golden Age. Along with industry, trade, and wealth, gastronomy flourished in the Netherlands during the late 16th and well into the 17th century. We know because, among other sources, art of the region, an extraordinary output in a short time span, is filled with luscious images of banquets. The wealthy had access to the best ingredients from all over the world, and city dwellers had an advantage over rural folk. Even the poor saw better diets, if only from a modest improvement in work and living conditions and an increase in charity. This was, too, a golden age for art lovers. Art acquisition, a rich person's sport, was more prevalent at this time, with burghers, the biggest group of urban residents, becoming affluent enough to purchase art. This was "anonymous" art—not commissioned but produced for the mass market and tied not to the name of any one artist but to the value of the individual work in the eyes of the buyer.

"The Dutch," wrote Daniel Defoe in A Plan of the English Commerce (1728), "must be understood as they really are, the Middle Persons in Trade, the Factors and Brokers of Europe… they *buy* again to *sell* again, *take* in to *send* out, and the greatest Part of their vast Commerce consists in being supply'd from All Parts of the World that they may supply All the World again." Defoe's interest was in the talent of the Dutch to buy and sell to advantage, but in terms of food, this extraordinary global exchange, celebrated and meticulously documented in art, foreshadowed and rivaled food commerce in our times.

During the Golden Age, northern Netherlands provinces were predominantly rural, unlike the southern, which were urban. Agriculture and fishing formed the basis of the economy, and animal husbandry ruled. Because the soil in many provinces did not favor wheat farming, livestock husbandry became the predominant form of agriculture. Grazing grounds found their way into art, but the farmers and their monotonous existence not so much, even though farmers were potent symbols of honest work and frugal living. Frequently featured livestock included cattle, goats, sheep, and chickens. Moral messages abounded, usually promoting hard work as a prerequisite to material wealth. Fishing, a traditional means of livelihood, expanded with technology, spreading outside the local market to become the very foundation of international trade. A frequent feature in art images, the herring became a symbol of Dutch prosperity, an example of "how great things grew from humble beginnings," although the fishermen themselves, at the bottom of the social ladder, rarely were central figures.

As urbanization spread, many farmers turned to market gardening to satisfy expanding demand for vegetables. This interaction of urban and agricultural sectors, an integral part of society, was reflected in art, both in the selection of topics and the style of execution. Vegetables played a big role in Dutch diet. They were brought into towns by barges and small boats from outlying farm areas to be sold at city markets. They were also shipped from France, Italy,

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

and Germany. Even more than fishermen and livestock farmers, horticulture farmers were focused on the market, with the prices depending on the ease of cultivation and seasonal availability.

Hardy fruits and vegetables were available year-round at low prices and made up the diet of the urban poor. These were also popular in still life paintings: apples, onions, squashes, turnips, artichokes, cabbages, and orange Horn carrots—which represented Dutch innovation in breeding new varieties. For the affluent, having pictures of these vegetables, or even eating them from time to time, served as reminder of the virtues of modest life. More elaborate and exotic vegetables, such as asparagus, also were featured, even if rarely eaten. Only the affluent could afford difficult crops and imports; yet images of opulent tables and extravagant fare, as in the works of Frans Snyders, Nicolaes Gillis, Pieter Claesz, and many others, also appealed to those with little access to such feasts.

Open air markets in major cities were gathering places. "Every morning, the wives of farmers and fishermen, though occasionally the men as well, would enter the cities to sell their goods…. People had incentives to form personal relationships, vendors wanting to maintain regular customers and buyers hoping for discounts." Urban markets were guided by city regulations that dictated hours of operation and vendor behavior. Separate markets existed for vegetables and fruits, fish, saltwater fish, and poultry, though not all municipalities could afford appropriate space and facilities or standards of cleanliness. In general, damaged vegetables were not allowed, and fish markets operated only in the morning before the sun could rot the fish. Some vendors, bakers for instance, had a clear advantage. Demand for their product was so high that they were prosperous enough to commission portraits.

Marketplace depictions were not idealized in art. Apart from occasionally placing exotic products in the wrong context or mixing fresh-water with salt-water fish, food was not altered. A bruised or damaged vegetable or fruit was an opportunity for a moral lesson. Poor quality questioned the integrity of the vendor, reminding the customer that one bad apple could damage the lot and favoring vendors who used stringent standards. And, of course, aging or damage held lessons about the ephemeral nature of all things.

Reconstructing the food history of a place from topical images is always challenging, even when the images come from a master of kitchen interiors and market scenes. Pieter Cornelisz van Rijck was one of the "important contemporary painters" listed by Karel van Mander, artist biographer and chronicler of the Dutch Golden Age. He encouraged local artists to travel abroad and learn from the world's heritage. Pieter Cornelisz van Rijck followed the advice. A student in his early days of Jacob Willemsz Delff, he later studied with Huybrecht Jacobsz Grimani, whom he accompanied to Italy, spending considerable time in Naples, where he continued to study and work. There he also came under the influence of Jacopo Bassano.

Pieter Cornelisz van Rijck's *Still Life with Two Figures* is a snap-shot of the food genre, a close-up of abundance and prosperity as well as the energy with which food was moved and promoted. The two figures capture unnamed farm workers in the midst of marketplace bustle. The man, a large basket strapped on his back, brings the goods to the stall. He is alert and purposeful. The woman in charge of this vibrant scene has stout arms and clear eyes. The produce is firm and fresh, and caged poultry hint at the range of ways by which one could make a living from the farm. The large birds in the foreground complement the colorful mess.

Market scenes around the world have not changed much, though trade of perishable foods and the urgency to move them from farm to table have changed. Dutch market scenes, the one by Cornelisz van Rijck among them, showed a sense of urgency. Perishables were intended to move as quickly as possible because blemishes and spoilage posed economic risks. Modern food technologies, such as refrigeration, permit a longer travel time. But as refrigerated perishable goods get manipulated en route, new risks are introduced. Pathogens are moving into new food items, modifying and expanding the nature and epidemiologic characteristics of foodborne disease. Despite the differences in emphasis from spoilage to microbiological contamination that causes disease outbreaks, the old rotten apple emblem still applies. Mishandled food from a single manufacturer can still spoil the lot as it raises havoc with the marketplace.

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Article Title
Listeriosis Outbreaks and Associated Food Vehicles, United States, 1998–2008

CME Questions

1. You are seeing a 25-year-old woman in her 10th week of pregnancy with a 2-day history of mild fever, loose stools, and vomiting. She reports that she has not eaten unusual foods in the past week. You wonder whether this patient might have listeriosis. What should you consider regarding the epidemiology of listeriosis?

A.   Infection with *Listeria* is marked by at least mild gastrointestinal symptoms among pregnant women
B.   There are fewer than 200 cases of listeriosis in the United States annually
C.   The incidence of listeriosis increased from 1996 to 2003
D.   The incidence of listeriosis has remained fairly stable since 2003

2. What should you consider regarding listeriosis outbreaks evaluated in the current study?

A.   There were 24 confirmed outbreaks between 1998 and 2008
B.   Less than one third of patients required hospitalization
C.   The median duration of outbreaks was 6 months
D.   Listeriosis most commonly affected infants

3. What was the most common food vehicle for listeriosis in the current study?

A.   Deli meats
B.   Cheese made from unpasteurized milk
C.   Frankfurters
D.   Sprouts and other vegetables

4. What else should you consider regarding outbreaks of listeriosis in the current study?

A.   Serotype 1/2a accounted for most outbreaks of listeriosis
B.   Serotype 4b was associated with the highest hospitalization rate
C.   Outbreaks recorded later in the study period involved more cases
D.   The number of outbreaks associated with processed meats increased throughout the study period

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

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Article Title
Staphylococcal Infections in Children, California, USA, 1985–2009

CME Questions

1. You are caring for an 18-month-old male child admitted with fever, lethargy, and decreased oral intake for 2 days. His blood culture is growing *Staphylococcus aureus*. What should you consider regarding temporal trends of pediatric hospitalizations related to staphylococcal species in the current study?

A. The biggest increase in the incidence of hospitalizations occurred between 1985 and 2002
B. The biggest increase in the incidence of hospitalizations occurred between 2006 and 2009
C. Hospitalizations for cellulitis declined between 2002 and 2006
D. The maximum incidence of hospitalization remained below 100 cases per 100,000 population

2. What was one the demographic risk factors for hospitalization with staphylococcal infection in the current study?

A. Female sex
B. African American race
C. Age 14 to 17 years
D. Low family income

3. What should you consider regarding outcomes of hospitalization related to staphylococcal infection in the current study?

A. Excluding neonates, the mean length of hospital stay was approximately 14 days
B. The length of hospital stay progressively increased over the entire study period
C. Staphylococcal infection did not independently increase the risk of mortality
D. African American race was associated with a higher risk of mortality due to staphylococcal infection

4. What should you consider regarding the types of infection with staphylococcal infections in the current study?

A. Bacteremia was the most common type of infection
B. Rates of infection with methicillin-susceptible *S. aureus* increased substantially from 2000 to 2009
C. Rates of infection with methicillin-resistant *S. aureus* (MRSA) increased substantially from 2000 to 2009
D. MRSA infections were most common among neonates

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

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Article Title

Pneumocystis jirovecii Genotype Associated with Increased Death Rate of HIV-infected Patients with Pneumonia

CME Questions

1. You are seeing a 30-year-old man with HIV infection and a new diagnosis of *Pneumocystis jirovecii* pneumonia (PCP). You are concerned regarding the possibility of genetic mutations in this organism. What was the overall rate of dihydropteroate synthase (DHPS) mutations among cases of PCP in the current study?

A. 4%
B. 33%
C. 74%
D. 83%

2. DHPS mutations were most associated with which of the following patient characteristics in the current study?

A. CD4 count less than 50 cells/μL
B. Age over 40 years
C. Male sex
D. Diagnosis of PCP after 1995

3. You initiate treatment with trimethoprim-sulfamethoxazole. Which of the following variables was most associated with sulfa resistance of *P. jirovecii* in the current study?

A. Need for mechanical ventilation at the time of PCP diagnosis
B. *P. jirovecii* type 10
C. *P. jirovecii* type 7
D. DHPS M2 mutation

4. Which of the following variables was most associated with a higher risk of death due to PCP in the current study?

A. DHPS M2 mutation
B. DHPS M3 mutation
C. *P. jirovecii* type 7
D. Older age

Activity Evaluation

1. The activity supported the learning objectives.

Strongly Disagree				Strongly Agree
1	2	3	4	5

2. The material was organized clearly for learning to occur.

Strongly Disagree				Strongly Agree
1	2	3	4	5

3. The content learned from this activity will impact my practice.

Strongly Disagree				Strongly Agree
1	2	3	4	5

4. The activity was presented objectively and free of commercial bias.

Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Broadening the Paradigm of Research and Development for Rift Valley Fever

Eastern Equine Encephalitis in Children, Massachusetts and New Hampshire, 1970–2010

Lessons and Challenges for Measles Control from Unexpected Large Outbreak, Malawi

Nosocomial and Corpse-to-Human Transmission of Nipah Virus, Bangladesh

Phylogenetic and Ecologic Analyses of Monkeypox Outbreak, South Sudan

Rift Valley Fever, Sudan, 2007 and 2010

Severe Lower Respiratory Tract Infection in Early Infancy and Pneumonia Hospitalizations among Children, Kenya

Laboratory-based Surveillance for Hepatitis E Virus Infection, United States, 2005–2012

Plague Outbreak in Libya, 2009, Unrelated to Plague in Algeria

Lessons from the History of Isolation and Quarantine

Avian Influenza Virus among Poultry Workers, Beijing, China

Typhus Group Rickettsiae in Mbeya Region, Southwestern Tanzania

Campylobacter spp. Infection among Employees at a Poultry Processing Plant, Virginia, 2008–2011

Environmental Analysis for Avian Influenza Surveillance in Wet Markets, Cambodia

Hepatitis E Virus in Pork Liver Sausage, France

Borrelia crocidurae Meningoencephalitis, West Africa

Kyasanur Forest Disease, India, 2011–2012

Genetic Variants of Echovirus 13, Northern India, 2010

Transmission and Maintenance Cycle of *Bartonella quintana* in Rhesus Macaques, China

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

Upcoming Infectious Disease Activities

February 15–18, 2013
4th International Meeting on Emerging Diseases and Surveillance (IMED)
Vienna, Austria
http://imed.isid.org

February 25–28, 2013
2013 ASM Biodefense and Emerging Diseases Research Meeting
Washington, D.C.
www.asmbiodefense.org

March 3–7, 2013
The Conference on Retroviruses and Opportunistic Infections (CROI) 2013
Georgia World Congress Center
Atlanta, GA, USA
http://www.retroconference.org

May 1–4, 2013
The Society for Healthcare Epidemiology of America (SHEA)
Spring 2013 Conference
Atlanta, GA, USA
http://shea2013.org

September 5–10, 2013
Options for the Control of Influenza VIII
Cape Town, South Africa
http://www.isirv.org

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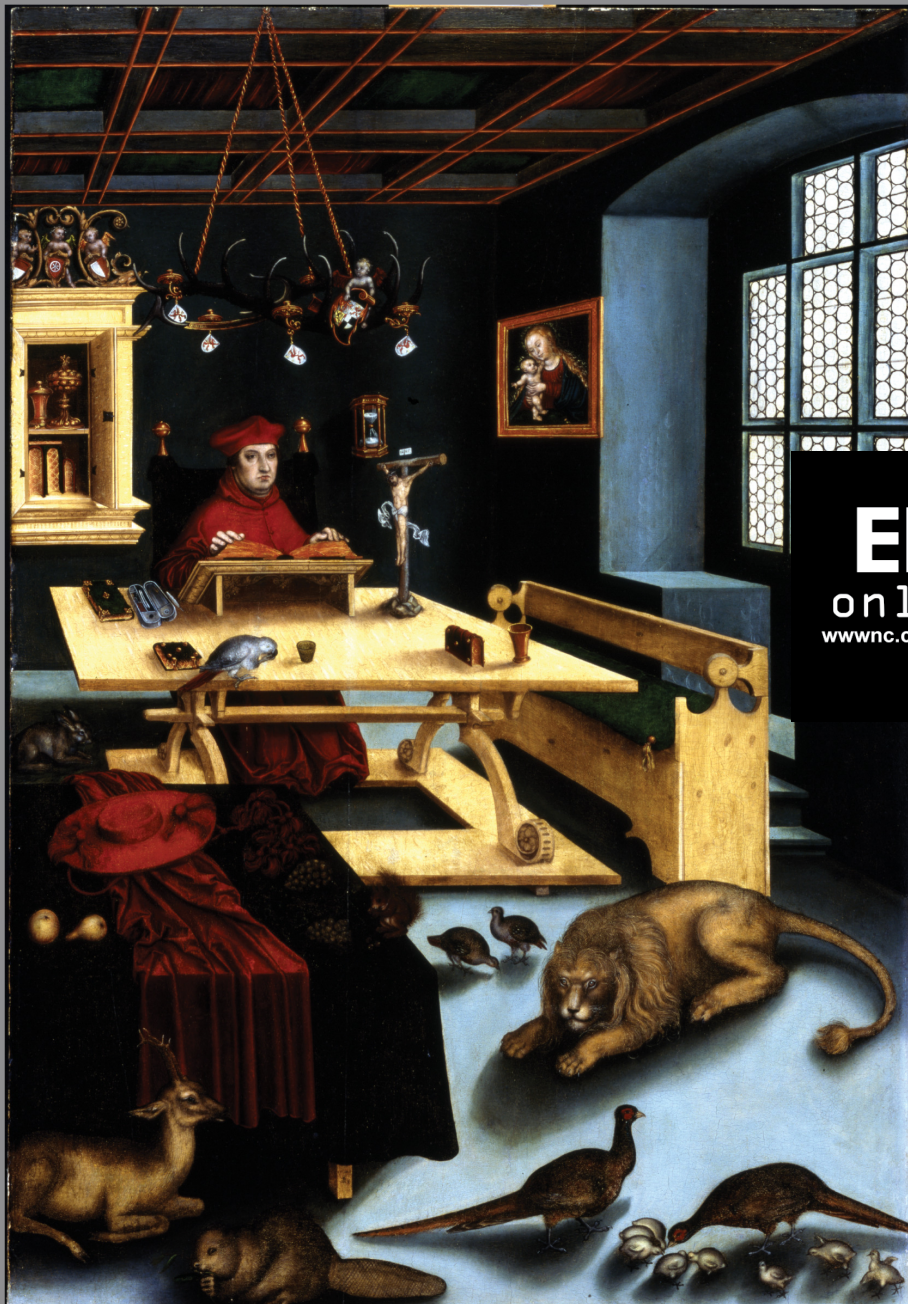   Peer-Reviewed
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Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's 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).

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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.

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Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit figures in black and white. If you wish to have color figures online, submit both in black and white and in color with corresponding legends. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpeg or .tif 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.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

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

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

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

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

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

Conference Summaries. Summaries of emerging infectious disease conference activities (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.

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.