EMERGING INFECTIOUS DISEASES®

November 2014

Foodborne Infections

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EMERGING INFECTIOUS DISEASES®

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On the Cover

Paul Cézanne 1839–1906
Still Life with Apples 1893–1894
Oil on canvas
65.4 × 81.6 cm (25 3/4 × 32 1/8 in.)

The J. Paul Getty Museum

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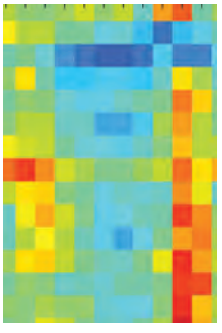

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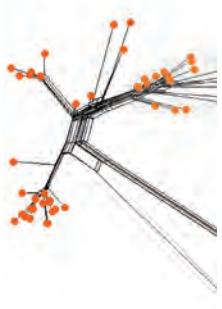

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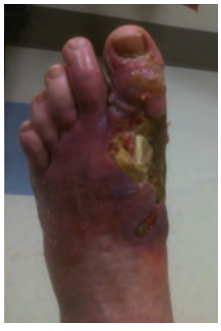

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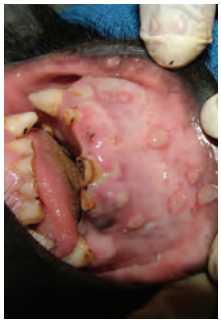

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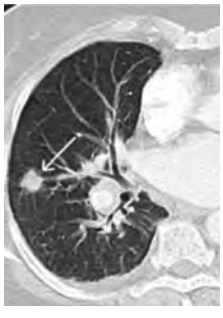

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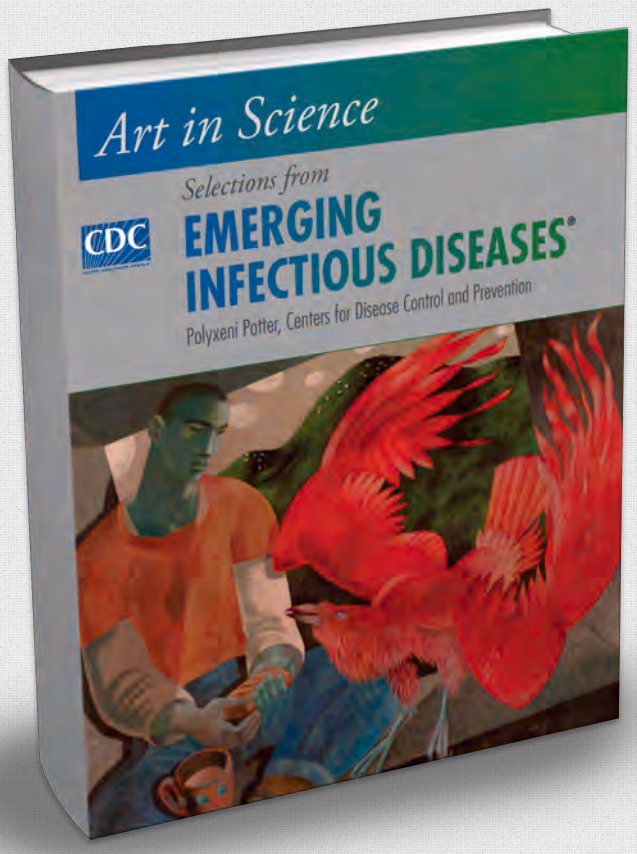

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This collection of 92 excerpts and covers from **Emerging Infectious Diseases** will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.

Blastomycosis Mortality Rates, United States, 1990–2010

Diana Khuu, Shira Shafir, Benjamin Bristow, and Frank Sorvillo

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Learning Objectives

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

1. Describe clinical features of blastomycosis, based on an epidemiologic study
2. Identify risk factors for blastomycosis-related mortality
3. Distinguish geographic differences in blastomycosis-related mortality.

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Jean Michaels Jones, Technical Writer/Editor, *Emerging Infectious Diseases. Disclosure: Jean Michaels Jones has disclosed no relevant financial relationships.*

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Authors

Disclosures: ***Diana Khuu, MPH; Shira Shafir, PhD, MPH; Benjamin Bristow, MD, MPH, DTM&H;*** *and* ***Frank Sorvillo, PhD,*** *have disclosed no relevant financial relationships.*

Blastomycosis is a potentially fatal fungal infection endemic to parts of North America. We used national multiple-cause-of-death data and census population estimates for 1990–2010 to calculate age-adjusted mortality rates and rate ratios (RRs). We modeled trends over time using Poisson regression. Death occurred more often among older persons (RR 2.11, 95% confidence limit [CL] 1.76, 2.53 for those 75–84 years of age vs. 55–64 years), men (RR 2.43, 95% CL 2.19, 2.70), Native Americans (RR 4.13, 95% CL 3.86, 4.42 vs. whites), and blacks (RR 1.86, 95% CL1.73, 2.01 vs. whites), in notably younger persons of Asian origin (mean = 41.6 years vs. 64.2 years for whites); and in the South (RR 18.15, 95% CL11.63, 28.34 vs. West) and Midwest (RR 23.10, 95% CL14.78, 36.12 vs. West). In regions where blastomycosis is endemic, we recommend that the diagnosis be considered in patients with pulmonary disease and that it be a reportable disease.

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Blastomycosis is a systemic infection caused by the thermally dimorphic fungus *Blastomyces dermatitidis* that can result in severe disease and death among humans and animals. *B. dermatitidis* is endemic to the states bordering the Mississippi and Ohio Rivers, the Great Lakes, and southern Canada; it is found in moist, acidic, enriched soil near wooded areas and in decaying vegetation or other organic material (*1*). Conidia, the spores, become airborne after disruption of areas contaminated with *B. dermatitidis*. Infection occurs primarily through inhalation of the *B. dermatitidis* spores into the lungs, where they undergo transition to the invasive yeast phase. The infection can progress in the lung, where the infection may be limited, or it can disseminate and result in extrapulmonary disease, affecting other organ systems (*2*).

The incubation period for blastomycosis is 3–15 weeks. About 30%–50% of infections are asymptomatic. Pulmonary symptoms are the most common clinical manifestations; however, extrapulmonary disease can frequently manifest as cutaneous and skeletal disease and, less frequently, as genitourinary or central nervous system disease. Liver, spleen, pericardium, thyroid, gastrointestinal tract, or adrenal glands may also be involved (*3*). Misdiagnoses and delayed diagnoses are common because the signs and symptoms resemble those of other diseases, such as bacterial pneumonia, influenza, tuberculosis, other fungal infections, and some malignancies (*4*). Accurate diagnosis relies on a high index of suspicion with confirmation by using histologic examination, culture, antigen detection assays, or PCR tests (*5*).

Antifungal agents, such as itraconazole for mild or moderate disease and amphotericin B for severe disease, can provide effective therapy, especially when administered early (*1*,*2*). With appropriate treatment, blastomycosis can be successfully treated without relapse; however, case-fatality rates of 4%–22% have been observed (*4*,*6*–*9*). Although spontaneous recovery can occur (*10*,*11*), case-patients often require monitoring of clinical progress and administration of drugs on an inpatient basis. Previous studies estimated average hospitalization costs for adults to be $20,000; that is likely less than the current true cost (*12*). Some reviews of outbreaks indicate a higher distribution of infection among persons of older age, male sex (*2*,*13*), black, Asian, and Native American racial/ethnic groups (*3*,*13*), and those who have outdoor occupations (*13*,*14*). Both immunocompetent and immunocompromised hosts may experience disease and death (*2*,*6*,*15*–*19*), although *B. dermatitidis* disproportionately affects immunocompromised patients, who tend to have more rapid and extensive pulmonary involvement, extrapulmonary infection, complications, and higher mortality rates (25%–54%) (*2*,*6*,*16*–*19*).

Past studies have expanded the knowledge about blastomycosis through focusing on cases documented in specific immunocompromised persons and statewide occurrences or in areas in which the disease is endemic (*4*,*6*–*9*,*16*–*18*); however, such studies may be limited for making definitive conclusions by their scope and small sample size. Much remains unknown about the public health burden of blastomycosis-related deaths in the United States. Reports suggest an increase in the number of blastomycosis cases in recent years (*13*,*20*). Clearer identification of risk factors from national data may raise awareness of blastomycosis in the United States and support adding it to the list of reportable diseases in regions where the pathogen is endemic to improve surveillance and control. In this study, we assessed the public health burden of blastomycosis-related deaths by examining US mortality-associated data and evaluating demographic, temporal, and geographic associations as potential risk factors.

Methods

Data Source

We used publicly available multiple-cause-of-death (MCOD) data from the National Center for Health Statistics to examine blastomycosis-related deaths in the United States during 1990–2010. These data are derived from US death certificates and include information on the causes of death coded by the International Classification of Diseases, 9th and 10th Revisions (ICD-9, ICD-10), demographic variables of age, sex, and race/ethnicity, date of death, and geographic region of residence.

Case Definition

We defined a case-patient as deceased US resident listed in the MCOD dataset during 1990–2010 whose death certificate listed blastomycosis as the underlying or contributing cause of death. The ICD-9 code 116.0 (years 1990–1998) and ICD-10 codes B40.0–B40.9 (years 1999–2010) were used to identify blastomycosis-related deaths.

Analysis

To ensure more stable estimates, we aggregated data for the study period. We calculated mortality rates and rate ratios (RRs) with 95% confidence limits (CLs) by age, sex, race/ethnicity, geographic region, and year of death using a maximum likelihood analysis presuming the response variable had a Poisson distribution (*21*), and with bridged-race population estimates data and designated geographic boundaries from the US census. We computed age-adjusted mortality rates using adjustment weights from the year 2000 US standard population data. We assessed temporal trends in age-adjusted mortality rates using a Poisson regression model of deaths per person-years in the population, designating year and age group dummy variables as independent variables, and the population as the offset.

We calculated the percentage change by year based on the estimated slope parameter and examined the Poisson regression models for overdispersion. We performed all analyses using SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

We identified 1,216 blastomycosis-related deaths among 49,574,649 deaths in the United States during 1990–2010. Among those 1,216 deaths, blastomycosis was reported as the underlying cause of death for 741 (60.9%), and as a contributing cause of death for 475 (39.1%). The overall age-adjusted mortality rate for the period was 0.21 (95% CL 0.20, 0.22) per 1 million person-years. Using Poisson regression, we identified a 2.21% (95% CL –3.11, s1.29) decline in blastomycosis-related mortality rates during the period (Figure).

Age

The mean age at death from blastomycosis was 60.8 years. Using 75 as the average age at death (*22*,*23*), we calculated that 19,097 years of potential life were lost. The mortality rates associated with blastomycosis increased with increasing age, peaking in the 75- to 84-year age group (Table 1). The mean age at death from blastomycosis was significantly lower among Hispanics ($p<0.01$), Native Americans ($p<0.01$), blacks ($p<0.01$), and Asians ($p<0.01$) than among whites based on the *t* test for difference in means.

Sex

Death related to blastomycosis was significantly more likely in men than in women ($p>0.05$). The average age at death was significantly lower for men than for women ($p = 0.02$) (Table 1). The annual mortality rate over the period obtained from using Poisson regression declined for both men and women (Table 2).

Race/Ethnicity

Native Americans and blacks were significantly more likely to die from blastomycosis-related complications than whites; overall, Asians and Hispanics were significantly less likely to die of blastomycosis than other groups (Table 1). The annual mortality rate over the period declined among blacks and whites (Table 2).

Geographic Region

Most (96.7%) of the blastomycosis-related deaths occurred in the southern and midwestern regions, and a small proportion of deaths occurred in the northeastern and western regions. The midwestern region had the highest mortality rate, followed by the southern, northeastern, and western regions (Table 1). Percentage changes in mortality rates per year over the period, calculated by using Poisson regression, showed an increase in mortality rates in the midwestern region, and a decline in the southern region (Table 2).

Table 3 shows the results of a subanalysis of the demographic characteristics of populations in the southern and midwestern regions. In the southern region, the mean age at death from blastomycosis was significantly lower among Native Americans ($p = 0.03$), blacks ($p<0.01$), and Hispanics ($p = 0.02$) than among whites based on a *t* test for difference in means. In the midwestern region, the mean age at death from blastomycosis was significantly lower among Native Americans ($p = 0.02$), Asians ($p<0.01$), blacks ($p<0.01$), and Hispanics ($p<0.01$) than among whites. Furthermore, the mean age at death from blastomycosis in the midwestern region was significantly lower among Asians than among Native Americans ($p<0.01$), blacks ($p<0.01$), and Hispanics ($p = 0.04$).

Discussion

Our findings indicate that blastomycosis is a noteworthy cause of preventable death in the United States. These findings confirm the demographic risk factors of blastomycosis indicated in previous case reports and extend these to mortality rates. Blastomycosis death occurred more often among older persons than among younger persons (*24*), and more often among men than women (*2*,*24*). The age association found likely represents waning age-related immune function and higher prevalence of

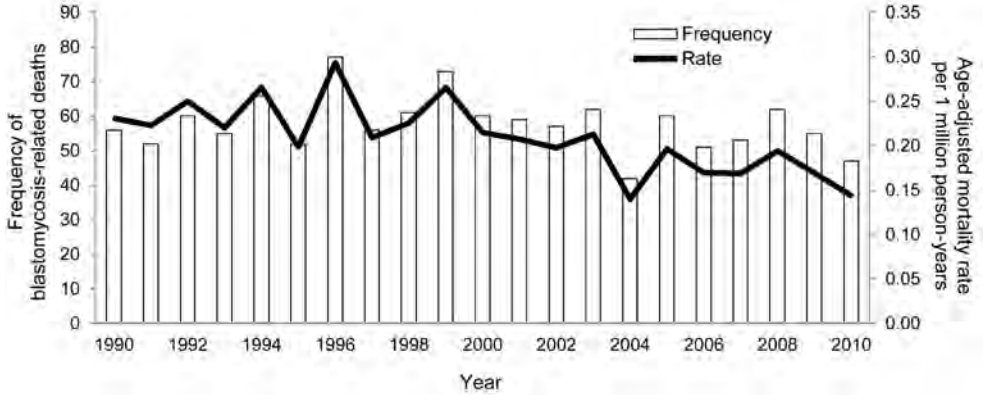

Figure. Number of blastomycosis-related deaths and age-adjusted mortality rates per 1 million person-years, by year, United States, 1990–2010.

Table 1. Blastomycosis-related deaths by sex, race/ethnicity, age group, and geographic region with corresponding age-adjusted mortality rates, United States, 1990–2010*

Characteristic	No. (%) deaths	Mean age at death, y	Age-adjusted mortality rate/1 million person-years (95% CL)†	Age-adjusted mortality rate ratio (95% CL)
Sex				
F	409 (33.6)	62.3	0.14 (0.13, 0.16)	1
M	807 (66.4)	60.1	0.35 (0.32, 0.37)	2.43 (2.19, 2.70)
Race/ethnicity				
White	918 (75.5)	64.2	0.22 (0.21, 0.23)	1
Hispanic	25 (2.1)	53.0	0.06 (0.03, 0.08)	0.25 (0.19, 0.33)
Black	223 (18.3)	50.6	0.41 (0.35, 0.46)	1.86 (1.73, 2.01)
Asian	20 (1.6)	41.6	0.11 (0.06, 0.15)	0.47 (0.41, 0.55)
Native American	30 (2.5)	52.9	0.91 (0.57, 1.25)	4.13 (3.86, 4.42)
Age, y‡				
<1	1 (0.1)	NA	0.01 (0.00, 0.04)	0.03 (0.00, 0.19)
1–4	1 (0.1)	NA	0.00 (0.00, 0.01)	0.01 (0.00, 0.05)
5–14	6 (0.5)	9.2	0.01 (0.00, 0.01)	0.02 (0.01, 0.04)
15–24	33 (2.7)	19.3	0.04 (0.03, 0.05)	0.09 (0.06, 0.13)
25–34	59 (4.9)	30.3	0.07 (0.05, 0.09)	0.16 (0.12, 0.21)
35–44	122 (10.0)	39.7	0.14 (0.11, 0.16)	0.31 (0.25, 0.39)
45–54	189 (15.5)	49.7	0.25 (0.21, 0.28)	0.56 (0.46, 0.68)
55–64	240 (19.7)	59.7	0.44 (0.38. 0.49)	1
65–74	257 (21.1)	69.9	0.65 (0.57, 0.73)	1.48 (1.24, 1.76)
75–84	235 (19.3)	78.9	0.92 (0.80, 1.04)	2.11 (1.76, 2.53)
≥85	73 (6.0)	88.1	0.81 (0.62, 0.99)	1.84 (1.42, 2.40)
Geographic region‡				
West	20 (1.7)	67.1	0.02 (0.01, 0.02)	1
South	643 (52.9)	60.5	0.31 (0.28, 0.33)	18.15 (11.63, 28.34)
Midwest	533 (43.8)	61.1	0.39 (0.36, 0.42)	23.10 (14.78, 36.12)
Northeast	20 (1.7)	61.6	0.02 (0.01, 0.02)	1.00 (0.54, 1.86)
Total	1,216 (100)	60.8	0.21 (0.20, 0.22)	

*CL, confidence limit; NA, not applicable. States in western region: AK, AZ, CA, CO, HI, ID, MT, NM, NV, OR, UT, WA, WY. States in southern region: AL, AR, DC, DE, FL, GA, KY, LA, MS, NC, OK, SC, TN, TX, VA, WV. States in midwestern region: IA, IL, IN, KS, MI, MN, MO, MS, NE, ND, OH, SD, WI. States in northeastern region: CT, MA, ME, NH, NJ, NY, PA, RI, VT.
†Standard reference population is US standard population during year 2000.
‡Age-specific rates.

immunocompromising conditions. The observed sex differences in blastomycosis mortality may be attributable to differences in occupational or recreational exposures that increase risk for infection (*14*). For example, those who work outdoors involving construction, excavation, or forestry, or participate in outdoor recreational activities such as hunting (*7*,*11*), may more likely be exposed than those who principally work indoors.

The disproportionate burden of blastomycosis deaths sustained by persons of Native American or black race is also consistent with previous reports (*3*,*24*). Increased exposure and prevalence of infection, reduced access to health care, and genetic differences may play a role in the observed race-specific disparities in blastomycosis mortality rates (*25*). A finding of the current study is that even though persons of Asian descent are at lower risk for dying from blastomycosis thanwhites, those who died from blastomycosis did so at a much younger age (22.6 years younger). This disparity is even greater in the midwestern region, where Asians died at an age 27.2 years younger than did whites.

Consistent with the recognized geographic distribution of *B. dermatitidis* (*1*–*2*), we found that death related to blastomycosis occurred more often among persons who resided in the midwestern or southern regions than among those in the western and northeastern regions. During the study period, the southern region showed decreases in mortality rates, and the midwestern region, which had the highest mortality rate, showed an increase in rate.

The use of population-based data and large numbers can provide insight, though some limitations associated with using MCOD data should be considered. First, potential underdiagnosis and underreporting of death related to blastomycosis may lead to underestimates of mortality rates and the true public health burden of blastomycosis in the United States. Low physician awareness of blastomycosis may be a contributor. Second, it was not possible to verify accuracy of recorded data or access supplemental data. For example, there may be reporting errors regarding correct race/ethnicity identification on death certificates and in population census reports. Third, we could not adjust for other possible confounders (i.e., smoking, socioeconomic factors, activity, lifestyle, occupation) because these data are not recorded on death certificates. These limitations must be considered along with our findings.

This study sheds light on the scope of the incidence of blastomycosis in the United States, though the true

Table 2. Age-adjusted time trends in blastomycosis-related mortality rate for sex, race/ethnicity, and geographic region, United States,1990–2010*

Variable	Annual percent change† in age-adjusted mortality rates (95% CL)	p value
Sex		
F	–2.28 (–3.84, –0.70)	<0.01
M	–2.32 (–3.43, –1.20)	<0.01
Race/ethnicity		
White	–1.57 (–2.62, –0.51)	<0.01
Hispanic	‡	
Black	–5.12 (–7.19, –3.01)	<0.01
Asian	‡	
Native American	‡	
Geographic region		
Northeast	‡	
South	–5.06 (–6.28, –3.28)	<0.01
Midwest	1.70 (0.27, 3.15)	0.02
West	‡	
Total	–2.21 (–3.11, –1.29)	<0.01

*CL, confidence limit.
†Annual percent change based on the Poisson model for death, with year and age group dummy variables as independent variables, and the base population as the offset. No other covariates were adjusted in the model.
‡Few deaths occurred in these categories, and thus, were not included in Poisson regression analyses to avoid inappropriate use of small numbers in Poisson regression modeling.

incidence may be greater than that reported here. Although *B. dermatitidis* infection may be difficult to prevent because of its widespread distribution in areas where blastomycosis is endemic, deaths resulting from blastomycosis can be prevented with early recognition and treatment of patients with symptomatic infection. The continued incidence of blastomycosis in the United States, as indicated by the observed modest decrease in the mortality rates over the 21-year study period, calls for improvement in provider and community awareness, which may lead to including blastomycosis as a diagnostic consideration in patients with pulmonary disease refractory to treatment. Our findings, recent reports of disproportionately high infection rates among Asians (*26*), and the lack of decline in the mortality rates in the midwestern region support further investigation. We also encourage improvements in blastomycosis surveillance that involve examining trends in incident cases, hospitalization (including length of stay), timely diagnosis, and treatment to further elucidate the burden of blastomycosis in the United States.

Acknowledgments

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Table 3. Demographic distribution and age-adjusted mortality rates for the Midwestern and Southern regions in the United States, 1990–2010

Characteristic	Midwest			South		
	No. (%) deaths	Mean age at death, y	Age-adjusted mortality rate per 1 million person-years (95% CL)	No. (%) deaths	Mean age at death, y	Age-adjusted mortality rate per 1 million person-years (95% CL)
Sex						
F	186 (34.9)	62.7	0.25 (0.21, 0.28)	212 (33.0)	61.7	0.18 (0.16, 0.21)
M	347 (65.1)	60.2	0.57 (0.51, 0.63)	431 (67.0)	59.9	0.46 (0.42, 0.50)
Race/ethnicity						
White	418 (78.4)	64.5	0.34 (0.31, 0.38)	470 (73.1)	64.0	0.29 (0.27, 0.32)
Hispanic	17 (3.2)	47.2	0.45 (0.20, 0.70)	4 (0.6)	50.3	0.02 (0.00, 0.04)
Black	63 (11.8)	50.7	0.55 (0.41, 0.69)	157 (24.4)	50.6	0.46 (0.39, 0.54)
Asian	16 (3.0)	37.3	0.66 (0.29, 1.02)	3 (0.5)	56.7	0.09 (0.00, 0.20)
Native American	19 (3.6)	52.7	0.34 (0.31, 0.38)	9 (1.4)	51.1	0.71 (0.23, 1.20)
Total	533 (100.0)	61.1	0.39 (0.36, 0.42)	643 (100.0)	60.5	0.31 (0.28, 0.33)

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etymologia

Blastomycosis [blas″ to-mi-ko′ sis]

From the Greek *blastós* ("germ, sprout") and *mykēs* ("fungus, mushroom"), this invasive fungal infection was first reported in 1894 by T. C. Gilchrist. Gilchrist initially believed the disease was caused by a protozoan, but in collaboration with Stokes, he subsequently isolated the organism, which he named *Blastomyces dermatitidis*. The infection became known as Chicago disease because most early cases were identified in the Chicago area, but it was subsequently shown to be endemic to much of eastern North America. Sporadic cases have also been reported in Africa, the Middle East, and India.

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Legionnaires' Disease Incidence and Risk Factors, New York, New York, USA, 2002–2011

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Learning Objectives

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

1. Analyze trends in the epidemiology of Legionnaires' disease.
2. Describe demographic variables associated with a higher risk for Legionnaires' disease.
3. Assess the clinical profile and prognosis of Legionnaires' disease cases in the current study.
4. Distinguish occupations associated with a high risk for Legionnaires' disease.

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Incidence of Legionnaires' disease in the United States is increasing. We reviewed case records to determine the the epidemiology of and risk factors for the 1,449 cases reported to the New York City Department of Health and Mental Hygiene, New York, New York, USA, during 2002–2011. The highest incidence (2.74 cases/100,000 population) occurred in 2009; this incidence was higher than national incidence for that year (1.15 cases/100,000 population). Overall, incidence of Legionnaires' disease in the city of New York increased 230% from 2002 to 2009 and followed a socioeconomic gradient, with highest incidence occurring in the highest poverty areas. Among patients with community-acquired cases, the probability of working in transportation, repair, protective services, cleaning, or construction was significantly higher for those with Legionnaires' disease than for the general working population. Further studies are required to clarify whether neighborhood-level poverty and work in some occupations represent risk factors for this disease.

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Legionnaires' disease, a bacterial infection caused primarily by the species *Legionella pneumophila*, was initially recognized as the cause of a 1976 outbreak of respiratory disease that resulted in 221 cases of illness, primarily among attendees of an American Legion convention in Philadelphia (*1*). In that outbreak, 34 people died, catapulting the previously unidentified disease to national attention (*1–4*). Infection with *Legionella* spp. is now classified into 2 clinically distinct diseases, Pontiac fever and Legionnaires' disease; Pontiac fever is a milder illness that does not involve pneumonia (*2*).

An estimated 8,000–18,000 persons are hospitalized for legionellosis each year in the United States; ≈5% 30% of case-patients die (*2*,*5*). During the 2000s, cases of legionellosis in the United States reported to the Centers for Disease Control and Prevention increased 279%, from 1,110 in 2000 to 4,202 in 2011. During the same period, the national incidence of legionellosis increased 249%, from 0.39 per 100,000 persons in 2000 to 1.36 per 100,000 persons in 2011 (*6*,*7*).

Most *Legionella* species live in water, and transmission to humans occurs through inhalation of small water droplets in which the pathogen is aerosolized or by aspiration of contaminated water into the lungs (*2*,*8*). Known host risk factors for legionellosis are smoking, chronic obstructive pulmonary disease, diabetes, immune system compromise, older age (>50 years), and receipt of a transplant or chemotherapy (*9*). Environmental risk factors associated with legionellosis outbreaks are travel, residence in a health care facility, and proximity to cooling towers, whirlpool spas, decorative fountains, and grocery produce misters (*2*,*6*,*10*,*11*). However, only limited studies have been done regarding socioeconomic and occupational risk factors for community-acquired cases; some studies have identified driving as a potential occupational risk factor (*12*,*13*).

To describe the epidemiology of Legionnaires' disease in New York, New York, we analyzed surveillance data for 2002–2011. In addition to overall incidence, we measured the associations between acquisition of *Legionella* infection and socioeconomic and occupational groups.

Materials and Methods

Routine Surveillance Data Collection

The New York City (NYC) Health Code requires providers and laboratories to report all cases of legionellosis in city residents to the NYC Department of Health and Mental Hygiene (DOHMH); these reports include positive results for *Legionella* in cultures, urine antigen tests, direct fluorescent antibody stains, and serologic testing. In addition, all *Legionella*-positive cultures are required to be sent to either the DOHMH Public Health Laboratory or the New York State Department of Health at Wadsworth Center for confirmation, speciation, and serogrouping. For this analysis, residents of the city of New York who had confirmed legionellosis during 2002–2011 were identified by using the Council of State and Territorial Epidemiologists criteria for confirmed cases (*14*). These criteria include radiographic or clinical pneumonia and laboratory diagnosis made by urinary antigen, culture, or 4-fold rise in *L. pneumophila* serum antibody titer. Three cases of Pontiac fever were found during 2002–2011 but were excluded from this study; all legionellosis cases we analyzed were classified as Legionnaires' disease.

Case Investigation

DOHMH investigates all urine antigen, culture, direct fluorescent antibody stain, or nucleic acid assay results positive for *Legionella* and all reports of 4-fold or greater rise in antibody titers between acute- and convalescent-phase serum specimens. Because single reports of elevated *Legionella* serum antibody titers are not diagnostic for legionellosis, those reports are investigated on a case-by-case basis. For the investigations, information from medical charts is abstracted, and patients undergo a standardized interview. Data collected are patient sex, age, race/ethnicity, pre-existing medical conditions, occupation, nights away from home, recreational water exposures, and other risk factors for acquiring *Legionella* infection.

All case-patients or case-patient proxies were asked about work, nights away from home, visits to and stays in health care facilities, exposure to water aerosols, and other possible exposures during the 14 days before symptom onset. For this analysis, cases were considered to be definitely health care facility–associated if the case-patient resided in a hospital or nursing home for the entire 10 days (for 2002–2007) or the entire 14 days (for 2008–2011) before onset. (This change in criteria was made in 2008 in consultation with the New York State Department of Health in light of the consensus at that time that the incubation period was 2–14 days. However, NYC DOHMH has since returned to a standard 2–10 day incubation period for this determination.) Cases were considered possibly health care facility–associated if the case-patient resided in a hospital or nursing home for part of the 2–9 days (for 2002–2007) or 2–13 days (for 2008–2011) before onset. All other cases were considered community acquired.

Death data were recorded by whether the case-patient had died at the time the investigation was closed. Investigations for confirmed cases are considered closed when diagnosis is confirmed and the patient or proxy interview is completed or it is determined that the interview cannot be completed. Death classification may therefore not have included some case-patients who died from legionellosis

after the investigation was closed and may have included some who died of causes other than legionellosis.

Data Sources

Intercensal population estimates for 2002–2009 were produced by DOHMH on the basis of the US Census Bureau Population Estimate Program and housing unit data obtained from the NYC Department of City Planning, available as of November 2012 (*15*). For 2010 and 2011, 2010 US Census data were used.

The Community Health Survey, a yearly cross-sectional telephone survey conducted by DOHMH that provides citywide public health surveillance data (*15*), was used to estimate the prevalence of diabetes in the general population. The American Community Survey, a yearly demographic survey conducted by the US Census Bureau (*16*), was used to calculate population denominators for occupational data. Occupational data collected during case-patient interviews were used to categorize case-patients into American Community Survey–defined occupational classifications.

Neighborhood-level poverty was assessed by using census tract poverty data provided by the US Census. Neighborhood-level poverty was defined as the percentage of residents with household incomes <100% of the federal poverty level on the basis of US Census data from 2000. Census tracts were classified into 6 poverty level categories: very low, <5%; low, 5%–9%; medium, 10%–19%; high, 20%–29%; very high, 30%–39%; and highest, ≥40%.

Statistical Analyses

Crude and age-adjusted population-based incidence rates were calculated, with age-adjustments standardized to the US Census 2000 population. Relative risks and their 95% CIs were used to compare the demographic characteristics of legionellosis case-patients with those of the general population of the city of New York. Student *t*-tests were used to compare average incidences between 2 population subgroups. Logistic regression was used to compare death outcomes across sex and acquisition setting while adjusting for age.

Case-patient addresses were geolocated to census tracts by using ArcMap 10 (http://www.esri.com/software/arcgis). Because health care facility–associated cases may be associated with different risk factor patterns, we restricted occupational and neighborhood-level poverty analyses to community-acquired cases. To assess the relationship between race/ethnicity and socioeconomic status, we calculated crude and age-adjusted legionellosis incidence by race/ethnicity in each neighborhood-level poverty group. All analyses were conducted by using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Results were considered statistically significant at $p<0.05$.

Results

Cases

In the city of New York, a total of 1,449 confirmed legionellosis cases were reported through routine surveillance for January 1, 2002–December 31, 2011. Cases were predominantly diagnosed by urinary antigen testing (n = 1,409, 97.2%); 5.7% (n = 82) of all cases were confirmed by culture. The method of diagnosis remained relatively constant over the study period. The crude incidence rate of legionellosis increased from 0.83 cases/100,000 population in 2002 to the highest incidence of 2.74 cases/100,000 population in 2009, a 230% increase (Figure 1). In 2010 and 2011, crude incidence rates remained high at 2.02 and 2.64 cases/100,000 population, respectively. Average yearly incidence for 2002–2011 was 1.75 cases/100,000 population.

Incidence of legionellosis increased in all age groups during the analysis period, but the largest increase (826%) was for the 70–79 age group from 2002 to 2010. Incidence of community-acquired cases was consistently higher during the summer and early fall months; 71.6% (n = 1,038) of cases were diagnosed during June–October.

Demographic Variables

The average incidence of legionellosis was higher for the male population than for the female population (2.29 cases/100,000 population vs. 1.26 cases/100,000 population; p = 0.0004) and was higher for older age groups (Figure 2). Median case-patient age was 61.0 years (mean 61.8 years, range 9 months to 103 years), and overall incidence rates increased for each age group, with the highest incidence among those ≥80 years of age (Figure 2). Cases were predominately acquired in the community (88.3%, n = 1,279) rather than definitely (7.0%, n = 102) or possibly (4.7%, n = 68) acquired in a health care facility.

Race/ethnicity was unknown for 279 (19%) case-patients. Average incidence per year for non-Hispanic blacks (2.15 cases/100,000 population) was higher than that for non-Hispanic whites (1.56 cases/100,000 population; p = 0.13)

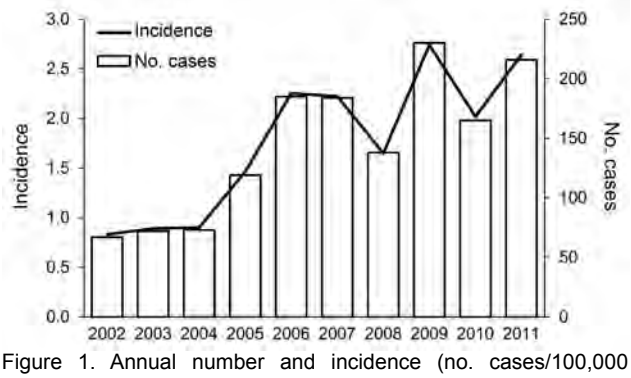

Figure 1. Annual number and incidence (no. cases/100,000 population) of Legionnaires' disease cases, New York, New York, USA, 2002–2011.

and significantly higher than that for Hispanics (1.02 cases/100,000 population; p = 0.003) or Asian/Pacific Islanders (0.41 cases/100,000 population; p = 0.0004).

Deaths

Overall, 12.8% (185/1,449) of legionellosis case-patients died before the DOHMH case investigation was closed. Death rates were significantly higher for case-patients with definitely health care facility–associated infections than for those with community-acquired infections (odds ratio [OR] 4.78, 95% CI 3.07–7.46). Overall, 35.3% (36/102) of case-patients with definitely health care facility–associated infections died, compared with 10.2% (131/1,279) of those with community-acquired infections. The crude odds for death for women were 1.48 times that for men for community-acquired cases (95% CI 1.09–2.02; p = 0.02), but after adjusting for age, the odds for death were not significantly different between men and women (OR 1.33, 95% CI 0.97–1.83; p = 0.07).

Medical Risk Factors

Of the 1,449 legionellosis cases, 1,278 (88.2%) patients had ≥1 underlying medical condition that was a known risk factor for legionellosis (*9*). Current or past smoking (n = 879, 60.7%) and diabetes mellitus (n = 506, 34.9%) were the most frequently reported underlying conditions. After stratifying patients by age, the risk for diabetes was higher for persons with legionellosis than for the general population in every age category; the risk for diabetes was 1.9 times higher for legionellosis case-patients ≥65 years of age, 2.5 times higher for those 45–64 years of age, 5.7 times higher for those 25–44 years of age, and 6.3 times higher for those 18–24 years of age (Table 1).

Neighborhood Poverty Level

To assess neighborhood poverty level, we restricted our analysis to community-acquired cases. Of the 1,279 community-aqcuired cases, 1,261 (98.6%) could be geolocated. After patient age was adjusted for, the incidence of legionellosis for community-acquired cases followed a gradient; incidence in the highest poverty areas (3.0 average yearly cases/100,000 population) was 2.5 times higher than that for the lowest poverty areas (1.2 average yearly cases/100,000 population) (Table 2). As shown in Table 3, the same gradient existed within each racial/ethnic group, with the highest incidence of disease in the highest poverty group. However, after age adjustment, rates of legionellosis among black non-Hispanics remained higher than those for other race/ethnic groups in each poverty group.

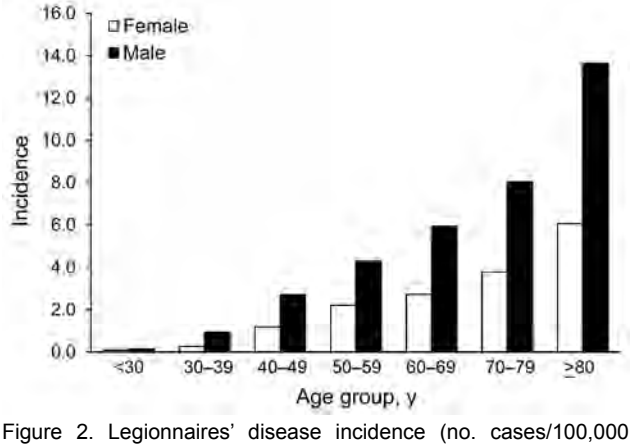

Figure 2. Legionnaires' disease incidence (no. cases/100,000 population) by sex and age group, New York, New York, USA, 2002–2011.

Occupation

Among the 1,279 community-acquired legionellosis cases, 375 (29.3%) case-patients reported working in the 2 weeks before disease onset. The average and median age of case-patients who worked was 53.0 years, and the average age of case-patients who did not work was 64.9 years (median 65.0 years), a mean difference of 11.9 years (95% CI 9.9–13.8 years; p<0.0001). A higher percentage of working case-patients were male than female (71.5% vs. 59.1%; p<0.0001). Compared with the general population, legionellosis case-patients were significantly more likely to work in transportation (crude relative risk [RR] 2.36, 95% CI 1.82–3.06), repair (crude RR 1.86, 95% CI 1.11–3.11), protective services (crude RR 1.77, 95% CI 1.15–2.71), cleaning services (crude RR 1.54, 95% CI 1.07–2.22), or construction (crude RR 1.49, 95% CI 1.03–2.16) (Table 4).

Discussion

Surveillance data show a sustained increase in legionellosis incidence in the city of New York during 2002–2011, a trend that is also reflected in data nationwide (*6*,*7*,*17*). During this period, legionellosis incidence in New York was highest in 2009 (2.74 cases/100,000 population), higher than overall US incidence in 2009 (1.5 cases/100,000 population) (*6*). For 2011, the last year of the study, legionellosis incidence in New York was 2.64 cases/100,000 population, higher than US overall incidence in 2011 (1.36 cases/100,000 population) (*7*).

Urinary antigen testing remained the primary method of legionellosis diagnosis during the study period, indicating that the increases are most likely not an artifact of changes in diagnostics. However, it is possible that increased awareness of the disease among clinicians led to increased testing over time. The overall epidemiology of legionellosis cases in New York is similar to that reported elsewhere, with higher incidence among men and in older age groups (*6*,*7*,*11*). In addition, several host, socioeconomic, and occupational factors were significantly associated with the risk of acquiring *Legionella* infection among case-patients in New York.

Table 1. Most common underlying medical conditions among Legionnaires' disease case-patients, New York, New York, USA, 2002–2011*

Condition	No. (%) case-patients	Relative risk (95% CI)†
Smoking history	879 (60.7)	
COPD	223 (15.4)	
Cancer	215 (14.8)	
Diabetes		
Overall	506 (34.9)	
By age group, y†		
18–24	1 (7.7)	6.3 (0.95–41.10)
25–44	31 (15.2)	5.7 (4.1–7.9)
45–64	208 (34.1)	2.5 (2.2–2.8)
≥65	264 (42.8)	1.9 (1.7–2.0)

*n = 1,449. COPD, chronic obstructive pulmonary disease.
†For case-patients with diabetes only. Population denominators for diabetes prevalence are from the American Community Survey. Relative risks for children age <18 y (2/5 case-patients <18 had diabetes) were not calculated because population denominators were not available for this age group.

More than 88% of legionellosis case-patients in this study had ≥1 underlying medical condition that is recognized as a risk factor for infection, a rate higher than that reported for legionellosis populations in Italy and France (*18*,*19*). A smoking history and diabetes were the most commonly reported risk factors for legionellosis patients in New York, a result that has also been seen elsewhere in the United States (*8*). Prevalence of diabetes among legionellosis patients in New York was higher than that for the general population of the city in every age category for which data were available, although the number of legionellosis cases in the 18–24-year-old group was small (n = 13), and prevalence of diabetes did not achieve statistical significance in this group. The prevalence of legionellosis case-patients with diabetes (34.9%) was also higher than that reported among persons with legionellosis in France (*19*). Given the available data for 2002–2011, we were unable to determine percentages of type 1 and type 2 diabetes among the case-patients with diabetes; because type 1 diabetes is not currently preventable, this limitation restricts the ability to make inferences about whether diabetes may be a preventable risk factor for acquisition of *Legionella* infection.

We found that socioeconomic factors were associated with increased incidence of legionellosis. The data show a distinct gradient in incidence according to neighborhood-level poverty, ranging from 3.0 average yearly cases/100,000 population in the highest-poverty areas to 1.2 average yearly cases/100,000 population in the lowest-poverty areas. Because >98% of community-acquired legionellosis cases in this study were geocoded to a census tract, these results are unlikely to be biased. Legionellosis incidence also varied by racial and ethnic groups, with the highest incidence among non-Hispanic blacks, a finding that was also noted in a review of nationwide legionellosis cases reported to the Centers for Disease Control and Prevention during 2000–2009 (*6*). Surveillance data included in our study indicate substantial disparities in incidence of legionellosis by race/ethnicity and socioeconomic factors. The association between race/ethnicity and neighborhood-level poverty suggests that socioeconomic factors may contribute to the disparity in incidence by race/ethnicity. It remains unclear whether the disparities in legionellosis incidence arise from differences in neighborhood environments, such as building construction and maintenance; from differences in access to preventive health care services that could potentially improve conditions such as diabetes; or from differences in the population itself, such as increased levels of diabetes and other host risk factors (*20*,*21*).

We also found that certain occupations might be associated with increased risk for community-acquired legionellosis. Occupations that may involve working with machinery or being outdoors (e.g., transportation, construction, manufacturing) were associated with increased risk of acquiring *Legionella* infection. The increased risk associated with cleaning and janitorial work may involve greater exposure to aerosolized water in the air, perhaps through contact with plumbing systems. The increased risk associated with being a protective services worker (e.g., police officer, crossing guard, security guard) has a less clear causal pathway but may involve a higher exposure to aerosolized water in the outdoors. Other studies in the United States regarding occupational risks for acquiring *Legionella* infection are lacking, making it difficult to determine whether these results are consistent with trends observed elsewhere. However, the results we found for transportation workers are consistent with studies regarding increased risk of *Legionella* infection in England, the Netherlands, and Japan (*12*,*22*,*23*).

Our study has several limitations. First, legionellosis is likely underdiagnosed, as has been documented elsewhere (*24*). Second, we did not have race/ethnicity data for 19% of the case-patients in our study; if the rate of missing data differs across race/ethnicity groups, the estimates of incidence

Table 2. Rates of community-acquired Legionnaires' disease by census tract poverty level, New York, New York, USA, 2002–2011*

Census tract poverty level†	No. cases	Rate/100,000 population	
		Crude	Age-adjusted‡
Very low	80	1.4	1.2
Low	229	1.5	1.4
Medium	298	1.3	1.4
High	247	1.4	1.6
Very high	196	1.9	2.2
Highest	211	2.3	3.0
Total	1,261	1.6	

*Eighteen community-acquired cases could not be geolocated to a census tract and were excluded from the total case count.
†By percentage of residents with household incomes <100% of the federal poverty level according to 2000 U.S. Census data: very low, <5%; low, 5%–9%; medium, 10%–19%; high, 20%–29%; very high, 30%–39%; highest, ≥40%.
‡Age-adjustment calculations were based on 2000 U.S. Census standard population.

Table 3. Sociodemographic characteristics of persons with community-acquired Legionnaires' disease, New York, New York, USA, 2002–2011*

Poverty level† and race/ethnicity	Cumulative no. cases	Average annual incidence/100,000 population	
		Crude	Age-adjusted‡
Very low			
White non-Hispanic	53	1.3	0.92
Black non-Hispanic	9	1.9	2.0
Hispanic	4	1.0	1.4
Asian/Native Hawaiian/Pacific Islander/American Indian non-Hispanic	4	0.83	1.5
Other/multirace non-Hispanic	0	0	0
Unknown	10		
Low			
White non-Hispanic	130	1.4	1.1
Black non-Hispanic	34	1.5	1.6
Hispanic	13	0.79	1.0
Asian/Native Hawaiian/Pacific Islander/American Indian non-Hispanic	11	0.74	1.1
Other/multirace non-Hispanic	3	0.62	1.2
Unknown	38		
Medium			
White non-Hispanic	112	1.3	0.98
Black non-Hispanic	76	1.7	1.8
Hispanic	45	0.92	1.2
Asian/Native Hawaiian/Pacific Islander/American Indian non-Hispanic	7	0.23	0.29
Other/multirace non-Hispanic	4	0.38	0.50
Unknown	54		
High			
White non-Hispanic	50	1.3	0.94
Black non-Hispanic	88	1.7	1.9
Hispanic	40	0.75	1.1
Asian/Native Hawaiian/Pacific Islander/American Indian non-Hispanic	6	0.31	0.39
Other/multirace non-Hispanic	3	0.43	0.60
Unknown	60		
Very high			
White non-Hispanic	24	2.3	1.8
Black non-Hispanic	78	2.2	2.5
Hispanic	48	1.0	1.4
Asian/Native Hawaiian/Pacific Islander/American Indian non-Hispanic	8	0.98	1.1
Other/multirace non-Hispanic	3	1.1	1.5
Unknown	35		
Highest			
White non-Hispanic	20	2.6	2.6
Black non-Hispanic	91	2.7	3.3
Hispanic	55	1.2	1.7
Asian/Native Hawaiian/Pacific Islander/American Indian non-Hispanic	0	0	0
Other/multirace non-Hispanic	1	0.45	0.87
Unknown	44		
Total	1,261		

*Eighteen cases could not be geolocated to a census tract and were excluded from the total case count.
†By percentage of residents with household incomes <100% of the federal poverty level according to 2000 U.S. Census data: very low, <5%; low, 5%–9%; medium, 10%–19%; high, 20%–29%; very high, 30%–39%; highest, ≥40%.
‡Age-adjustment calculations were based on 2000 U.S. Census standard population.

by race/ethnicity may be biased and could underestimate the true incidence of disease in at least some groups. Third, during the analysis period, we identified some health care facility–associated outbreaks and some community-associated clusters that may or may not have been outbreaks involving a point source exposure. Although most cases included in the review period were sporadic rather than outbreak-related, the inclusion of both types of cases in this review obscures possible differences between sporadic and outbreak-related cases in terms of exposure and host-patient factors. Fourth, travel-associated cases were not excluded from this analysis. Surveillance staff did ask case-patients if they spent a night away from home during the incubation period, but we cannot determine whether the case was "travel-associated" on the basis of this question because it would have included respondents who did not travel outside the city.

Fifth, although the data indicate that an association between occupation and socioeconomic status is likely, we were unable to adjust for this possibility because of small numbers. The low percentage of case-patients with community-acquired legionellosis who reported working in the 2 weeks before illness onset (n = 375, 29.3%) probably reflects the role of advanced age (i.e., many who acquire this infection are retired), medical conditions, or both as risk factors for disease. If underdiagnosis is higher

Table 4. Incidence of and risk for Legionnaires' disease by case-patient occupation, New York, New York, USA, 2002–2011

Occupational category	No. (%) working case-patients	% Total working population*	Mean crude annual disease incidence†	Crude relative risk (95% CI)‡
Transportation	49 (13.1)	5.5	1.9	2.36 (1.82–3.06)
Repair	14 (3.7)	2.0	1.5	1.86 (1.11–3.11)
Protection	20 (5.3)	3.0	1.4	1.77 (1.15–2.71)
Cleaning	27 (7.2)	4.7	1.3	1.54 (1.07–2.22)
Construction	26 (6.9)	4.7	1.2	1.49 (1.03–2.16)
Service	24 (6.4)	5.0	1.0	1.28 (0.87–1.89)
Legal	10 (2.7)	2.1	1.0	1.27 (0.69–2.34)
Office	59 (15.7)	15.0	0.9	1.05 (0.83–1.33)
Entertainment	17 (4.5)	4.6	0.8	0.98 (0.61–1.55)
Production	14 (3.7)	3.8	0.8	0.99 (0.59–1.65)
Counsel	7 (1.9)	2.1	0.7	0.91 (0.43–1.89)
Finance	8 (2.1)	2.7	0.6	0.78 (0.39–1.55)
Medical	12 (3.2)	4.2	0.6	0.76 (0.43–1.32)
Food	16 (4.3)	5.3	0.7	0.80 (0.50–1.29)
Health	10 (2.7)	4.1	0.5	0.66 (0.36–1.21)
Sales	27 (7.2)	11.3	0.5	0.64 (0.44–0.92)
Engineering	2 (0.5)	0.9	0.5	0.58 (0.15–2.33)
Management	11 (2.9)	8.1	0.3	0.36 (0.20–0.65)
Education	6 (1.6)	5.9	0.2	0.27 (0.12–0.60)
Computer	1 (0.3)	2.0	0.1	0.13 (0.02–0.95)
Uncategorized/missing	15 (4.0)	0.0	NA	
Total working	375 (100.0)	100.0	0.8	

*Percentage of total city population working in occupational category (American Community Survey occupational survey).
†Per 100,000 population, based on number of persons working in occupational category, according to 2005–2009 American Community Survey population data.
‡Comparison of risk for being in each occupational category for working case-patients versus the general working population.

for certain occupational groups, possibly because of socioeconomic factors and differences in access to health care or access to diagnostic tests for *Legionella* infection, a decreased association between certain occupations and risk for acquiring infection may result. Some correlation likely exists between occupational group and socioeconomic status, but it is difficult to make assumptions about individual income levels on the basis of occupational category. Another limitation is the lack of exact measurement of the occupational risk factors for each individual; this study makes assumptions about the average levels of exposures in each occupational category that may not hold true for individual patients within each category, especially because the occupational categorization was done after data collection. In addition, because of the small numbers within some occupational categories and the nature of surveillance data, these analyses could not be adjusted for confounders that may be related to occupation and risk of acquiring *Legionella* infection. However, among the occupational groups for which there was a univariate association with risk of acquiring *Legionella* infection, the frequency of smoking and frequency of diabetes were not significantly elevated. In view of the limitations of the data, interpretation of these findings should be used primarily for hypothesis generation. More careful measurement of occupational risk factors during routine surveillance may help clarify the causal pathway between occupation and risk of disease.

Despite these limitations, our findings suggest systemic differences in the risk for acquiring *Legionella* infection based on neighborhood-level poverty; these socioeconomic disparities in disease should be of concern to public health policy makers. If environmental issues in high-poverty neighborhoods contribute to the disparity, greater effort may be warranted, for example, on the upkeep of cooling towers and water systems in the buildings in these areas. If occupations such as working in transportation, construction, and plumbing involve increased exposure to *Legionella* and risk for acquiring infection, public health interventions that target these occupational groups (e.g., use of personal protective equipment such as respirators under certain working conditions) may be effective in reducing legionellosis incidence. Future studies are needed to clarify the exact mechanisms by which these host, socioeconomic, and occupational exposures may contribute to *Legionella* infection to help guide public health interventions. Socioeconomic and occupational factors represent important and understudied potential sources of exposure among community-acquired cases of legionellosis.

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Death Patterns during the 1918 Influenza Pandemic in Chile

Gerardo Chowell, Lone Simonsen, Jose Flores, Mark A. Miller, and Cécile Viboud

Scarce information about the epidemiology of historical influenza pandemics in South America prevents complete understanding of pandemic patterns throughout the continent and across different climatic zones. To fill gaps with regard to spatiotemporal patterns of deaths associated with the 1918 influenza pandemic in Chile, we reviewed archival records. We found evidence that multiple pandemic waves at various times of the year and of varying intensities occurred during 1918–1921 and that influenza-related excess deaths peaked during July–August 1919. Pandemic-associated mortality rates were elevated for all age groups, including for adults ≥50 years of age; elevation from baseline was highest for young adults. Overall, the rate of excess deaths from the pandemic was estimated at 0.94% in Chile, similar to rates reported elsewhere in Latin America, but rates varied ≈10-fold across provinces. Patterns of death during the pandemic were affected by variation in host-specific susceptibility, population density, baseline death rate, and climate.

The 1918 influenza A(H1N1) pandemic was one of the most devastating epidemic events in recent history; an estimated ≈1% of the global population (20–50 million persons) died (*1*), including >14 million in India alone (*2*). Our understanding of the epidemiologic patterns of this pandemic has improved over the past decade as a result of intensive efforts to locate, digitize, and analyze archival disease records (*3*). In particular, studies focusing on the United States (*4*,*5*), Mexico (*6*), Colombia (*7*), Brazil (*8*), and Peru (*9*) have shed light on geographic variation in the patterns of timing, intensity, and patient age during successive pandemic waves across the Americas. Yet there have been no reports from temperate locales of South America; this gap prevents a complete understanding of the epidemiology of the pandemic throughout the continent and across different climatic zones.

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A consistent finding across reports from North America, Europe, Latin America, and Asia (*4–7*,*9–12*) is the disproportionate increase in mortality rates among young adults during the pandemic period compared with prepandemic years. Further reports from the United States and Europe have shown that influenza-related deaths among seniors (≥50 years of age) were significantly reduced during the lethal 1918–19 pandemic wave relative to baseline periods. In contrast, this protective effect for seniors did not apply to Mexico, Colombia, and remote island populations, probably because of differences in prior immunity between regions (*6*,*13*). In addition, large geographic variations in mortality rates from the pandemic have been documented (*14–16*).

Although recent studies have highlighted latitudinal and climatic variations in contemporary influenza epidemics and pandemics (*17–20*), little is known about the role of climate in the severity or timing of the 1918 pandemic. Chile is a unique country; it spans an extensive latitudinal gradient, has a variety of climatic zones, and has preserved historical records dating back to the 1918 pandemic period. We characterized geographic variation in pandemic influenza–related death patterns in Chile by using national and regional death statistics combined with a unique dataset of individual-level death certificates from Concepción, a large city in southern Chile. We discuss our findings in relation to those reported elsewhere in the Americas and globally.

Material and Methods

Geographic Setting

Chile covers a long and narrow strip between the Andes mountains (to the east) and the Pacific Ocean (to the west), located at latitude 17°S–56°S and longitude 66°W–76°W. The climatic zones of Chile include dry and desert areas in the north, Mediterranean regions in the center, and rainy temperate areas in the south. According to census information, the 1917 population of Chile was 3.9 million (*21*), and Chile was divided into 24 contiguous administrative provinces (Figure 1). In the period surrounding the 1918 pandemic, hygienic conditions were poor and the infant mortality rate was very high (rate among children <1 year of age was 25%–30%) (*21*).

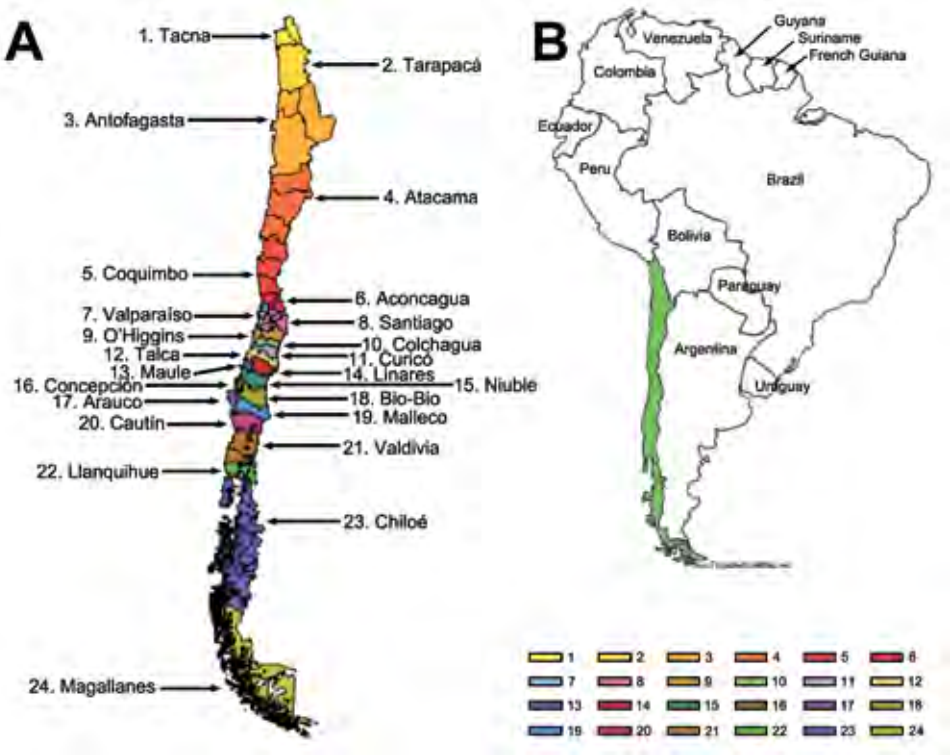

Figure 1. The 24 contiguous administrative provinces in Chile in 1918 (A) and location of Chile in South America.

Data Sources

Vital Statistics, Chile, Anuarios Estadísticos, 1915–1921

To explore the spatiotemporal patterns of pandemic-related deaths in Chile, we compiled monthly all-cause death statistics for 1915–1921 for the 24 administrative provinces from official publications and a variety of sources (*21*). We obtained province-level population size estimates (1917 census), population density estimates (1920), and infant (<1 year of age) mortality rates (*21*) (Table 1). To explore pandemic timing and effect along a latitudinal gradient, we compiled geographic coordinates for province-level population centers.

Historical Death Certificates in Concepción, Chile, 1915–1920

Given the lack of age detail in official vital statistics records, we used a different data source to document the age patterns of persons who died during the pandemic. Concepción is the third largest city in the country and has kept well-preserved individual death certificates. All-cause death records comprising 18,805 individual death certificates corresponding to January 1915–December 1920 were manually retrieved from the civil registry of the city. For each record, we tabulated the person's age at death, cause of death, and exact date of death. Most death certificates listed a unique cause of death. We then created a weekly time series for deaths from all causes and respiratory illnesses, stratified into 4 age groups (<20, 20–29, 30–49, and ≥50 years). Deaths ascribed to influenza, pneumonia, bronchopneumonia, or bronchitis were classified as respiratory. Information about age or cause of death was missing on <1% of all records. We obtained age-specific population data for Concepción from the Instituto Nacional de Estadísticas and used estimates from 1920 to derive age-specific mortality rates for the study period (*22*). The total population of the city was 82,662 in 1920, and it increased ≈1% annually over the study period (*22*)

Local Newspapers, 1918–1919

We gathered anecdotal information on the temporal course and severity of the pandemic during 1918–1919 by examining the most popular daily newspapers published in the capital city of Santiago (El Mercurio) and in Concepción (El Sur). We identified 49 issues of El Mercurio and 45 issues of El Sur that contained articles or commentaries relating to pandemic influenza during this period.

Statistical Analyses

To quantify the mortality rate associated with the influenza pandemic by province and age group during 1918–1921, we estimated excess deaths as the number of deaths occurring above a seasonal baseline of expected deaths in the absence of pandemic influenza activity (*23*). To estimate baseline deaths in the absence of influenza activity,

Table 1. Estimates of absolute excess mortality rates attributable to pandemic influenza across 24 provinces of Chile, 1918–1921*

Province†	Pandemic period, no. excess deaths/10,000 population				Cumulative absolute excess mortality rate
	Jul 1918–Mar 1919	Jun 1919–Mar 1920	Nov 1920–Mar 1921	Jun 1921–Dec 1921	
Tacna	8.8	64.8	0.0	0.0	73.6
Tarapacá	14.3	0.0	10.5	0.0	24.8
Antofagasta	0.0	0.0	0.0	0.0	0.0
Atacama	27.0	27.5	0.0	42.3	96.8
Coquimbo	0.0	59.2	0.0	38.3	97.5
Aconcagua	0.0	44.6	0.0	0.0	44.6
Valparaiso	20.6	36.5	0.0	0.0	57.1
Santiago	48.0	57.9	0.0	99.7	205.6
O' Higgins	15.1	37.4	8.0	0.0	60.5
Colchagua	12.7	57.6	0.0	0.0	70.3
Curicó	9.7	44.5	0.0	0.0	54.2
Talca	0.0	53.0	0.0	0.0	53
Maule	0.0	56.8	0.0	0.0	56.8
Linares‡	0.0	25.6	0.0	0.0	25.6
Niuble	7.2	70.0	0.0	0.0	77.2
Concepción	0.0	57.0	0.0	0.0	57
Arauco	0.0	41.0	8.5	0.0	49.5
Bio-Bio	0.0	125.2	0.0	0.0	125.2
Malleco	0.0	76.6	0.0	0.0	76.6
Cautin	6.4	52.6	0.0	0.0	59
Valdivia	0.0	56.7	11.2	30.0	97.9
Llanquihue	7.6	80.2	0.0	36.3	124.1
Chiloé	0.0	84.1	0.0	62.3	146.4
Magallanes	21.8	18.9	0.0	46.0	86.7
Total Chile	13.6	54.4	1.4	24.5	93.9

*Excess mortality estimates were based on a seasonal regression model applied to monthly deaths.
†Provinces are sorted in geographic order from northern to southern Chile.
‡Model baseline to estimate excess deaths excluded the high-death months of Sep 1917–Mar 1918.

we fitted a cyclical regression model to monthly mortality data for the prepandemic period 1915–1917 and included temporal trends and harmonic terms for seasonality (*5*,*24*–*26*). For the province of Linares, we excluded observations for months with atypically high numbers of deaths during September 1917–March 1918 from the baseline model. Periods with statistically significant elevations in deaths over the model baseline were interpreted as periods of pandemic-related excess deaths.

Pandemic periods for each province were defined as the months when all-cause deaths exceeded the upper 95% confidence limit of the baseline model. We then summed the excess deaths above the baseline model during each pandemic period identified during 1918–1921 to estimate the absolute mortality burden of the pandemic.

We also calculated the relative risk (RR) for pandemic-associated death, defined as the ratio of excess deaths during the pandemic periods to the expected baseline number of deaths for these periods. The RR measure facilitates comparison between countries, regions, and patient age groups with different background risks for death (*25*,*27*).

Geographic Patterns

We analyzed geographic variations in pandemic timing and severity across 24 provinces spanning the entire latitudinal gradient of Chile (Figure 1). For each geographic area, we recorded the timing of the pandemic all-cause death peak, defined as the month with maximal elevation of deaths during 1918–1921. We also used univariate analyses and multivariate stepwise regression models to explore the association between the variables of province-level estimates of peak timing, excess mortality rate, and RR for death and the variables of latitude, population size, population density, and infant mortality rates.

Spatial Autocorrelation

We quantified the extent of spatial autocorrelation in estimates of excess deaths and RR in the 24 provinces by using the Moran I statistic and a nearest-neighbor spatial mixing matrix (*28*) via randomization tests (*29*). This analysis evaluates whether pandemic mortality rates in contiguous provinces are more similar than those obtained for any pair of provinces selected at random.

Results

Newspaper Information

The first newspaper report of the pandemic in Santiago, Chile, appeared in the El Mercurio newspaper on October 16, 1918. Although during the early stages of the pandemic, the etiology was suspected to be typhus exanthematicus, the clinical characteristics readily favored a respiratory disease, reminiscent of influenza illnesses that had affected other areas of the world several months

earlier. By October 29, 1918, some hospitals in Santiago City had reached maximum capacity. In Concepción, the first reports referring to the pandemic were published on October 24, 1918, in the local newspaper, El Sur.

Geographic Patterns, Pandemic Waves, and Excess Deaths

According to province-level all-cause monthly mortality rates, the pandemic virus spread heterogeneously in multiple waves across Chile (Figure 2). The first pandemic wave spanned from October 1918 through February 1919, peaked in January 1919, and was relatively mild. The mean excess mortality rate was 13.6 deaths per 10,000 population across provinces. Latitude and population size explained 33% of the variability in the timing of peak pandemic activity from northern to southern Chile; deaths peaked earlier in the northern provinces ($p = 0.02$) (Figure 2).

The highest absolute excess mortality rates in this period occurred in Santiago (Table 1; Figure 3). Baseline mortality rates and population density explained 89% of the variability in excess mortality rates across provinces during the 1918–1919 wave ($p<0.0001$). The province in which RR for excess pandemic deaths was highest was the southernmost province of Magallanes (RR 81.5%) (Figure 4).

Chile experienced the brunt of pandemic deaths during the second wave in the winter–spring of July 1919–February 1920; deaths peaked in August 1919. The average excess mortality rate was 54.4 deaths per 10,000 population across provinces (Figure 3); all provinces except the northern provinces of Tarapacá and Antofagasta experienced substantial excess mortality rates during this period (Table 1). Baseline mortality rates and population density explained 29.9% of the variability in excess mortality rates ($p = 0.03$). The RR for death ranged from 0 (Tarapacá) to 221% (Bio-Bio in southern Chile); the RR trend increased significantly from northern to southern Chile, even after population size was controlled for (Spearman $r = 0.56$, $p = 0.007$) (Figure 4).

During spring–summer 1920–1921, a third minor pandemic wave affected 4 provinces in northern ($n = 1$), central ($n = 1$), and southern Chile ($n = 2$) (Table 1). This mild wave was followed by a fourth wave of pandemic activity during June–December 1921, which generated excess deaths in 7 provinces (Table 1, Figure 3). The effect of this fourth pandemic wave was highest in Santiago; excess mortality rate was 99.7 deaths per 10,000 population, corresponding to a 42.8% elevation over baseline (Figures 3, 4).

The cumulative excess mortality rate during the 1918–1921 study period was estimated at 93.9 deaths per 10,000 population (RR 129%), representing 34,978 excess deaths nationally. Cumulative excess pandemic death rates varied by ≈10-fold across provinces, from 24.8 deaths per 10,000 population in Tarapacá to 205.6 per 10,000 in Santiago, the hardest hit province. Stepwise multivariate regression identified baseline mortality rates and population density as

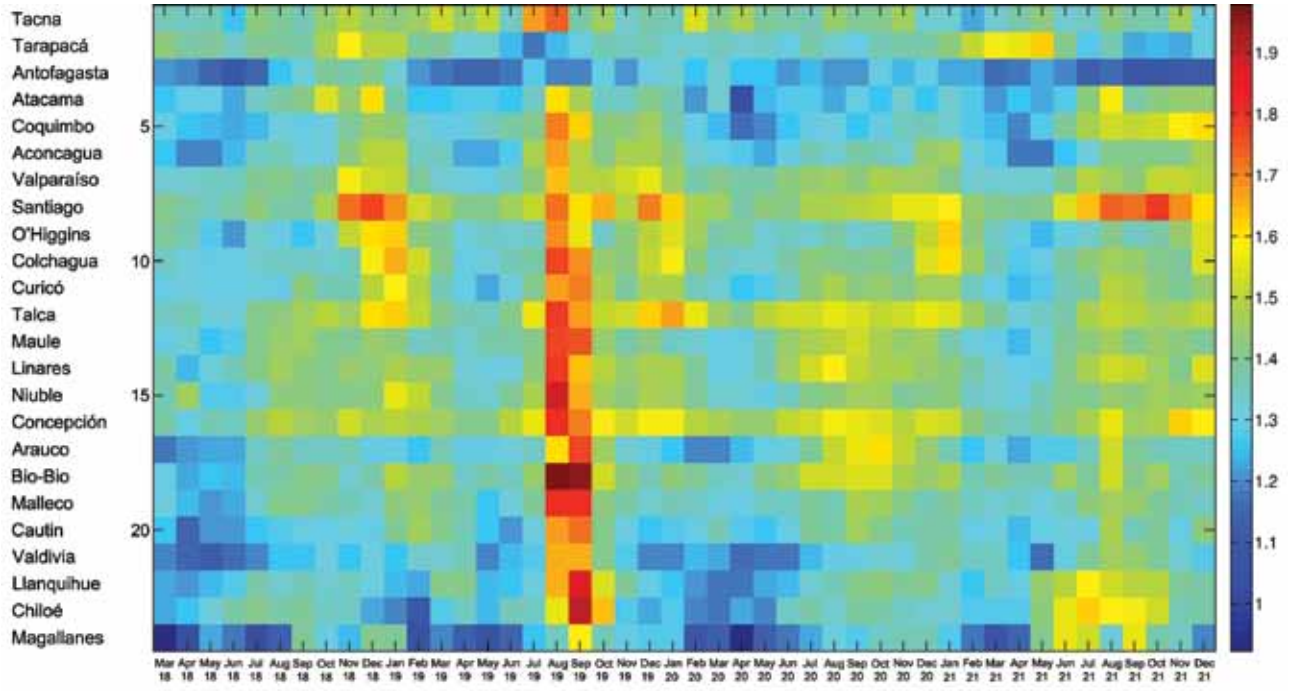

Figure 2. Temporal evolution (March 1918–December 1921) of all-cause mortality rates during the 1918 influenza pandemic across 24 provinces of Chile, sorted in geographic order from northern to southern Chile. For visualization purposes, the time series are log transformed.

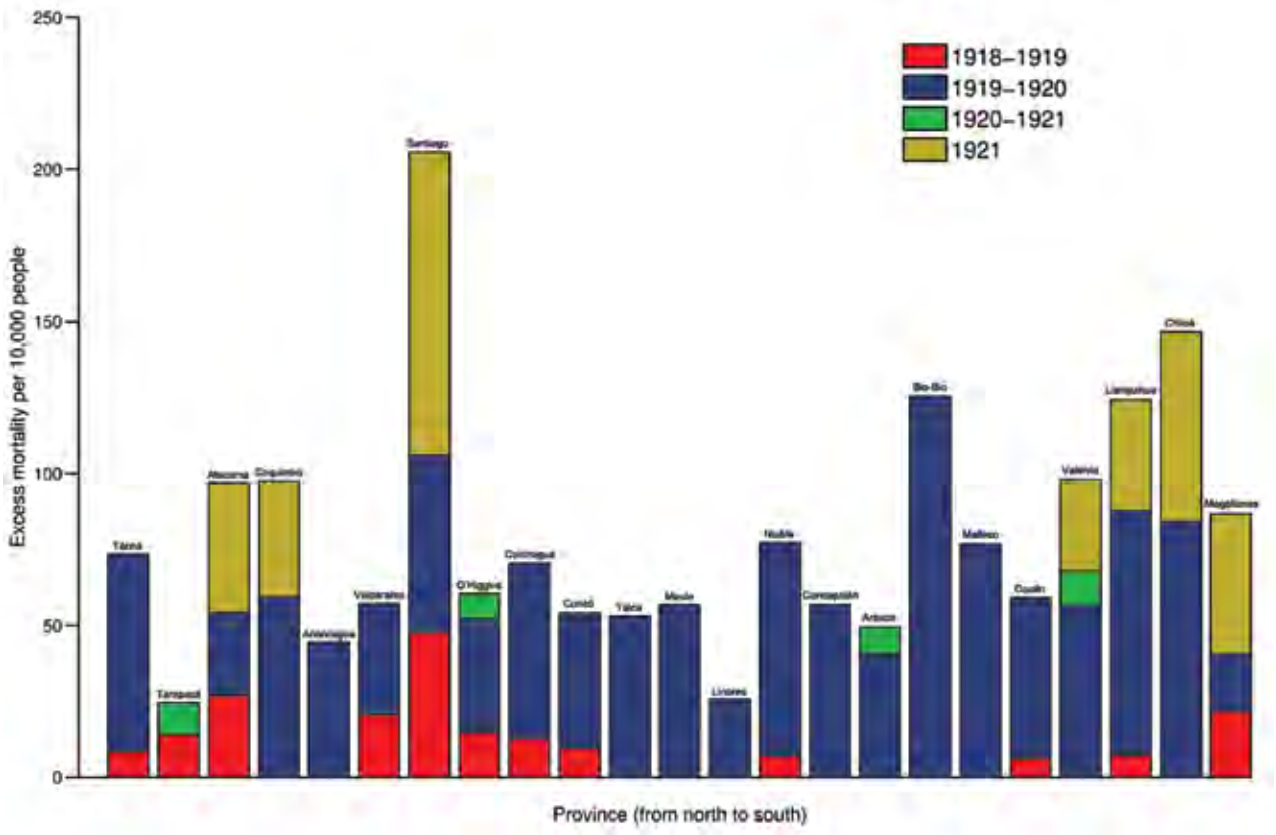

Figure 3. Excess deaths per 10,000 population across 24 provinces of Chile according to pandemic periods (July 1918–March 1919, June 1919–March 1920, November 1920–March 1921, and June 1921–December 1921) in geographic order from northern to southern Chile. Excess deaths are above the upper limit of the baseline mortality curve calibrated by using all-cause monthly deaths before the 1918 influenza pandemic.

significant and positively associated predictors of cumulative excess mortality rates across provinces (R^2 = 71.4%, p<0.0001). There was no association between cumulative excess mortality rates and latitude (Spearman r = 0.32, p = 0.14).

In contrast to absolute excess mortality rates, the estimates of cumulative pandemic RR for death during 1918–1921 were moderately associated with latitude; risk was highest in southern Chile (Spearman r = 0.48, p = 0.02; Figure 4) and provinces with low population density (Spearman r = –0.46, p = 0.03). Stepwise multivariate regression identified latitude, baseline mortality rates, and population density as predictors of cumulative RR for pandemic deaths across provinces (R^2 = 51.5%, p = 0.003).

Timing of Pandemic Waves and Patterns of Death by Age Group in Concepción

To explore timing of pandemic waves and patterns of death by age group in detail, we analyzed cause- and age-specific death rates for the city of Concepción during 1915–1920. Concepción experienced 3 periods of significantly elevated deaths from respiratory illness during 1918–1920: winter 1918 (July–September 1918), spring 1918–1919 (December 1918–January 1919), and winter–spring 1919–1920 (August 1919–February 1920). For these 3 putative pandemic waves, all-age rates of excess deaths from respiratory illness were estimated at 4, 7, and 34 deaths per 10,000 population, respectively (Table 2). Although the first local newspaper reports referring to the pandemic date back to October 24, 1918, the pandemic virus may have circulated at low levels 1–3 months earlier, July–September 1918, because all-age deaths from respiratory illness increased 31% above prepandemic baseline levels during this period (Figure 5, http://wwwnc.cdc.gov/EID/article/20/11/13-0632-F5.htm). The age patterns for this putative pandemic wave were mixed; significant excess deaths from respiratory illness occurred among children and persons ≥50 years of age (Figure 6, http://wwwnc.cdc.gov/EID/article/20/11/13-0632-F6.htm), and a high number of all-cause deaths occurred among young adults 20–29 years of age.

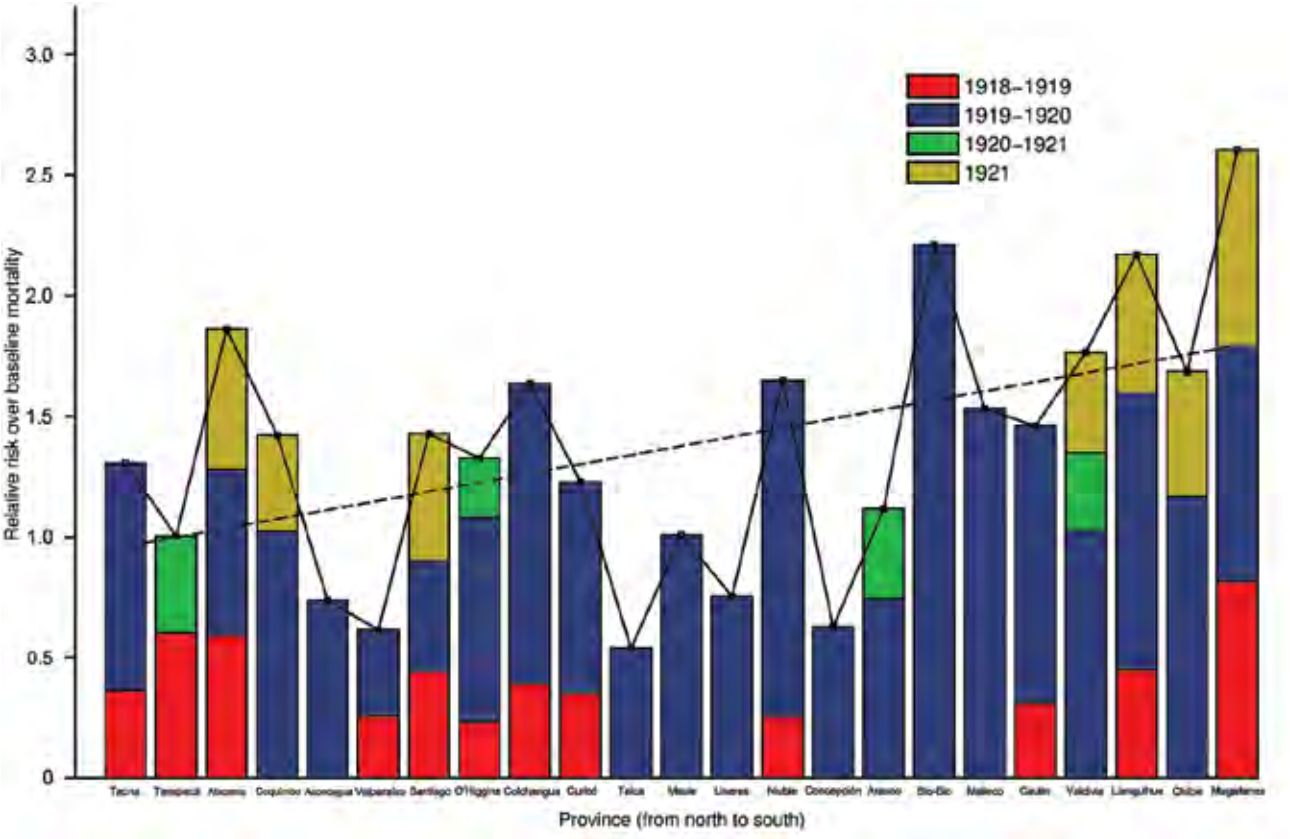

Figure 4. Relative risk for death over the baseline risk across provinces of Chile according to pandemic periods (July 1918–March 1919, June 1919–March 1920, and June 1921–December 1921). The relative risk for death is based on the ratio of excess deaths to baseline deaths, facilitating comparisons across provinces with different background risks for death. Solid line shows how relative risk varies with latitude by connecting observations for individual pro-vinces; dashed line is a fitted linear trend.

The occurrence of a subsequent pandemic wave in Concepción during December 1918–January 1919 is supported by newspaper reports. Although rates of absolute excess deaths were highest for older adults (Table 2) during this pandemic wave, the RR for death was highest among young adults 20–29 years of age, 54%–107% above baseline (Figure 6).

The onset of a third, severe and prolonged, pandemic wave in the city of Concepción was first noted in the local news on August 18, 1919. The August 1919–February 1920 pandemic wave in Concepción was synchronous with the major onslaught of the pandemic in other Chilean locales and was associated with the most deaths of the 3 pandemic waves. Excess death rates for respiratory illness and all causes were estimated at 34.1 and 64.2 deaths per 10,000 population, respectively. During this third wave, RR remained highest for young adults 20–29 years of age.

We cannot rule out the occurrence of a later pandemic wave in 1921 in Concepción because refined mortality

Table 2. Estimated rates of absolute excess pandemic deaths, by pandemic wave, age group, and cause of death, Concepción, Chile, 1918–20*

Cause of death	Age group, no. excess deaths/10,000 population				
	<20 y	20–29 y	30–49 y	≥50 y	All ages
Respiratory					
Jul 1918–Sep 1918	7.8	0.2	0.1	5.5	3.9
Nov 1918–Mar-1919	3.3	4.6	13.7	13.8	6.9
Aug 1919–Mar 1920	53.9	8.9	27.1	28	34.1
Total pandemic period	65.0	13.7	40.9	47.3	44.9
All-cause					
Jul 1918–Sep 1918	6.8	7.1	0.7	12.5	5.6
Nov 1918–Mar-1919	2.4	9.8	17.0	12.6	6.0
Aug 1919–Mar 1920	73.0	25.5	58.8	116.8	64.2
Total pandemic period	82.2	42.4	76.5	141.9	75.8

*Estimates of excess deaths were based on a seasonal regression model applied to weekly respiratory and all-cause mortality.

data for this city were limited to 1915–1920. Overall, on the basis of respiratory and all-cause death data, we estimate that the cumulative effect of the pandemic during 1918–1920 in Concepción was 44.9–75.8 deaths per 10,000 population. Estimates for Concepción were similar, whether we aggregated city-level mortality data by week or month (within 90% of each other). Furthermore, our city-specific estimates were consistent with all-cause estimates obtained from the official vital statistics for the broader province.

Discussion

Our analysis of extensive archival mortality statistics, death certificates, and contemporaneous newspaper reports reveals the substantial impact of the 1918–1921 pandemic in Chile. We found evidence that during 1918–1921, a total of 4 pandemic waves of varying timing and intensity occurred; in most areas of Chile, the highest rates of pandemic-related excess deaths were for July 1919–February 1920. Santiago was by far the hardest hit in terms of cumulative mortality burden. Pandemic-related excess mortality rates for 1918–1921 varied 10-fold across the 24 provinces; these differences were partly explained by latitude, baseline mortality rates, and population density. In agreement with previous reports (*4*–*7*,*9*–*12*), we found that among all age groups, the RR for pandemic death was highest among young adults 20–29 years of age. Our findings also indicate substantial excess mortality rates for senior populations, in agreement with previous reports from central Mexico (*6*) and Colombia (*7*).

We used monthly all-cause mortality data to estimate province-level mortality burden of the pandemic, as did prior studies (*30*–*32*). Because of the unusual severity of the 1918 pandemic, use of rather crude death indicators such as monthly all-cause deaths provides good agreement with more detailed cause-specific data (*4*,*26*,*30*,*31*). We cannot, however, rule out the possibility that underreporting (*1*) might have biased some of the geographic differences evidenced here. However, estimates of pandemic peak timing and excess deaths derived from refined death certificate data for the city of Concepción aligned with those derived from official all-cause mortality statistics for the broader province.

The temporal course of the pandemic in Chile differed markedly from that reported for the Northern Hemisphere. Although peak pandemic-related deaths occurred during October–November 1918 in temperate regions of the Northern Hemisphere (autumn) (*4*–*6*, *10*–*11*), the most lethal pandemic wave in Chile peaked in midwinter (August 1919). This pattern of delayed pandemic deaths in Chile echoes patterns reported from Argentina and Uruguay (*33*,*34*). Chile also experienced an earlier and milder wave of deaths during the warmer months (November 1918–February 1919), as did other South American localities including Peru (*9*), Colombia (*7*), Argentina (*33*), and Uruguay (*35*).

We suspect that a mild "herald" pandemic wave might have occurred in Concepción during July–September 1918, as did a concurrent mild pandemic wave in Lima, Peru (*9*). Indeed, we identified a significant elevation in total deaths among young adults 20–29 years of age during mid-1918 in Concepción but no increase in respiratory deaths in this signature age group. It is possible that the low and potentially unrecognized effect of this pandemic wave precluded identification of an increase in cause-specific deaths or that, alternatively, this elevation in deaths might be associated with a different pathogen. Herald waves occurring during March–August 1918 and associated with excess deaths concentrated among young adults have been reported for New York City (*4*), Mexico (*6*), Geneva (*36*), Copenhagen (*26*), the US military (*26*), the United Kingdom (*14*), and Singapore (*11*); these waves align with the laboratory identification of the pandemic influenza A(H1N1) virus from US soldiers in May 1918 (*37*). It is possible that other areas of Chile experienced mild pandemic waves during mid-1918 but that such waves could not be detected by using all-age, all-cause death data. In fact, the herald pandemic wave of 1918 in Denmark was only clearly evident from a sharp and unseasonal elevation in respiratory illness; there was little concurrent increase in all-cause or respiratory deaths (*26*). If the 1918 pandemic virus had indeed reached Chile by July 1918, only ≈4 months after the earliest putative outbreaks in New York City (*4*), this finding would indicate rapid transcontinental diffusion of the virus via boats and railways, reminiscent of the 1889 pandemic (*38*).

We found substantial variation in pandemic excess mortality rates across provinces of Chile. In particular, both relative and absolute risks for pandemic death during 1918–1921 were associated with baseline mortality rates. Furthermore, the highest relative mortality burden of the pandemic was found in southern Chile. Similarly, excess mortality rates associated with the 1918–1919 influenza pandemic were significantly higher in southern than in northern Europe (*30*). More broadly, substantial geographic heterogeneity in pandemic-associated mortality rates was reported globally and linked to socioeconomic factors (*15*). In addition to baseline death rates as predictors, we found that higher population density was predictive of higher absolute excess mortality rates.

Cumulative pandemic RR followed a north–south gradient for 1918–1921; this relative measure of pandemic death burden, which takes into account prepandemic baseline deaths, was highest in the southernmost provinces of Chile. We did not detect a southward gradient in total or infant mortality rates for the baseline prepandemic years, indicating that the pandemic-associated

gradient was truly unique to this period and was not driven by reporting artifacts or socioeconomic conditions. Experimental studies indicate that influenza transmission is favored by lower temperatures and humidity levels (*39*), and we speculate that the higher relative death rate estimated for these areas could be explained by more favorable climate conditions in southern Chile (*18*,*40*). Moreover, the southward gradient in severity of the 1918 pandemic aligns with the patterns of the 2009 influenza A(H1N1) pandemic in Chile (*28*) and in Brazil (*23*). In contrast to patterns in RR for death, we did not find any association between latitude and cumulative absolute pandemic excess mortality rates during 1918–1921. Variations in absolute mortality rates seem to be mainly driven by underlying differences in baseline death rates and population density across Chile and hence do not support the climate hypothesis. Overall, the association between climate and influenza disease burden remains elusive, especially during pandemic seasons (*20*).

Excess mortality rates associated with the 1918 pandemic show substantial variability throughout Latin America, ranging from 0.4% in Boyacá, Colombia (*7*), to 2.9% in Iquitos, Peru (*9*). Our estimated cumulative mortality rate for Chile during 1918–1921 is 0.94%, which lies in the middle of the range of previous estimates for the broader region. At the province level, the highest pandemic excess mortality rate estimated was 2.1% for Santiago, which is comparable to that for Toluca, Mexico (*6*). In contrast, reports from the United States and Europe indicate lower mortality rates for these regions (0.5%–1.1%) (*4*,*5*,*30*).

Our detailed historical data from the city of Concepción, Chile, enabled us to analyze age-specific deaths and confirm the signature mortality risk among young adults (*4*–*7*,*10*,*12*,*26*). Furthermore, our data indicate that pandemic excess mortality rates were elevated among adults ≥50 years of age in Concepción, in agreement with data from Mexico (*6*) and Colombia (*7*), supporting a lack of senior sparing in Latin America, in contrast to the United States (*4*,*5*) and Europe (*10*,*26*). We and others have proposed that these age profile discrepancies originate from regional differences in prior immunity to the 1918 pandemic virus among seniors, resulting from the heterogeneous global circulation of influenza viruses in the 19th century (*6*). It is plausible that in the middle of the 19th century, remoteness could have affected the probability of introduction and dissemination of influenza viruses to Chile and the broader Latin American region.

In conclusion, our historical analysis reveals that the excess mortality rate of the 1918–1921 influenza pandemic was substantial in Chile, the southernmost region of Latin America, and that the major mortality burden was concentrated in the colder months of July 1919–February 1920, more than a year after the identification of the virus in the United States. Difference in pandemic excess mortality rates between Chilean provinces were >10-fold, and the gradient of excess deaths relative to baseline deaths increased from northern to southern Chile. Pandemic excess deaths were elevated for persons in all age groups, including seniors; these data are in agreement with data from other Latin American settings, although the signature atypical severity of the disease among young adults remained. Our findings suggest that a combination of local factors affected pandemic death patterns in Chile: variations in host-specific susceptibility, population density, baseline death rate, and climate conditions. We conclude that predicting death patterns for future pandemics is complex and multifactorial and that additional studies in undersampled areas are needed to refine our understanding of the predictors of mortality burden on a local scale.

This research was conducted in the context of the Multinational Influenza Seasonal Mortality Study, an ongoing international collaborative effort to understand influenza epidemiological and evolutionary patterns, led by the Fogarty International Center, National Institutes of Health (http://www.origem.info/misms/index.php). Funding for this project comes in part (L.S.) from the RAPIDD (Research and Policy for Infectious Disease Dynamics) program of the Science & Technology Directorate, Department of Homeland Security, and from the Office of Global Affairs, International Influenza Unit, in the Office of the Secretary of the US Department of Health and Human Services.

Dr Chowell is an associate professor in the School of Human Evolution and Social Change at Arizona State University and Research Fellow at the Fogarty International Center, National Institutes of Health. His research interests include mathematical modeling of infectious disease transmission with a focus on influenza and the role of public health interventions. He is also interested in the quantitative characterization of the 1918 influenza pandemic in the Americas.

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Genomic Definition of Hypervirulent and Multidrug-Resistant *Klebsiella pneumoniae* Clonal Groups

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Multidrug-resistant and highly virulent *Klebsiella pneumoniae* isolates are emerging, but the clonal groups (CGs) corresponding to these high-risk strains have remained imprecisely defined. We aimed to identify *K. pneumoniae* CGs on the basis of genome-wide sequence variation and to provide a simple bioinformatics tool to extract virulence and resistance gene data from genomic data. We sequenced 48 *K. pneumoniae* isolates, mostly of serotypes K1 and K2, and compared the genomes with 119 publicly available genomes. A total of 694 highly conserved genes were included in a core-genome multilocus sequence typing scheme, and cluster analysis of the data enabled precise definition of globally distributed hypervirulent and multidrug-resistant CGs. In addition, we created a freely accessible database, BIGSdb-Kp, to enable rapid extraction of medically and epidemiologically relevant information from genomic sequences of *K. pneumoniae*. Although drug-resistant and virulent *K. pneumoniae* populations were largely nonoverlapping, isolates with combined virulence and resistance features were detected.

Klebsiella pneumoniae is a frequent cause of nosocomial infections and has also emerged as an agent of severe community-acquired infections, including pyogenic liver abscess, pneumonia, and meningitis (*1*,*2*). The rise of antimicrobial drug resistance in *K. pneumoniae*, a member of the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) of bacterial pathogens (*3*), raises serious therapeutic challenges. Most multidrug-resistant (MDR) *K. pneumoniae* isolates, which produce extended-spectrum β-lactamases (ESBLs) and/or carbapenemases in combination with quinolone and aminoglycoside resistance, belong to particular clones (*4*–*6*). Invasive community-acquired isolates are predominantly of capsular serotypes K1 and K2 and appear to differ in clonal background from MDR isolates (*7*–*11*). Controlling the emergence of these 2 types of high-risk clones and mitigating the alarming prospect of strains that would combine high virulence with multidrug resistance requires a precise definition of clonal groups (CGs) and rapid identification of their medically relevant features. *K. pneumoniae* clones have been recognized so far by using multilocus sequence typing (MLST) based on 7 housekeeping genes (*4*,*8*,*12*). However, MLST fails to draw clear discontinuities between CGs (*4*–*6*). Rapid, high-throughput sequencing promises to revolutionize medical microbiology and molecular epidemiology (*13*,*14*) by improving discriminatory power and providing access to the resistome and virulome of clinical isolates. However, it remains challenging to extract medically relevant information from genome sequences in a timely manner. The objectives of this work were to delineate precisely, based on genome-wide genotyping, CGs corresponding to highly virulent and MDR *K. pneumoniae* isolates; extract the antimicrobial drug resistance and virulence-associated genomic features of those CGs by using a rapid and simple bioinformatics tool; and detect potential dual-risk isolates carrying virulence and resistance genes.

Materials and Methods

Isolate Selection for Genome Sequencing

We sequenced 48 nonredundant *K. pneumoniae* isolates (Table S1, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). Forty-two of the isolates were of capsular serotype K1 or K2, the 2 serotypes predominantly associated with community-acquired invasive

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infections (*2*,*15*). To conduct genome sequencing, we used the HiSeq 2000 Sequencing System (Illumina, San Diego, CA, USA) with a 2 × 100 nt paired-end strategy (see Supplemental Methods section at http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf).

Genomes from Sequence Databases

Genomic sequences available as of July 15, 2013, for the entries *Klebsiella*, *K. pneumoniae*, and *K. variicola*, were downloaded from the NCBI (National Center for Biotechnology Information) genome sequence repository (http://www.ncbi.nlm.nih.gov/genome/). The downloaded sequences comprised 7 complete genomes and 115 whole-genome shotgun sequences available as scaffolds or contigs. Of these 115 draft genomes, 2 were discarded because of the poor quality of assembly and 1 was discarded because it corresponded in fact to the more distant species *K. oxytoca*. Thus, a total of 119 publicly available genome sequences were included (Table S1, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf).

Serotype Determination and MLST Data Generation

The capsular serotype was determined by PCR, using K1- and K2-specific PCR primers (*15*). The serotype of some comparative isolates was initially determined by using classical serology methods and/or by determining their C-pattern (*8*). MLST data were generated by using the international *K. pneumoniae* MLST typing scheme (*8*,*12*).

Definition of MLST and Core Genome MLST (cgMLST) Schemes

A set of 634 genes, defined as the strict cgMLST set, was obtained by using stringent conservation and synteny criteria to maximize typeability and to minimize paralogous or xenologous loci (see Supplemental Methods section at http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). Two typing schemes, defined as sets of predefined loci, were implemented by using the BIGSdb genome database software (*16*) in a newly created database named BIGSdb-Kp (http://bigsdb.web.pasteur.fr). First, the MLST scheme, with its reference sequences and sequence types (STs), was imported from the international MLST database (http://www.pasteur.fr/mlst) into BIGSdb-Kp, which now acts as the reference. Second, a 694-gene cgMLST scheme was defined as the combination of the 7 MLST genes, the 53 ribosomal MLST genes (*17*), and the 634 strict cgMLST genes.

Genes Associated with Virulence and Heavy Metal Resistance or with Drug Resistance

Sequences of genes previously associated with virulence or heavy metal resistance were used to define virulence-associated loci, and all currently described variants of major antimicrobial drug resistance determinants (namely, β-lactamases, aminoglycoside resistance–conferring enzymes and fluoroquinolone-resistance loci) were included in the BIGSdb-Kp database. For selected strains, the antimicrobial drug resistance phenotype was analyzed by using conventional methods (see Supplemental Methods section at http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf).

Data Analysis

Phylogenetic networks were constructed by using SplitsTree v4.13.1 (*18*) based on comparison of allelic profiles using distance matrices corresponding to the percentage of distinct loci (excluding missing alleles), which were obtained using BioNumerics v6.6 (Applied-Maths, Sint-Martens-Latem, Belgium). Clonal complexes (CCs) were defined as groups for which MLST profiles showed only 1 allelic mismatch with at least 1 other member of the group. Phylogenetic reconstruction based on cgMLST genes was performed by using minimum evolution analysis after discarding putative homologous recombination biases (see Supplemental Methods section at http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). Selected genomes were annotated by using the MicroScope annotation and comparative genomics platform (*19*).

Data Availability

The annotated genomic sequences of strains BJ1-GA, T69, SA1, and cur15505 (SB2390) were deposited in the European Nucleotide Archive (ENA) and are available under accession nos. CBTU010000000, CBTV010000000, CBTW010000000, and CCBO010000000, respectively. The sequences of CIP 52.145 chromosome and plasmids are available under accession nos. FO834904 and FO834905 and FO834906, respectively. Sequence reads corresponding to the remaining 43 isolates have been deposited in the ENA Sequence Read Archive under study accession nos. ERS500935–ERS500961, ERS503301–ERS503315, and ERS508075. The 48 assembled genomic sequences generated in this study are accessible from the BIGSdb-Kp database through Institut Pasteur's whole-genome MLST home page (http://bigsdb.web.pasteur.fr).

Results

Diversity of Virulent and MDR *K. pneumoniae* Isolates as Determined by MLST

The average number of contigs and the N50 (i.e., the length for which half of the bases of a draft genome are situated in contigs of that length or longer) of the 48 genomes generated in the present study were similar to those of the 112 publicly available draft genomes (Table S2,

http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). These 2 characteristics quantify the level of fragmentation of the sequence assemblies. MLST allele sequences were retrieved from genomic sequences imported into the BIGSdb-Kp database. For 46 of the 48 sequenced strains, MLST alleles were also obtained by the classical Sanger sequencing method, which, in all cases, confirmed the genomic data. This result corresponds to <1 error in 138,552 nt (i.e., 0.00072%), demonstrating the high quality of consensus base calls in the assemblies.

Forty-three different STs were found in the entire dataset. Among the isolates we sequenced, the 20 K1 isolates represented 2 STs, among which ST23 (n = 18) predominated, whereas the 22 K2 isolates represented 10 distinct STs, among which ST380 (n = 5) and ST86 (n = 6) predominated (Table S1, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). Isolates belonging to these 3 predominant STs, as well as ST57, ST65, and ST375, were previously reported from invasive infections (*7*–*11*,*20*). Reference strains NTUH-K2044 and CG43 were found to belong to ST23 and ST86, respectively. Publicly available genomes predominantly included (85/119, 71%) STs previously associated with multidrug resistance: ST11, ST14, ST15, ST258, and ST512 (*4*,*5*,*21*,*22*). Therefore, the dataset comprised multiple representative isolates of both types of high-risk *K. pneumoniae*.

Definition of CGs based on cgMLST

Sequences corresponding to the 694 cgMLST loci were extracted from the 167 genomes by using BIGSdb, and allelic profiles were compared (Figure 1). Sharp discontinuities were apparent: groups of isolates were clustered in homogeneous branches that were well separated from other groups. These results demonstrate the existence of clearly delineated *K. pneumoniae* clones. To define CGs nonarbitrarily, we analyzed the distribution of the number of allelic mismatches (loci at which sequences differ) among all pairs of genomes (Figure S1, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). The results showed a high number of genome pairs with <100 mismatches or 500–600 mismatches; almost no genome pairs had 100–300 mismatches. Therefore, we propose to define *K. pneumoniae* cgMLST CGs a groups of cgMLST profiles having ≤100 allelic mismatches (i.e., 14.4% of the 694 alleles) with at least 1 other member of the group.

By using this definition, we defined 14 CGs (Figure 1). The MDR-associated ST258 and its MLST single-locus variants belonged to a single well-demarcated group, designated CG258. On the basis of MLST results, ST258 was previously associated with many other STs in a single heterogeneous CC (*4*,*6*). We showed that most of these STs do not belong to CG258, demonstrating the power of cgMLST to discard spurious MLST associations. Within CG258, isolates of ST258 and ST512 formed a main cluster with, on average, only 2.1% allelic mismatches, whereas strains of ST11 and ST340 differed from this cluster by 11.0%, indicating genetic substructure within this CG. The other CC associated with multidrug resistance, CC14 (*5*), was split into 2 distinct CGs: CG14 and CG15 (Figure 1).

Figure 1. Phylogenetic network of the 167 *Klebsiella pneumoniae* genomes as determined on the basis of the allelic profiles of the 694 core genome multilocus sequence typing (cgMLST) genes. The network was constructed by using the neighbor-net method implemented in SplitsTree v4.13.1 (*18*). Nodes are colored according to the clonal group (CG). Only the 10 most relevant CGs are highlighted; note that ST35 was subdivided into 2 CGs (CG35-A and CG35-B). Gray shading indicates CG258. Gray dots indicate phylogroups KpII-B and KpIII (*K. variicola*). Bold indicates reference strains. Scale bar represents 100 allelic mismatches. ATCC, American Type Culture Collection; ST, sequence type.

Most isolates of serotype K1, which is associated with pyogenic liver abscess, belong to ST23 and ST57 (*7*,*8*,*11*). All isolates of these 2 STs were placed together in CG23, a compact, clearly delineated CG. CG23 was highly homogeneous (3.0% distinct alleles on average), even though epidemiologically unrelated isolates from distinct continents were included (Table S1, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). This result suggests that these strains recently emerged from a common ancestor, which is in agreement with epidemiologic evidence for the recent emergence of liver abscess caused by *K. pneumoniae* (*2*). STs associated with serotype K2 were distributed into 3 main CGs: CG86, CG375, and CG380. In addition, K2 isolates of MLST-defined CC65 (ST25, ST65, and ST375) represented 3 distinct CGs, which differed by at least 32%. This result shows that MLST classification into CCs can conflate members of distinct CGs (Figure 2; Table S1, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf), and it illustrates the inability of MLST to reliably recognize *K. pneumoniae* CGs. In our study, the use of cgMLST demonstrated that K2 isolates associated with severe community-acquired infections are genetically more diverse than K1 isolates. The relative prevalence of these serotype K2–associated CGs, and the differential clinical characteristics of infections they cause, remain to be determined by using prospective collections of isolates.

To determine the population structure of *K. pneumoniae*, we performed phylogenetic analyses of cgMLST sequences (Figure 2). The results showed that most branches leading to CGs were long and branched deep in the tree. This pattern suggests an evolutionary radiation of most groups at approximately the same time, presumably when *K. pneumoniae* expanded into a novel niche. Whereas CG23 and CG380 branched off early, CG375 and CG86 were placed on a single branch together with ST25 and CG65, suggesting a common ancestry for these 4 latter clones.

Reproducibility and Epidemiologic Relevance of cgMLST

One pair of public genomes in fact corresponded to the same strain: BAA-2146. This isolate, an MDR NDM-1–producing isolate belonging to ST11, was independently sequenced twice and was assembled by using 2 distinct methods (*23*) (GenBank accession no. APNN01). The 2 genomic assemblies did not show a single allelic difference. This observation indicates that cgMLST is highly reproducible.

To explore the ability of cgMLST to cluster epidemiologically associated isolates and distinguish them from

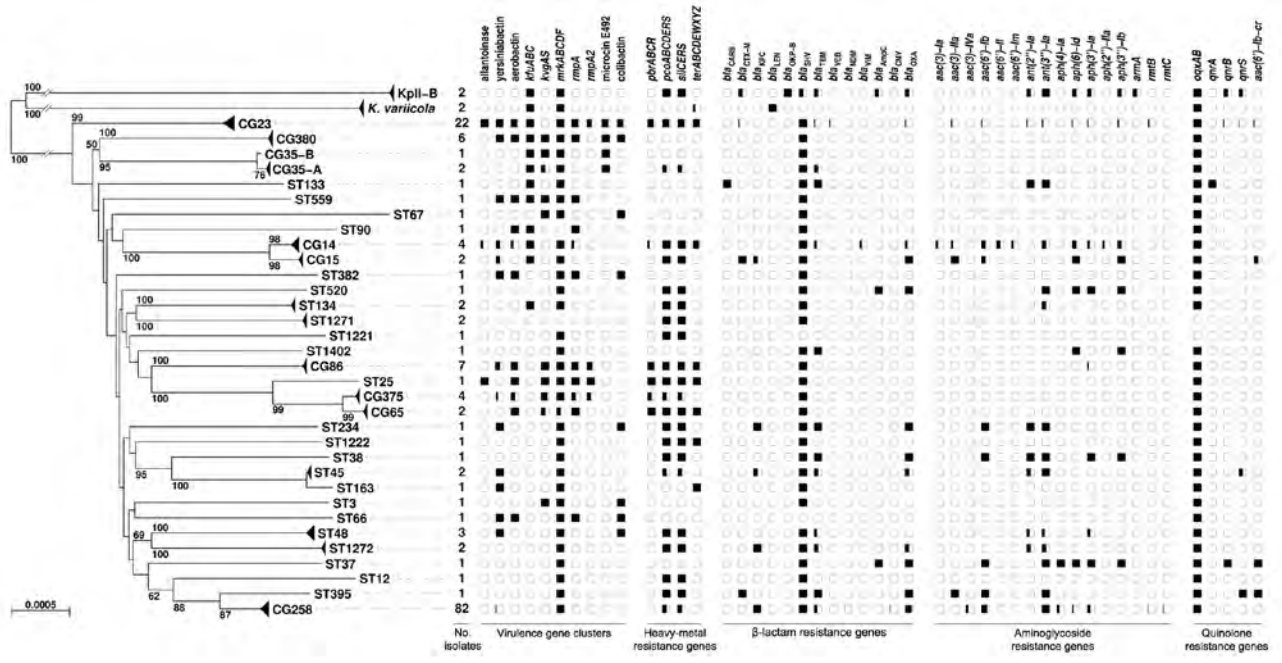

Figure 2. Phylogenetic tree of the 167 *Klebsiella pneumoniae* genomes as determined on the basis of core genome multilocus sequence typing (cgMLST) genes and distribution of virulence and resistance features. The tree was inferred from minimum evolution analysis based on aligned cgMLST sequences, with *K. variicola* and KpII-B sequences as outgroups. Terminal branches corresponding to different taxa from the same clonal group (CG) or sequence type (ST) are shown as triangles of depth proportional to internal diversity. Bootstrap values ≥50% based on 1,000 gene-by-gene replicates are given at branches. Scale bar represents 0.05% estimated sequence divergence. The virulence and resistance gene content (indicated along the top of the figure) of identified clones is represented by squares, which are colored in black proportionally to the percentage of presence of a gene or cluster among members of a given CG or ST.

other genetically closely related isolates, we included in our analysis 20 published genomes of isolates from a 2011 outbreak at the National Institutes of Health Clinical Center (Bethesda, Maryland, USA) (*24*). The outbreak isolates formed a unique cluster nested within the diversity of CG258 (Figure 3), suggesting that cgMLST may be useful for short-term epidemiologic questions and outbreak investigation. Additional studies on well-defined sets of outbreaks will be needed to define levels of variation among epidemiologically related and unrelated isolates.

Detection of Virulence-Associated Genes in *K. pneumoniae* Genomes

Genes previously associated with virulence and gene clusters coding for resistance to heavy metals, which can be encoded on the virulence plasmid (*25*), were searched by using BIGSdb (Figure 2; Table S3, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). The type-3 fimbriae cluster *mrkABCDF* was almost universally present, and plasmid-associated clusters *pcoABCDERS* (copper resistance) and *silCERS* (silver resistance) were widely distributed. In contrast, the other genes were mainly associated with particular CGs. The allantoinase cluster was present only in members of CG23 and ST25. Some or all isolates of the major CGs associated with community-acquired invasive infections (CG23, CG86, CG375, and CG380) harbored the 2 well-recognized virulence factors *iucABCDiutA* (aerobactin synthesis) and *rmpA* (the positive regulator of mucoid phenotype) in addition to the yersiniabactin siderophore cluster and (except for CG23) the 2-component system *kvgAS*. These results reinforce the view that CG23, CG86, CG375, and CG380 represent hypervirulent CGs of *K. pneumoniae* (*7–11*,*20*,*26*). Several other STs and CGs (ST25, CG65, ST66 [corresponding to the virulent K2 reference strain CIP 52.145], ST90, and ST382) harbored virulence genes, suggesting additional hypervirulent clones, which is also supported by the clinical origin of some isolates of these STs (Table S1, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). In contrast, CG258 was almost entirely devoid of virulence genes. Microcin E492 and colibactin synthesis clusters were present in almost all isolates of CG23 and CG380. These 2 secreted molecules, which cause damage to eukaryotic cells in vitro, may contribute to the virulence of these 2 CGs.

Detection of Drug Resistance–Associated Genes in *K. pneumoniae* Genomes

We investigated the presence of genes associated with resistance to β-lactams, aminoglycosides, and quinolones. Because single amino acid changes can have medically relevant phenotypic effects, we searched the known protein variants of the major β-lactamase families (Figure 2; Table S4, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). At least 1 variant of the SHV enzyme was found in 160 isolates, among which 159 belonging to phylogroup KpI (*27*). The *K. variicola* and KpII-B isolates harbored bla_{LEN} and $bla_{OKP\text{-}B}$, respectively,

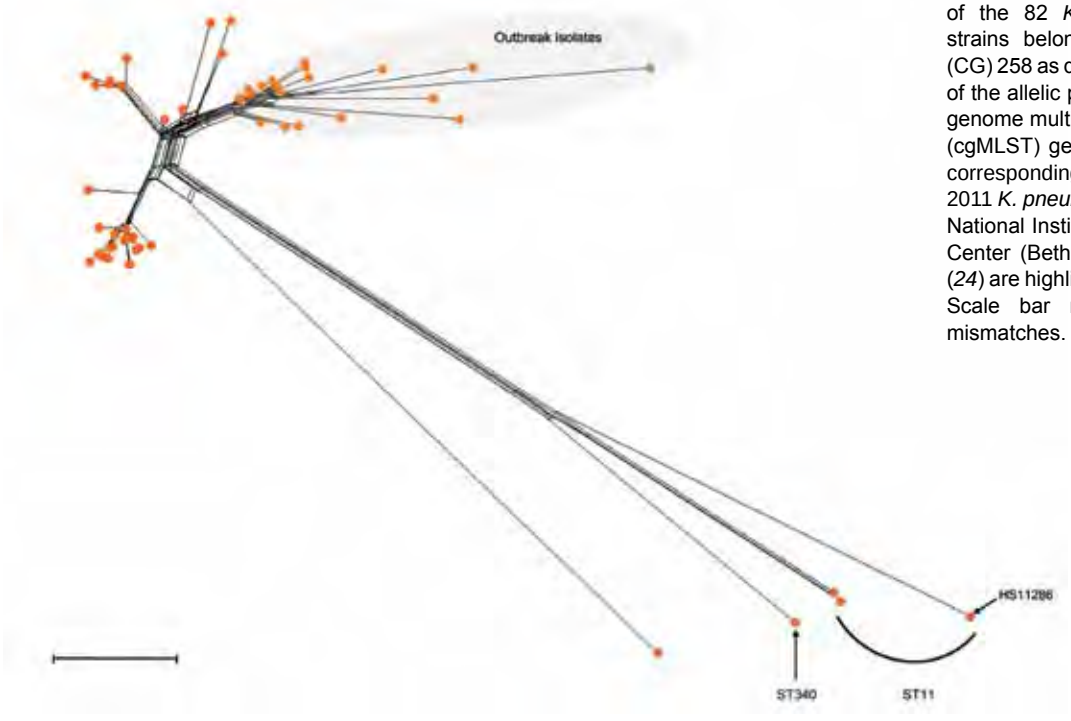

Figure 3. Phylogenetic network of the 82 *Klebsiella pneumoniae* strains belonging to clonal group (CG) 258 as determined on the basis of the allelic profiles of the 694 core genome multilocus sequence typing (cgMLST) genes. The 20 genomes corresponding to isolates from the 2011 *K. pneumoniae* outbreak at the National Institutes of Health Clinical Center (Bethesda, Maryland, USA) (*24*) are highlighted by gray shading. Scale bar represents 10 allelic mismatches. ST, sequence type.

and 1 KpII-B isolate also contained the ESBL-encoding bla_{SHV-18} gene. These results are consistent with previous associations of *K. pneumoniae* phylogroups KpI, KpII, and KpIII with chromosomally encoded β-lactamase families SHV, OKP, and LEN, respectively (*27*,*28*). No gene corresponding to a chromosomal β-lactamase was detected in the genomes of CIP 52.145 (ampicillin susceptible) and 4_1_44.

In addition, bla_{TEM} and bla_{OXA} were detected in 45 and 80 genomes, respectively. In 48 of the latter, an internal stop codon was present within bla_{OXA-9}, leading to a truncated protein. This truncation was previously described on the multiple antimicrobial drug resistance plasmid pRMH712 isolated from strain 4003 (ENA accession no. GU553923). Nine genomes contained a gene encoding the ESBL CTX-M-15. As expected, all 20 genomes from the 2011 outbreak at the National Institutes of Health Clinical Center were found to harbor a gene encoding the carbapenemase KPC-3 (*24*). A bla_{KPC} gene was also detected in 62 other genomes. Several other β-lactamase–encoding genes, including bla_{NDM-1}, were detected (Table S1, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf), consistent with earlier findings (*29*).

Regarding loci associated with resistance to quinolones, genes *qnrB* and *qnrS* were each present in 5 genomes, whereas a *qnrA1* gene was found in the genome of strain 1162281, consistent with previous work (*30*) (Figure 2; Table S5, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). Genes coding for the enzymatic targets of quinolones were detected in all genomes, except for gene *gyrB*, which was absent in 2 genomes. The *gyrA* and *parC* genes were mutated in their quinolone resistance–determining region in all isolates of the MDR clone CG258, whereas no mutation of these genes was found among the hypervirulent clones. In addition, the *oqxAB* locus encoding an efflux pump (*31*) was detected in 163 of the 167 genomes, demonstrating that this locus is highly conserved in *K. pneumoniae* (*32*).

We identified 16 aminoglycoside resistance–associated loci in the genomes studied (Figure 2; Table S6, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). The most frequently represented gene (100 genomes) was *ant(3″)-Ia*. The *aac(6′)-Ib-cr* variant, coding for an aminoglycoside- and quinolone-modifying enzyme, was found in 6 genomes (Table S5, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). Genes conferring resistance to aminoglycosides were mainly present in the MDR clones CG258 and CG14.

For all strains except cur15505, antimicrobial drug resistance phenotypes were highly concordant with detected genes (Table S1, at http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). Strain cur15505 was resistant to ceftriaxone and ceftazidime, an antimicrobial drug resistance phenotype compatible with the production of an SHV-type ESBL. However, the bla_{SHV} allele present in this strain's genome could not be precisely identified by BIGSdb-Kp because it was situated at a contig extremity.

Although isolates of hypervirulent CGs harbored almost no resistance genes (Figure 2), we did identify 2 isolates of CG23 (BG130 and BG141) that displayed genomic features responsible for high-level resistance to β-lactams, aminoglycosides, and quinolones, as confirmed by analysis of the resistance phenotype (Table S1, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). The first strain, BG130, which was isolated in Vietnam in 2008, contained an array of genes identical to a part of the Tn*6061* transposon from *Pseudomonas aeruginosa* and to the VEB-1-encoding region found in a *K. pneumoniae* isolate from Greece (*33*); these genes were bla_{VEB-1}, *ant(2″)-Ia* (*aadB*), *arr-2*, *cmlA5*, bla_{OXA-10}, *ant(3″)-Ia* (*aadA1*), *qacEΔ1*, *sulIΔ*, *cmlA9*, *tetR*, and *tetA*. Moreover, downstream of this gene array, strain BG130 carried an additional region comprising gene *rmtB*, encoding an rRNA methylase, and genes bla_{TEM-1} and $bla_{CTX-M-15}$. Strain BG130 also harbored gene *qnrS1*; the gene had the same surroundings as those in plasmid pHS8, which was described in a *K. pneumoniae* isolate from China (*34*). The second strain, BG141, which was isolated in Madagascar in 2007, also carried numerous resistance genes. Those genes included $bla_{CTX-M-15}$, bla_{TEM-1}, bla_{OXA-1}, *aac(6′)-Ib-cr*, and *qnrB1*, and the gene order was identical to that found in plasmids pKDO1 and pKPX-2, which were identified in MDR *K. pneumoniae* strains from the Czech Republic and Taiwan, respectively (*35*,*36*). These results are evocative of horizontal gene transfer that may have occurred between virulent and MDR *K. pneumoniae* strains.

Discussion

K. pneumoniae clinical isolates are evolving toward increasing levels of antimicrobial drug resistance, placing this species among the infectious bacterial pathogens that are most challenging to control (*3*). In parallel, even though most infections still occur opportunistically in debilitated patients, *K. pneumoniae* is emerging as a cause of severe community-acquired invasive infections (*2*). Recognizing the resistant or hypervirulent CGs is a prerequisite to better understand and control their global emergence. We implemented a genome database for *K. pneumoniae*, BIGSdb-Kp, and defined 694 genomic loci suitable for genotyping as well as loci associated with virulence and antimicrobial drug resistance. A genome-wide gene-by-gene approach based on the BIGSdb system (*16*) was previously applied to *Neisseria meningitidis* (*37*) and *Campylobacter* species (*38*). The BIGSdb system provides a simple tool for rapidly extracting medically relevant information.

The detection of genes with the BIGSdb system faces 2 limitations. First, for multicopy genes, only 1 occurrence might be detected. In *K. pneumoniae*, this problem is posed by the possible co-occurrence of chromosomal and plasmid-borne bla_{SHV} genes. However, this issue has been recently addressed since version 1.8.0 (http://sourceforge.net/p/bigsdb/news/; K. Jolley, pers. comm.). Second, the number of loci for which allelic number attribution fails is strongly dependent on the assembly fragmentation (Figure S2, http://bigsdb.web.pasteur.fr/klebsiella/archives/Bialek_TechnicalAppendix.pdf). Therefore, it seems advisable to exclude highly fragmented genomes from the analysis.

Several *K. pneumoniae* international clones associated with multidrug resistance or hypervirulence were described by using MLST (*4–8*,*21*). However, the low level of nucleotide sequence divergence among *K. pneumoniae* isolates (*8*) makes it difficult to define borders between clones (*4–6*). The 694-gene cgMLST genotyping scheme represents ≈100 times more sequence information than that provided by the 7-gene MLST. cgMLST demonstrated clear discontinuities in the *K. pneumoniae* genotypic space and showed that high-risk CGs were remarkably distinct from their closest genotypic neighbors. This result is in striking contrast to the genotypic continuum obtained by using MLST. Retrospectively, the failure of MLST to disclose the sharp discontinuities among CGs can be attributed to a severe lack of resolution.

The discovery of recognizable *K. pneumoniae* CGs opens the way to studying their genomic and biologic specificities. Different combinations of virulence and resistance genes were found among CGs, providing identification markers and hinting that these clones had distinct host–pathogen relationships and antimicrobial drug exposure. The genomic signatures of CGs show that MDR and hypervirulent populations of *K. pneumoniae* are, so far, mostly nonoverlapping. However, genes encoding resistance to β-lactams, quinolones, and aminoglycosides were detected in 2 isolates of hypervirulent clone CG23: BG130 (Vietnam, 2008) and BG141 (Madagascar, 2007). These 2 isolates are among the first documented members of CG23 shown to harbor $bla_{CTX\text{-}M\text{-}15}$ and genes conferring high-level resistance to aminoglycosides and fluoroquinolones. A CTX-M-15–producing ST23 strain was also reported from South Korea (*39*). These results show that the gloomy prospect of dual-risk *K. pneumoniae* strains, combining virulence and multidrug resistance features, is becoming a reality.

Genome-wide genotyping is a powerful approach to address fine-scale epidemiologic questions (*37*). Our findings show that the discriminatory power of cgMLST, based on 694 genes, makes it possible to distinguish a subgroup of isolates involved in the 2011 *K. pneumoniae* outbreak in Bethesda (*24*) from epidemiologically unrelated CG258 isolates. However, local epidemiologic investigations will require more resolution to decipher recent transmission events. Because the typical *K. pneumoniae* genome harbors ≈5,300 genes, there is ample room for defining additional loci to improve discriminatory power. The BIGSdb-Kp database can host additional loci and schemes curated at a distance by distinct laboratories (*16*).

Our findings demonstrate the existence of clearly distinguishable *K. pneumoniae* CGs and show that MDR and hypervirulent populations of this species are largely nonoverlapping. However, hypervirulent *K. pneumoniae* are beginning to evolve toward increasing levels of antimicrobial drug resistance and may represent a new and serious threat to public health (*40*). The freely accessible BIGSdb-Kp database represents a novel tool for monitoring the emergence of high-risk clones and for global collaboration on the population biology, epidemiology, and pathogenesis of *K. pneumoniae*.

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Dr Bialek-Davenet is a microbiologist working as a postdoctoral fellow in the Microbial Evolutionary Genomics Unit, Institut Pasteur. Her research interests include genomic epidemiology and resistance and virulence determinants of *K. pneumoniae*.

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Respiratory Viruses and Bacteria among Pilgrims during the 2013 Hajj

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Pilgrims returning from the Hajj might contribute to international spreading of respiratory pathogens. Nasal and throat swab specimens were obtained from 129 pilgrims in 2013 before they departed from France and before they left Saudi Arabia, and tested by PCR for respiratory viruses and bacteria. Overall, 21.5% and 38.8% of pre-Hajj and post-Hajj specimens, respectively, were positive for ≥1 virus (p = 0.003). One third (29.8%) of the participants acquired ≥1 virus, particularly rhinovirus (14.0%), coronavirus E229 (12.4%), and influenza A(H3N2) virus (6.2%) while in Saudi Arabia. None of the participants were positive for the Middle East respiratory syndrome coronavirus. In addition, 50.0% and 62.0% of pre-Hajj and post-Hajj specimens, respectively, were positive for *Streptococcus pneumoniae* (p = 0.053). One third (36.3%) of the participants had acquired *S. pneumoniae* during their stay. Our results confirm high acquisition rates of rhinovirus and *S. pneumoniae* in pilgrims and highlight the acquisition of coronavirus E229.

More than 2 million Muslims gather annually in Saudi Arabia for a pilgrimage to the holy places of Islam known as the Hajj. The Hajj presents major public health and infection control challenges. Inevitable overcrowding within a confined area with persons from >180 countries in close contact with others, particularly during the circumambulation of the Kaaba (Tawaf) inside the Grand Mosque in Mecca, leads to a high risk pilgrims to acquire and spread infectious diseases during their time in Saudi Arabia (*1*), particularly respiratory diseases (*2*). Respiratory diseases are a major cause of consultation in primary health care facilities in Mina, Saudi Arabia, during the Hajj (*3*). Pneumonia is a leading cause of hospitalization in intensive care units (*4*).

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Numerous studies have shown a high prevalence of respiratory symptoms among pilgrims (*5*–*7*). Respiratory viruses, especially influenza virus, are the most common cause of acute respiratory infections among pilgrims (*8*–*11*). We recently reported the acquisition of rhinovirus (*5*) and *Streptococcus pneumoniae* infections (*12*) by French pilgrims during the 2012 Hajj season and highlighted the potential for spread of these infections to home countries of pilgrims upon their return. However, none of the French pilgrims were positive for Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 (*13*) and 2013 (*14*).

In this study, we collected paired nasal and throat swab specimens from adult pilgrims departing from Marseille, France to Mecca, Saudi Arabia, for the 2013 Hajj season. The primary objective was to determine the prevalence of the most common respiratory viruses and bacteria upon return of pilgrims from the Hajj. The secondary objective was to evaluate the potential yearly variation of the acquisition of these respiratory pathogens by comparing results from the 2012 and 2013 Hajj seasons.

Methods

Participants

Pilgrims who planned to participate in the 2013 Hajj were recruited on September 15, 2013, at a private specialized travel agency in Marseille, France, which organizes travel to Mecca. Potential participants were asked to participate in the study on a voluntary basis if they were ≥18 years of age and were able to provide consent.

Study Design

In this prospective cohort study, participants were sampled and followed up before departing from France (on October 2, 2013) and immediately before leaving Saudi Arabia (on October 24, 2013). Upon inclusion in the study, participants were interviewed by Arabic-speaking investigators who used a standardized pre-travel questionnaire that collected information on the demographic characteristics and medical history of each participant. A post-travel questionnaire that collected clinical data and information

on vaccination status and compliance with preventive measures was completed during a face-to-face interview 2 days before the pilgrims returned to France by a single investigator who joined the pilgrims after the Hajj. Health problems that occurred during the pilgrims' stay were also recorded by a physician who traveled with them during the entire stay in Saudi Arabia, including during the rituals.

Subjective fever was defined as a feverish feeling according to the pilgrims' report. Influenza-like illness (ILI) was defined as the presence of cough, sore throat, and subjective fever (*15*). The study protocol was approved by the Aix Marseille Université institutional review board (July 23, 2013; reference no. 2013-A00961–44) and by the Saudi Ministry of Health Ethical Review Committee. The study was performed in accordance with the good clinical practices recommended by the Declaration of Helsinki and its amendments. All participants gave written informed consent.

Respiratory Specimens

Paired nasal and throat swab specimens were collected from each participant by using rigid cotton-tipped swab applicators (Medical Wire and Equipment, Corsham, UK) 10 days (September 22, 2013) before participants departed from France (pre-Hajj specimens) and only 1 day (October 23, 2013) before they left Saudi Arabia (post-Hajj specimens). Nasal and throat swab specimens collected from participants were placed in viral transport media (Virocult and Transwab, respectively; Sigma, St. Louis, MO, USA) at the time of collection and kept at 20°C before being transported to a laboratory in Marseille for storage at –80°C within 48 h of collection.

Detection of Respiratory Viruses

Nasal swab samples were independently tested as described (*5*) for influenza virus A/H3N2 (*16*), influenza B virus (*16*), influenza C virus (*17*), and A(H1N1)pdm09 virus (*18*); human adenovirus (*19*); human bocavirus (*20*), human cytomegalovirus (*21*); human coronaviruses (HCoVs); human enterovirus (*22*); human metapneumovirus (*23*); human parainfluenza viruses (HPIVs); human parechovirus (*24*); human respiratory syncytial virus (*25*); and human rhinovirus (HRV) (*26*) by using real-time reverse transcription PCRs. HCoVs and human HPIVs were detected by using an HCoV/HPIV R-Gene Kit (Argene/bioMérieux, Marcy l'Etoile, France) (*27*). HCoV-positive samples were then genotyped by using the FTD Respiratory Pathogens 21 Kit (Fast Track Diagnostics, Luxembourg, Luxembourg).

Detection of Respiratory Bacteria

Throat swab samples were independently tested as described (*12*) by using quantitative real-time PCRs for *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Bordetella pertussis*, and *Mycoplasma pneumoniae*. Sequences of all primers and probes have been reported (*28*). In the present study, reactions were performed by using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

The Pearson χ^2 and Fisher exact tests, as appropriate, were used to analyze categorical variables. Statistical analyses were performed by using SPSS software package version 17 (SPSS Inc., Chicago, IL, USA). p values ≤0.05 were considered significant.

Results

Characteristics of Study Participants

A total of 129 persons were invited to participate in the study. All persons agreed to participate in the study and responded to the pre-travel questionnaire. The participants were 77 women (59.7%) and 52 men (40.3%) who had a mean (SD) age of 61.7 (9.8) years (age range 34–85 years) (Table 1). Although most (94.6%) participants were born in northern Africa, most (94.5%) had lived for years in Marseille or the surrounding cities. More than half of the participants (52.7%) reported having ≥1 chronic disease, as described (*14*).

Clinical Features

All post-travel questionnaires were completed. During the 3-week stay in Saudi Arabia (October 3–24, 2013), most (90.7%) pilgrims had ≥1 respiratory symptom, including cough (86.8%), sore throat (82.9%), rhinorrhea (72.1%), myalgia (50.4%), fever (49.6%), and dyspnea (21.7%), and 47.3% met the criteria for self-reported ILI (41.3% in 2012 vs. 47.3% in 2013; p = 0.325). Onset of respiratory symptoms peaked in the second week (week 41) after the arrival of the pilgrims in Mecca and decreased thereafter. However, 90 (69.8%) pilgrims still had respiratory symptoms before leaving Saudi Arabia at the time of sampling (week 43). Only 1 pilgrim (0.8%) was hospitalized during the stay in Saudi Arabia (for undocumented pneumonia). No deaths occurred.

Regarding preventive measures, 51.2% of participants reported receiving pneumococcal vaccination (Pneumo 23) in the past 5 years, which was significantly higher than the rate in 2012 (35.9% in 2012 vs. 51.2% in 2013; p = 0.013). None had received the 2013 influenza vaccine before departing for the Hajj, but 44.2% reported having received the seasonal influenza vaccine in 2012 (31.8% among participants <65 years of age vs. 65.8% among participants >65 years of age; p = 0.001). During the stay in Saudi Arabia, 53.5% of pilgrims reported either frequent use (9.3%) or occasional use (44.2%) of facemasks; 93.0% used disposable handkerchiefs; 49.6% reported frequent handwashing;

Table 1. Demographic and baseline characteristics of pilgrims during the 2012 and 2013 Hajj*

Characteristic	2012 study cohort, n = 169	2013 study cohort, n = 129	p value
Mean age, y (SD, range)	59.3 (12.4, 21–83)	61.7 (9.8, 34–85)	0.079
Age groups, y, no. (%)			
20–40	13 (7.8)	5 (3.9)	NA
41–60	67 (40.1)	50 (38.8)	NA
61–80	85 (50.9)	71 (55.0)	NA
>80	2 (1.2)	3 (2.3)	NA
Sex, no. (%)			0.669
M	64 (38.3)	52 (40.3)	NA
F	103 (61.7)	77 (59.7)	NA
Birthplace, no. (%)			
Algeria	116 (69.5)	90 (69.8)	NA
Tunisia	17 (10.2)	17 (13.2)	NA
Morocco	15 (9.0)	15 (11.6)	NA
Metropolitan France	13 (7.8)	5 (3.9)	NA
Egypt	6 (3.6)	2 (1.6)	NA
Location of residence in France, no. (%)			
Marseille	110 (65.9)	91 (70.5)	NA
Southern France (outside Marseille)	48 (28.7)	31 (24.0)	NA
Other	7 (5.4)	7 (5.4)	NA
Duration of stay in France, y, no. (%)†			
5–10	8 (5.4)	13 (11.1)	NA
11–20	13 (8.8)	11 (9.4)	NA
>20	127 (85.8)	93 (79.5)	NA

*NA, not applicable.
†Immigrants only.

and 67.4% used hand sanitizer. ILI symptoms were less frequently reported by persons who reported receiving the influenza vaccine in 2012 compared with reports by unvaccinated persons (34.1% vs. 61.5%, respectively; p = 0.009) (odds ratio 0.32, 95% CI 0.14–0.76). In contrast, none of the other preventive measures was found to be effective in preventing ILI symptoms during the stay in Saudi Arabia.

Detection of Respiratory Viruses

Pre-Hajj and post-Hajj nasal swab specimens were obtained from 121 (93.8%) and 129 (100%) participants, respectively. A total of 26 (21.5%) of 121 pre-Hajj specimens tested were positive for ≥1 virus compared with 50 (38.8%) of 129 post-Hajj specimens tested (p = 0.003) (Table 2). Moreover, 36 (29.8%) participants had acquired ≥1 virus during the stay in Saudi Arabia (Figure 1). The prevalence of human coronavirus E229 (HCoV-E229) was significantly higher in post-Hajj specimens than in pre-Hajj specimens (12.4% vs. 0%; p<0.001). A high prevalence of HRV was observed in pre-Hajj and post-Hajj specimens (14.0% and 14.7%, respectively; p = 0.88). Of 19 participants whose post-Hajj specimens were positive for HRV, 17 (89.5%) had acquired the infection during their stay in Saudi Arabia (Figure 1).

The prevalence of influenza A and B viruses was significantly higher in post-Hajj specimens than in pre-Hajj specimens (7.8% vs. 0%; p = 0.002); further details are described elsewhere (*14*). Coronaviruses HKU1, NL63, and OC43; human enterovirus; human metapneumovirus; HPIV; and human respiratory syncytial virus were also acquired during the stay in Saudi Arabia by a low proportion of participants (Table 2). Of 50 participants whose post-Hajj specimens were positive for ≥1 respiratory virus, 43 (86.0%) reported ≥1 respiratory symptom during their stay in Saudi Arabia, of whom 37 (86.0%) still had respiratory symptoms at the time of sampling. Also, of 79 participants whose post-Hajj specimens were negative for respiratory viruses, 74 (93.7%) reported ≥1 respiratory symptom during their stay Saudi Arabia, of whom 53 (71.6%) still had respiratory symptoms at the time of sampling. None of the preventive measures was found to be effective in preventing respiratory viruses in post-Hajj specimens.

Detection of Respiratory Bacteria

Pre-Hajj and post-Hajj throat swab specimens were obtained from 126 (97.7%) and 129 (100%) participants, respectively. None of the participants were positive for *N. meningitidis*, *B. pertussis*, or *M. pneumoniae* at any point in the study period (Table 2).

A total of 63 (50.0%) of 126 pre-Hajj specimens tested and 80 (62.0%) of 129 post-Hajj specimens tested were positive for *S. pneumoniae* (p = 0.053) (Table 2; Figure 2). Of 80 participants whose post-Hajj specimens were positive for *S. pneumoniae*, 29 (36.3%) had acquired the infection during their stay in Saudi Arabia (Figure 2). In addition, of 63 participants whose pre-Hajj specimens were positive for *S. pneumoniae*, 12 (19.0%) subsequently had post-Hajj specimens that were negative for *S. pneumoniae* (Figure 2), of whom 10 (83.3%) reported having received antimicrobial drugs during their stay in Saudi Arabia: 7 received amoxicillin, 2 received amoxicillin and ciprofloxacin,

Table 2. Prevalence of respiratory viruses and bacteria among participants before departing from France and before leaving Saudi Arabia, 2012 and 2013 Hajj*

Respiratory pathogen	2012 study, n = 169			2013 study, n = 129		
	Before departing from France, no. (%)	Before leaving Saudi Arabia, no. (%)	p value	Before departing from France, no. (%)	Before leaving Saudi Arabia, no. (%)	p value
Virus						
Influenza virus A (H3N2)	0	0	NA	0	8 (6.2)	0.007†
Influenza virus B	0	2 (1.3)	0.23	0	1 (0.8)	1
Influenza virus C	1 (0.6)	0	1	2 (1.7)	0	0.23
A(H1N1)pdm09	0	0	NA	0	1 (0.8)	1
Human adenovirus	1 (0.6)	3 (1.9)	0.36	2 (1.7)	0	0.23
Human bocavirus	ND	ND	NA	2 (1.7)	0	0.23
Human coronavirus E229	ND	ND	NA	0	16 (12.4)	$<10^{-3}$†
Human coronavirus HKU1	ND	ND	NA	0	5 (3.9)	0.06
Human coronavirus NL63	ND	ND	NA	0	1 (0.8)	1
Human coronavirus OC43	ND	ND	NA	0	5 (3.9)	0.06
Human cytomegalovirus	ND	ND	NA	0	0	NA
Human enterovirus	1 (0.6)	1 (0.6)	1	1 (0.8)	3 (2.3)	0.62
Human metapneumovirus	0	0	NA	2 (1.7)	1 (0.8)	0.61
Human parainfluenza viruses	ND	ND	NA	4 (3.3)	1 (0.8)	0.20
Human parechovirus	ND	ND	NA	0	0	NA
Human respiratory syncytial virus	0	0	NA	0	1 (0.8)	1
Human rhinovirus	5 (3.0)	13 (8.4)	0.036†	17 (14.0)	19 (14.7)	0.88
At least 1 virus	8 (4.3)	17 (11.0)	0.040†	26 (21.5)	50 (38.8)	0.003†
Bacteria‡						
Bordetella pertussis	0	0	NA	0	0	NA
Mycoplasma pneumoniae	0	0	NA	0	0	NA
Neisseria meningitidis	0	0	NA	0	0	NA
Streptococcus pneumoniae	12 (7.3)	30 (19.5)	0.001†	63 (50.0)	80 (62.0)	0.053

*In 2012, 146 pre-Hajj samples (88.5%) were collected in the month before departing from France and were stored at ambient temperature (stabilized by air conditioning at 20°C) for a mean time of 13 d (range 5–37 d) before being transported to a laboratory in Marseille for storage at −80°C. In addition, 19 pre-Hajj samples (11.5%) were collected on the day of departure from France (at the airport) and were stored at ambient temperature for 30 d after collection before being transported to a laboratory in Marseille for storage at −80°C. In 2013, all samples collected during the study were kept at ambient temperature before being transported to a laboratory in Marseille for storage at −80°C within 48 h of collection. NA, not applicable; ND, not determined.
†Statistically significant difference.
‡In the 2012 study, nasal swab specimens were collected from participants instead of throat swab specimens, which were used in the present study conducted in 2013.

and 1 received azithromycin. Of 80 participants whose post-Hajj specimens were positive for *S. pneumoniae*, 73 (91.2%) reported ≥1 respiratory symptom during their stay in Saudi Arabia, of whom 56 (76.7%) still had respiratory symptoms at the time of sampling.

Among 66 participants who reported having received a pneumococcal vaccination in the 5 years before traveling to Saudi Arabia, 37 (56.1%) had post-Hajj specimens that were positive for *S. pneumoniae*. The prevalence of *S. pneumoniae* in post-Hajj specimens was significantly lower in persons who reported using hand sanitizer during their stay in Saudi Arabia than in remaining participants (55.2% vs. 76.2%; p = 0.021) (odds ratio 0.39, 95% CI 0.17–0.88) and slightly lower in persons who reported more frequent handwashing than usual during their stay in Saudi Arabia than in persons who reported usual handwashing (54.7% vs. 69.2%; p = 0.08).

Of 80 participants whose post-Hajj specimens were positive for *S. pneumoniae*, 27 (33.8%) were co-infected with ≥1 virus (Figure 2). Of 49 participants whose post-Hajj specimens were negative for *S. pneumoniae*, 23 (46.9%) were infected with ≥1 virus (33.8% vs. 46.9%; p = 0.14) (Figure 2).

Discussion

For the second consecutive year, we conducted a prospective longitudinal study of respiratory viruses and bacteria in respiratory specimens collected from a single cohort of pilgrims before departing from Marseille, France, to Mecca, Saudi Arabia, for the Hajj and immediately before leaving Saudi Arabia. By collecting samples from pilgrims before their departure from Saudi Arabia, we were able to rule out acquisition of infections acquired as a result of travel through the international airports of Jeddah, Saudi Arabia, and Istanbul, Turkey, as part of the return trip to Marseille. Close monitoring for respiratory symptoms and compliance with preventive measures was also performed by the investigators accompanying the group.

In this study, we confirmed that performing the Hajj pilgrimage is associated with an increased occurrence of respiratory symptoms in most pilgrims; 8 of 10 pilgrims showed nasal or throat acquisition of respiratory pathogens. This acquisition may have resulted from human-to-human transmission through close contact within the group of French pilgrims because many of them were already infected with HRV or *S. pneumoniae* before departing from France. Alternatively, the French pilgrims may

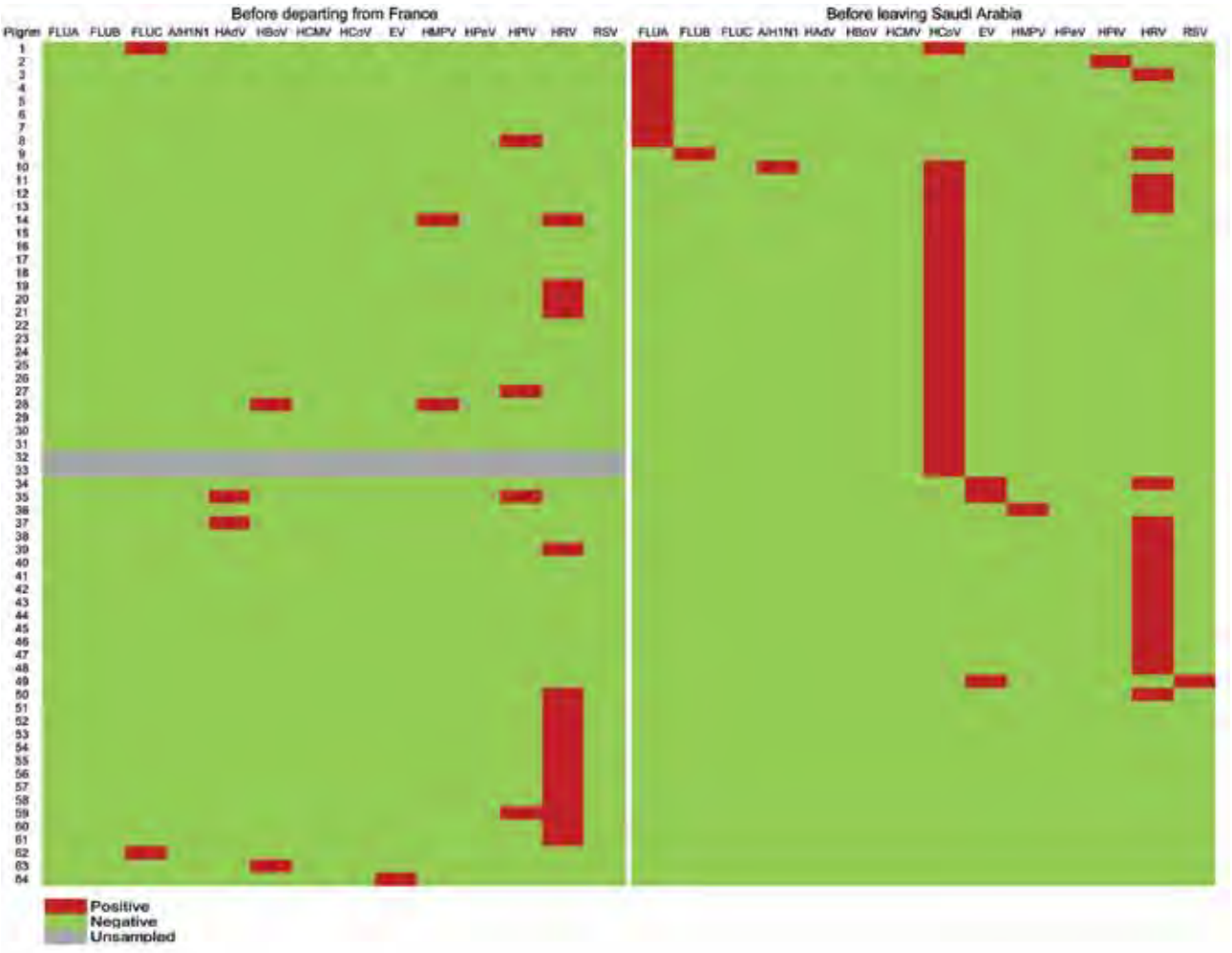

Figure 1. Patterns of respiratory viruses detected among 64 pilgrims who were positive for viruses during the study period before departing from France and before leaving Saudi Arabia, 2013 Hajj. FLUA, influenza A(H3N2) virus; FLUB, influenza B virus; FLUC, influenza C virus; A/H1N1, A(H1N1)pdm09 virus; HAdV, human adenovirus; HBoV, human bocavirus; HCMV, human cytomegalovirus; HCoV, human coronaviruses; EV, human enterovirus; HMPV, human metapneumovirus; HPeV, human parechovirus; HPIV, human parainfluenza virus; HRV, human rhinovirus; RSV, human respiratory syncytial virus.

have acquired these respiratory pathogens from other pilgrims, given the extremely high crowding density to which persons from many parts of the world are exposed when performing Hajj rituals. Finally, contamination originating from an environmental source might have played a role. Sequencing of these pathogens would be required to determine how often new infections were acquired during the stay in Saudi Arabia. However, detection of nasal carriage of coronaviruses other than MERS-CoV and influenza A and B viruses in only the post-Hajj specimens supports the hypotheses that infection occurred during the Hajj.

We confirmed the predominance of HRV and *S. pneumoniae* among pathogens acquired during the pilgrims' stay (*5*,*12*). We also highlighted acquisition of coronaviruses other than MERS-CoV, most notably HCoV-E229, by pilgrims during the 2013 Hajj pilgrimage. In 2012 and 2013, results of screening for MERS-CoV infection in different cohorts of pilgrims, including the present cohort, were negative (*13*,*14*,*29*). Finally, we found that compared with acquisition of HRV and HCoV-E229, influenza viruses were acquired at a lower frequency among pilgrims.

The present study is a continuation of our previous study in 2012 (*5*). We extended the investigation to additional viruses, including human bocavirus, human cytomegalovirus, coronaviruses, human parechoviruses, and HPIV, and showed a high frequency of HCoV-E229 infection in pilgrims returning from the Hajj. The prevalence of HRV was lower in 2012 than in 2013, both before departing from France (3.0% in 2012 vs. 14.0% in 2013; $p = 0.001$) and before leaving Saudi Arabia (8.4% in 2012 vs. 14.7% in 2013; $p = 0.092$). However, samples that were obtained from pilgrims before departing from France during the 2012 study were stored at room temperature (20°C) for

≤30 days before being processed. This protocol may have resulted in degradation of genetic material, which probably contributed to underestimation of frequencies of infection in 2012. In 2013, all samples collected during the study period were stored at –80°C within 48 h of collection.

The prevalence of *S. pneumoniae* was also significantly lower in 2012 than in 2013 before pilgrims departed from France (7.3% vs. 50.0%; p<0.001) and before they left Saudi Arabia (19.5% vs. 62.0%; p<0.001). However, in the 2012 study, nasal swab specimens were collected from participants instead of throat swab specimens, which were used in the 2013 study. In addition, the period of the storage of samples before freezing differed between the 2012 and the 2013 studies, as mentioned earlier in this report.

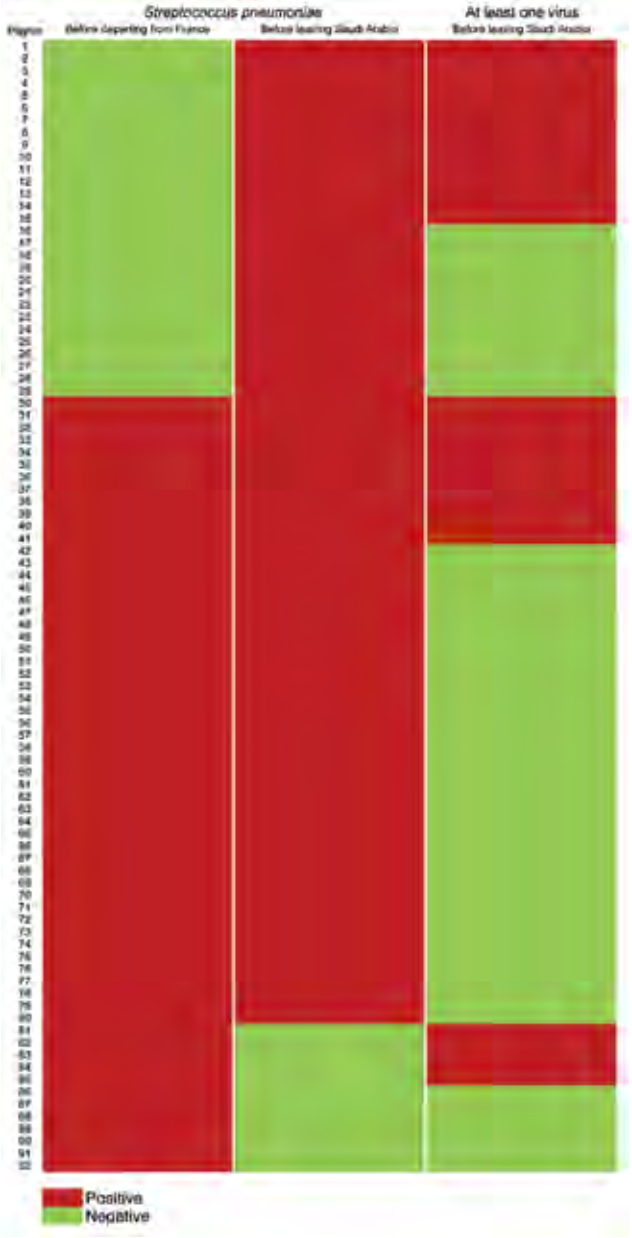

Figure 2. Detection of *Streptococcus pneumoniae* among 92 pilgrims who were positive for viruses during the study period before departing from France and before leaving Saudi Arabia, 2013 Hajj. A color version of this figure is available online (wwwnc.cdc.gov/eid/article/20/11/14-0600-f2.htm).

Our results confirm that various respiratory viruses might be acquired by pilgrims during their stay in Saudi Arabia and introduced into home countries of pilgrims on their return, thus contributing to potential international spread of these viruses. However, detection of other human coronaviruses does not enable any conclusions regarding MERS-CoV, for which the available data to date, although limited, indicate different epidemiologic characteristics. We could not demonstrate whether pathogens detected in respiratory specimens were responsible for observed symptoms because nasal carriage was observed in asymptomatic pilgrims in certain instances, and symptoms might have resulted from infection by pathogens that were not investigated in our study. In future studies, checking pilgrims at more frequent intervals might provide useful information. Nevertheless, we believe that Hajj cough likely results from infection of the respiratory tract by various respiratory viruses, including HRV and HCoV-E229, which are known to cause mild or serious lower respiratory tract infections (*30*,*31*). However, our results cannot be extrapolated to all pilgrims. A large-scale study based on a similar design and conducted in a large number of pilgrims from many countries would be useful.

We found that pilgrims who had received influenza vaccine in 2012 were less likely to report ILI symptoms during their stay in Saudi Arabia in 2013. Thus, availability of seasonal influenza vaccine for all persons attending the Hajj is crucial. Vaccination with a conjugate pneumococcal vaccine should be considered for persons with medical risk factors for invasive pneumococcal disease. In addition, use of hand sanitizer during the stay in Saudi Arabia was reported by more than two thirds of pilgrims in our survey and was associated with a lower prevalence of *S. pneumoniae* carriage. Interventional studies are urgently needed that evaluate efficacy of influenza and pneumococcal vaccines and use of hand sanitizer and closely monitor respiratory symptoms and carriage of respiratory pathogens in large cohorts of pilgrims. It is expected that results of such studies will lead to implementation of evidence-based recommendations about preventive measures during the Hajj.

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Dr Benkouiten is a researcher at the Institut Hospitalo–Universitaire Méditerranée Infection, Marseille, France. His research interests focus on the epidemiology of respiratory infections in the context of mass gatherings.

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Seroprevalence of Norovirus Genogroup IV Antibodies among Humans, Italy, 2010–2011

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Noroviruses (NoVs) of genogroup IV (GIV) (Alphatron-like) cause infections in humans and in carnivorous animals such as dogs and cats. We screened an age-stratified collection of serum samples from 535 humans in Italy, using virus-like particles of genotypes GIV.1, circulating in humans, and GIV.2, identified in animals, in ELISA, in order to investigate the prevalence of GIV NoV-specific IgG antibodies. Antibodies specific for both genotypes were detected, ranging from a prevalence of 6.6% to 44.8% for GIV.1 and from 6.8% to 15.1% for GIV.2 among different age groups. These data are consistent with a higher prevalence of GIV.1 strains in the human population. Analysis of antibodies against GIV.2 suggests zoonotic transmission of animal NoVs, likely attributable to interaction between humans and domestic pets. This finding, and recent documentation of human transmission of NoVs to dogs, indicate the possibility of an evolutionary relationship between human and animal NoVs.

Noroviruses (NoVs) are a major cause of epidemic gastroenteritis in children and adults. They cause nearly half of all gastroenteritis cases and >90% of nonbacterial gastroenteritis epidemics worldwide (*1*). NoVs belong to the genus *Norovirus* in the family *Caliciviridae* (*2*,*3*). NoV virions are nonenveloped and ≈30 to 35 nm in diameter. The icosahedral capsid surrounds a 7.7-kb positive-sense single-stranded RNA genome covalently linked to viral protein g (VPg) at the 5′ end and polyadenylated at the 3′ end (*4*). The RNA genome is organized into 3 open reading frames (ORFs). ORF1 encodes a polyprotein that is cleaved by the virus-encoded protease to produce several nonstructural proteins, including the RNA-dependent RNA polymerase; ORF2 encodes a major capsid protein, VP1; and ORF3 encodes a small basic protein (VP2) that has been associated with the stability of the capsid (*4*,*5*). Based on the full-length VP1 aa sequence, NoVs have been divided into 6 genogroups (GI to GVI) and multiple genotypes (*6*,*7*). However, only GI, GII, and GIV NoVs have been shown to infect humans; GII strains are the most prevalent worldwide (*4*). Human GIV NoV (Alphatron-like) strains have been identified at low prevalence from either sporadic cases or outbreaks of human gastroenteritis (*8–10*). However, analysis of wastewater, sewage, and seafood in Japan and Italy has revealed, indirectly, that GIV NoVs are common in humans (*11–14*).

GIV.2 NoVs (strain GIV.2/Pistoia/387/06/ITA) were first detected in the feces of a captive lion cub with severe hemorrhagic enteritis in Italy (*15*). Subsequently, similar NoVs were identified in fecal samples of dogs and cats with diarrhea (*16*,*17*). Sequence comparison in the VP1 of human and animal GIV NoVs has revealed that, although they are genetically related, the 2 groups of viruses represent 2 distinct genotypes: GIV.1 viruses predominate in humans and GIV.2 in animals (*7*,*15*). Historical evidence shows that viruses genetically and antigenically closely related to human NoVs might infect animals (*15–19*). Also, a human GII.4 NoV strain has been found to replicate and cause clinical signs and lesions in experimentally-infected gnotobiotic pigs and calves (*20*,*21*). These findings have raised public health concerns about potential cross-species transmission and generation of novel human NoV strains by recombination. The close genetic relatedness (*17*,*22*) of human and animal GIV NoVs indicates that they may have originated from a common ancestor. Interspecies transmission between humans and pets might have been facilitated by the social interactions established since domestication of small carnivores. This eventuality has been demonstrated firmly in a recent study in Finland, which reported the

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detection of GII.4 and GII.12 NoVs in the dogs belonging to human patients hospitalized with NoV gastroenteritis (*19*). To address whether cross-species transmission of GIV.2 might occur between carnivores and humans, we investigated the prevalence of antibodies against GIV.1 and GIV.2 NoVs in a representative population in Italy spanning all age groups.

Materials and Methods

Human Serum Samples

Human serum samples were collected from a random sampling of inpatients and outpatients seeking medical attention for various clinical conditions at the Microbiology Unit of the University Hospital "P. Giaccone" of Palermo, Sicily, Italy, during September 2010–June 2011. All patients were enrolled in the study after giving informed consent. Serum samples from a total of 535 persons were tested. For our analysis, samples were divided on the basis of patient age groups: <1 year, 1–5 years, 5-year age groups from 6–79, and >80 years of age.

Virus-like Particles

The recombinant baculoviruses carrying the genes for the viral capsid proteins of the lion/NoV/GIV.2/Pistoia/387/06/ITA and Hu/NoV/GIV.1/SaintCloud624/1998/U.S. strains were obtained as previously described (*23*,*24*). For large-scale production of virus-like particles (VLPs), 100 mL of *Sf9* cells (1×10^6 cell/mL) suspension culture were inoculated with the recombinant baculovirus at a multiplicity of infection of 3 PFU/cell. Assembled VLPs were isolated from the culture medium of infected cells at 48 h postinfection by centrifugation at 4,000 rpm for 20 min. The recombinant capsid proteins were concentrated by ultracentrifugation through a 17% sucrose cushion in 50 mmol/L Tris-HCl, pH 7.5; 1 mmol/L EDTA; and 100 mmol/L NaCl, aka TEN-buffer, and purified on a discontinuous 20%–60% (wt/vol) sucrose gradient, as previously described (*24*). The collected fractions were dialyzed against phosphate-buffered saline (PBS), and the protein concentration of VLP preparations was determined by measuring the optical density at 280 nm (OD_{280}) and visually by running aliquots containing bovine serum albumin standards on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. The presence of VLPs was confirmed by electron microscopy.

Antigenic Relationships of VLPs

To evaluate the antigenic relationship between GIV.1 and GIV.2 VLPs, we tested polyclonal rabbit serum produced against the lion GIV.2 strain (*24*) for GIV.1 and GIV.2 antigen reactivity by using Western blot (WB) testing to limit dilution analysis (not shown). Although a modest reactivity with the heterologous GIV.1 antigen was observed at dilution ≤1:100, the GIV.2 antiserum showed the highest levels of reactivity with the homologous antigen. Two experiments were performed to investigate serologic cross-reactions between GIV VLPs and human NoVs belonging to genetic groups GI and GII. First, we tested GIV.1 and GIV.2 VLPs using an antigen-ELISA kit (Ideia Norovirus, Oxoid, Basingstroke, UK). Second, we assessed the reactivity of the GIV.2 antiserum with GII.4 VLPs (Hu/NoV/GII.4/MD145–12/1987/U.S.) (*25*) in WB analysis. The GIV.1 and GIV.2 VLPs were not detected by the commercial antigen-ELISA kit even at concentrations ≤10 μg of protein/mL; the GIV.2-specific rabbit antiserum did not show reactivity with GII.4 VLPs in WB analysis.

ELISA

For the development of the antibody detection ELISA, we diluted the supernatant containing mock infected cells GIV.1 and GIV.2 VLPs to a final concentration of 1 μg/mL in carbonate-bicarbonate buffer (0.05 M, pH 9.6) and 100 mL was added to each well of a 96-well EIA plate (Costar, Bio-Rad Laboratories, Segrate, Italy). The plates were incubated at 4°C overnight. The wells were washed 5 times with 0.1% Tween-PBS (PBS-T) and then blocked with 200 mL of PBS containing 2% bovine serum albumin at room temperature for 2 hours. After the 5 washings, each serum sample (100 mL), diluted to 1:100 in 1% dried milk (Blotto, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) in PBS, was added to the antigen-coated wells, and the plates were incubated at 37°C for 1 h. Plates were washed 5 times with 0.1% PBS-T and then incubated with horseradish peroxidase-conjugated goat anti-human IgG (Sigma-Aldrich, Milan, Italy) at 1:5,000 dilution for 30 min at 37°C. The reaction developed after the addition of 100 μL per well of 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) substrate for 15 min and stopped after addition of an equal volume of 1 M/L phosphoric acid. We measured absorbance at 405 nm using a Multiskan automatic plate reader (ThermoLabsystems, Abu Gosh, Israel). The cutoff point of the ELISA was established as the mean of the OD_{405} readings of 50 human serum samples negative in WB for both GIV.1 and GIV.2 antigens plus 2 standard deviations. For each tested sample, a positive/negative ratio (OD_{405} of VLPs/OD_{405} of mock infected cells) ≥2.0 was used to evaluate the background binding. All samples that had OD_{405} values ≥0.5 at the initial dilution of 1:100 were considered positive and titrated in 2-fold dilutions. Mean ELISA antibody titers were calculated and expressed as the reciprocal of the highest serum dilution that had positive absorbance (OD_{405}≥0.5) for GIV.1 and/or GIV.2 antigens. The data were analyzed by using GraphPad Prism Software (GraphPad Software, La Jolla, CA, USA). We used a χ^2 test for trend to determine the trend of age-class prevalence of

IgG antibodies to GIV.1 and GIV.2 VLPs, and Fisher exact test to determine the difference between the seroprevalence rates for the 2 antigens and the differences in prevalence among the age groups. A p value of <0.05 was considered statistically significant.

Results

Of 535 human serum samples tested at the initial dilution of 1:100, 151 (28.2%) were positive for the presence of GIV NoV-specific antibodies: 107 (20.0%) samples reacted with both GIV.1 and GIV.2 VLPs, 39 (7.3%) with GIV.1, and 5 (0.9%) with GIV.2. When all the positive serum samples were rescreened by endpoint titration, 118 samples (22.0%) reacted with the GIV.1 antigen at rates of 14.5% (78/535) at dilution of 1:200 and 7.4% (40/535) at dilution of 1:400. Twenty-six (4.8%) samples reacted with GIV.2 at rates of 2.2% (12/535) at a dilution of 1:200 and 2.6% (14/535) at 1:400. Seven (1.3%) samples were positive for antibodies against the antigens tested at final dilutions of 1:200 and 1:400 (3 and 4 samples, respectively) (Table).

We further examined age-related patterns of seroprevalence for GIV.1 and GIV.2 NoVs (Figure). The rate of IgG antibodies against GIV.1 NoVs was 6.6% in infants <1 year of age. This rate of GIV-reactive serum samples increased from 6.6%–37.9% in the 1- to 5-year age group and reached a peak of 43.3% in children 6–10 years of age. The prevalence then declined, reaching the lowest values in the 11- to15-year age group and in young adults who were 26–30 years of age (10.7% and 9.6%, respectively). The rate of GIV.1-positive NoVs gradually increased from 14.9% in the 31- to 35-year age group to 44.8% in the 61- to 65-year age group. The prevalence in the older age groups gradually declined to 10.0% in persons >80 years of age.

Compared with prevalence of IgG antibodies against GIV.1 NoVs, the overall prevalence for IgG antibodies against GIV.2 NoVs was low in most age groups, varying from 6.8% in preschool-age children (1–5 years) to 10.7% in the 11- to 5-year age group, reaching peaks in young adults who were 16–20 years (13.8%) and 31–35 years of age (15.1%), and its prevalence ranged 9.37%–12.5% in persons 56–70 years of age. The lowest level of seropositivity was in persons in the age groups 26–30, 41–45, and 76–80 years (3.2%–5.9%, respectively). Antibodies against GIV.2 NoVs were not identified in samples from the following age groups: <1 year, 21–25 years, 36–40 years, 46–55 years, and 71–75 years.

Table. Seroprevalence of IgG antibody against norovirus GIV.1 and GIV.2 in human serum specimens, Italy, 2010–2011*

NoV GIV virus-like particles	Serum dilutions 1:200 (%)	1:400 (%)	Total (%)
GIV.1	78 (14.5)	40 (7.4)	118/535 (22.0)
GIV.2	12 (2.2)	14 (2.6)	26/535 (4.8)
GIV.1+GIV.2	3 (0.5)	4 (0.7)	7/535 (1.3)

*GIV; genogroup IV; NoV, norovirus.

To perform statistical analysis, we combined data from several age groups because, in some age groups, no serum samples tested positive for GIV.2 NoVs, and we found that the positive GIV.1 trend, when charted, showed a significant shape ($\chi^2 = 16.50$ $p<0.01$) of seroprevalence. Two peaks were observed in the age groups 1–10 and 56–70 years. Likewise, statistically significant differences were found for the GIV.1 seroprevalence in age group 1–10 years when compared with that in the age groups 11–25 ($p = 0.03$) and 26–40 ($p = 0.02$) years. The differences were not significant when the GIV.1 seroprevalence in age groups 1–10 years was compared to that in the age groups 41–55 ($p = 0.38$), 56–70 ($p = 0.61$,), and >70 years ($p = 0.06$).

Similar analyses were performed for GIV.2 NoV antibodies, but no statistical significance was found ($\chi^2 = 10.74$, $p = 0.0568$), likely because of the low number of seropositive persons. Two peaks in age groups were also observed for GIV.2 NoV antibodies: in the age groups 56–70 and 11–25 years. When comparing the seroprevalence rates

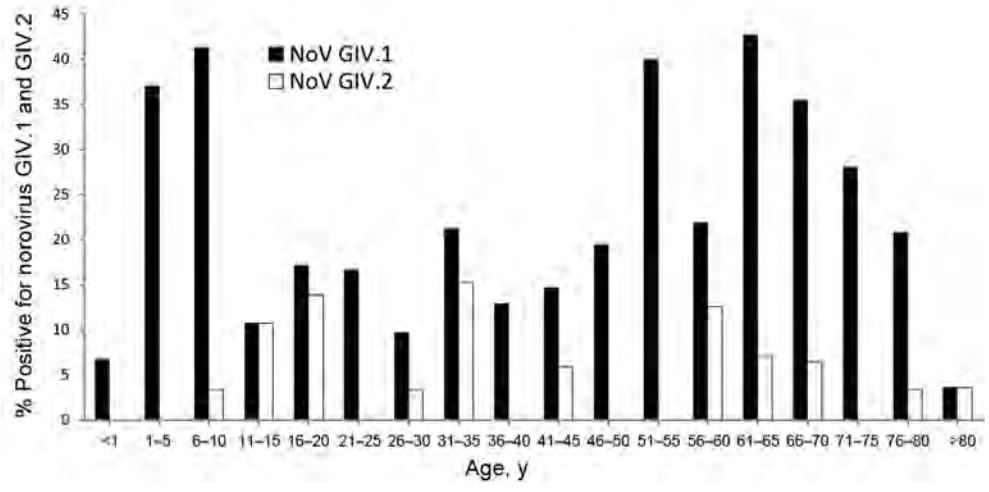

Figure. Age-related prevalence of antibodies against norovirus genogroup IV, genotypes GIV.1 and GIV.2, in human serum specimens, Italy, 2010–2011.

between the NoV antigens GIV.1 and GIV.2, statistically significant differences were observed ($p<0.01$), suggesting that the seroprevalence values reported for the human serum specimens are not related.

Discussion

In vitro expression of viral proteins is essential to gathering information on the epidemiology of noncultivable NoVs in humans and animals (*26*–*28*). Serologic studies documenting the seroprevalence of GI and GII NoVs in humans have been performed since the early 1990s (*27*,*29*,*30*); however, serologic studies on GIV NoVs have not been performed, thus limiting the understanding of the epidemiology of viruses in this genogroup.

We examined the prevalence of IgG antibodies against GIV.1 and GIV.2 NoVs and found an overall seroprevalence rate of 28.2%. The majority of the positive serum samples ($OD_{405} \geq 0.5$) reacted at the initial dilution of 1:100 with both GIV.1 and GIV.2 VLPs. This could be accounted for by the existence of highly conserved epitopes between genotype GIV.1 and GIV.2 NoVs. Heterologous sero-responses among strains of the same genogroups have been reported frequently in ELISA-based investigations (*31*,*32*), although greater sero-responses were detected against the homologous strains (*33*). Accordingly, to rule out the cross-reactivity between GIV.1 and GIV.2 NoVs, all the samples with OD_{405} values ≥ 0.5 were further assessed by endpoint titration. Antibodies specific for GIV NoV genotypes GIV.1 and GIV.2 were detected. These findings support previous hypothesis that humans may be exposed to NoVs from carnivores (*34*) or to antigenically related strains.

In general, the seroprevalence for GIV.1 NoVs detected in this investigation was lower than those reported for GI and GII NoVs in previous studies. In an earlier study in Italy, the seropositivity was 51.0% for GI.2 NoVs and 91.2% for GII.4 NoVs (*35*). In Japan, the seroprevalence rates were 82.0% for GI.1 NoVs and 88.0% for GII.3 NoVs (*36*), and in South Korea (*37*), the seroprevalence rates for GI.4, GII.3, and GII.4 NoVs were 84.1%, 76.3%, and 94.5%, respectively. However, consistent with the age-related patterns reported for GI and GII NoVs, we observed a positive association between the seropositivity for GIV.1 NoVs and age: the highest rates of antibodies were in children 5–10 years of age and in adults >50 years. GIV.1 NoVs have been documented in Italy, including in samples from children hospitalized with symptoms of acute gastroenteritis and in wastewater samples (*10*,*12*,*14*).

An age-related seroprevalence pattern was also observed for GIV.2 NoVs, although this profile differed from the seroprevalence profile of GIV.1 NoVs. The highest positive rate for GIV.2 NoV was detected in persons 15–35 years of age, although this pattern was not supported statistically. The overall prevalence rate for GIV.2 NoVs (4.8%) was markedly lower than the prevalence rates reported for other NoVs in humans (*27*,*31*,*37*), correlating with the lower prevalence of GIV viruses when compared with GII and GI viruses. Mesquita et al. (*34*) found IgG antibodies against GVI.2 viruses in 22.3% of small animal veterinarian practitioners and in 5.8% of the age-matched population controls, suggesting that veterinarians are more exposed to these viruses.

In conclusion, age-stratified serologic investigation revealed that GIV.1 NoVs are common in humans, although the prevalence of these viruses is somewhat low as documented in direct epidemiologic investigations. Also, antibodies specific for animal (GIV.2) NoVs were identified in human serum samples. This was not unexpected, because social interactions exist between humans and pets in all human populations and societies, and companion animals may transmit several zoonotic diseases to humans. Recent studies have also revealed that dogs can be infected, in turn, with human NoVs of different genotypes (*19*). Altogether, these findings seem to indicate that the evolution of human and animal NoVs is tightly intermingled.

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Dr Di Martino is a researcher at the Faculty of Veterinary Medicine of Teramo, Italy. Her research interests include the study of human and animal noroviruses, with particular emphasis on development of recombinant diagnostic tools.

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Drug-Resistant *Candida glabrata* Infection in Cancer Patients

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Cancer patients are at risk for candidemia, and increasing *Candida* spp. resistance poses an emerging threat. We determined rates of antifungal drug resistance, identified factors associated with resistance, and investigated the correlation between resistance and all-cause mortality rates among cancer patients with ≥1 *C. glabrata*–positive blood culture at MD Anderson Cancer Center, Houston, Texas, USA, during March 2005–September 2013. Of 146 isolates, 30 (20.5%) were resistant to fluconazole, 15 (10.3%) to caspofungin, and 10 (6.8%) to multiple drugs (9 caspofungin-resistant isolates were also resistant to fluconazole, 1 to amphotericin B). Independently associated with fluconazole resistance were azole preexposure, hematologic malignancy, and mechanical ventilation. Independently associated with caspofungin resistance were echinocandin preexposure, monocytopenia, and total parenteral nutrition. Fluconazole resistance was highly associated with caspofungin resistance, independent of prior azole or echinocandin use. Caspofungin resistance was associated with increased 28-day all-cause mortality rates. These findings highlight the need for good stewardship of antifungal drugs.

Patients with cancer are often at risk for candidemia because of indwelling catheters, abdominal surgery, use of cytotoxic chemotherapy, parenteral nutrition, antibacterial drugs, and corticosteroids (*1*–*5*). Increasing drug resistance among *Candida* spp. poses an emerging threat to these patients. Moreover, the widespread prophylactic use of azoles in patients with hematologic malignancies and a reduced threshold for empiric initiation of antifungal treatment among critically ill patients have led to a notable shift from infections with *C. albicans* to infections with non-*albicans Candida* species (*2*–*4*). Among cancer patients, one of the most common *Candida* species isolated is *C. glabrata* (*3*–*5*), which is the main species exhibiting multiazole, echinocandin, and multidrug resistance (resistance to at least 2 classes of antifungal drugs) (*6*–*9*).

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Recently, on the basis of the integration of epidemiologic, molecular, and limited clinical data, the Clinical Laboratory Standards Institute (CLSI) updated antifungal susceptibility break points for *Candida* spp. (*10*,*11*). According to the new definitions, rates of caspofungin nonsusceptibility among *C. glabrata* clinical isolates range from <10% (*12*) to as high as 62% (*13*). Previous use of azoles or echinocandins are strong predictors of resistance to the respective classes (*3*,*5*,*6*,*14*,*15*), but little is known about the current rates of cross-resistance between azoles and echinocandins in patients with cancer or about additional clinical factors that could be associated with resistance. In a contemporary cohort of cancer patients with *C. glabrata* fungemia, we determined rates of in vitro resistance and cross-resistance to azoles and echinocandins, identified factors associated with resistance, and investigated the association between antifungal resistance and all-cause mortality rates.

Patients and Methods

We included patients seen at MD Anderson Cancer Center from March 2005 through September 2013, for whom ≥1 blood culture was positive for *C. glabrata* and who had symptoms, signs, or laboratory findings consistent with infection. We retrospectively reviewed electronic medical records for demographic, clinical, and laboratory data for the day of candidemia (defined as day of blood collection for culture), and we reviewed pharmacy records and clinical notes for previous use of antifungal drugs and cumulative doses. The study was approved by the MD Anderson Cancer Center institutional review board.

Isolation and identification of *C. glabrata* isolates in blood culture were performed by using standard microbiological procedures (*2*–*4*). We determined MICs of fluconazole, voriconazole, posaconazole, caspofungin, and amphotericin B by using the broth microdilution method described in CLSI M27-A documents (*10*,*16*), according to prominent (50%) reduction in turbidity and 100% growth inhibition for amphotericin B. In a subset

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of caspofungin-resistant isolates, we also tested in vitro susceptibility to micafungin and anidulafungin.

Susceptibility to antifungal drugs was defined according to clinical break points for *C. glabrata* (*10*,*11*); isolates for which fluconazole MIC was ≤32 mg/L were considered dose dependent, whereas those for which MIC was ≥64 mg/L were considered resistant. Because clinical break points for voriconazole and posaconazole are undefined, *C. glabrata* isolates in which MIC was >1 dilution above the epidemiologic cutoff values (0.5 and 2.0 mg/L, respectively) were considered potentially resistant. Caspofungin/anidulafungin and micafungin resistance was defined as MICs ≥0.5 mg/L and ≥0.25 mg/L, respectively (*10*,*11*). Strains for which caspofungin or anidulafungin MIC was 0.25 mg/L or micafungin MIC was 0.125 mg/L were classified as intermediate. Resistance to amphotericin B was defined as MIC ≥2 mg/L (*10*,*11*).

Continuous variables were compared by using the Student *t*-test or the Mann-Whitney U criterion for variables that were not normally distributed. Categorical variables were compared by using the χ^2 test, Fisher exact test, and linear-by-linear associations for trend. Binary and ordinal (after testing the parallel lines assumption) logistic regression analyses were used to identify variables independently associated with fluconazole, caspofungin, and multidrug resistance. Survival curves were compared by using the log-rank test and Cox regression analysis. The proportional hazards assumption was tested graphically and by building time-dependent variables. For univariate analyses, clinically relevant parameters ($p<0.1$) were included at model entry. Variables were retained in the final model if $p<0.05$; p values >0.05 but <0.1 were noted as indicating trends. All analyses were performed by using SPSS, version 21 (IBM Corporation, Chicago, IL, USA).

Results

Patient Population

We studied 146 candidemia episodes (first positive blood culture per hospitalization) in 144 patients (Table 1).

Table 1. Basic demographic, clinical, and laboratory characteristics for 144 cancer patients with 146 episodes of *Candida glabrata* fungemia, MD Anderson Cancer Center, Houston, Texas, USA, March 2005–September 2013*

Characteristic	No. (%)
Host	
Age, y, mean (± SD), range	55.5 (± 14.52), 12–85
Male sex	74 (51.38)
Solid tumor†	98 (68.05)
Hematologic malignancy	46 (31.95)
Leukemia	22 (15.3)
Acute myeloid leukemia	17 (11.81)
Acute lymphoblastic leukemia	5 (3.47)
Lymphoma	14 (9.72)
Multiple myeloma	4 (2.77)
Myelodysplastic syndrome	2 (1.38)
Myelohyperplastic syndrome	4 (2.77)
Hematopoietic stem cell transplantation	16 (11.11)
Clinical disease	
Intensive care unit stay	59 (40.41)
Mechanical ventilation	27 (18.49)
Presence of a central line	131 (89.72)
Total parenteral nutrition	36 (24.65)
Recent (within 1 mo before the day of candidemia) drug exposures	
Chemotherapy	69 (47.26)
Any corticosteroids	85 (58.21)
Antibacterial drugs	144 (98.63)
Azoles	44 (30.13)
Echinocandins	32 (21.91)
Laboratory findings	
Neutropenia, cells/μL	
<500	28 (19.17)
100–500	9 (6.16)
<100	19 (13.14)
Lymphopenia, cells/μL	
<500	86 (58.9)
<100	30 (20.54)
Monocytopenia, <100 cells/μL	39 (26.71)

*All parameters were present on the day of candidemia, defined as the day of blood culture collection. Data are presented as absolute numbers (%) unless otherwise indicated for normally distributed variables or median numbers (25th–75th percentile) for variables that were not normally distributed.
†Tumor types were as follows: 47 (32.63%) gastrointestinal, 12 (8.33%) gynecologic, 9 (6.25%) genitourinary, 6 (4.16%) breast, 6 (4.16%) lung, 3 (2.08%) thyroid, 4 (2.77%) sarcomas, 3 (2.08%) head and neck, 2 (1.38%) central nervous system, and 6 (4.16%) other.

A second episode occurred for 2 patients, >2 months after the first episode. Most (68%) patients had solid tumors, whereas during 1999–2003, of 150 *C. glabrata* bloodstream isolates (*3*), 64 (42.6%) were from patients with solid tumors (p<0.001).

Azole Resistance

Of the 146 isolates, 30 (20.5%) were resistant to fluconazole. For those 30, the voriconazole MIC was ≥1 mg/L (epidemiologic break point [EB] 0.5 mg/L) for 28 (93.3%) isolates, and the posaconazole MIC was ≥4 for 26 (86.6%) isolates and ≥2 mg/L (EB) for 29 (96.6%) isolates. For 1 isolate that was resistant to fluconazole (MIC 128 mg/L), MICs for voriconazole and posaconazole were both below the EB (0.25 and 0.5 mg/L, respectively). Therefore, 29 (96.6%) of the 30 fluconazole-resistant isolates could be characterized as multiazole resistant. A total of 20 (66.7%) fluconazole-resistant strains were isolated from patients with hematologic malignancies, and 10 (33.3%) were isolated from patients with solid tumors.

Factors significantly associated with fluconazole resistance are summarized in Table 2. The observed association of azole exposure with fluconazole resistance resulted mostly from recent administration of voriconazole; 14 (46.6%) of 30 patients from whom fluconazole-resistant isolates were obtained had received voriconazole within 1 month before the day of candidemia, as opposed to 10 (8.6%) of 116 from whom dose-dependent isolates were obtained (p<0.001). In comparison, 6 (20%) of the 30 patients from whom fluconazole-resistant isolates were obtained had received fluconazole within 1 month, as opposed to 19 (16%) of the 116 from whom fluconazole dose-dependent isolates were obtained (p = 0.639). Of the 30 patients from whom fluconazole-resistant isolates were obtained, 2 (6.6%) had received posaconazole within 1 month, as opposed to 1 (0.9%) of 116 from whom fluconazole dose–dependent isolates were obtained (p = 0.107). Factors independently associated with fluconazole resistance were recent azole exposure, hematologic malignancy, and mechanical ventilation (Table 2).

Echinocandin Resistance

Of the 146 isolates, 24 (16.4%) were intermediate and 15 (10.3%) were resistant to caspofungin. On the basis of the 2008 break point of ≤2 mg/L, 11 (73.3%) of the 15 resistant isolates and 35 (90%) of the 39 intermediate or resistant isolates would have been considered susceptible (*16*). Of 11 caspofungin-resistant isolates that were available for repeat testing, 10 were also resistant to micafungin or anidulafungin. One caspofungin-resistant isolate was intermediate to micafungin and susceptible to anidulafungin (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/20/11/14-0685-Techapp1.pdf). Factors independently associated with caspofungin resistance were recent echinocandin exposure, total parenteral nutrition (TPN), and monocytopenia (absolute monocyte count <100 cells/mL, Table 3) or severe lymphopenia (absolute lymphocyte count <100 cells/mL [online Technical Appendix Table 2]).

Multidrug Resistance

Caspofungin resistance (MIC ≥0.5 mg/mL) was independently associated with fluconazole resistance (Tables 2,3). Among 44 isolates with recent (within 1 month) azole exposure, fluconazole resistance was found for approximately one-third (13 [35.1%] of the 37) that were caspofungin intermediate or susceptible and for all 7 (100%) that were caspofungin resistant (p = 0.002). Among 102 isolates without recent azole exposure, fluconazole resistance was found for 8 (8.5%) of 94 that were caspofungin intermediate or susceptible and for 2 (25%) of 8 that were caspofungin resistant (p = 0.17).

Fluconazole resistance was also independently associated with caspofungin resistance. Among 32 isolates with recent echinocandin exposure, caspofungin resistance was found for 3 (17.6%) of 17 fluconazole dose–dependent

Table 2. Factors present at the time of candidemia and associated with fluconazole resistance, in cancer patients with *Candida glabrata* fungemia, MD Anderson Cancer Center, Houston, Texas, USA, March 2005–September 2013*

	No. (%) cases			Multivariate analysis	
Factor	Dose-dependent, n = 116	Resistant, n = 30	p value	Odds ratio (95% CI)	p value
Hematologic malignancy	27 (23.27)	20 (66.66)	<0.001	3.63 (1.18–11.17)	0.024
Leukemia	12 (10.34)	10 (33.33)	<0.001		
HSCT	6 (5.17)	10 (33.33)	<0.001		
Monocytopenia, <100 cells/μL	26 (22.41)	13 (43.33)	0.021		
Any corticosteroids†	60 (51.72)	25 (83.33)	0.002		
Intensive care unit stay	42 (36.21)	17 (56.66)	0.042		
Mechanical ventilation	17 (14.66)	10 (33.33)	0.019	3.96 (1.16–13.51)	0.028
Presence of a central line	101 (87.06)	30 (100)	0.047		
Azole exposure†	24 (20.68)	20 (66.66)	0.001	5.09 (1.66–15.64)	0.004
Echinocandin exposure†	17 (14.65)	15 (50)	<0.001		
Echinocandin resistance	6 (5.17)	9 (30)	<0.001	5.23 (1.31–20.78)	0.019

*Blank cells indicate that the respective variables did not contribute significantly and were not retained in the final multivariate model (p>0.1). HSCT, hematopoietic stem cell transplantation.
†Within 1 month before the day of candidemia (day of blood collection for culture).

Table 3. Factors present at the time of candidemia and associated with caspofungin resistance in cancer patients with *Candida glabrata* fungemia, MD Anderson Cancer Center, Houston, Texas, USA, March 2005–September 2013*

	No. (%) patients				Multivariate analysis	
Factor	Susceptible, n = 107	Intermediate, n = 24	Resistant, n = 15	p value	Odds ratio (95% CI)	p value
Hematologic malignancy	27 (25.23)	12 (50.00)	8 (53.33)	0.012		
Leukemia	12 (11.21)	4 (16.66)	6 (40.00)	0.012		
Hematopoetic stem cell transplantation	6 (5.610)	5 (20.830)	5 (33.330)	0.013		
Neutropenia, <500 cells/μL	16 (14.95)	6 (25.00)	6 (40.00)	0.016		
Lymphopenia, <500 cells/μL	59 (55.14)	15 (62.50)	12 (80.00)	0.069		
Monocytopenia, <100 cells/μL	20 (18.69)	10 (41.66)	9 (60.00)	<0.001	3.53 (1.44–8.65)	0.006
Mechanical ventilation	17 (15.89)	3 (12.50)	7 (46.66)	0.024		
Any corticosteroids†	56 (52.33)	17 (70.83)	12 (80.00)	0.004		
Total parenteral nutrition	22 (20.56)	5 (20.83)	9 (60.00)	0.005	3.37 (1.37–8.24)	0.008
Echinocandin exposure†	15 (14.02)	6 (25.00)	11 (73.33)	<0.001	2.75 (1.09–6.95)	0.032
Fluconazole resistance	15 (14.02)	6 (25.00)	9 (60.00)	<0.001	3.16 (1.13–7.88)	0.013

isolates and for 8 (53.3%) of 15 fluconazole-resistant isolates (p = 0.034). Among 114 isolates without recent echinocandin exposure, caspofungin resistance was found for 3 (3%) of 99 fluconazole dose-dependent isolates and for 1 (6.7%) of 15 fluconazole-resistant isolates (p = 0.516).

A total of 10 (6.8%) isolates exhibited multidrug resistance (*9*); 2 exhibited in vitro resistance to amphotericin B, 9 exhibited resistance to caspofungin and fluconazole, and 1 was resistant to caspofungin and amphotericin B. Multidrug resistance was found for 30% of fluconazole-resistant strains and 66.6% of caspofungin-resistant strains. All 7 multidrug-resistant isolates that were available for testing were also resistant to micafungin and/or anidulafungin. We did not observe any significant increase in the rates of fluconazole, echinocandin, or multidrug resistance over the 8-year study.

In a separate analysis comparing multidrug-resistant isolates with other isolates, recent (within 1 month before the day of candidemia) echinocandin exposure and TPN were independently associated with multidrug resistance. Values for recent echinocandin exposure were adjusted odds ratio (aOR) 39.9, 95% CI 4.61–345.73, p = 0.001 when compared with all other isolates and aOR 57.22, 95% CI 6.32–517.93, p<0.001 when compared with fluconazole-intermediate and caspofungin-susceptible isolates. Values for TPN were aOR 7.32, 95% CI 1.5–32.83, p = 0.014 when compared with all other isolates and aOR 4.58, 95% CI 0.8–26.26, p = 0.088 when compared with fluconazole-intermediate and caspofungin-susceptible isolates.

In additional analyses, we entered antifungal exposure within 1 year instead of 1 month as an independent variable, and we entered cumulative doses of drug within 1 month or 1 year before the date of candidemia either as continuous or categorical (above vs. below the mean for all patients or those with prior antifungal exposure) independent variables. All associations remained significant, and no increase in predictive value was found for any model.

Resistance without Prior Exposure to Antifungal Drugs

A total of 11 *C. glabrata* isolates with no documented exposure to the respective classes of antifungal drugs were classified as resistant. A total of 8 isolates with no documented azole exposure were resistant to fluconazole. One of those 8, and 3 additional isolates, were classified as caspofungin resistant, without any documented exposure to echinocandins. Of those 4, the caspofungin MIC was 0.5 mg/L for 3, all of which were fluconazole dose-dependent, and 8 mg/L for 1, which was multidrug resistant. Of those 4 strains, 3 were available for testing of susceptibility to other echinocandins (the multidrug-resistant isolate was not available); 2 were resistant to either micafungin or anidulafungin, and 1 was intermediate to micafungin and anidulafungin. Classification of that 1 isolate as intermediate did not change the results.

All-Cause Mortality Rates

The 28-day all-cause mortality rate was 39.7% (58/146) among all patients, and 32.3% (30/93) among those who received echinocandins. There was no association between death (log-rank p>0.2) and age (≥65 vs. <65 years), type of malignancy (solid vs. hematologic), or TPN. Among all patients (online Technical Appendix Figure) and among the 93 who received echinocandins, caspofungin MIC was inversely associated with 28-day survival rate. Specifically, among patients who received echinocandins, the 28-day crude mortality rates were 25.4% (17/67), 41.7% (5/12), 50% (5/10), and 75% (3/4) for those with isolates with echinocandin MICs of ≤0.125, 0.25, 0.5, and >2 mg/L (the 2008 CLSI break point) (*16*), respectively (log-rank p = 0.001 for linear trend, Figure).

Among patients who received echinocandins, the association between caspofungin MIC and all-cause mortality rates remained significant (adjusted hazards ratio [aHR] for MIC ≥0.5 mg/L = 2.59, 95% CI 1.08–6.19, p = 0.033) after adjustment for intensive care unit stay (aHR = 3.8, 95% CI 1.71–8.45, p = 0.001) and monocytopenia (aHR = 4.02, 95% CI 1.89–8.55, p<0.001). Those associations remained significant after reclassification of 1 isolate as intermediate;

that isolate was resistant to caspofungin, intermediate to micafungin, and anidulafungin (online Technical Appendix Table 1).

Discussion

In this contemporary series of cancer patients with *C. glabrata* fungemia, the rates of in vitro caspofungin resistance and multidrug resistance are among the highest reported to date. By comparing the updated (*10*) with the previous, non–species-specific CLSI definitions of in vitro susceptibility (*16*), we found that 90% of caspofungin-intermediate or -resistant *C. glabrata* bloodstream isolates would have been previously classified as susceptible. Caspofungin resistance was associated with previous exposure to echinocandins, use of TPN, and all-cause mortality rate.

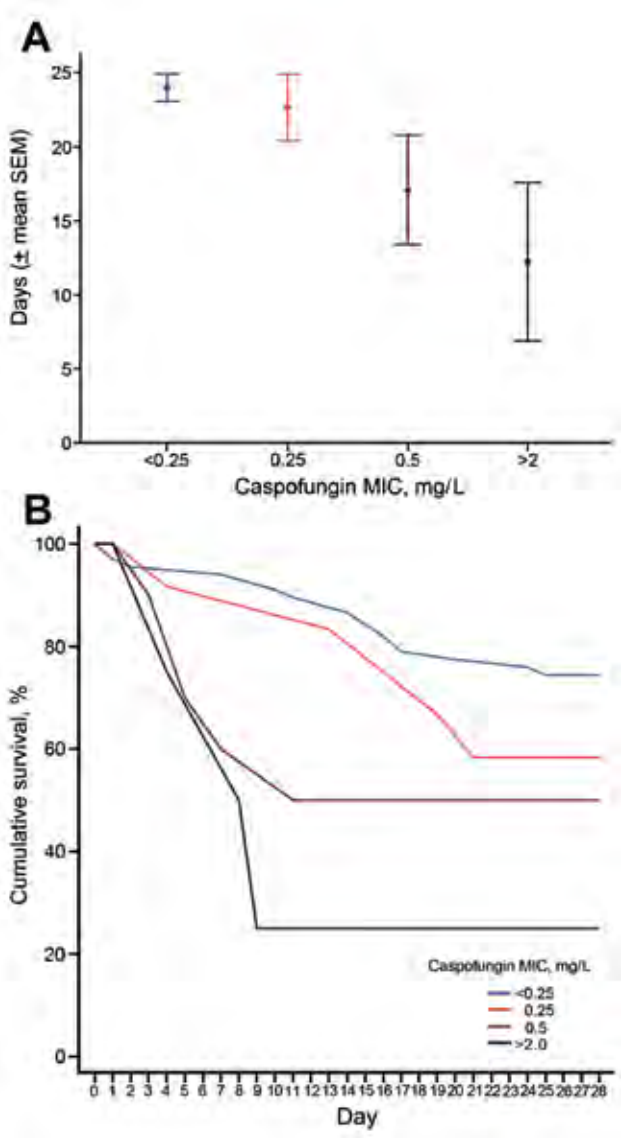

Figure. A) Mean 28-day survival (days, mean ± SE) and B) Kaplan-Meier survival curves, relative to caspofungin MIC and susceptibility in *Candida glabrata* isolates, according to the updated definitions (susceptible: MIC<0.25 mg/L, intermediate: MIC = 0.25 mg/L, resistant: MIC ≥0.5 mg/L) and previous definitions (susceptible: MIC ≤2 mg/L, nonsusceptible: MIC >2 mg/L) among 93 patients who received an echinocandin, MD Anderson Cancer Center, Houston, Texas, USA, March 2005–September 2013; log-rank p = 0.001 for linear trend.

Contrary to previous findings from our institution (*3*), most patients with *C. glabrata* fungemia in the series reported here had solid tumors rather than hematologic malignancies. One third of fluconazole-resistant isolates and half of those with decreased susceptibility to caspofungin were isolated from patients with solid malignancies. These results probably reflect an overall increase in solid tumors; however, our findings also confirm that *C. glabrata* bloodstream infections have become major clinical problems among all patients at risk for candidemia (*6*,*9*,*14*,*15*,*17*).

In agreement with previously reported findings, our study indicated that broad use of azoles—mainly voriconazole—and echinocandins was strongly associated with *C. glabrata* fluconazole and caspofungin resistance (*3*,*5*,*6*,*14*,*15*). In our study, 11 *C. glabrata* isolates were classified as resistant without having had any previous documented exposure to the respective classes of antifungal drugs. This finding is in agreement with a recent report of isolation of 4 *C. glabrata* FKS mutants from patients who had not received echinocandins (*17*). Because several factors place cancer patients at risk for candidemia and clinical failure of antifungal drugs (*1*–*5*), we sought to identify those clinical factors associated with in vitro resistance. On the basis of our results, we consider it likely that poor host defense mechanisms associated with the presence of hematologic malignancy, myelosuppression, and critical illness are independently associated with resistance.

We also observed an independent association between TPN and caspofungin resistance or multidrug resistance. TPN is an established risk factor for candidemia and a marker of intestinal dysfunction (*18*). Moreover, TPN causes atrophy of the intestinal mucosa, facilitating microperforations and *Candida* translocation, and it is associated with thick biofilm formation and catheter-related infections (*18*,*19*). Whether our observed association between TPN and caspofungin resistance is reflective of critical illness or whether the above mechanisms also promote the development of resistance remains to be determined.

In our study, almost one third of fluconazole-resistant strains and two-thirds of caspofungin-resistant strains were multidrug resistant. These rates of cross-resistance are significantly higher than those previously reported from multi-institutional registries (*20*,*21*) and another tertiary academic hospital (*6*). Specifically, investigators from Duke University Hospital reported a 25% rate of fluconazole resistance over a 10-year period, which is similar

to our rate of 21%. In the same report (*6*), the overall rate of resistance to at least 1 echinocandin was lower (6.7%) than that found in our study (10.7%), although by 2010 it had increased to 12.7% (*6*). In another study, 11% of *C. glabrata* bloodstream isolates were resistant to caspofungin and 18% had FKS mutations (*17*). Notably, the rates of multidrug resistance determined by the study from Duke (3.5%) (*6*), the Centers for Disease Control and Prevention SENTRY Antimicrobial Surveillance Program (1%) (*20*), and another recent multi-institutional study (1%) (*21*) were substantially lower than the rates of multidrug resistance determined in our study (6.8%). These data document a worrisome trend for concomitant resistance of *C. glabrata* clinical isolates to azoles and echinocandins, which seems to be more prominent in our population of patients with cancer.

In our study, resistance to fluconazole was highly associated with caspofungin resistance, independent of prior use of antifungal drugs; this finding is in agreement with our institution's previously reported findings for different *Candida* species (*22*). Echinocandin, but not azole, exposure was a significant independent predictor of multidrug resistance. These findings could reflect a worrisome potential for development of multidrug resistance in *C. glabrata*, a versatile, haploid species (*7*). In a recent study, serial exposures of a *C. glabrata* laboratory strain to low-dose micafungin led to the development of a single-point mutation conferring multiazole and echinocandin resistance with preserved virulence (*23*). Moreover, in an analysis of molecular events leading to echinocandin resistance of *C. glabrata* isogenic isolates consecutively obtained from a patient receiving chronic TPN, a multidrug-resistant strain emerged after multiple courses of treatment with caspofungin but no previous azole exposure (*8*). Selective pressure from antifungal drugs, along with other factors, such as chemotherapy (*24*) and broad-spectrum antibacterial drugs (*25*), might lead to the expansion of similar phenotypes.

By applying the updated clinical break points to our patient population, we captured a strong and potentially independent correlation of all-cause mortality rates with in vitro caspofungin MICs but not with other factors classically associated with poor outcomes such as advanced age and hematologic malignancy (*2*,*4*,*5*). Although other residual confounders cannot be ruled out, this finding is in agreement with previously reported significant associations between clinical failure of echinocandins and elevated in vitro echinocandin MICs (*6*,*8*,*14*,*17*). In some animal studies, FKS mutations leading to echinocandin resistance were associated with decreased fitness (*8*,*26*). Nevertheless, a recent study that used an immunocompromised murine model of systemic candidiasis showed that caspofungin was ineffective against *C. glabrata* isolates with MIC ≥1 mg/L (*27*). Furthermore, investigators have also described the development of compensatory mechanisms that override the decreased virulence resulting from clinical exposure of an FKS mutant *C. glabrata* isolate to an echinocandin (*8*). Clinical (*8*,*28*) and laboratory (*23*) strains that exhibit high-level antifungal resistance without decreases in fitness have been described. What remains incompletely characterized are the spectrum of mutations predisposing to azole and/or echinocandin resistance, the role of epigenetic mechanisms, and the virulence of resistant (compared with susceptible) *Candida* strains in humans. According to our results, lowering the MIC break point for caspofungin resistance in *C. glabrata* bloodstream isolates to 0.5 mg/L is clinically relevant.

Our study has several limitations. It was a retrospective study performed at a single institution, and our patient population was rather small and selected. Therefore, our observations might not be applicable to different patient groups at risk for serious *Candida* infections. The number of caspofungin-resistant isolates was small, and we used in vitro caspofungin MIC alone to define echinocandin resistance, without molecular confirmation of underlying mutations. The interlaboratory variability in caspofungin MICs is substantial, (*29*,*30*), and there is evidence that micafungin and anidulafungin MICs correlate better with the presence of FKS mutations and clinical outcomes (*15*). However, testing the micafungin and anidulafungin MICs of available caspofungin-resistant isolates did not change our conclusions. Moreover, our most striking finding was the high percentage of multidrug-resistant *C. glabrata* isolates. In a previous study (*20*), 100% of such multidrug-resistant isolates had an FKS mutation; in the study reported here, all multidrug-resistant isolates that were available for testing were resistant to 2 echinocandins. Therefore, we believe that the substantial number of multidrug-resistant strains harbored molecular mechanisms of resistance. It should be noted that the reference for assessing sensitivity and specificity of in vitro MICs has been the presence of mutations within the FKS1 and FKS2 hot spot regions. Nevertheless, there is emerging evidence that non–FKS-related mechanisms might be operative or might predispose to the development of echinocandin resistance and even multidrug resistance (*8*,*23*). Recently, a high in vitro caspofungin MIC (≥0.5 mg/L) was shown (*17*) to have a higher positive predictive value for echinocandin failure than the presence of FKS hot spot mutations, in agreement with our findings and contrary to previously reported findings (*6*,*31*).

In summary, the rate of in vitro caspofungin and multidrug resistance of *C. glabrata* bloodstream isolates in our patient population is, to our knowledge, among the highest reported. Our findings might indicate a worrisome propensity of *C. glabrata* strains for multidrug resistance in cancer patients and should prompt awareness of the need for good

stewardship of antifungal drugs. Prospective, large-scale clinical registries, with molecular data on mutations that confer resistance to antifungal drugs, are needed.

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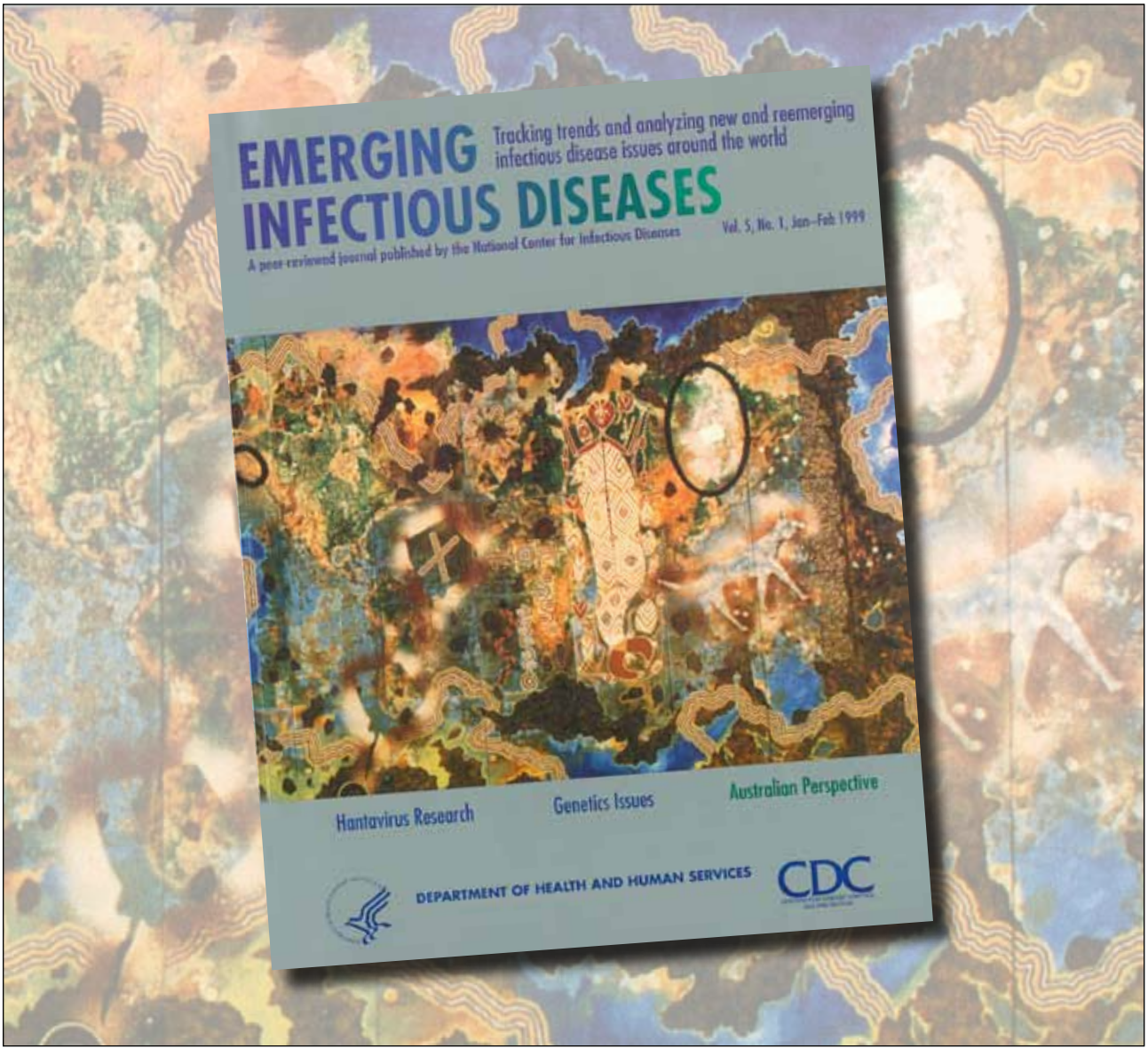

Novel *Chlamydia trachomatis* Strains in Heterosexual Sex Partners, Indianapolis, Indiana, USA

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Chlamydia trachomatis causes a high number of sexually transmitted infections worldwide, but reproducible and precise strain typing to link partners is lacking. We evaluated multilocus sequence typing (MLST) for this purpose by detecting sequence types (STs) concordant for the *ompA* genotype, a single-locus typing standard. We tested samples collected during April 2000–October 2003 from members of established heterosexual partnerships (dyads) in the Indianapolis, Indiana, USA, area who self-reported being coital partners within the previous 30 days. *C. trachomatis* DNA from 28 dyads was tested by MLST; sequences were aligned and analyzed for ST and phylogenetic relationships. MLST detected 9 *C. trachomatis* STs, 4 unique to Indianapolis; STs were identical within each dyad. Thirteen unique strains were identified; 9 (32%) dyads harbored novel recombinant strains that phylogenetically clustered with strains comprising the recombinants. The high rate of novel *C. trachomatis* recombinants identified supports the use of MLST for transmission and strain diversity studies among at-risk populations.

Chlamydia trachomatis, a bacterium that can infect both men and women, is most commonly sexually transmitted. In 2008, approximately 105.7 million new *C. trachomatis* sexually transmitted infections (STIs) occurred worldwide (*1*); an estimated 2.86 million incident cases occurred in the United States (*2*). The last surveillance study of STIs in the United States, in 2011, reported 1,412,791 chlamydial infections, the largest case number for any disease ever reported to the Centers for Disease Control and Prevention (*3*).

C. trachomatis infections in men and women are mostly asymptomatic; thus, continued sexual activity among persons unaware of their infection status facilitates further transmission. Gaps in knowledge of chlamydial STIs include how measures of immunity, bacterial load, condom use, and other factors relate to transmission risk. Longitudinal studies of these factors are needed to inform treatment and prevention strategies (*4*). The tools required include careful ascertainment of sexual history and behavioral determinants and reproducible and discriminating biomarkers to strengthen the case for transmission between sex partners linked by partner tracing.

The standard biomarker for these studies is *ompA* genotyping, but this method lacks precision because the gene is under immune selection and represents only 0.1% of the genome. Because large-scale whole-genome sequencing of clinical samples is not yet feasible, multilocus sequence typing (MLST) for *C. trachomatis* has been developed to provide greater insight into strain types; 3 such MLST methods have been reported in the literature (*5*–*10*). The scheme we developed, on the basis of analysis of 19 reference strains and 68 geographically diverse clinical isolates, identified 44 MLST sequence types (STs), compared with only 20 *ompA* genotypes (*11*). In our scheme, we were also able to discriminate single-nucleotide polymorphisms (SNPs) that correlate with disease phenotypes attributable to *C. trachomatis*: lymphogranuloma venereum (LGV), trachoma, and non-LGV urogenital diseases (*11*). Our scheme has since been expanded to encompass 192 geographically and clinically diverse samples.

For this study, we applied our MLST scheme to a subset of a well-defined heterosexual partnership (dyad) cohort in Indianapolis, Indiana, USA, comprising 28 dyads for which concordance of the *ompA* genotype existed between partners. The purpose of the study was to determine whether MLST, which provides a more detailed level of strain typing than *ompA* genotyping, would also show strain concordance between partners, as would be expected if transmission had occurred within the dyads. In addition, we sought to identify additional *C. trachomatis* strain types,

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beyond those identified by *ompA* genotyping, that might be unique to Indianapolis, because this geographic region has not previously been included in any MLST database.

Materials and Methods

Study Population

A study of *C. trachomatis* concordance in heterosexual partnerships (dyads) was conducted in Indianapolis during April 2000–October 2003; participants were sexually active heterosexual men and women 15–25 years of age who visited an urban STI clinic (*12*). Written informed consent was obtained, and the study was approved by the Indiana University–Purdue University Institutional Review Board. Eligibility was defined as self-reported sexual activity between the partners during the previous 30 days. A total of 210 heterosexual dyads were established by research disease intervention specialists and enrolled. *C. trachomatis* infection was identified by Amplicor CT/NG (Roche Diagnostics, Indianapolis, IN, USA) nucleic acid amplification test and cell culture, as previously described (*12*). Of the 210 dyads, 130 contained ≥1 *C. trachomatis*–infected partner; for 45 dyads, both partners were infected and had identical *ompA* genotypes.

For the MLST study, we used remainder samples from 56 members of 28 dyads who were concordant for *C. trachomatis* infection and *ompA* genotype. These samples were provided to investigators at Children's Hospital Oakland Research Institute (CHORI) in a de-identified and blinded fashion. Thus, CHORI research was considered not to involve human subjects, and informed consent was not required.

Reference and Clinical Samples

We used 56 samples (from cervix in women and urethra in men) from 28 dyads in which persons within each dyad were concordant for *C. trachomatis* infection and *ompA* genotype. Additionally, we used for analysis MLST data for 20 *C. trachomatis* reference strains (A/Sa1, A/HAR13, B/TW5/OT, Ba/Apache2, C/TW3/OT, D/UW3/Cx, Da/TW448, E/Bour, F/ICCal3, G/UW57/Cx, H/UW4/Cx, I/UW12/Ur, Ia/UW202, J/UW36/Cx, Ja/UW92, K/UW36/Cx, L_1/440, L_2/434, L_2a/UW396, L_3/404) and 172 clinical samples in the MLST database (http://www.mlst.net).

ompA Genotyping and MLST Analyses

ompA genotyping of the samples had been previously performed at Indiana University (*13*,*14*) as part of the earlier *C. trachomatis* concordance study. Cultured and noncultured clinical samples were sent to CHORI for analysis (*11*). DNA was extracted, and MLST for 7 housekeeping genes was performed by using primers as described (*11*; http://www.mlst.net; online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/20/11/14-0604-Techapp1.pdf). A consensus sequence was created from forward and reverse sequences, and the genes were concatenated and queried against all 202 MLST sequences in the database (*15*). Sequence output was used to identify each unique allelic profile to assign an ST, and all STs were deposited in the *C. trachomatis* database (http://chlamydia.mlst.net). The concatenated sequences of the 7 MLST loci and the allelic profiles for each sample were used to identify sample relatedness.

ompA genotypes were defined on the basis of homology with reference strains of *C. trachomatis*. If ≥1 SNP was identified when sequences were compared to those of the closest hit reference strain, a number was used to denote the presence of the SNP(s) (e.g., Ia4) (*15*).

Strain Clustering and SNP Analyses

Strain clustering and SNP analyses were performed as described (*11*). Briefly, clusters of related and singleton STs as well as evolutionary patterns among the isolates and for the entire dataset were determined by using eBURST (http://eburst.mlst.net). Neighbor-joining and minimum evolution methods in MEGA4 (http://www.megasoftware.net) were used to construct the trees along with multiple substitution models, including p-distance and Jukes-Cantor; all methods gave similar results. To test support for each node in the tree, we performed 1,000 bootstrap replicates.

All SNPs were identified for each ST by using the PROC FREQ tool in SAS software (SAS Institute, Cary, NC, USA). The probability of association of a SNP with an ST was determined by using a classification index (*16*). Variance across the dataset was determined using the Levene test (*17*). A p value <0.05 was considered significant.

Results

MLST Discrimination of *C. trachomatis*

The Table shows the distribution of MLST STs and *ompA* genotypes for each dyad with SNP location(s), if present, for each of the 7 MLST housekeeping genes. We noted that in some cases, the DNA extracted directly from the patient sample was sufficient for MLST, whereas in other cases, the cultured sample was required because the patient sample did not yield sufficient DNA for MLST. We found no differences in MLST results when we compared DNA directly extracted from the patient sample with DNA extracted from the culture of the same sample.

For each of the 28 dyads, both partners had the same MLST ST. A total of 9 STs were found in the 56 samples from the 28 dyads: ST15, ST19, ST23, ST34, ST39, ST45, ST46, ST47, and ST55. Twenty-one dyads harbored MLST STs that matched STs of samples obtained from geographically diverse areas currently included in the

MLST database, whereas 7 dyads harbored newly identified STs not present in the database (online Technical Appendix Table 2). Among these 7 dyads, 4 MLST STs were unique to Indianapolis: ST45 (dyad 22), ST46 (dyads 23–26), ST47 (dyad 27), and ST55 (dyad 28); these results reflect the SNPs in various alleles (Table).

Table. *Chlamydia trachomatis ompA* genotypes, MLSTs, and SNPs for samples from heterosexual patient pairs (dyads) in Indianapolis, Indiana, USA, April 2000–October 2003*

Dyad no.	Sample nos.	*ompA* genotype	ST	*ompA* genotype(s) associated with ST–closest hit genotype homology (SNPs)†
1	**J/112i** **J/113i**	**J/UW36/Cx**	**15**	**J/UW36/Cx & K/UW36/Cx–K/42nl & K/49nl**
2	K/186i K/187i	K/UW36/Cx	15	J/UW36/Cx & K/UW36/Cx–K/42nl & K/49nl
3	**H/114i** **H/115i**	**H/UW4/Cx**	**19**	**D/UW3/Cx, G/UW57/Cx, H/UW4/Cx, I/UW12/Ur, J/UW36/Cx–G/SotonG1**
4	Ia/94i Ia/95i	Ia/UW202	23	D/UW3/Cx, Ia/UW202–Ia/UW202
5	Ia/118i Ia/119i	Ia/UW202	23	D/UW3/Cx, Ia/UW202–Ia/UW202
6	Ia4/177i Ia4/180i	Ia4	23	D/UW3/Cx, Ia/UW202, Ia4–Ia/UW202
7	Ia/178i Ia/179i	Ia/UW202	23	D/UW3/Cx, Ia/UW202–Ia/UW202
8	Ia/183i Ia/184i	Ia/UW202	23	D/UW3/Cx, Ia/UW202–Ia/UW202
9	**D2/96i** **D2/97i**	**D2**	**34**	**D2, D/UW3/Cx, E/Bour, F/ICCal3, Ja/UW92–F/ICCal3**
10	F/98i F/99i	F/ICCal3	34	D2, D/UW3/Cx, E/Bour, F/ICCal3, Ja/UW92–F/ICCal3
11	F/181i F/182i	F/ICCal3	34	D2, D/UW3/Cx, E/Bour, F/ICCal3, Ja/UW92–F/ICCal3
12	**D2/189i** **D2/190i**	**D2**	**34**	**D2, D/UW3/Cx, E/Bour, F/ICCal3, Ja/UW92–F/ICCal3**
13	F/191i F/192i	F/ICCal3	34	D2, D/UW3/Cx, E/Bour, F/ICCal3, Ja/UW92–F/ICCal3
14	E/88i F/89i	E/Bour	39	E/Bour–E/Bour
15	E/102i E/103i	E/Bour	39	E/Bour–E/Bour
16	E/106i E/107i	E/Bour	39	E/Bour–E/Bour
17	E/116i E/117i	E/Bour	39	E/Bour–E/Bour
18	E6/120i E6/121i	E/Bour	39	E/Bour–E/Bour
19	E/108i E/109i	E/Bour	39	E/Bour–E/Bour
20	E/110i E/111i	E/Bour	39	E/Bour–E/Bour
21	E/171i E/172i	E/Bour	39	E/Bour–E/Bour
22	**D1/90i** **D1/91i**	**D1**	**45**	**D1–F/ICCal3 (*glyA:* 176, 264)**
23	**E/92i** **E/93i**	**E/Bour**	**46**	**E/Bour–Da/TW448 (*leuS:* 58, 96)**
24	**E/104i** **E/105i**	**E/Bour**	**46**	**E/Bour–Da/TW448 (*leuS:* 58, 96)**
25	**E/173i** **E/174i**	**E/Bour**	**46**	**E/Bour–Da/TW448 (*leuS:* 58, 96)**
26	**E/185i** **E/188i**	**E/Bour**	**46**	**E/Bour–Da/TW448 (*leuS:* 58, 96)**
27	E/100i E/101i	E/Bour	47	E/Bour–E/Bour (*hybG:* 257, 289, 451; *pykF:* 317, 384)
28	F4/175i F4/176i	F4	55	F4–F/ICCal3 (*leuS*: 44)

*Boldface indicates putative recombinant strains. MLST, multilocus sequence typing; SNP, single-nucleotide polymorphism; ST, sequence type.
†*ompA* genotypes that are associated with each ST are listed; after the dash, the genotype that is the closest hit for the 7 MLST housekeeping genes is listed. In some cases, the closest hit is a reference strain with SNPs in a gene; the location of the SNP is listed based on the start position of the gene as designated by the genome sequence of D/UW3/Cx (GenBank accession no. AE001273.1).

Eleven *ompA* genotypes were represented in the study and, by study design, were identical within dyads. The *ompA* genotypes included E, F, H, Ia, J, and K sequences that were identical to those of reference strains and 5 variant *ompA* genotypes of D1, D2, E6, F4, and Ia4 that had SNPs compared with the reference strains.

By combining MLST ST and *ompA* genotype data, we identified 13 unique strains (9 by MLST and 4 by *ompA* genotype) among the samples from our study group. Among these 13 strains, 8 were unique to Indianapolis and were found in 12 dyads (dyads 1, 6, 9, 12, 18, 22, 23, 24, 25, 26, 27, and 28) (Table; online Technical Appendix Table 2). Moreover, of the 13 strains identified among the dyad samples, 9 (69%) contained gene sequences that suggested recombination within the genome, meaning that the *ompA* genotype was different from the *ompA* genotype that should be associated with the MLST ST if the genome were just 1 strain. For example, for strains from dyads 10 and 12, the *ompA* was D2, but the sequences of the 7 housekeeping genes (MLST ST34) matched the 7 housekeeping genes of strain F/ICCal3 from the MLST database. Putative recombinants (boldface in Table) represented a rate of 32% (9 of 28 samples).

MLST STs and *ompA* genotypes for each sample in the MLST dataset are shown in online Technical Appendix Table 2. We found substantial variability of *ompA* genotypes associated with MLST STs, as shown previously (*11*). For the STs for the 56 Indianapolis samples, ST15 was associated with *ompA* genotypes J and K; ST34 was associated with *ompA* genotypes D2, E, and F; and ST19 was associated with *ompA* genotypes D, G, H, I, and J. online Technical Appendix Table 3 shows the characteristics of the alleles for each MLST locus based on the inclusion of the Indianapolis dataset in the MLST database.

Phylogeny of STs by Disease Phenotype and Evidence for Recombination

The association of disease phenotype with 3 clonal complexes (CCs) was identified by eBURST (Figure 1), similar to those we reported previously: *C. trachomatis* strains that cause trachoma A, B, Ba, and C (CC-A); noninvasive STIs with low population prevalence (CC-B); and noninvasive, globally prevalent D/Da, E, and F STIs (CC-C). The Indianapolis strains were confined to noninvasive STI CCs (B and C), as expected. The strains associated with 3 of the 4 unique STs in the Indianapolis samples are seen in CC-C (Figure 1).

The minimum-evolution tree also displayed 3 disease clusters (Figure 2); each of the Indianapolis strains is denoted next to the corresponding ST. Cluster I grouped noninvasive, low-prevalence STIs (eBURST CC-B), including a subcluster of strains that cause trachoma (eBURST CC-A). Cluster II grouped only invasive LGV strains. Cluster III grouped noninvasive, prevalent D/Da, E, and F STIs (eBURST CC-C). The tree constructed based on amino acid analysis showed similar clustering (data not shown).

The 9 putative Indianapolis recombinants were localized on the MLST tree with strains of the same *ompA* genotype and were recombinants of strains within the same cluster. Most recombinants were in cluster III. Four dyads with ST46 (unique to Indianapolis) had *ompA* genotype E and homology of the 7 housekeeping genes to reference strain Da/TW448 but with SNPs in *leuS*. D1/90i and D1/91i (ST45) and D2/96i, D2/97i, D2/189i, and D2/190i (ST34) were recombinants with homology to F/ICCal3 with SNPs in *glyA* and F/ICCal3, respectively. In cluster I, J/112i and J/113i shared the same ST as K/186i and K/187i and were recombinants with K/42nl and K/49nl. H/114i and H/115i were recombinants with G/SotonG1.

Discussion

We investigated a well-defined, epidemiologically linked partner cohort of persons with *C. trachomatis* infection in which members of each dyad shared strains with identical *ompA* genotype and found that MLST ST was identical as well. This study confirms the reproducibility of MLST and short-term (≈30 days) stability of MLST in the context of a sexual partnership in which transmission has likely occurred. Whereas the identification of 8 unique *C. trachomatis* strains in Indianapolis was not surprising, given that samples from this city had not previously been subjected to MLST, the rate of 32% (9/28 samples) for recombinants was striking. We only considered 28 dyads in our analyses of strain diversity because of the closely defined epidemiologic link within the partnerships and the fact that strains were identical within dyads.

Because MLST provides ≥3 times the genetic data of *ompA*, the additional discriminatory power of this typing method is not surprising. We found that 8 of the 28 Indianapolis dyads (dyads 1 and 22–28) contained *ompA* genotypes that did not match our previous associations of *ompA* genotype with MLST STs in the MLST database (Table). For example, a J strain by *ompA* genotyping was associated with the MLST ST of a K strain (dyad 1); an *ompA* E strain was associated with the MLST ST of a Da strain (dyads 23–26); an *ompA* D1 strain was associated with the MLST ST of an F strain with SNPs in *glyA* (dyad 22); an *ompA* E strain was associated with the MLST ST of an E strain with 3 SNPs in *hybG* and 2 in *pykF* (dyad 27); and an *ompA* strain F4 matched the MLST of an F strain with an SNP in *leuS* (dyad 28).

ompA genotyping should not be considered a formal part of an MLST scheme because it is under immune selection (*18*), and housekeeping genes provide a stable evolutionary marker for STs. However, *ompA* genotyping remains a useful tool because it has been the mainstay

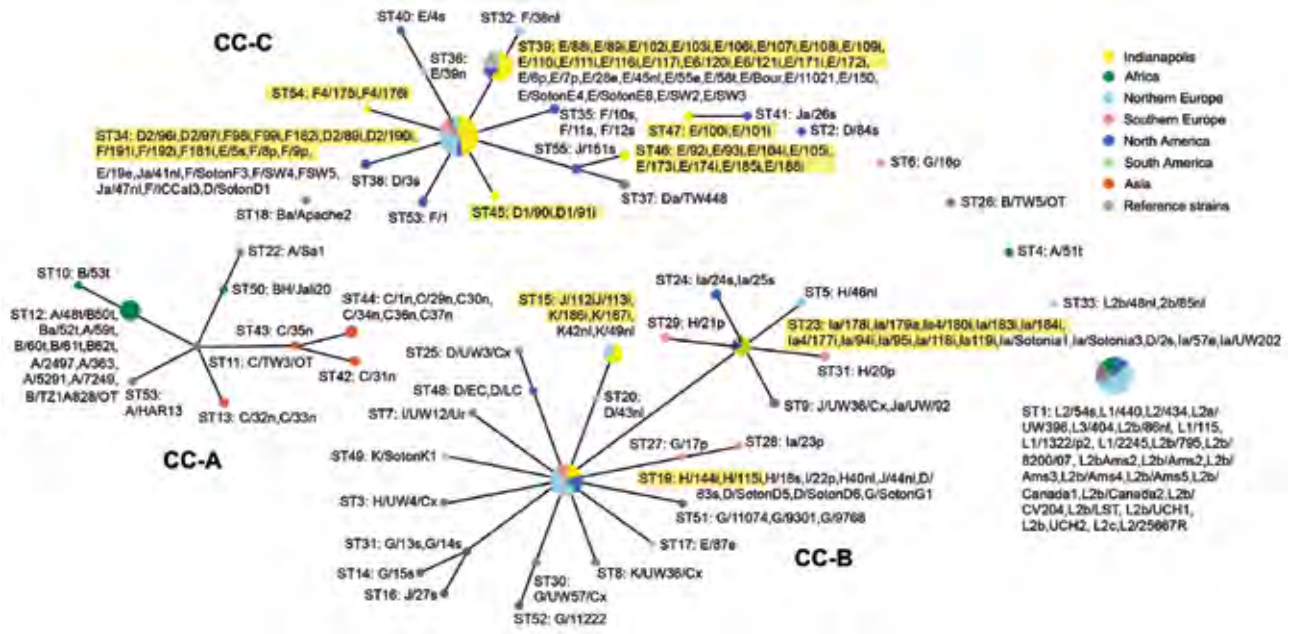

Figure 1. Population snapshot for *Chlamydia trachomatis* samples collected during April 2000–October 2003 from members of heterosexual partnerships (dyads) in Indianapolis, Indiana, USA, compared with reference strains. Data were compiled in eBURST (http://www.mlst.net). Three distinct clonal complexes (CCs) are shown, along with numerous singletons of various sizes and 1 doublet. CC-A, strains causing trachoma; CC-B, noninvasive, nonprevalent urogenital strains; CC-C, noninvasive, globally prevalent urogenital strains. Samples from Indianapolis are highlighted in yellow (shown with sample identification number) and are restricted to clusters I and III. Each circle represents a sequence type (ST) at the point where linked STs within each CC are likely to have descended from the same recent ancestor. The area of the circle denotes the number of samples for that ST. The primary founder of the CC is at the hub; subgroup founders are represented as secondary hubs (e.g., C/35n).

of typing *C. trachomatis* for >20 years and is valuable for comparison with strains typed only by this method. Furthermore, *ompA* genotyping, but not MLST, was able to identify 2 dyads in which partners were infected with strains exhibiting mutations in *ompA* that had not previously been detected: E6 (dyad 18) and Ia4 (dyad 6) (Table). This result indicates utility in continuing *ompA* genotyping as a separate but adjunctive method with MLST for epidemiologic and transmission studies and for establishing strain concordance among members of less well-defined partnerships.

We further identified 3 clonal complexes that correlated with phenotypic disease, similar to previous findings (*11*). Most Indianapolis samples clustered with noninvasive D/Da, E, and F strains in CC-C (Figure 1), a result that is expected, given that these strains are the most prevalent worldwide (*19*–*22*). Whereas the Indianapolis samples were represented in 9 STs, 4 of these STs were distinct for this city, which suggests some clonal expansion of those unique strains in this area.

Genomic characteristics may also drive specific events, such as recombination, that may result in clonal expansion within a relatively small sexual network. Recombinants of the most prevalent urogenital *ompA* genotypes E, F, and D have previously been reported (*23*,*24*). Several reports have also been published regarding recombinants between genotypes D, E, and F and *ompA* genotype J (*11*,*24*,*25*); recombinants of LGV and D strains have also been documented (*6*,*10*,*23*), and previous MLST studies have shown evidence for recombination (*7*,*10*,*11*). In a previous study, we found 9 (17%) of 53 urogenital samples, excluding all ocular samples from patients with trachoma, were recombinant among a geographic distribution that included the western United States, Portugal, the Netherlands, and Ecuador (*11*).

In this study, members of 9 (32%) of the 28 dyads were infected with strains that contained gene sequences suggesting recombination within the genome (Table); this was the case for 2 of 4 STs that were unique to Indianapolis (STs 45 and 46). The 9 putative recombinants were localized on the MLST tree with strains of the same *ompA* genotype and, not surprisingly, were recombinants of strains within the same cluster (Figure 1). Our sample size was small, but the high rate of recombination suggests emerging diversity within a tight sexual network. This hypothesis is supported by historic studies of *ompA* genotypes among patients attending inner city STD clinics; in one such example, Ia genotypes that have much lower prevalence in other parts of the United States predominated among patients in Birmingham, Alabama, and were more prevalent than

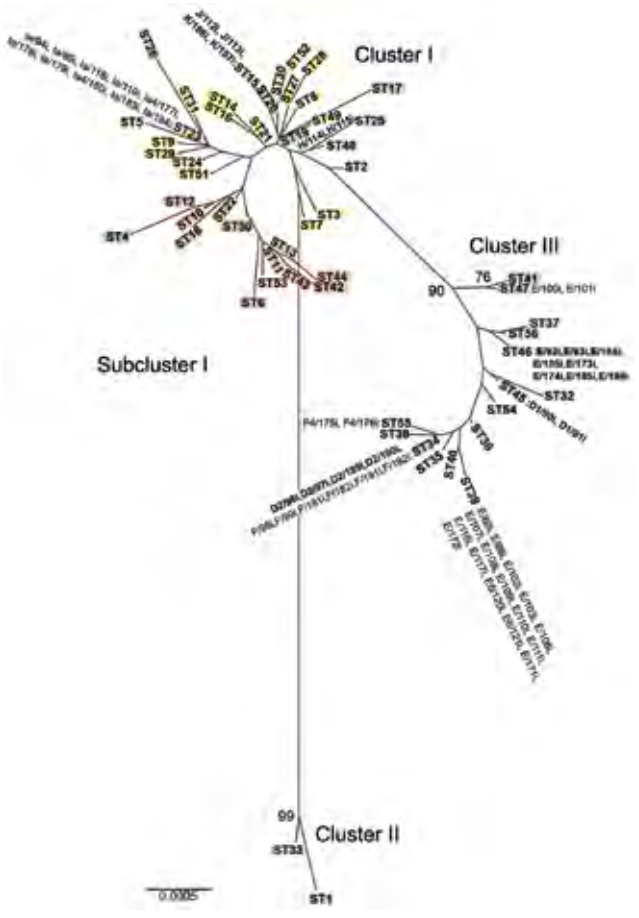

Figure 2. Minimum evolution tree of *Chlamydia trachomatis* samples collected during April 2000–October 2003 from members of heterosexual partnerships (dyads) in Indianapolis, Indiana, USA, compared with reference strains. The tree was constructed by using the 192 concatenated sequences in the MLST database (http://www.mlst.net) for the 7 loci. Bootstrap values (1,000 replicates) >70% are shown. Three clusters and 1 subcluster are shown: cluster I, yellow, noninvasive, nonprevalent sexually transmitted infection (STI) strains; subcluster I, red, trachoma strains; cluster II, purple, invasive lymphogranuloma venereum strains; and cluster III, blue, noninvasive, highly prevalent STI strains. Green denotes putative recombinant stains. Samples from Indianapolis are indicated next to sequence types; those in boldface are putative recombinants. Scale bar indicates number of substitutions per site.

genotype D, which was the third most prevalent genotype for all other cities studied (*26*).

Our study has several strengths. Availability of the concordance study with carefully defined sexual partnerships, application of MLST to confirm the concordance of samples between dyads and to identify unique strains, and use of full-length *ompA* sequences enabled us to identify *ompA* variants and compare and combine the results of the 2 strain typing methods. The weaknesses of our study include the relatively small numbers of sexual partners and that only concordant dyads with epidemiologically linked strains were studied, limiting our conclusions about the overall diversity of strains in Indianapolis, which are likely much larger than what we discovered here.

In summary, our findings validate the discriminatory power of MLST for partnership and transmission studies of *C. trachomatis* infections among at-risk populations locally and globally. Larger partner and population studies that use MLST and *ompA* genotyping will provide valuable data on transmission concordance or discordance that will inform interventions and public health policy to better control *C. trachomatis* transmission. Furthermore, applying these tools globally will expand our knowledge of *C. trachomatis* strain diversity and their emergence among populations at risk for chlamydial STIs.

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Spread of *Streptococcus pneumoniae* Serotype 8-ST63 Multidrug-Resistant Recombinant Clone, Spain

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Since 2004, a total of 131 isolates of *Streptococcus pneumoniae* multidrug-resistant invasive serotype 8 have been detected in Spain. These isolates showed resistance to erythromycin, clindamycin, tetracycline, and ciprofloxacin. All isolates were obtained from adult patients and shared a common genotype (sequence type [ST]63; penicillin-binding protein 1a [*pbp1a*], *pbp2b*, and *pbp2x* gene profiles; *ermB* and *tetM* genes; and a ParC-S79F change). Sixty-eight isolates that required a ciprofloxacin MIC ≥16 μg/mL had additional *gyrA* gene changes. Serotype 8-ST63 *pbp2x* sequences were identical with those of antimicrobial drug–susceptible serotype 8-ST53 isolates. Serotype 8-ST63 *pbp2b* sequences were identical with those of the multidrug-resistant Sweden 15A-ST63 clone. Recombination between the capsular locus and flanking regions of an ST53 isolate (donor) and an ST63 pneumococcus (recipient) generated the novel 15A-ST63 clone. One recombination point was upstream of *pbp2x* and another was within *pbp1a*. A serotype 8-ST63 clone was identified as a cause of invasive disease in Spain.

Streptococcus pneumoniae is a frequent cause of community-acquired pneumonia, meningitis, bacteremia, and otitis media in children. The diverse biochemical composition of the capsular polysaccharide results in ≥94 serotypes (*1*). However, only a few serotypes cause most invasive disease episodes worldwide. Serotype 8 pneumococci cause invasive pneumococcal disease in adults and have been occasionally associated with outbreaks. Nevertheless, isolates of this serotype are rarely found in children as a cause of invasive disease or as colonizers of the nasopharynx (*2*,*3*). Few lineages have been identified among serotype 8 pneumococci; the major clone is Netherlands 8-ST53, which has been detected worldwide and is typically susceptible to antimicrobial drugs (*4*).

The capsular polysaccharide is the major virulence factor of pneumococci and usually determines their ability to act as invasive or colonizing microorganisms (*3*). In addition to exhibiting the capsule, pneumococci can show low or high genetic diversity, but genotype–serotype association is common. However, this association could be disrupted because of capsular switching caused mainly by recombination of capsular genetic loci. This finding could be a sporadic event, but the recombinant occasionally spreads and could cause pneumococcal disease.

Capsular switching was associated with emergence of a serotype 19A variant of a formerly serotype 4 clone (*5*) related to immunity pressure sustained after a 7-valent pneumococcal conjugate vaccine was introduced into the United States. This well-known phenomenon was the origin of the major penicillin-resistant clone (serotype 14 variant of the Spain 9V-ST156 clone), which caused invasive pneumococcal disease in Spain in the 2000s (*4*,*6*).

Data from the Spanish Reference Laboratory for Pneumococci, which has received pneumococci from Spain since 1979, showed rates of 2.5% to 6.5% for serotype 8 invasive isolates in the last 3 decades. These rates did not show any association with introduction of therapeutic or preventive measures (*7*,*8*). Over these decades, serotype 8 pneumococci were usually susceptible to antimicrobial drugs, although some isolates were resistant to erythromycin or tetracycline. Moreover, serotype 8 pneumococci were isolated mainly from adult patients (*7*,*8*). However, since 2004, serotype 8 pneumococci have been identified that showed resistance to erythromycin, clindamycin, tetracycline, and ciprofloxacin.

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In the present study, we analyzed the evolution and molecular epidemiology of these multidrug-resistant serotype 8 pneumococci. We determined whether the increase in these isolates was associated with dissemination of a new recombinant clone (serotype 8-ST63) capable of causing invasive pneumococcal disease in different areas of Spain.

Materials and Methods

Bacterial Strains

Isolates received at the Spanish Reference Laboratory for Pneumococci were serotyped by using the quellung reaction and antisera provided by the Staten Serum Institute (Copenhagen, Denmark) (*8*). During January 2004–December 2012, this laboratory received 22,228 invasive pneumococcal isolates (4,274 from children <15 years of age, 16,506 from persons ≥15 years of age, and 448 from persons for whom age data were not available). Of these isolates, 767 were serotype 8 (3.2%). The proportion of serotype 8 was 4.4% among isolates from adults and 0.4% among isolates from children (*8*). Of the 767 serotype 8 pneumococci, 131 isolates were resistant to ≥3 antimicrobial drugs (all isolates were from adults). Of these isolates, 119 were available for molecular characterization.

Reference Strains

American Type Culture Collection (ATCC) BAA-661, the reference strain of the Sweden 15A-ST63 clone (resistant to erythromycin, clindamycin, and tetracycline, and decreased susceptibility to penicillin), was used as a control for pulsed-field gel electrophoresis (PFGE) and PCR–restriction fragment length polymorphism (PCR-RLFP) analysis of penicillin-binding protein (PBP) genes. Strains CSUB8370, CSUB8757, and CSUB5364 (all 3 strains are serotype 8, susceptible to antimicrobial drugs, and ST53) were also used as controls for PFGE and PCR-RFLP of PBPs (*4*,*9*,*10*).

Antimicrobial Drug Susceptibility Testing

MICs of antimicrobial drugs for drug-resistant isolates were determined by using the microdilution method, 2%–5% lysed horse blood, and commercially available panels (STRHAE1; Sensititre, East Grunstead, UK) and following the recommendations of the Clinical Laboratory Standards Institute (*11*,*12*). *S. pneumoniae* ATCC 49619 was used for quality control testing. When ciprofloxacin or levofloxacin MICs were >2 μg/mL, these MICs were tested by using the E test (AB Biodisk, Solna, Sweden).

Molecular Typing

Genomic DNA was embedded in agarose plugs and digested with *Sma*I. Fragments were separated by PFGE in a CHEF-DRIII apparatus (Bio-Rad, Hercules, CA, USA as described (*9*). PFGE patterns were compared visually with patterns of reference and control strains. Seventeen strains selected from different areas and years were studied by using multilocus sequencing typing (MLST) as described (*13*). Allele numbers and sequence types (STs) were assigned by using the pneumococcal MLST website (http://www.mlst.net).

PBP Fingerprinting

The analysis of *pbp1a*, *pbp2b*, and *pbp2x* genes was performed by using PCR-RFLP. We used primers described by du Plessis et al. (*14*) for *pbp1a* and those described by Gherardi et al. (*15*) for *pbp2b* and *pbp2x*. PCR products were digested with *Hinf*I, and digestion patterns were visually compared. In addition, both strands of PCR PBP gene products of 3 selected strains were purified and sequenced by using amplification primers for *pbp2b* and *pbp2x*. For *pbp1a* amplification, primers and PBP1ASq1 (5′-TAAGGTCTACATGTCTAAT-3′) were used for sequencing purposes.

Resistance Phenotype Characterization

The presence of macrolide resistance genes (*ermB*, *mefA*/*E*) and the tetracycline resistance gene (*tetM*) was tested by PCR described elsewhere (*16*). To characterize quinolone resistance for all strains, we used a PCR-RFLP method that detects point mutations at the main quinolone resistance determinant region (QRDR) positions (*17*,*18*). In brief, after PCR amplification, *parC* amplicons were digested with *Hinf*I or *Sfa*NI to detect changes in S79 or D83, respectively. PCR products of *parE* were digested with *Hinf*I to detect changes in D435. Finally, *gyrA* amplicons were digested with *Hinf*I (S81) or *Mbo*II (E85) (*17*,*18*). Control strains with known QRDR mutations were used in each run (*19*).

PCR Amplification and DNA Sequencing and Analysis

QRDRs sequences were determined for 43 strains (15 with low-level ciprofloxacin resistance [LLCipR] [MIC ≤8 μg/mL] and 28 with high-level ciprofloxacin resistance [HLCipR] [MIC ≥16 μg/mL)]. In brief, *gyrA*, *parC*, *parE*, and *gyrB* genes were amplified by using PCR and primers and conditions previously described (*19*). PCR products were purified, and both strands were sequenced by using primers for PCR amplification (*19*).

Analysis of Recombination Site Upstream of *pbp2x*

We used sequence alignments of ≈20 kb located upstream of the *pbp2x* gene of 2 serotype 8 (ST53) strains (2071247 [GenBank accession no. ALBK01000004); and 2081685 [ALBN01000001]), 1 serotype 15A (ST63) strain (GA47179 [AIKX01000002]), and strain G54 (serotype

19F, ST63, CP001015) (*20*) pneumococcal genomes to design 7 sets of PCR primers. These primers were used to identify the putative recombination event upstream of *pbp2x* (Table).

Results

Description and Spread of Multidrug-Resistant Strain

During 2003–2012, among invasive strains collected from adults, the prevalence of serotype 8 ranged between 3.6% and 5.0% (Figure 1). In 2004, four invasive multidrug-resistant serotype 8 isolates were detected at the Spanish Reference Laboratory from 3 hospitals in Madrid (central Spain). Multidrug-resistant serotype 8 isolates were also detected in eastern (2005), southern (2006), and northwestern (2007) Spain. During 2009-2012, multidrug-resistant serotype 8 isolates were detected among invasive isolates from 7 autonomous communities. In the Madrid area, serotype 8-ST63 isolates were detected mainly in adult HIV-infected patients (*21*). All multidrug-resistant serotype 8 isolates were from adults (78.7% from male patients). Sources of isolation of multidrug-resistant serotype 8 pneumococci were blood (n = 116), pleural fluid (n = 6), cerebrospinal fluid (n = 2), fluid from eyes with endophthalmitis (n = 6), and abscess fluid (n = 1).

All 131 multidrug-resistant serotype 8 pneumococci were susceptible to penicillin (MIC <0.03 μg/mL), cefotaxime (MIC <0.03 μg/mL), and amoxicillin (MIC <0.06 μg/mL) and resistant to erythromycin (MIC >128 μg/mL), clindamycin (MIC >128 μg/mL), and tetracycline (MIC >64 μg/mL). Ciprofloxacin MICs ranged from 2 μg/mL to 64 μg/mL, and 68 strains showed HLCipR (MIC ≥16 μg/mL).

Molecular Typing

A common PFGE pattern (nearly identical to that of the reference Sweden 15A-ST63 clone) was found among all 119 available serotype 8 isolates with multidrug resistance, which suggested a capsular switching event. This PFGE pattern was different from those of other antimicrobial drug–susceptible serotype 8-ST53 isolates (related to the Netherlands 8-ST53 clone) (Figure 2, panel A). These PFGE patterns were confirmed after MLST characterization of 17 selected multidrug-resistant serotype 8 isolates (all were ST63).

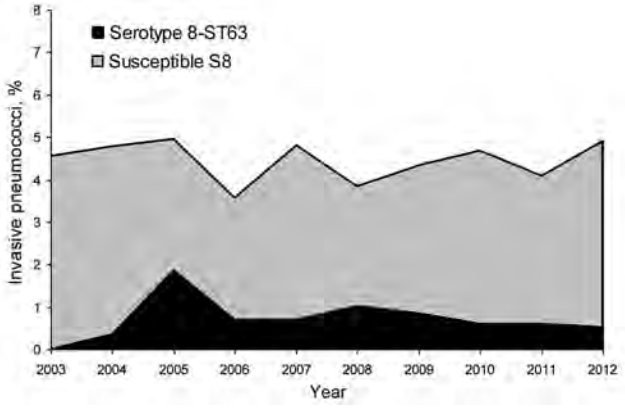

Figure 1. *Streptococcus pneumoniae* serotype 8 among invasive pneumococci isolated from adults in Spain, 2003–2012.

Table. Primers used to map the putative recombination event upstream of the *pbp2x* gene of *Streptococcus pneumoniae*, Spain, 2004–2012*

Primer	Sequence, 5′ → 3′	Position†
F1	AAATCCGAAGAAGGCCAAAT	278234–278253
R1	GTACTTGAGATTGGCGTGTTTG	278834–278813
F2	TCAATGACTGTGATGCCTGTT	290699–290719
R2	TGTCAGACAAATAGGACAAGGAGA	291315–291292
F3	GTCAATGACACCAACCTCTTG	282168–282188
R3	GCTATGAGCCATTCTAGCAAAGA	283037–283015
F4	TGAATGTAAAGACACACGAGGAA	273278–273300
R4	CAGTGATAACGAATACCATACAGAA	274128–274104
F5	CAGCTCTATGAACACCGGACT	289177–289197
R5	TTCCTAGTCGTAACCATCATTTCA	289927–289904
F6	CCTTGGATACGGGTATTCGTT	287148–287168
R6	GCAGTCGCTTGACCTTTTCT	287756–287737
F7	GTGGACAGGAAGCAAAGCTC	275192–275211
R7	GGCAGTCAGATTTGCAGACA	276056–276037

*The penicillin-binding protein 2x gene (*pbp2x*) (SPG_0305) of *S. pneumoniae* G54 strain (serotype 19F-ST63; GenBank accession no. CP001015) is located between positions 292638 and 294890 of its genome. F, forward; R, reverse.
†Corresponding to the genome of the G54 strain.

PBP Typing and Analysis

Results of PCR-RFLP analysis of *pbp1A*, *pbp2b*, and *pbp2x* genes of serotype 8-ST63 and antimicrobial drug–susceptible serotype 8-ST53 and of the multidrug-resistant Sweden 15A-ST63 reference strain (ATCC BAA-661) are shown in Figure 2, panel B. Amino acid sequence variation of the PBP1A, PBP2B, and PBP2X proteins of the epidemic strain and 2 control strains is shown in Figure 2, panel C. All serotype 8-ST63 isolates showed the same restriction profile for each *pbp* gene analyzed, which suggested clonal homogeneity. The restriction profile of *pbp2b* of the new strain was identical with that of the Sweden 15A-ST63 clone and different from those of serotype 8-ST53 drug-susceptible strains according to PCR-RFLP results. These results were confirmed after sequencing the *pbp2b* gene amplicons of 3 serotype 8-ST63 isolates, the Sweden 15A-ST63 reference strain, and 2 serotype 8-ST53 isolates (Figure 2, panel C).

The PCR-RFLP profile of *pbp2x* of serotype 8-ST63 isolates was identical with those of serotype 8-ST53 isolates (related to the Netherlands 8-ST53 clone) and different from that of the Sweden 15A-ST63 reference strain. These results were confirmed after sequencing the *pbp2b* gene amplicons of 3 serotype 8-ST63 isolates, the Sweden 15A-ST63 reference strain, and the serotype 8-ST53 isolates.

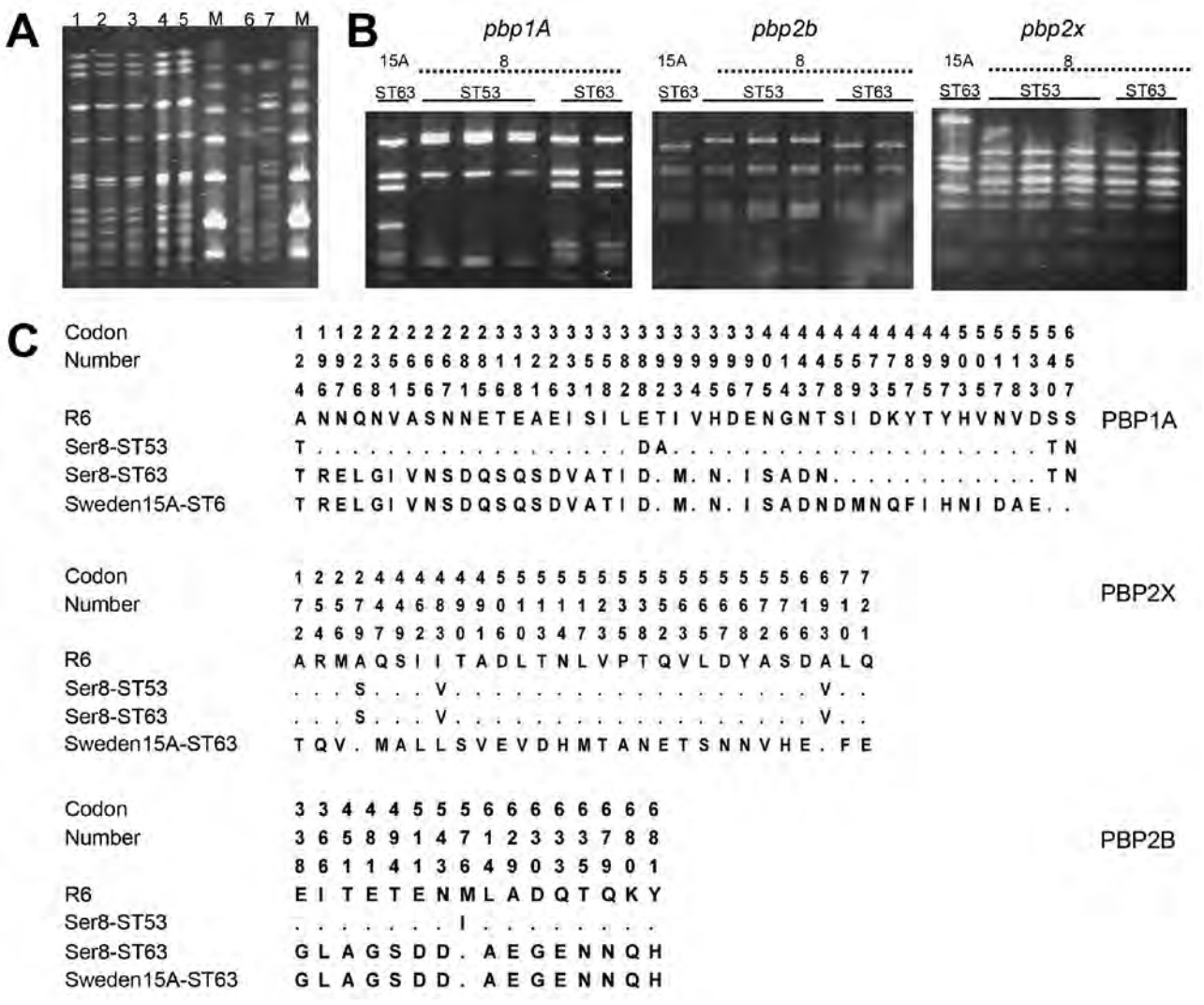

Figure 2. A) Pulsed-field gel electrophoresis patterns of chromosomal DNA of *Streptococcus pneumoniae* isolates after digestion with *Sma*I. Lane 1, Sweden 15A-ST63 (American Type Culture Collection [ATCC] BAA-661); lanes 2–5, serotype 8-ST63; lanes 6 and 7, serotype 8-ST53; lane M, molecular mass markers. B) PCR–restriction fragment length polymorphism patterns of penicillin-binding protein genes *pbp1A, pbp2b*, and *pbp2x*. Lane 1, Sweden 15A-ST63 (ATCC BAA-661) lanes 2–4: serotype 8-ST63; lanes 5 and 6, serotype 8-ST53. C) Amino acid sequence variations among PBP1A, PBP2B, and PBP2X proteins. Row 1, *S. pneumoniae* R6; row 2, serotype 8-ST53; row 3, serotype 8-ST63; row 4, Sweden 15A-ST63 reference strain. Codon numbers of polymorphic sites are numbered in a vertical format. Amino acids are numbered according to their positions in the corresponding protein. Only those amino acids that differ from those of the strain R6 sequence are shown. Dots indicate an amino acid residue that is identical with that in the strain R6 sequence.

All serotype 8-ST63 isolates had the same PCR-RFLP profile for *pbp1a*. This profile was different from those of antimicrobial drug–susceptible serotype 8-ST53 and nearly identical with the profile of Sweden 15A-ST63. Nucleotide sequence variation in the *pbp1a* gene of serotype 8-ST53, serotype 8-ST63, and Sweden 15A-ST63, determined by using *S. pneumoniae* R6 as a reference, is shown in Figure 3. Using R6 nomenclature for the *pbp1a* gene, we found that sequences of serotype 8-ST63 and Sweden 15A-ST63 were identical for nt 1–1341. Sequences of serotype 8-ST53, serotype 8-ST63, and Sweden 15A-ST63 were identical at nt 1342–1353. Sequences of *pbp1a* for nt 1354–1970 of serotype 8-ST63 were identical with those of antimicrobial drug–susceptible serotype 8-ST53. These results suggest that a recombination point was located in the 3′ end of the *pbp1a* gene.

Recombination Event Upstream of *pbp2x*

To determine the position of the recombination event that gave rise to the serotype 8-ST63 strain, we searched the public databases for genomic sequences located upstream of the *pbp2x* gene corresponding to serotype 8-ST53 and

serotype 15A-ST63 pneumococcal strains. Draft genomic sequences of 2 serotype 8-ST53 strains (2071247 [ALBK01000004]) and 2081685 [ALBN01000001]) were found. These sequences differed only at 5 positions in an ≈20-kb region. The draft sequence of the genome of a single serotype 15A-ST63 (strain GA47179 [AIKX01000002]) was also used. Moreover, when we compared this sequence (serotype 15A-ST63) with complete genomes of *S. pneumoniae* strains, we found an excellent match with that of strain G54 (serotype 19F; ST63); only 2 nt changes occurred in a 20,792-bp overlap.

The G54 strain has been shown to be a type 19F transformant of a serogroup 15 isolate (*20*). Nucleotide alignment of the 4 strains mentioned above showed several polymorphic sites, which enabled clear (although somehow limited) discrimination between ST53 and ST63 strains (Figure 4, panel A). Different fragments of the ≈20-kb region located upstream of *pbpx2* were amplified by PCR and sequenced (Table; Figure 4, panel A). Sequence comparisons showed that the recombination event between the donor DNA of a serotype 8 (ST53) strain and a recipient strain of serotype 15A (ST63) had occurred in a ≈3.8-kb region that contained genes SPG_0286 and SPG_0290 (using the strain G54 notation). Further attempts to determine more precisely the location of the recombination event were not made because this region has only 17 polymorphic sites. A scheme of the possible recombination event in which capsular loci and flanking regions (*pbp2x* and *pbp1a*) of a serotype 8-ST53 strain were acquired by ST63 pneumococci is shown in Figure 4, panel B.

Molecular Characterization of Drug Resistance

The *ermB* gene (which confers the macrolide–lincosamide–streptogramin B phenotype) and the *tetM* gene (which confers tetracycline resistance) were detected by PCR. Both genes are usually present in pneumococci related to the Sweden 15A-ST63 clone (*16*). These genes are located in the composite element Tn*5251* (Figure 4, panel B) (*20*).

Among 131 serotype 8-ST63 strains, 63 showed LL-CipR (MICs 2 μg/mL–8 μg/mL) and 68 showed (HLCipR (MICs ≥16 μg/mL). An increase in the proportion of serotype 8-ST63 isolates with HLCipR was observed: from 19.5% (8/41) during 2004-2006 to 86.7% (26/30) during 2010-2012 (p<0.001). PCR-RFLP analysis showed that all 119 available serotype 8-ST63 isolates that were studied lacked the restriction site for *Hin*fI in *parC*, which is

Figure 3. Nucleotide sequence variations in the penicillin-binding protein 1A (*pbp1a*) gene of *Streptococcus pneumoniae*. Row 1, *S. pneumoniae* R6; row 2, serotype (Ser) 8-ST53; row 3, serotype 8-ST63; row 4, Sweden 15A-ST63 reference strain. Polymorphic sites are numbered in a vertical format. Nucleotides are numbered according to their positions in the gene. Only polymorphic sites are shown. Dots indicate a nucleotide that is identical to that in the R6 sequence. The putative recombination site is shaded in gray.

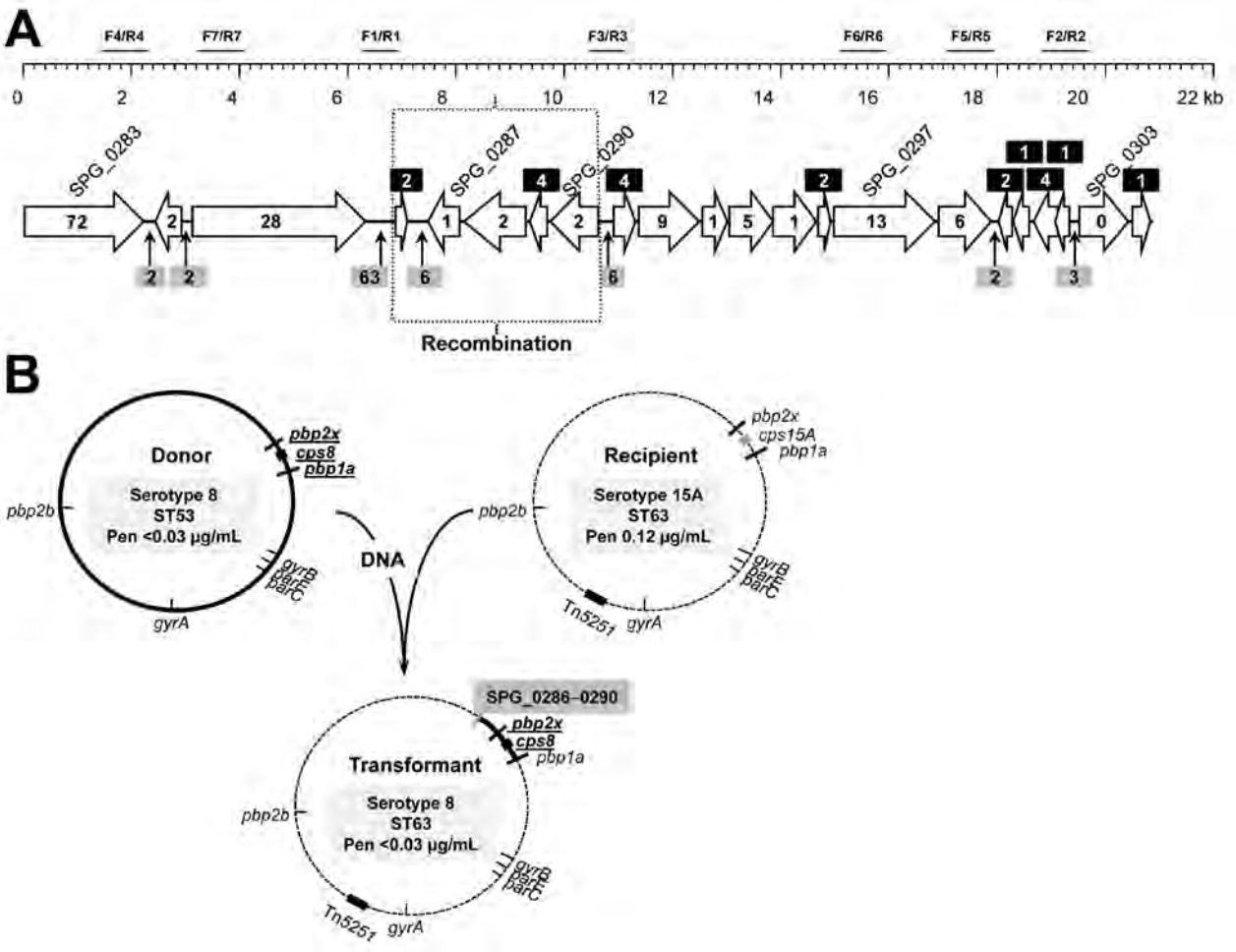

Figure 4. Chromosome location of the recombination region located upstream of the penicillin-binding protein 2x (*pbp2x*) gene of *Streptococcus pneumoniae* (A) and antimicrobial drug resistance determinants, capsular loci of the putative donor, recipient, and recombinant strains (B). In the genomic region upstream of *pbp2x* (SPG_0305), genes have been named as in the G54 genome (ST63) (GenBank accession no. CP001015). Number of polymorphic sites located either within the genes (inserted into the arrows or into black boxes) or in their intergenic regions (gray boxes) is shown. The approximate location of the recombination event is indicated by the dotted box. Amplified and sequenced fragments are indicated in relationship to primer pair designations (Table). MICs for penicillin (Pen) are shown.

associated with a change in S79. This change was confirmed to be an S79F after sequencing 43 isolates (15 LLCipR and 28 HLCipR). Fifty-nine of 61 available LLCipR isolates (all with the S79 change) had levofloxacin MICs ≤2 µg/mL (susceptible).

A total of 58 of 68 HLCipR isolates were available for analysis. Of these 58 isolates, 47 lacked the *Hin*fI restriction site in *gyrA*, which is associated with a mutation at S81; 10 isolates lacked the *Mbo*II restriction site related to a *gyrA* change at E85; and 1 isolate lacked the *Hin*fI and *Mbo*II restriction sites that were assumed to be caused by changes in S81 and E85 in *gyrA*. The results of *gyrA* QRDR sequencing of 28 selected HLCipR isolates showed various *gyrA* changes (S81F [n = 23]; S81Y [n = 2]; E85K [n = 3]; and S81F and E85K [n = 1 each]). No *parE* changes were detected by PCR-RFLP or sequencing. Serotype 8-ST63 isolates for which QRDRs were sequenced showed polymorphisms for *parC* (G77[GGA]), *parE* (I460V), and *gyrA* (Y75[TAT]) that were identical with those of the Sweden 15A-ST63 reference strain and other pneumococci of this lineage (*21*,*22*).

Discussion

In this study, we described the emergence and spread of a pneumococcal clone that originated by recombination of a ciprofloxacin-resistant and multidrug-resistant strain related to the Sweden 15A-ST63 clone, which acted as a recipient of DNA, and a serotype 8 strain with ST53, which acted as a donor of DNA. The new recombinant clone (serotype 8-ST63) was initially confined to the metropolitan area of Madrid and was mainly associated with HIV-infected patients (*21*). However, in the past 5 years, the new

clone has been detected in 9 other regions in Spain, which indicates an ability to spread. Two characteristics merged in this new clone, the invasive disease potential of serotype 8 and the antimicrobial drug resistance of ST63, which suggested that the recombinant could be a new antimicrobial drug–resistant clone.

The capsular polysaccharide is the major factor in determining the invasive disease potential of a given isolate. Serotype 8 has been associated with high invasive disease potential when a large collection of invasive and colonizing isolates were compared (*23*). Moreover, capsular serotype 8 was associated with high attack rates; 30 cases of invasive pneumococcal disease/100,000 carriage acquisitions were detected (*2*). Conversely, serotype 15A pneumococci show low invasive potential and are usually associated with ST63 and resistance to macrolides, clindamycin (*ermB*) and tetracycline (*tetM*) (*2*,*16*). The overall frequency of clonal complex (CC) 63 among invasive isolates from adults was low: 2.5% (22/899) in 1997–2008 (*4*). Moreover, in 3 studies conducted in Spain with quinolone-resistant and macrolide-resistant pneumococci, CC63 was 1 of the most prevalent clones (*16*,*19*,*22*).

The results of the present study suggest that the new strain could be the result of a recombination event between a serotype 15A-ST63 pneumococcal isolate that contained the S79F change in ParC and a serotype 8-ST53 pneumococcus. In this event, the recombination fragment included the *pbp2x* gene and part of the *pbp1a* gene. The recombination of the *cps* locus and flanking regions has been observed by other authors, (*6*,*24*,*25*), which suggests that it is a common biologic process in the evolution of pneumococci.

The new strain, serotype 8-ST63, is penicillin susceptible (MIC <0.01 μg/mL), and ST63 isolates with other serotypes (15F, 15A, 15B, 19F, and 19A) are usually penicillin resistant (MIC range 0.12 μg/mL–0.5 μg/mL) (*10*,*16*,*22*). The penicillin resistance of CC63 isolates is associated with a Q552E change in PBP2X (*26*). The acquisition of *pbp2x* genes from serotype 8-ST53, without changes involved in b-lactam resistance, by the recombinant strain explains its penicillin susceptibility.

There have been other examples of recombinant clones caused by capsular switching that successfully spread. For instance, in the past decade, acquisition of serotype 14 by pneumococci of serotype 9V of ST156 has been associated with the worldwide increase of serotype 14 as a cause of invasive pneumococcal disease (*4*,*6*). Moreover, exchange of capsular genes with *pbp* genes has been documented in the serotype 19A-ST320 clone that spread worldwide and is a major cause of multidrug-resistant invasive and noninvasive disease (*27*,*28*). In the United States, recombination of capsular and *pbp* genes has been observed as a vaccine escape of a penicillin-susceptible serotype 4 that acquired the capsular genes and PBPs of a serotype 19A strain. This recombinant strain had a nonvaccine serotype and an antimicrobial drug resistance pattern that favored its spread in the United States. (*5*). To the best of our knowledge, the new recombinant clone (serotype 8-ST63) described here has not been detected outside Spain.

A report from South Africa described the spread of 2 quinolone-resistant strains that caused invasive disease and colonized children; most of these children were receiving treatment with antimicrobial drugs (including levofloxacin) for multidrug-resistant tuberculosis (*29*). Moreover, in previous studies, our group and others have observed some clustered infections, with a limited number of cases caused by quinolone-resistant pneumococci in a specific geographic area (*19*,*30*). The recombinant strain (serotype 8-ST63) described in the present study has been detected in several cities in Spain, which demonstrates its ability to disseminate.

Most isolates of this new clone with ciprofloxacin resistance required MICs of levofloxacin that were in the susceptible range according to recommendations of the Clinical and Laboratory Standards Institute (*12*). Because these strains had a first-step mutation, use of fluoroquinolones to treat infections could become a problem. At least 1 death, resulting from therapeutic failure of levofloxacin, has been documented: the patient had pneumococcal pneumonia caused by a serotype 8-ST63 pneumococci in Seville, Spain. In this case, the initial strain had the S79F change in ParC and required a levofloxacin MIC of 1 μg/mL. After levofloxacin therapy, high-level drug resistance developed in this strain after a second change in GyrA (*31*).

In the 1990s, use of antimicrobial drugs was high in Spain. During this period, penicillin-resistant and multidrug-resistant clones spread over the country, caused invasive disease, and colonized healthy children. Since that time, use of antimicrobial drugs has decreased in Spain (*32*). Together with vaccination with the 7-valent pneumococcal conjugate vaccine, the decrease in use of b-lactams probably contributed to the recent decrease in penicillin-resistance and multidrug-resistance rates (*4*,*8*). In contrast, data from the Spanish Medicines Agency (http//agemed.es) indicate that levofloxacin consumption has increased from 0.2 defined daily doses/1,000 persons/day (DDD) in 2002 to 0.4 DDD in 2006 and 0.6 DDD in 2012. This antimicrobial drug pressure could have favored spread of the fluoroquinolone-resistant clone in Spain and resulted in the increase in the proportion of HLCipR among serotype 8-ST63 isolates.

Children are the main reservoir for pneumococci, but quinolones are seldom prescribed for this population. Thus, isolation of quinolone-resistant pneumococci from children is rare. We have not detected any serotype 8-ST63 pneumococci in isolates from children. However,

in a plausible scenario, the recombinant clone could colonize the nasopharynx of children and become a major source for dissemination.

In conclusion, emergence and spread of the serotype 8-ST63 clone that originated by genetic interchange of capsular genes and their flanking regions, including *pbp1a* and *pbp2x* genes, have been detected in Spain. The 2 clones involved in capsular switching were Netherlands 8-ST53 and Sweden 15A-ST63. Surveillance is needed to clarify the dynamics of this new multidrug-resistant clone as cause of pneumococcal disease.

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Foodborne Illness, Australia, **Circa 2000 and Circa 2010**

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Foodborne disease is a major public health problem worldwide. To examine changes in foodborne illness in Australia, we estimated the incidence, hospitalizations, and deaths attributed to contaminated food circa 2010 and recalculated estimates from circa 2000. Approximately 25% of gastroenteritis cases were caused by contaminated food; to account for uncertainty we used simulation techniques to estimate 90% credible intervals. We estimate that circa 2010, 4.1 million foodborne gastroenteritis cases occurred, and circa 2000, 4.3 million cases occurred. Circa 2010, contaminated food was estimated to be responsible for 30,840 gastroenteritis-associated hospitalizations, 76 associated deaths, and 5,140 nongastrointestinal illnesses. Cases of salmonellosis and campylobacteriosis increased from 2000 to 2010 and were the leading causes of gastroenteritis-associated hospitalizations; *Listeria monocytogenes* and nontyphoidal *Salmonella* spp. infections were the leading causes of death. Although the overall incidence of foodborne illnesses declined over time in Australia, cases of foodborne gastroenteritis are still common.

Foodborne illness is a major public health problem and a common cause of illness and death worldwide. Outbreaks linked to contaminated food can affect the public's trust and financially harm implicated businesses and associated food industries. Estimates of the effects of foodborne illnesses and individual pathogens provide evidence for policy interventions and food safety regulation. In addition, estimates of changes in the incidence of foodborne illnesses and hospitalizations over time provide information on the effectiveness of changes to food safety standards and regulation.

Many agents can cause foodborne illness; some of these agents are transmitted to humans by other routes as well as by food. Most foodborne illnesses manifest as gastroenteritis, but other presentations, such as meningitis and hepatitis may also result from infection, and sequelae may occur weeks after the acute infection.

Many countries have estimated the incidence of foodborne diseases (*1*–*5*). In Australia in 2000, foodborne incidence, hospitalizations, and deaths were estimated to cost 1.25 billion Australian dollars annually (*6*,*7*). However, since 2000, surveillance has substantially improved, data availability has increased, and methods have been refined. To inform current public health decisions and policies in Australia, we used new methods and datasets to estimate the incidence of infectious gastroenteritis and associated hospitalizations and deaths in Australia circa 2010. We then applied these refined methods to circa 2000 data so that estimates from the 2 periods could be directly compared.

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Methods

We estimated the incidence of illness and the number of hospitalizations and deaths associated with 23 potentially foodborne pathogens or agents in Australia circa 2010 (online Technical Appendix 1 Table 1, http://wwwnc.cdc.gov/EID/article/2011/13-1315-Techapp1.pdf). Pathogens we did not consider relevant were those acquired only overseas (e.g., *Vibrio cholerae*, *Trichinella spiralis)* and those that cause gastroenteritis but are not proven agents of foodborne disease (e.g., *Clostridium difficile*). Estimates of chronic sequelae from foodborne illnesses are discussed elsewhere in this issue (*8*).

When possible, data for the circa 2010 study period covered 2006–2010, and all denominator data were based on the Australian population during that period (*9*). Estimates of incidence relied on data obtained from 4 sources: notifiable disease surveillance at the national and state levels; outbreak surveillance through the OzFoodNet Outbreak Register; the National Gastroenteritis Survey II (NGSII; http://www.ozfoodnet.gov.au/), a cross-sectional survey; and the Water Quality Study (WQS), a randomized controlled trial (conducted during 1997–1999) of household water treatment to prevent gastroenteritis (*10*,*11*). Estimates of severe illness were determined by using hospitalization and death data. This study was approved by the Australian National University Human Research Ethics Committee. Further details of the data sources and methods are in online Technical Appendix 1.

To estimate incidence, hospitalizations, and deaths, we built on our previous methods (*7*), making them similar to those used in the United States (*2*,*3*). We calculated estimates

by using simulation techniques in @Risk version 6 (http://www.palisade.com/) with multiple inputs, each with different levels of uncertainty. We used empirical, lognormal, and PERT (program evaluation review technique) probability distributions to model uncertainty in source data and multipliers. Estimates are expressed as probability distributions summarized by a median point estimate with a 90% credible interval (CrI) (online Technical Appendix 2, http://wwwnc.cdc.gov/EID/article/2011/13-1315-Techapp2.pdf).

Incidence Circa 2010

To estimate the annual incidence of infectious gastroenteritis in Australia circa 2010, we used symptoms included in the NGSII telephone survey conducted during February 2008–January 2009. Case definition has a considerable effect when determining the incidence of gastroenteritis (*12*). To enable a valid comparison of circa 2000 and circa 2010 gastroenteritis estimates, we used the case definition from the earlier study (*13*,*14*). In NGSII, persons were considered case-patients if they had ≥3 episodes of diarrhea or ≥2 episodes of vomiting within a 24-h period during the preceding 4 weeks and did not report a noninfectious cause for their illness. However, for persons who had concomitant respiratory symptoms, we applied a stricter definition: ≥4 episodes of diarrhea and/or ≥3 episodes of vomiting (*15*). In NGSII, 4.5% (341/7,578) of survey respondents reported gastroenteritis in the preceding 4 weeks, equating to 0.74 gastroenteritis episodes per person per year (95% CI 0.64–0.84) or 15.9 million cases annually in Australia.

We used 2 main approaches to estimate the incidence of foodborne illness caused by specific pathogens or illnesses. Our preferred approach was the surveillance approach, in which we estimated the community incidence of illness by applying an underreporting multiplier to scale up data from notifiable disease surveillance. When these data were not available, we used a pathogen fraction approach, in which we estimated the percentage of overall gastroenteritis caused by specific pathogens. When data were unavailable by either of these approaches, we used other surveillance data, such as outbreak data. Approach-specific flow charts are provided in online Technical Appendix 2.

Using the surveillance approach, we adjusted for underreporting of community cases to public health surveillance. We used findings from an underreporting multiplier study in Australia (*16*) for moderate illnesses and bloody diarrhea. For serious illnesses, we assumed the underreporting factor as 1 illness reported for every 2 that occurred in the community, as used by Mead et al. (*17*) and Scallan et al. (*2*). We applied another multiplier to outbreak surveillance data to adjust for underreporting when only outbreak cases were notified (online Technical Appendix 2).

When we used the pathogen fraction approach, our main data source was the WQS (*10*,*11*). The WQS provided data on the proportion of gastroenteritis episodes caused by specific pathogens, and we applied those proportions to total foodborne illness incidence data from the NGSII. However, the WQS was conducted before rotavirus vaccine was added to the Australian vaccination schedule. To account for the effect of the vaccine on infection incidence, we calculated a time-trend multiplier by using age-specific hospitalization data from before and after introduction of rotavirus vaccine (*18*).

We used the surveillance approach for cases caused by 16 pathogens, of which 11 were from the National Notifiable Diseases Surveillance System (NNDSS; http://www.health.gov.au/internet/main/publishing.nsf/Content/cda-surveil-nndss-nndssintro.htm) and 5 were from outbreak data. We used the pathogen fraction approach for cases caused by 6 pathogens. In addition, because local data were lacking, we applied US seroprevalence data to the Australian population data to estimate the incidence of toxoplasmosis (online Technical Appendix 2).

Incidence Circa 2000

Methods for calculating incidence have changed since the circa 2000 estimates were determined (*7*); the changes include updated underreporting multipliers (*16*), more rigorous expert elicitation (*19*), and new estimates of the foodborne multipliers for some pathogens. These changes could result in a potentially misleading comparison of circa 2010 and circa 2000 findings. We recalculated estimates for circa 2000 by using the original data with methods identical to those used for circa 2010 data. Updated estimates of the total incidence of foodborne gastroenteritis were determined by using the original 2001 National Gastroenteritis Survey, together with the 2010 foodborne proportion of 25% (compared with 32% in the circa 2000 study). To recalculate the circa 2000 estimates, we replaced multipliers used in that study with circa 2010 multipliers and applied them to 1996–2000 data from NNDSS for *Campylobacter* spp., nontyphoidal *Salmonella enterica* serotypes (hereafter referred to as nontyphoidal *Salmonella* spp.), *S. enterica* serotype Typhi, *Shigella* spp., hepatitis A virus, and *Listeria monocytogenes* infections and to 1996–2000 surveillance data from the state of Victoria, Australia, for *Giardia lamblia*. Only pathogens for which we had surveillance data from both periods were included in this analysis.

Hospitalizations and Deaths

We estimated the annual number of hospitalizations for foodborne illnesses by using 2006–2010 state and territory hospitalization data (http://www.aihw.gov.au/hospitals/australian-hospital-statistics/) for which principal and additional diagnoses were based on the Australian modification of the 10th International Classification of Diseases (*20*), and we estimated the annual number of deaths by using 2001–2010

Australian Bureau of Statistics' national death data for underlying or contributing cause (http://www.aihw.gov.au/deaths/). Reports for a large number of hospitalizations and deaths caused by gastrointestinal illnesses that were presumed infectious did not identify a specific pathogen.

We adjusted for travel-associated cases and estimated the proportions of foodborne disease–associated hospitalizations and deaths (online Technical Appendix 3, http://wwwnc.cdc.gov/EID/article/2011/13-1315-Techapp3.pdf). Because the recorded hospitalizations and deaths associated with each pathogen reflect only laboratory-confirmed cases, we applied an underdiagnosis multiplier of 2 (range 1–3). This multiplier has been used in other studies (*2*,*7*,*17*) but never validated. Assuming that outbreaks provide a representative denominator population from which to calculate the proportion of hospitalized case-patients, we confirmed the appropriateness of the multiplier by using the OzFoodNet Outbreak Register (http://www.ozfoodnet.gov.au/) to calculate, for a number of pathogens, the proportion of hospitalized case-patients. For the included pathogens, we compared this proportion with the ratio of our estimated yearly hospitalizations to yearly illnesses.

Domestically Acquired Multiplier

To exclude infections acquired overseas, we applied a domestically acquired multiplier to all pathogens to adjust the total incidence data. For many pathogens, this multiplier was estimated from surveillance data from states and territories that recorded illnesses acquired overseas; variability by state and by year was used to inform uncertainty in the multiplier. Other pathogens causing illness of short duration were assumed to be 100% domestically acquired. Details, by pathogen, are provided in online Technical Appendix 4 (http://wwwnc.cdc.gov/EID/article/2011/13-1315-Techapp4.pdf).

Proportion Foodborne Multiplier

To estimate the total number of foodborne infections caused by each pathogen, we applied a pathogen-specific proportion foodborne multiplier to all pathogens (online Technical Appendix 2 Table 2). The proportion foodborne multiplier was estimated for 9 pathogens in 2009 by using an expert elicitation process (*19*), and the multipliers for another 9 pathogens were estimated by using a similar expert elicitation study in 2005 (*21*). All illnesses due to seafood toxins were assumed to be caused by food, and multipliers for 3 viruses were assumed to be equal to those for similar pathogens.

The estimated annual number of gastroenteritis cases caused by 18 known pathogens/parasites for the circa 2010 study period is listed in Table 1. An estimated 25% of the cases were caused by contaminated food, of which 36%, 16%, and 11% were caused by bacteria, viruses, and parasites, respectively. Given an absence of other data sources, we applied this overall foodborne proportion of 25% to the total number of gastroenteritis cases to determine the number caused by contaminated food (*3*,*17*,*22*).

Results

Incidence

Foodborne Gastroenteritis Circa 2010

We estimated that each year circa 2010, 4.1 million domestically acquired cases (90% CrI 2.3–6.4) of foodborne gastroenteritis occurred in Australia. Of those annual cases, 0.8 million were caused by the 18 pathogens that were known agents of gastroenteritis, and the remaining 3.3 million cases were caused by unknown or unidentified pathogens (Table 1; online Technical Appendix 5, http://wwwnc.cdc.gov/EID/article/2011/13-1315-Techapp5.pdf). Pathogenic *Escherichia coli*, norovirus, *Campylobacter* spp., and nontyphoidal *Salmonella* spp. were the most common causes of foodborne gastroenteritis; together, they were responsible for 93% of the foodborne illnesses caused by known pathogens.

Foodborne Nongastrointestinal Illness Circa 2010

In addition to causing foodborne gastroenteritis, contaminated food also caused 5,140 cases (90% CrI 3,530–7,980) of nongastrointestinal illness in Australia circa 2010 (Table 2). Toxoplasmosis was the most common foodborne nongastrointestinal illness; 3,750 cases (90% CrI 1,400–7,150) occurred each year. The percentage of foodborne illnesses caused by nongastroenteric agents ranged from a low of 12% for hepatitis A infection to a high of 100% for scombrotoxicosis and ciguatera.

Comparison of Circa 2010 Estimates with Circa 2000 Estimates

When we applied the newer estimation methods, including the new proportion foodborne multiplier (i.e., 25%), to circa 2000 data, the annual number of foodborne gastroenteritis cases was 4.3 million (90% CrI: 2.2–7.3). That total translates to a circa 2000 incidence of 224,000 cases/million population (90% CrI 116,000–374,000). Comparison of the circa 2010 incidence (186,000 cases/million population; 90% CrI 105,000–289,000) with the circa 2000 incidence showed a 17% decreased incidence of foodborne gastroenteritis between 2000 and 2010, although the CrI included 1 (rate ratio [RR] 0.83, 90% CrI 0.4–1.8). Similar recalculation of circa 2000 estimates for key gastrointestinal pathogens showed a total of 28,000 cases (90% CrI 15,000–50,000) of foodborne salmonellosis each year (incidence 1,500 cases/million population, 90% CrI 800–2,700) and 139,000 cases (90% CrI 82,500–227,000) of

Table 1. Estimated number of gastroenteritis cases caused by domestically acquired pathogens, Australia, circa 2010*

Causative agent	Total no. cases, median (90% CrI)	% Cases caused by contaminated food, median (90% CrI)	No. cases caused by contaminated food, median (90% CrI)
Bacterium			
Bacillus cereus	3,350 (900–10,100)	100 (98–100)	3,350 (900–10,100)
Campylobacter spp.	234,000 (147,000–374,000)	77 (62–89)	179,000 (108,500–290,000)
Clostridium perfringens	16,500 (2,600–53,400)	98 (86–100)	16,100 (2,550–50,600)
STEC	4,300 (2,050–9,500)	56 (32–83)	2,350 (950–5,850)
Other pathogenic *E. coli*	1,100,000 (833,000–1,450,000)	23 (8–55)	255,000 (85,800–632,000)
Salmonella spp, nontyphoidal	56,200 (31,900–101,000)	72 (53–86)	39,600 (21,200–73,400)
Salmonella enterica ser. Typhi	20 (8–45)	75 (2–97)	15 (5–30)
Shigella spp.	3,000 (1,650–5,400)	12 (5–23)	350 (150–850)
Staphylococcus aureus	1,300 (200–7,050)	100 (95–100)	1,300 (200–7,000)
Vibrio parahaemolyticus	60 (15–170)	75 (5–96)	40 (10–120)
Yersinia enterocolitica	1,500 (900–2,500)	84 (28–94)	1,150 (650–1,950)
Virus			
Adenovirus	88,400 (28,800–205,000)	2 (1–3)	1,650 (500–4,650)
Astrovirus	67,100 (20,900–155,000)	2 (1–3)	1,300 (350–3,400)
Norovirus	1,550,000 (1,220,000–1,940,000)	18 (5–35)	276,000 (78,100–563,000)
Rotavirus	44,800 (18,500–90,800)	2 (1–3)	850 (300–2,000)
Sapovirus	81,600 (63,400–102,000)	18 (5–35)	15,000 (7,450–24,300)
Parasite			
Cryptosporidium spp.	17,900 (8,150–39,800)	10 (1–27)	1,700 (150–6,100)
Giardia lamblia	32,800 (19,800–56,400)	6 (1–50)	3,700 (800–10,600)
Subtotal	3,090,000 (2,810,000–3,900,000)	25 (13–42)	798,000 (528,000–1,310,000)
Unknown etiology	12,800,000 (10,500,000–14,500,000)	25 (13–42)	3,310,000 (1,800,000–5,152,000)
Total	15,900,000 (13,700,000–18,000,000)	25 (13–42)	4,110,000 (2,330,000–6,390,000)

*All estimates were based on an empirical distribution of the Australian population in the June quarter of 2006–2010; for the parameters of these distributions, see online Technical Appendix 4 (http://wwwnc.cdc.gov/EID/article/2011/13-1315-Techapp4.pdf). CrI, credible interval; *E. coli*, *Escherichia coli*; STEC, Shiga toxin–producing *E. coli.*

foodborne campylobacteriosis each year (incidence 7,400 cases/million population, 90% CrI 4,500–12,200) (Table 3). Comparison of the circa 2000 and circa 2010 incidence rates showed RRs of 1.24 (90% CrI 0.5–2.8) for foodborne salmonellosis and 1.13 (90% CrI 0.5–2.3) for foodborne campylobacteriosis, although the CrI included 1. CrIs include uncertainty derived from incidence multipliers and were considerably wider than intervals for ratios derived from raw surveillance data.

Hospitalizations

Circa 2010, there were an estimated 30,600 hospitalizations (90% CrI 28,000–34,000) for foodborne gastroenteritis and 240 hospitalizations (90% CrI 180–350) for nongastrointestinal foodborne illnesses (Table 4). Approximately 5,900 of all hospitalizations for gastroenteritis were for illnesses caused by known pathogens, of which *Campylobacter* spp. and nontyphoidal *Salmonella* spp. were the leading causes of hospitalization, and *L. monocytogenes* was the leading cause of nongastrointestinal illnesses requiring hospitalization. The remaining 24,700 hospitalizations were for gastroenteritis of unknown etiology.

Deaths

For circa 2010, we estimated that there were 60 deaths (90% CrI 53–63) due to foodborne gastroenteritis and 16 deaths (90% CrI 10–21) due to nongastrointestinal foodborne illnesses (Table 4). Nontyphoidal *Salmonella* spp. and *L. monocytogenes* were the most commonly identified causes of all illnesses that resulted in death; each year, these pathogens were each responsible for an estimated 15 foodborne illness–associated deaths. Gastroenteritis of unknown etiology as an underlying or contributing cause of death resulted in 39 deaths each year.

Discussion

Foodborne illness is extremely common in Australia: on average, each person in Australia experiences an episode of foodborne gastroenteritis approximately every 5 years. Although foodborne gastroenteritis is often not serious, the cost to society is considerable through direct medical costs and days of lost work. Approximately 1 in 5 persons with gastroenteritis seeks medical attention. Thus, up to 1 million medical visits a year could be for foodborne illnesses (*23*).

We examined changes in foodborne illness in Australia over time, a key reason for repeating studies to estimate incidence. Our findings showed a slight decline in the rate of foodborne gastroenteritis between the circa 2000 and circa 2010 study periods, but our findings also showed increases in the rates of illness caused by some specific pathogens. Changed estimates were driven by differences in estimates of total gastroenteritis and by pathogen-specific surveillance trends. In Australia from 2006 onward, the number of raw egg–associated salmonellosis outbreaks has markedly increased (*24*), and since 2000, the numbers of

Table 2. Estimated number of acute foodborne illness cases caused by domestically acquired pathogens and agents that do not result in gastroenteritis, Australia, circa 2010*

Illness	% Foodborne, median (90% CrI)	No. illnesses, median (90% CrI)
Hepatitis A virus infection	12 (5–24)	40 (10–100)
Listeriosis	98 (90–100)	150 (50–200)
Toxoplasmosis	31 (4–74)	3,750 (1,400–7,150)
Ciguatera	100 (100–100)	150 (40–300)
Scombrotoxicosis	100 (100–100)	1,050 (0–2,450)
Total	40 (25–59)	5,140 (3,530–7,980)

*All estimates were based on an empirical distribution of the Australian population in the June quarter of 2006–2010; for the parameters of these distributions, see online Technical Appendix 4 (http://wwwnc.cdc.gov/EID/article/20/11/13-1315-Techapp4.pdf). CrI, credible interval.

notified laboratory-confirmed cases of campylobacteriosis and salmonellosis have increased (*25*). Estimates of rotavirus cases for circa 2010 were lower than those for circa 2000, reflecting the success of the vaccination program (*18*). Also, the estimated number of foodborne illness cases caused by hepatitis A virus declined from 245 cases/year circa 2000 to 40 cases/year circa 2010, reflecting improved disease control through vaccination (*24*). Although these interventions were not targeted at foodborne disease, our findings highlight the benefits of vaccination programs in reducing circulation of enteric pathogens and transmission through food.

It must be noted that where we observed changes over time, they were often not significant due to the many sources of uncertainty. When we examined the CrIs, over half of the uncertainty arose from the distribution for the foodborne multiplier estimated from expert elicitation; most of the other sources of uncertainty arose from the distributions for the underreporting and pathogen fraction multipliers. Further studies to estimate foodborne multipliers for high-incidence pathogens (in particular, norovirus and other pathogenic *E. coli*) would help reduce this uncertainty in overall estimates. Scallan et al. (*3*) highlighted the profound effect that changes in these proportions of foodborne transmission can have on overall estimates of disease incidence. We identified similar effects when we used updated methods to recalculate estimates for circa 2000; in particular, the estimates for foodborne gastroenteritis illnesses declined from 5.4 to 4.3 million cases. New approaches should be examined for estimating the relative importance of different modes of transmission for pathogens that are potentially foodborne.

Similar studies estimating the incidence of foodborne disease have been conducted in the United States (*2*,*3*,*17*), United Kingdom (*4*), Canada (*22*), and the Netherlands (*5*). We estimated that 25% of all gastroenteritis cases in Australia were caused by contaminated food; this percentage is similar to estimates for the United Kingdom and to the most recent estimates for the United States but lower than estimates for the Netherlands. Although the Canadian study does not report an overall proportion of foodborne transmission, analysis of the study results puts it at ≈20% (*22*). In the United States, Scallan et al. (*2*) estimated that 9.4 million (26%) of 36.4 million domestically acquired illnesses caused by known pathogens were transmitted via contaminated food, and in the United Kingdom, Adak et al. (*4*) estimated that 26% of infectious intestinal illnesses were caused by pathogens transmitted via contaminated food. The estimate for the Netherlands was higher at 39% (*5*). These overall estimates of the proportion of gastroenteritis caused by contaminated food depend on the pathogens included in the estimates, the incidence of common pathogens in the study area, and the proportion of those common pathogens that are considered to be foodborne.

Table 3. Comparison of estimates of the annual number of cases and incidence rates for foodborne gastroenteritis and key foodborne pathogens, Australia, circa 2000 and circa 2010*

	Circa 2000		Circa 2010		
Foodborne illness/pathogen	No. cases, median (90% CrI)	Rate per million population (90% CrI)	No. cases, median 90% (CrI)	Rate per million population (90% CrI)	RR (90% CrI)
Gastroenteritis	4.3 million (2.2–7.3 million)	224,000 (116,000–374,000)	4.1 million (2.3–6.4 million)	186,000 (105,000–289,000)	0.83 (0.4–1.8)
Campylobacter spp.	139,000 (82,500–227,000)	7,400 (4,500–12,200)	179,000 (108,500–290,000)	8,400 (5,050–13,650)	1.13 (0.5–2.3)
Salmonella spp., nontyphoidal	28,000 (15,000–50,000)	1,500 (800–2,700)	39,600 (21,200–73,400)	1,850 (1,000–3,350)	1.24 (0.5–2.8)
Salmonella enterica ser. Typhi	9 (3–21)	0.5 (0–1)	15 (5–30)	0.6 (0–1)	1.2 (0.5–2.6)
Shigella spp.	515 (175–1,300)	28 (9–70)	350 (150–850)	16 (6–40)	0.57 (0.2–2.3)
Hepatitis A virus	245 (65–725)	13 (3–40)	40 (10–100)	2 (1–5)	0.15 (0.06–0.4)
Listeria monocytogenes	125 (70–185)	7 (4–10)	150 (50–100)	7 (3–10)	1 (0.4–1.9)
Giardia lamblia	2,600 (565–7,400)	140 (30–405)	3,700 (800–10,600)	175 (35–490)	1.25 (0.5–1.9)

*Estimates are based on an empirical distribution of the Australian population in the June quarter of 1996–2000 (circa 2000 estimates) and 2006–2010 (circa 2010 estimates); for the parameters of these distributions, see online Technical Appendix 4 (http://wwwnc.cdc.gov/EID/article/2011/13-1315-Techapp4.pdf). CrI, credible interval; RR, rate ratio.

Table 4. Estimated annual number of hospitalizations and deaths resulting from domestically acquired foodborne pathogens, parasites, and diseases, Australia, circa 2010*

Illness, causative agent/illness	ICD-10-AM code	No. hospitalizations, median (90% CrI)	No. deaths, median (90% CrI)
Gastrointestinal illness, cause			
Bacterium			
Bacillus cereus	A05.4	25 (4–45)	0
Campylobacter spp.	A04.5	3,200 (2,100–4,500)	3 (2–4)
Clostridium perfringens	A05.2	0 (0–2)	1 (0–1)
STEC	A04.3	7 (2–15)	0
Other pathogenic *E. coli*	A04.0, A04.1, A04.4	20 (6–50)	0 (0–1)
Salmonella spp., nontyphoidal	A02.0-A02.9	2,100 (1,300–3,000)	15 (8–20)
Salmonella enterica ser. Typhi	A01.0	15 (6–35)	0
Shigella spp.	A03	25 (9–50)	0
Staphylococcus aureus	A05.0	10 (7–20)	0
Vibrio parahaemolyticus	A05.3	1 (0–1)	0
Yersinia enterocolitica	A04.6	35 (10–65)	1 (0–1)
Virus			
Adenovirus	A08.2	15 (8–25)	0
Astrovirus	NA	NA	NA
Norovirus	A08.1	150 (35–350)	1 (0–2)
Rotavirus	A08.0	50 (30–100)	0 (0–0)
Sapovirus	NA	NA	NA
Parasite			
Cryptosporidium spp.	A07.2	40 (6–100)	0
Giardia lamblia	A07.1	100 (25–300)	0
Subtotal		5,900 (4,700–7,500)	21 (14–26)
Unknown etiology	A08.4, A09, A09.0, A09.9	24,700 (22,600–27,800)	39 (27–54)
Total		30,600 (28,000–34,000)	60 (53–63)
Nongastrointestinal illness			
Hepatitis A	B15.9	20 (6–50)	0 (0–2)
Listeriosis	A32	150 (100–250)	15 (9–20)
Toxoplasmosis	B58	30 (10–60)	1 (0–2)
Ciguatera	T61.0	25 (10–40)	0
Scombrotoxicosis	T61.1	8 (5–10)	0
Total		240 (180–350)	16 (10–21)

*All estimates based on an empirical distribution of the Australian population in the June quarter of 2006–2010 for hospitalizations and 2001–2010 for death; see online Technical Appendix 3 (http://wwwnc.cdc.gov/EID/article/2011/13-1315-Techapp3.pdf) for the methods used to determine these estimates. CrI, credible interval; ICD-10-AM, Australian modification of the 10th International Classification of Diseases; NA, not applicable. *E. coli*, *Escherichia coli*; STEC, Shiga toxin–producing *E.coli.*

The methods we used to calculate estimates in this study were refined from those used for the circa 2000 study, and in the intervening years, surveillance has improved and data availability has increased. In addition, we used national data to incorporate variations in foodborne disease patterns to provide more representative estimates. A further improvement was our use of more detailed hospitalization data. Previous hospitalization estimates for foodborne gastroenteritis were determined by using the hospital principal diagnosis data with a multiplier to adjust for additional diagnoses. In this study, we used the principal plus additional diagnoses data so that we could identify different diagnosis patterns by pathogen; for example, we found that 77% of the hospital diagnoses for salmonellosis were listed as principal diagnoses, whereas 37% of the diagnoses for norovirus infection were listed as principal diagnoses. Our new approach better captures different diagnosis patterns, especially for illnesses with multiple concomitant conditions (e.g., listeriosis) (*26*).

We also incorporated new expert elicitations into our methods to determine the circa 2010 estimates, further improving data quality (*19*). These expert elicitations were undertaken in 2009 to decide which pathogens/agents should be included in the estimates and to determine the proportion of cases caused by foodborne transmission. Compared with estimates obtained by using the Delphi process in 2005 (*21*), the estimated proportion of foodborne transmission in the circa 2010 study was generally lower, and uncertainty bounds were generally wider. In particular, our estimates showed a lower proportion of foodborne transmission for *Clostridium perfringens*, other pathogenic *E. coli*, norovirus, nontyphoidal *Salmonella* spp., and Shiga toxin–producing *E. coli* (STEC). This finding may reflect that environmental sources of gastrointestinal infection have been somewhat neglected and that health departments have a primary focus on foodborne diseases (*19*). Compared with previously published estimates for 2000 (*7*), our estimates for circa 2000 showed fewer illnesses attributed to food; this difference was due to our use of lower foodborne proportions for some pathogens.

When estimating the community incidence of foodborne illness, we used underreporting multipliers to adjust

for the proportion of infected persons who did not seek treatment or submit specimens for testing. We used previously published estimates (*16*) of pathogen-specific multipliers for nontyphoidal *Salmonella* spp., *Campylobacter* spp., and STEC. The underreporting multiplier used for nontyphoidal *Salmonella* spp. (7, 95% CrI 4–14) was extrapolated to all other moderate illnesses, except *Campylobacter* spp. and STEC. These new underreporting multipliers were smaller than those used in previously published estimates for Australia (15, 95% CrI 5–25) (*7*).

The underreporting multiplier for serious illnesses and the underdiagnosis multiplier for hospitalizations and deaths remained at 2 (CrI 1–3), consistent with usage in other studies (*2*,*17*,*27*). The use of this multiplier for hospitalizations and deaths was validated by comparing data from the OzFoodNet Outbreak Register with hospital and death data, which suggested that a multiplier of at least 2 was necessary to account for underdiagnosis. Data on pathogen-specific underdiagnosis are limited, and further studies are required to thoroughly validate this multiplier and assess whether there are pathogen-specific differences in the underdiagnosis of severe illness.

The incidence of cases, hospitalizations, and deaths associated with foodborne pathogens in Australia does not show the complete burden from these pathogens because infection with some of them (i.e., *Campylobacter* spp., nontyphoidal *Salmonella* spp., and STEC) may lead to sequelae. The estimates in this study, together with our estimates of sequelae (*8*), highlight the considerable effect of foodborne *Campylobacter* spp. infection in Australia (*28*).

In a complex study of this type, there are several gaps and limitations in the data. While NNDSS and the OzFoodNet Outbreak Register are nationally representative, jurisdictions may have reported or coded their data differently. In addition, there were no available Australian data on toxoplasmosis, so we relied on data from the United States (*29*). We used data from the WQS (*10*,*11*) for pathogens that were not nationally notifiable or had limited outbreak data. The WQS study was the best of its kind in Australia; however, the data are now >15 years old, and the study population was based on families in Melbourne with children. We adjusted WQS data for changes over time and weighted the data for the age structure of the general population (online Technical Appendix 2). In addition, cohort study participants may be reluctant to provide fecal samples; in the WQS, only one third of persons with gastroenteritis submitted a fecal sample (*11*). Furthermore, the WQS did not test for all known foodborne pathogens, and a pathogen was identified for only 17% of the fecal specimens that were examined (*10*).

The estimated incidence of foodborne disease in Australia circa 2010 was considerable: 4.1 million cases (90% CrI 2.3–6.4) of foodborne gastroenteritis and 5,140 cases (90% CrI 3,530–7,980) of nongastrointestinal foodborne illness occurred annually. Most foodborne illness occurs as gastroenteritis, but the effect of nongastrointestinal illnesses and sequelae are substantial because they can result in hospitalization and, occasionally, death. We identified that over time, the incidence of all foodborne gastroenteritis declined, but the incidences of salmonellosis and campylobacteriosis increased, although changes were not significant due to amount of uncertainty inherent in our estimates. These findings should assist policy makers to advocate for improved regulation and control of foodborne disease for specific pathogens.

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Sequelae of Foodborne Illness Caused by 5 Pathogens, Australia, Circa 2010

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In Australia circa 2010, 4.1 million (90% credible interval [CrI] 2.3–6.4 million) episodes of foodborne gastroenteritis occurred, many of which might have resulted in sequelae. We estimated the number of illnesses, hospitalizations, and deaths from Guillain-Barré syndrome, hemolytic uremic syndrome, irritable bowel syndrome, and reactive arthritis that were associated with contaminated food in Australia. Data from published studies, hospital records, and mortality reports were combined with multipliers to adjust for different transmission routes. We used Monte Carlo simulation to estimate median estimates and 90% CrIs. In Australia, circa 2010, we estimated that 35,840 (90% CrI 25,000–54,000) illnesses, 1,080 (90% CrI 700–1,600) hospitalizations, and 10 (90% CrI 5–14) deaths occurred from foodborne gastroenteritis–associated sequelae. *Campylobacter* spp. infection was responsible for 80% of incident cases. Reducing the incidence of campylobacteriosis and other foodborne diseases would minimize the health effects of sequelae.

Foodborne gastroenteritis is a major source of illness in Australia, causing an estimated 4.1 million (90% credible interval [CrI] 2.3–6.4 million) illnesses, 30,600 (90% CrI 28,000–34,000) hospitalizations, and 60 (90% CrI 53–63) deaths each year (*1*). In addition to the direct effects of these illnesses, infection with some pathogens can result in sequelae, which can be severe, require multiple hospitalizations, and be costly to society (*2*). We report on the effects of sequelae associated with Guillain-Barré syndrome (GBS), hemolytic uremic syndrome (HUS), irritable bowel syndrome (IBS), and reactive arthritis (ReA) from 5 pathogens acquired from contaminated food in Australia.

Each of these 4 sequel illnesses are preceded by different gastrointestinal infections and have unique characteristics. GBS, a rare but serious autoimmune illness, affects the nervous system and causes acute flaccid paralysis. GBS can occur as a sequel to *Campylobacter* spp. infection 10 days–3 weeks after gastrointestinal illness (*3*,*4*). HUS is characterized by acute renal failure, hemolytic anemia, and thrombocytopenia and can result from infection with Shiga toxin–producing *Escherichia coli* (STEC) ≈4–10 days after onset of gastroenteritis (*5*,*6*). IBS is a gastrointestinal disorder that causes abdominal pain and bowel dysfunction. It is not life threatening, but it can cause substantial health effects after illness with *Campylobacter* spp., nontyphoidal *Salmonella enterica* serotypes (hereafter referred to as nontyphoidal *Salmonella* spp.), or *Shigella* spp. (*7*,*8*). ReA is a type of spondyloarthritis that can develop up to 4 weeks after an enteric infection from *Campylobacter* spp., nontyphoidal *Salmonella* spp., *Shigella* spp., or *Yersinia enterocolitica* (*9*). We estimated the number of illnesses, hospitalizations, and deaths resulting from GBS, HUS, IBS, and ReA from selected foodborne pathogens in Australia in a typical year circa 2010.

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Methods

We estimated the effects of foodborne sequelae acquired in Australia circa 2010 using data from multiple sources in Australia and from international peer-reviewed literature. We defined foodborne sequelae as illnesses occurring after bacterial gastroenteritis caused by eating contaminated food. Sequelae were defined as the secondary adverse health outcomes resulting from a previous infection by a microbial pathogen and clearly distinguishable from the initial health event (*10*). Illness can be acute, such as with HUS, or chronic (lasting for many years), as with IBS. We estimated incidence, hospitalizations, and deaths with uncertainty bounds using Monte Carlo simulation in @Risk version 6 (http://www.palisade.com/), which incorporates uncertainty in both data and inputs. Each stage of our calculation was represented by a probability distribution, and our final estimates of incidence, hospitalizations, and deaths were summarized by the median and 90% CrI. Similar to a recent study in the United States (*11*), we used empirical distributions for source distributions, such as the number of hospitalizations or deaths, to avoid assumptions about the expected shape of these distributions. All other inputs were modeled by using the PERT (project evaluation and review technique) distribution, which enables the input of a minimum, maximum, and modal value, or 3 percentile points, such as a median value and 95% bounds. We used this distribution widely in our analyses because

it enables asymmetric distributions and can be produced from many data sources, including expert elicitation data. The Australian National University Human Research Ethics Committee approved the study.

Incidence of Sequelae

Several pathogens are associated with the development of sequelae. Community estimates of foodborne illness from Kirk et al. (*1*) for *Campylobacter* spp., nontyphoidal *Salmonella* spp., *Shigella* spp., STEC, and *Y. enterocolitica* were used for estimating the incidence of foodborne sequelae (Table 1). Although *Shigella* spp. and nontyphoidal *Salmonella* spp. have been associated with HUS and STEC has been associated with IBS and ReA, data on which to base estimates are limited. In addition, although other pathogens, such as *Chlamydia trachomatis*, *Clostridium difficile*, *Giardia lamblia*, and norovirus, have been associated with these sequelae (*12*–*15*), we assessed only pathogens commonly associated with sequelae, domestically acquired, and with a foodborne transmission pathway. A "sequelae multiplier," which is the proportion of sequelae cases that develop after enteric infection with a specific bacterial pathogen, was applied to our estimates of domestically acquired foodborne gastroenteritis cases caused by that pathogen (*1*). For each sequel illness, we reviewed relevant studies published during 1995–2012 using systematic reviews and studies using Australian data where possible to estimate the relevant sequelae multipliers. We reviewed articles about sequelae after infection with *Campylobacter* spp., *E. coli*, nontyphoidal *Salmonella* spp., *Shigella* spp., and *Y. enterocolitica*, and we estimated sequelae multipliers for GBS, HUS, IBS, and ReA after bacterial gastrointestinal infection on the basis of these reviews (Table 2). Relevant articles and additional information are documented in online Technical Appendix 1 (http://wwwnc.cdc.gov/EID/article/20/11/13-1316-Techapp1.pdf).

Our sequelae multiplier for GBS was based on 30.4 (range 19.2–94.5) cases of GBS per 100,000 cases of campylobacteriosis using data from studies from the United Kingdom, Sweden, and the United States (*16*–*18*). For HUS, the sequelae multiplier used was 3% (95% CI 1.7%–5.4%) from a South Australian study on STEC and HUS notifications during 1997–2009 (*19*). On the basis of data from Haagsma et al. (*20*), we assumed that 8.8% (95% CI 7.2%–10.4%) of foodborne disease caused by *Campylobacter* spp., nontyphoidal *Salmonella* spp., and *Shigella* spp. result in IBS. We used a separate sequelae multiplier for each pathogen that resulted in ReA. We assumed that 7% (range 2.8%–16%) of foodborne cases of *Campylobacter* spp., 8.5% (range 0%–26%) of foodborne cases of nontyphoidal *Salmonella* spp., 9.7% (range 1.2%–9.8%) of foodborne cases of *Shigella* spp., and 12% (range 0%–23.1%) of foodborne cases of *Y. enterocolitica* result in ReA (see full reference list in online Technical Appendix 1). Total foodborne IBS and ReA cases reflect the sum of modeled IBS and ReA cases from these 3 and 4 pathogens, respectively. Details on the sequelae multipliers and incidence estimation methods are in online Technical Appendix 1 and online Technical Appendix 2 (http://wwwnc.cdc.gov/EID/article/20/11/13-1316-Techapp2.pdf).

We compared the incidence of sequelae circa 2010 to that of sequelae circa 2000 by applying the same sequelae multipliers to estimates of the incidence of acute gastroenteritis to specific pathogens in 2006–2010 and 1996–2000, respectively. The estimates of incidence of acute gastroenteritis were based on notification data for *Campylobacter* spp., nontyphoidal *Salmonella* spp., *Shigella* spp., STEC, and *Y. enterocolitica* (*19*,*21*,*22*), (online Technical Appendix 3, http://wwwnc.cdc.gov/EID/article/20/11/13-1316-Techapp3.pdf).

Hospitalizations and Deaths

To estimate hospitalizations associated with IBS from foodborne *Campylobacter* spp., nontyphoidal *Salmonella* spp., and *Shigella* spp. and hospitalizations associated with ReA from foodborne *Campylobacter* spp., nontyphoidal *Salmonella* spp., *Shigella* spp., and *Y. enterocolitica*, we used hospitalization data for 2006–2010 from all Australian states and territories, according to the International Classification of Diseases, Tenth Revision, Australian Modification (ICD-10-AM) codes. To estimate deaths for all 4

Table 1. Pathogens associated with GBS, HUS, IBS, and ReA included in this study, Australia, circa 2010*

Pathogen	GBS	HUS	IBS	ReA
Campylobacter spp.	X		X	X
Nontyphoidal *Salmonella* spp.†			X	X
Shigella spp.			X	X
Shiga toxin–producing *Escherichia coli*		X		
Yersinia enterocolitica				X

*GBS, Guillain-Barré syndrome; HUS, hemolytic uremic syndrome; IBS, irritable bowel syndrome; ReA, reactive arthritis.
†Nontyphoidal *S. enterica* serotypes.

Table 2. Sequelae multipliers extracted from the literature about domestically acquired foodborne bacterial gastroenteritis*

Sequelae	ICD-10-AM code	Incidence after bacterial infection, %
GBS, median (range)	G61.0	0.0304 (0.0192–0.0945)
HUS, median (95% CI)	D59.3	3 (1.7–5.1)
IBS, median (90% CrI)	K58.0 K58.9	8.8 (7.2–10.4)
ReA, median (range)	M02.1 M02.3 M02.8 M03.2	7–12 (0–26)

*CrI. credible interval; GBS, Guillain-Barré syndrome; HUS, hemolytic uremic syndrome; IBS, irritable bowel syndrome; ICD-10-AM, International Classification of Diseases, Tenth Revision, Australian Modification; ReA, reactive arthritis.

sequelae illnesses resulting from the respective foodborne pathogens, we used national death data for 2001–2010 from the Australian Bureau of Statistics, using ICD-10-AM codes (online Technical Appendix 4, http://wwwnc.cdc.gov/EID/article/20/11/13-1316-Techapp4.pdf). Principal diagnosis and additional diagnoses were included for hospitalizations, and underlying and contributing causes were included for deaths. Because we had only 1 year of hospitalization data for Victoria and 2 years for New South Wales, we extrapolated from these data to derive a distribution of the number of hospitalizations across all states, which was modeled as an empirical distribution. For these states, we assumed the same number of hospitalizations each year to adjust for missing data. Because of the severity of GBS and HUS, hospitalization estimates for these illnesses were not modeled, and all persons with estimated incident cases from contaminated food were considered to have been hospitalized.

We estimated incidences of hospitalization and death using a statistical model that incorporates uncertainty in case numbers and in multipliers using probability distributions (Figure), which is adjusted from the hospitalization estimation flow chart in Kirk et al. (*1*). We assumed that all estimated incident foodborne *Campylobacter*-associated GBS and STEC-associated HUS case-patients were hospitalized, so those cases were not modeled; however, multipliers were still needed for GBS and HUS to estimate deaths. Sequelae-associated deaths were estimated by using the same methods as for hospitalizations (Figure). Input data arose from the data sources discussed above or from multipliers that are discussed below.

Domestically Acquired Multiplier

The "domestically acquired multiplier" adjusted for the proportion of case-patients who acquired their infection in Australia. We estimated domestically applied multipliers for the antecedent bacterial gastrointestinal pathogens using notifiable surveillance data from each state, extrapolated to give national estimates (*1*). We adopted the domestically acquired multiplier for *Campylobacter* spp. of 0.97 (90% CrI 0.91–0.99) for GBS and the domestically acquired multiplier for STEC 0.79 (90% CrI 0.73–0.83) for HUS (*1*). For IBS and ReA, a combined domestically acquired multiplier for *Campylobacter* spp., nontyphoidal *Salmonella* spp., and *Shigella* spp. for IBS and *Campylobacter* spp., nontyphoidal *Salmonella* spp., *Shigella* spp. and *Y. enterocolitica* for ReA was calculated as a weighted average of the domestically acquired multipliers for each pathogen, weighted by the total number of IBS and ReA cases for each pathogen, respectively (online Technical Appendix 4; online Technical Appendix 5, http://wwwnc.cdc.gov/EID/article/20/11/13-1316-Techapp5.pdf).

Proportion Foodborne Multiplier

For each of the 4 sequelae, we calculated the proportion of hospitalizations and deaths from foodborne pathogens using 2 multipliers: a "bacterial multiplier" to attribute the proportion of overall cases of each of the sequelae illnesses to specific pathogens and a "foodborne multiplier" to attribute illnesses to foodborne exposure. The bacterial multiplier, which was the proportion of sequel cases attributable to their antecedent bacterial pathogen, was extracted from systematic reviews for GBS and HUS (*4*,*23*) and multiplied by the foodborne proportion for *Campylobacter* spp. and STEC, respectively. For IBS and ReA, from the literature we extracted a midpoint and range of the proportion of cases that resulted from infectious gastroenteritis (*12*,*20*,*24*). The IBS bacterial multiplier was then further multiplied by a foodborne multiplier for *Campylobacter* spp., nontyphoidal *Salmonella* spp., and *Shigella* spp., which was calculated as a weighted average of the foodborne multipliers for each pathogen, weighted by the total number of IBS cases for each pathogen. The ReA bacterial multiplier was then also multiplied by the foodborne multiplier for *Campylobacter* spp., nontyphoidal *Salmonella* spp., *Shigella* spp., and *Y. enterocolitica* by using a weighted average of the foodborne multipliers for each pathogen as was done for IBS (online Technical Appendices 4 and 5).

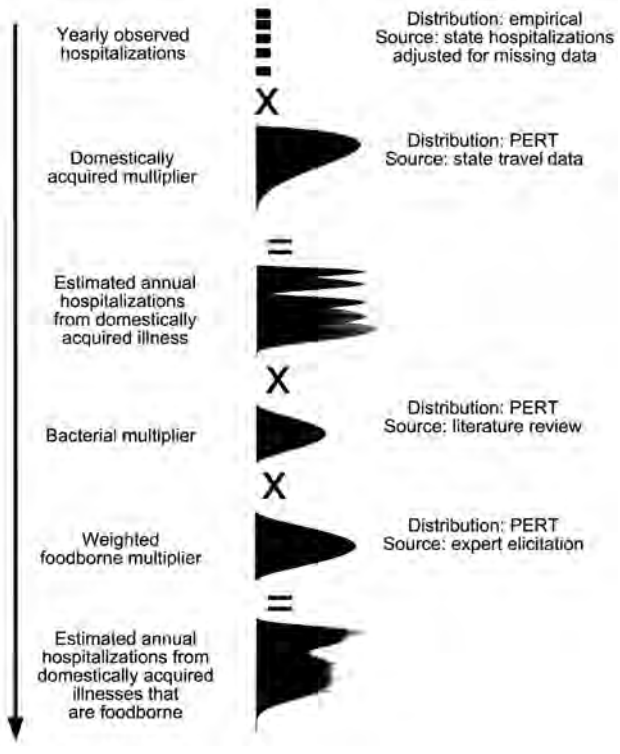

Figure. Flow chart for the approach used to calculate the estimated annual number of hospitalizations for sequelae associated with foodborne illness caused by 5 pathogens, Australia, circa 2010.

Results

Incidence

We estimated that, circa 2010 in Australia, 70 (90% CrI 30–150) new cases of *Campylobacter*-associated GBS, 70 (90% CrI 25–200) new cases of STEC-associated HUS, 19,500 (90% CrI 12,500–30,700) new cases of *Campylobacter*-, nontyphoidal *Salmonella*– and *Shigella*-associated IBS, and 16,200 (90% CrI 8,750–30,450) new cases of *Campylobacter*-, nontyphoidal *Salmonella*-, *Shigella*-, and *Y. enterocolitica*–associated ReA were domestically acquired and caused by contaminated food (Table 3). We estimated that 35,840 (90% CrI 25,000–54,000) domestically acquired sequel illnesses resulted from foodborne gastroenteritis—an incidence rate of 1,620 (90% CrI 1,150–2,450) sequelae cases per million population. *Campylobacter* spp. infection resulted in the largest number of sequelae cases annually; ≈80% of the 36,000 sequel illnesses were attributable to *Campylobacter* spp. alone.

Comparison with Estimates Circa 2000

Using data circa 2000, we estimated that 50 GBS cases, 55 HUS cases, 14,800 IBS cases, and 12,500 ReA cases occurred each year. Elsewhere, we estimated that the rate of foodborne campylobacteriosis was approximately 13% higher in 2010 than 2000 (*1*); this increase led to a 13% increase in *Campylobacter*-associated GBS in 2010 over 2000. Similarly, we estimated that the rate of foodborne salmonellosis was 24% higher in 2010 than in 2000 (*1*). These factors combine to explain much of the increase in IBS and ReA. The rate of STEC-associated HUS remained about the same in 2000 and 2010 (online Technical Appendix 3).

Hospitalizations and Deaths

We estimated that, circa 2010 in Australia, 1,080 (90% CrI 700–1,600) hospitalizations for sequelae illnesses occurred from domestically acquired foodborne gastroenteritis, equating to 50 (90% CrI 30–70) hospitalizations per million population per year (Table 4). We estimated a total of 10 (90% CrI 5–14) deaths from sequelae to domestically acquired foodborne gastroenteritis—a rate of 0.5 (90% CrI 0.2–0.6) deaths per million population per year (Table 4).

Discussion

Our study demonstrates that foodborne gastroenteritis in Australia results in substantial severe and disabling sequelae. We estimated a yearly rate of 1,620 incident cases of sequelae illnesses, 50 hospitalizations, and 0.5 deaths per million population circa 2010. In addition, a comparison with estimates recalculated for 2000 indicates an increase in the rates of GBS, IBS, and ReA since 2000, which is consistent with and directly related to rising levels of antecedent foodborne illnesses caused by *Campylobacter* spp. and nontyphoidal *Salmonella* spp. during this period (*1*). This increase highlights the importance of quantifying sequelae when estimating the effects of foodborne disease and provides further impetus for reducing illness from foodborne bacterial pathogens.

The impact of *Campylobacter* spp. infection in the community is high. Approximately 179,000 cases of foodborne campylobacteriosis occur in Australia each year (*1*), and *Campylobacter* spp. was responsible for 80% of the foodborne sequelae illness estimated in this study. The reported rate of infection from *Campylobacter* spp. in Australia has increased since 2010 (*1*) and is higher than in many other industrialized countries. For example, the rate of *Campylobacter* spp. for Australia was ≈10 times higher than that for the United States (*25*), double that for the Netherlands (*26*), and slightly higher than that for the United Kingdom (*27*). In the Netherlands, a lower rate of acute *Campylobacter* spp. gastroenteritis has contributed to lower estimates of rates of sequel illnesses than our estimates for GBS, IBS, and ReA (*26*).

In New Zealand, food safety interventions have been effective in lowering campylobacteriosis rates and sequelae. In 2006, high campylobacteriosis notification rates (>3,800 cases per million population) prompted increased research on *Campylobacter* spp., which resulted in the introduction of food safety and poultry industry interventions, including *Campylobacter* spp. performance targets at primary processing plants and promotion of freezing all fresh poultry meat (*28*). By 2008, the rate of campylobacteriosis notifications decreased by 54% to 1,615 cases per million population (*28*). In addition, after these interventions in New Zealand, the rate of GBS hospitalizations decreased by 13% (*29*). The less dramatic decrease in GBS than in campylobacteriosis might be explained by the fact that *Campylobacter* spp. is not the only cause of GBS. If Australia were to experience decreases similar to those in New Zealand, we would expect the rate of foodborne campylobacteriosis in the community to drop from approximately 8,400 to 3,864 cases per million population. Sequelae would decrease from 1,620 to 870 cases per million population per year. Furthermore, total GBS-associated hospitalizations, including GBS from all causes and readmissions, would decrease from ≈73 to 63 hospitalizations per million population annually.

A comparison of our foodborne *Campylobacter*-associated GBS incidence estimates with raw hospitalization data showed many more hospitalizations than incident cases. This finding probably is attributable to repeat hospitalizations. We took a conservative approach by basing incidence estimates on community estimates of campylobacteriosis and assuming that all persons with incident cases were hospitalized. A yearly median of 1,536 (range

Table 3. Estimated number of sequelae illnesses resulting from domestically acquired foodborne bacterial gastroenteritis, Australia, circa 2010*

Sequelae, pathogen	Median no. Illnesses (90% CrI)	Median rate (90% CrI)†
GBS, *Campylobacter* spp.	70 (30–150)	3.1 (2–6)
HUS, STEC	70 (25–200)	3.3 (1–9)
IBS		
Campylobacter spp	15,600 (9,000–26,500)	915 (570–1,440)
Nontyphoidal *Salmonella* spp.‡	3,500 (1,900–6,500)	
Shigella spp.	30 (10–80)	
Total§	19,500 (12,500–30,700)	
ReA		
Campylobacter spp.	12,500 (5,500–25,500)	765 (415–1,375)
Nontyphoidal *Salmonella* spp.‡	3,250 (700–9,000)	
Shigella spp.	29 (10–75)	
Yersinia enterocolitica	150 (50–300)	
Total§	16,200 (8,500–30,000)	
Total	35,840 (25,000–54,000)	1,620 (1,150–2,450)

*CrI, credible interval; GBS, Guillain-Barré syndrome; HUS, hemolytic uremic syndrome; IBS, irritable bowel syndrome; ReA, reactive arthritis; STEC, Shiga toxin–producing *Escherichia coli*.
†No. cases per million population.
‡i.e., nontyphoidal *S. enterica* serotypes.
§Simulated values, which might not add to total because of rounding and variation over simulations.

1,428–1,632) primary and additional GBS diagnoses occurred in Australian hospitals during 2006–2010 (including GBS from all causes and readmissions) and equates to a median rate of 73.1 (range 64.7–77.4) GBS-associated hospitalizations per million population each year. This rate is within the range from a New Zealand study, which found a median rate of 56.3 (range 42.1–75.9) GBS-associated hospitalizations during a 13-year period, with ≈41% of case-patients being readmitted, resulting in 23.2 (range 15.3 29.3) incident GBS hospitalizations per million population each year (*29*). If we assume that 41% of Australia's 1,536 GBS hospitalizations are readmissions and apply the domestically acquired multiplier and foodborne proportion multiplier used to estimate GBS-associated deaths (online Technical Appendix 4), we would estimate 170 (90% CrI 60–265) incident foodborne *Campylobacter*-associated GBS hospitalizations. This point estimate is higher than our current estimate of 70, although the credible interval includes our estimate. A validation study of medical records of persons with GBS would enable us to better characterize readmissions for GBS.

Our approach has several limitations. First, our comparison of sequelae estimates for 2000–2010 assumes a constant rate of sequelae illness after gastrointestinal infection over time. Although our methods provide an indirect method of assessing changes in sequelae incidence over time, the approach is useful because it enables comparison of the population-level effect of sequelae at these 2 time points. Second, our study measured incidence and not prevalence of sequelae. We estimated the number of new cases every year and did not quantify the long-term effects of these sequelae. Third, our study does not estimate all sequelae illness from foodborne disease pathogens. We did not include sequelae, such as end-stage renal disease, inflammatory bowel disease, and encephalitis, in our estimates. We chose GBS, HUS, IBS, and ReA for this study because they were known, well studied, and well characterized in available data sources. These provide a good basis to begin to understand the effects of foodborne sequelae and the policy implications of reducing illness from preceding bacterial pathogens.

Our estimates for GBS, HUS, IBS, and ReA incidence relied heavily on the quality of the literature we reviewed. We used Australian data and systematic reviews wherever possible. The Australian hospitalization and deaths data we used were of high quality and included both principal and additional diagnoses from all states. However, because data were missing from some states in some years, we extrapolated from these data to the remaining years. Finally, ICD-10 and ICD-10-AM coding can be problematic when co-morbid conditions are present, when hospital transfers occur, or when diagnostic criteria are inconsistent. Therefore, our estimates for sequelae hospitalizations and deaths may be conservative because they do not account for these coding errors.

Table 4. Estimated number of sequelae-associated hospitalizations and deaths caused by domestically acquired foodborne bacterial gastroenteritis, Australia, circa 2010*

	Hospitalizations		Deaths	
Sequelae	Median no. (90% CrI)	Rate (90% CrI)†	Median no. (90% CrI)	Rate (90% CrI)†
GBS	70 (30–150)	3.1 (2–6)	6 (2–10)	0.3 (0.1–0.5)
HUS	70 (25–200)	3.3 (1–9)	2 (1–3)	0.1 (0.03–0.12)
IBS	915 (550–1,400)	43 (25–70)	2 (1–2)	0.1 (0.05–0.11)
ReA	25 (20–40)	1 (1–2)	0	0
Total	1,080 (700–1,600)	50 (30–70)	10 (5–14)	0.5 (0.2–0.6)

*CrI, credible interval. GBS, Guillain-Barré syndrome; HUS, hemolytic uremic syndrome; IBS, irritable bowel syndrome; ReA, reactive arthritis; STEC, Shiga toxin–producing *Escherichia coli*.
†Cases per million population.

The sequelae estimates from this study showed that the impact of foodborne *Campylobacter* spp., nontyphoidal *Salmonella* spp., *Shigella* spp., STEC, and *Y. enterocolitica* was much greater then when consideration is given simply to the initial acute illness. *Campylobacter* spp. infection, in particular, was highlighted as an increasing problem in Australia. Our estimates provide a basis for costing studies, which can be useful for developing food safety policies and interventions. Finally, our study highlights the need for better data from large population-based studies in Australia to further characterize sequelae, as well as foodborne pathogens.

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Increasing Prevalence and Intensity of Foodborne Clonorchiasis, Hengxian County, China, 1989–2011

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During 1989–2011, three parasitic disease surveys were conducted in Hengxian County, China, where soil-transmitted helminthiases and foodborne clonorchiasis are endemic. We compared the data and found that the prevalence of helminthiases decreased and the prevalence and intensity of clonorchiasis increased over time, especially among men. Clonorchiasis control/intervention measures are urgently needed in this area.

Soil-transmitted helminthiases (STHs), parasitic diseases of humans, are caused by a group of intestinal nematodes, including roundworms (*Ascaris lumbricoides)*, whipworms (*Trichuris trichiura*), and hookworms (*Ancylostoma duodenale* and *Necator americanus*) (*1*,*2*). Clonorchiasis, another parasitic disease of humans, is caused by ingestion of raw freshwater fish harboring infective *Clonorchis sinensis* metacercariae (*3*,*4*). STHs and clonorchiasis are endemic to China. STHs occur mainly in western and southern China, where environmental conditions are more favorable to helminths and social infrastructure is limited; clonorchiasis occurs predominantly in southeastern and northeastern China, where residents commonly eat raw fish. According to 2 national parasitic disease surveys conducted during 1988–1992 and 2001–2004, the prevalence of STHs in China decreased from 53.6% to 19.6% and prevalence of clonorchiasis increased from 0.3% to 0.6% between surveys (*5*,*6*).

Hengxian County (2010 population 860,000 persons), located in southern China, was one of the areas sampled in 1989 (survey 1) and 2002 (survey 2), and a third survey was conducted in the county in 2011. We used data from surveys 1–3 to determine epidemiologic changes in STHs and clonorchiasis over time in Hengxian County.

The Study

Survey 1 sampled 1 natural village from each of 5 different towns in Hengxian County. (Towns in China have several administrative villages, the basic level of administration, which often comprise ≥1 natural villages.) Each sampled natural village had 500–600 residents, all of whom were eligible for survey participation. The same procedures were followed in survey 2, but only 3 towns were sampled. Survey 3 sampled 8 towns and 3 administrative villages within each town. Dozens of households were surveyed from each administrative village, and all household members ≥5 years of age were eligible for participation; ≈200 villagers were selected in each administrative village. In each survey, a single fecal sample from each participant was used to prepare a Kato-Katz thick smear to determine the presence of helminths and count *C. sinensis* eggs.

Prevalence was determined by sex and age groups. Participants were categorized as ≤4 years, 5–9 years, 10–14 years, 15–19 years, 20–29 years, 30–39 years, 40–49 years, 50–59 years, 60–69 years, or ≥70 years of age. Overall prevalence was standardized according to data from the 2010 census for Hengxian County. We defined infection intensity as the geometric mean number of eggs per gram of feces (EPG) in the egg-positive survey participants; lg(EPG + 1) transformation was used to calculate infection intensity in all survey participants. We used Student *t* test and analysis of variance to compare infection intensities between sexes and among surveys, respectively; Fisher least significant difference test was used to compare between individual surveys. Logarithmic correlation was used to explore the relationship between prevalence and infection intensity in age groups.

Survey 1 had 2,623 participants; surveys 2 and 3 had 1,748 and 3,437, respectively (Table 1). During surveys 1–3, the standardized prevalences were 19.7%, 30.5%, and 46.5%, respectively, for clonorchiasis and 86.3%, 25.1%, and 7.0%, respectively, for STHs. The corresponding standardized prevalences were 69.8%, 13.8%, and 0.5% for roundworm infections; 55.7%, 11.2%, and 1.4% for whipworm infections; and 24.3%, 6.3%, and 5.3% for hookworm infections.

During surveys 1–3, the standardized prevalences of clonorchiasis were 10.4%, 20.8%, and 29.2%, respectively, among females and 28.5%, 39.6%, and 62.9%, respectively,

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Table 1. Standardized prevalence of parasitic diseases, as determined from 3 surveys, Hengxian County, China, 1989–2011*

Infection	Prevalence, %								
	Survey 1, 1989			Survey 2, 2002			Survey 3, 2011		
	Female, n = 1,134†	Male, n = 1,227‡	Total, n = 2,361§	Female, n = 775¶	Male, n = 925 ‖	Total, n = 1,700#	Female, n = 1,641	Male, n = 1,796	Total, n = 3,437
Clonorchiasis	10.4	28.5	19.7	20.8	39.6	30.5	29.2	62.9	46.5
Soil-transmitted helminthiases**	86.2	86.3	86.3	29.5	20.9	25.1	9.4	4.7	7.0
Roundworms	70.1	69.6	69.8	16.9	10.9	13.8	0.5	0.4	0.5
Whipworms	57.0	54.5	55.7	13.1	9.4	11.2	1.7	1.0	1.4
Hookworms	27.8	21.1	24.3	7.8	4.9	6.3	7.5	3.2	5.3

*Population data were derived from the 2010 census for Hengxian County.
†117 children <5 years of age were excluded.
‡145 children <5 years of age were excluded.
§262 children <5 years of age were excluded.
¶24 children <5 years of age were excluded.
‖ 24 children <5 years of age were excluded.
#48 children <5 years of age were excluded.
**Some participants were infected with >1 type of helminth.

among males. The corresponding standardized prevalences of STHs were 86.2%, 29.5%, and 9.4% among females and 86.3%, 20.9%, and 4.7% among males. The corresponding standardized prevalences of roundworm, whipworm, and hookworm infections among males and females showed a decreasing trend similar to that for the total STHs.

In surveys 1–3, clonorchiasis prevalence increased fairly steadily by age group to middle age, after which, prevalence continued to increase with age in survey 1 and decreased with age in surveys 2 and 3 (Figure 1, panel A). STHs prevalence changed irregularly by age group in surveys 1 and 2 but increased gradually by age group in survey 3 (Figure 1, panel B). Prevalences of roundworm and whipworm infections usually peaked among children 5–14 years of age; hookworm infections peaked in middle-aged age groups in surveys 1 and 2 and in older age groups in survey 3.

In *C. sinensis* egg–positive participants, the geometric mean numbers of EPG were 367.3 EPG, 833.3 EPG, and 942.9 EPG in surveys 1–3, respectively (F = 36.2, p<0.001) (Table 2). Differences were significant between survey 1 and surveys 2 and 3 (p<0.001) but not between survey 2 and survey 3 (p = 0.210). In surveys 1–3, geometric mean numbers of EPG were 241.7 EPG, 364.9 EPG, and 405.1 EPG, respectively, among females (F = 3.3, p<0.05) and 419.8 EPG, 1,199.7 EPG, and 1,363.2 EPG, respectively, among males (F = 43.6, p<0.001). Among all participants, the geometric mean numbers of EPG were 1.6 EPG, 6.3 EPG, and 38.2 EPG during surveys 1–3, respectively (F = 522.9, p<0.001); differences between surveys were all significant (p<0.001). In surveys 1–3, geometric mean numbers of EPG were 0.6 EPG, 2.2 EPG, and 6.8 EPG, respectively, among females (F = 137.6, p<0.001) and 3.1 EPG, 13.5 EPG, and 171.5 EPG, respectively, among males (F = 474.4, p<0.001). Among egg-positive participants and all participants, infection intensities were all significantly lower among females than males, and infection intensities increased as

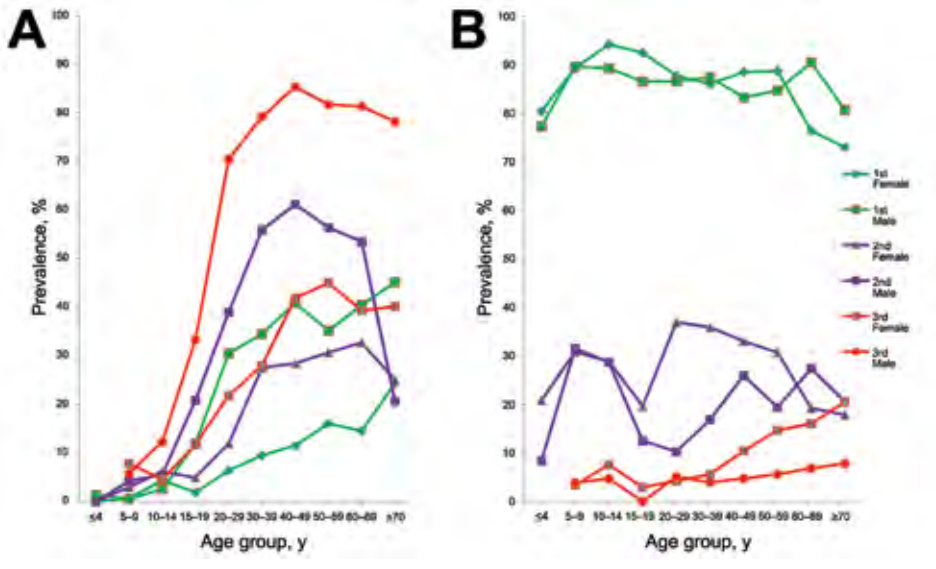

Figure 1. Prevalence of clonorchiasis (A) and soil-transmitted helminthiases (STHs) (B) among sex and age groups during 3 parasitic disease surveys, Hengxian County, China, 1989–2011. Green indicates the first survey (1989); purple indicates the second survey (2002); red indicates the third survey (2011).

Table 2. Differences in infection intensity between male and female participants in 3 parasitic disease surveys, Hengxian County, China, 1989–2011*

Sex	Geometric mean no. EPG among *Clonorchis sinensis* egg–positive participants			Geometric mean no. EPG among all participants		
	Survey 1, 1989	Survey 2, 2002	Survey 3, 2011	Survey 1, 1989	Survey 2, 2002	Survey 3, 2011
Female	241.7	364.9	405.1	0.6	2.2	6.8
Male	419.8	1,199.7	1,363.2	3.1	13.5	171.5
Total	367.3	833.3	942.9	1.6	6.3	38.2

*Geometric mean number of EPG was used as the indicator of infection intensity. Children <5 years of age were excluded. EPG, eggs per gram of feces.

ages increased, reaching a peak in middle-aged persons and then decreasing in older persons (Figure 2).

In surveys 1–3, clonorchiasis prevalence in different age groups was significantly correlated with the corresponding infection intensity in terms of the geometric mean number of EPG after logarithmic transformation ($p<0.01$). Determination coefficients (R^2) were 0.709, 0.891, and 0.748 in surveys 1–3, respectively, when only egg-positive participants were included in the analysis; corresponding R^2 were 0.901, 0.980, and 0.997, respectively, for all participants.

Conclusions

Analysis of data from 3 parasitic disease surveys conducted in Hengxian County over the last 22 years showed substantial decreases in the trend of STHs prevalence and substantial increases in the patterns of clonorchiasis prevalence and infection intensity. These findings indicate a transitioning pattern of the most prominent parasitic disease changing from STHs to clonorchiasis. Distribution patterns of prevalence and infection intensity among sex and age groups showed that men have been most affected by increases in *C. sinensis* transmission and infection. Thus, control measures and health education programs are urgently needed, especially among men, to reduce *C. sinensis* disease and transmission.

National STHs surveillance showed a similar decreasing trend (*7*). This trend may partly be explained by massive health education programs and chemotherapy for STHs, which were implemented in schools in China after the first national parasitic disease survey (*8*). In addition, over the last 30 years, rapid economic development has occurred in China, resulting in increased availability of safe water and establishment of sanitary lavatories for improved hygiene (*9*). Furthermore, the increased use of chemical fertilizer has reduced the use of human feces as fertilizer (a source of helminth transmission) (*10*). However, economic development has also promoted development of aquaculture and made it economically possible for more residents to include freshwater fish in their diets, a source of *C. sinensis* metacercariae when consumed raw (*11*).

Clonorchiasis is a neglected parasitic disease in China: no nationwide intervention or control programs have been implemented to reduce the caseload (*12*). A third national survey is expected to be conducted beginning in 2014, which will provide updated data regarding the national status of clonorchiasis and an impetus for public health control/intervention and education programs. A national clonorchiasis surveillance system should also be established to gather up-to-date information and inform public health policymakers (*13*).

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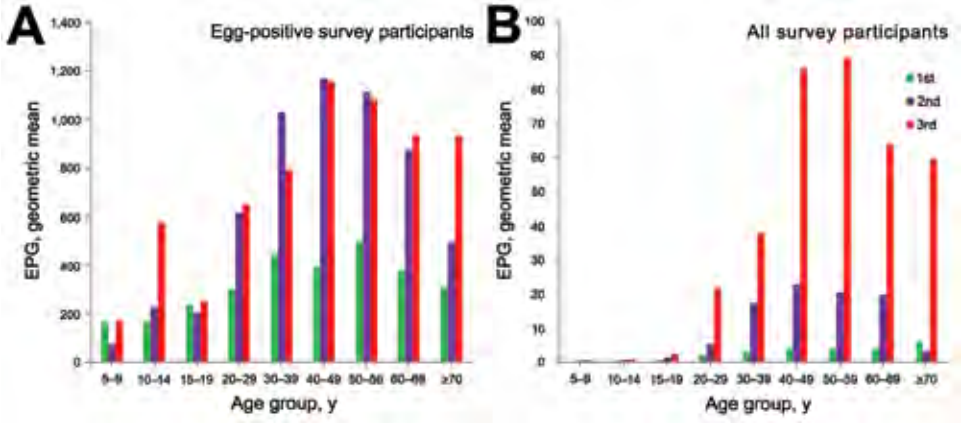

Figure 2. Infection intensity of clonorchiasis among persons in different age groups during 3 parasitic disease surveys, Hengxian County, China, 1989–2011. A) *Clonorchis sinensis* egg–positive survey participants. B) All survey participants. EPG, eggs per gram of feces. Green indicates the first survey (1989); purple indicates the second survey (2002); red indicates the third survey (2011).

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Mycobacterium ulcerans Infection Imported from Australia to Missouri, USA, 2012

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Buruli ulcer, the third most common mycobacterial disease worldwide, rarely affects travelers and is uncommon in the United States. We report a travel-associated case imported from Australia and review 3 previous cases diagnosed and treated in the United States. The differential diagnoses for unusual chronic cutaneous ulcers and those nonresponsive to conventional therapy should include *Mycobacterium ulcerans* infection.

Buruli ulcer disease, caused by *Mycobacterium ulcerans*, is the third most common mycobacterial disease worldwide. Cases are concentrated in sub-Saharan Africa but also occur in subtropical and nontropical regions (e.g., Australia and Japan) (*1*). *M. ulcerans* disease is not endemic to the United States, but rare cases have been reported in travelers returning from regions where the organism is endemic (*2–4*). We describe *M. ulcerans* infection in a man who returned to the United States after living abroad and review *M. ulcerans* cases reported in 3 travelers.

The Case

In December 2012, after unsuccessful treatment elsewhere, a 63-year-old white man was referred to the Veterans Affairs Medical Center, St. Louis, Missouri, USA, for evaluation of skin ulcers. Previous medical history indicated hypertension and hyperlipidemia. The patient sought care in April 2012 for a persistent, nonpainful ulcer (1-cm diameter) on his right medial calf (Figure). No previous episodes or other symptoms of infection were reported. The patient had lived abroad for >10 years and had recently returned to the United States from Queensland, Australia, where he spent his last month hiking in the Daintree Rainforest. His health care providers recommended topical antimicrobial drugs and wound care, and a punch biopsy was obtained but the specimen was not stained or cultured for acid-fast bacilli (AFB). Pathologic findings on the biopsy specimen were nonspecific (reactive epidermal changes and ulceration).

Between April and July 2012, the ulcer increased to 4 cm despite multiple debridements. In September a painful, warm, erythematous lesion developed on the patient's left great toe. He consulted physicians in numerous subspecialties and underwent 2 more biopsies; specimens were not initially stained or cultured for AFB. Pathologic findings from the specimens showed extensive necrosis without granulomas. The ulcers were diagnosed as pyoderma gangrenosum with bacterial superinfection, and treatment was initiated with oral antimicrobial drugs (amoxicillin/clavulanate 875/125 mg twice daily) and prednisone (60 mg/day). Transient improvement occurred, but in late November, the toe lesion worsened.

When the patient was referred to the Veteran's Affairs Medical Center in December 2012, biopsies were again obtained; pathologic evaluation showed extensive necrosis (without granulomas) and numerous AFB. Fresh frozen tissue was sent to the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) for mycobacterial testing. Empiric treatment with levofloxacin, doxycycline, and azithromycin was initiated for nontuberculous mycobacterial infection, and the previously prescribed prednisone was rapidly tapered and stopped. At CDC, immunohistochemical testing of tissue for mycobacteria showed extensive bacilli. DNA extracted from biopsy samples was evaluated by PCR targeting 16s rRNA and the IS*2402* insertion element (*5*). Sequences of IS*2404* amplicons were 100% identical with those for *Mycobacterium ulcerans*. Tissue cultured (30°C) at CDC grew AFB after 3 months (toe specimen) and 4 months (calf specimen); 16S rRNA gene sequencing confirmed a *Mycobacterium* species, most closely matching *M. marinum* or *M. ulcerans*.

Therapy, guided by World Health Organization recommendations (*1*), was changed to rifampin (900 mg/day or 10 mg/kg bodyweight), clarithromycin (1,000 mg/day), and moxifloxacin (400 mg/day). In February 2014, after 15 months' of antimicrobial drug treatment, debridement, and skin grafting to the left foot, the lesions were completely healed.

Conclusions

Imported *M. ulcerans* disease is exceedingly rare, even in today's age of global travel. When the infection does

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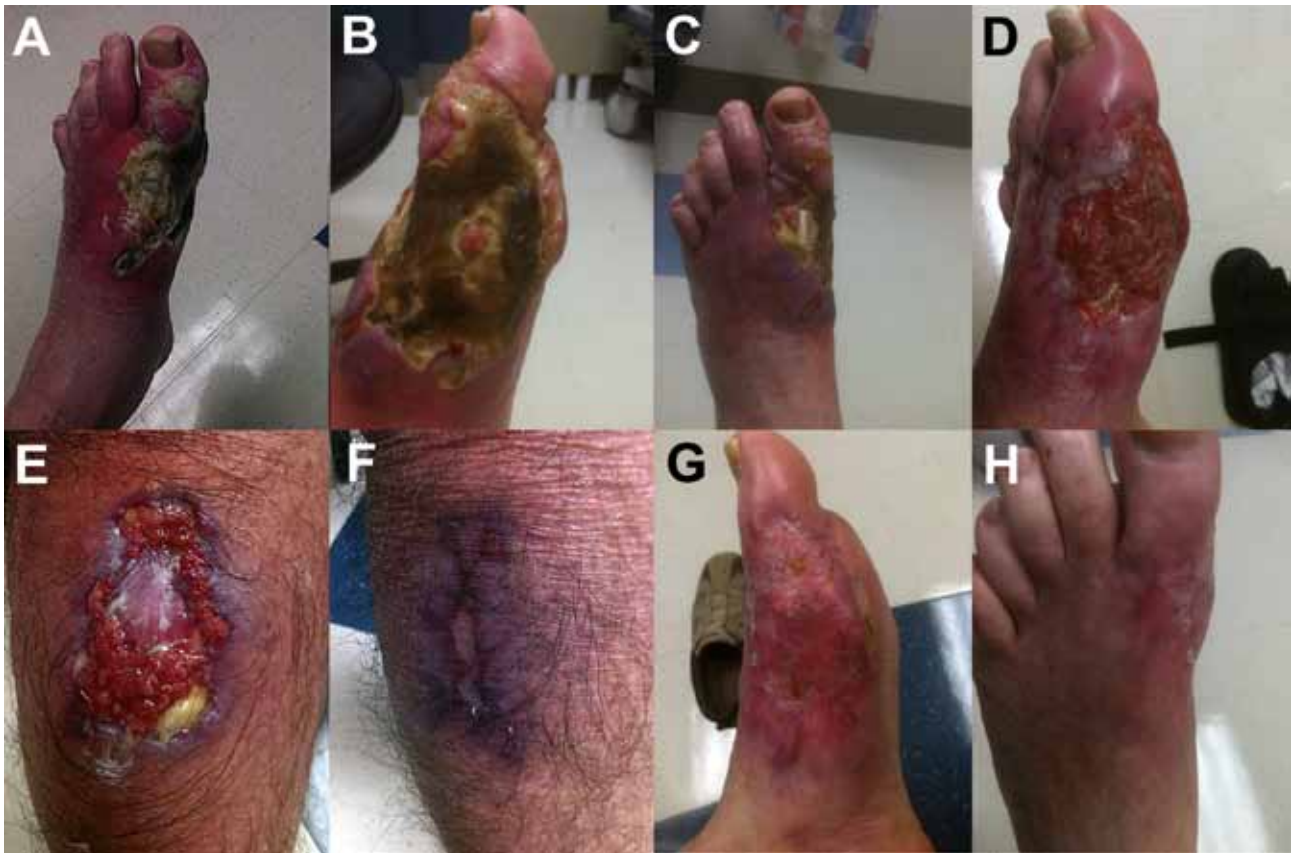

Figure. Progression of lesions caused by *Mycobacterium ulcerans* infection before, during, and after treatment. A–C) Left foot before treatment. D) Left lower leg during treatment. E) Right calf before treatment. F) Right calf after treatment. G, H) Left foot after treatment.

occur, diagnosis is often delayed. In addition to the case reported here, 3 other cases imported to the United States have been reported in the literature since 1967 (*2*–*4*).

The 4 *M. ulcerans* cases diagnosed in the United States were in men (median age 35 years, range 20–63) (Table). Lesions were located on the upper (25%) and lower (75%) extremities, similar to cases in Africa, where lesions commonly develop on the extremities of adults and on the trunk, head, neck, and upper limbs of children (*6*). Specific exposures were not identified in 3 of the US patients; the fourth had exposure to fresh water, a known risk factor (*6*). The 2012 imported US case is similar to cases in Australia, where patients have a median age of 61 years at diagnosis, and most have single lesions (95%) involving lower limbs (61%). However, in Australia, the median time from symptom onset to diagnosis is 42 days, consistent with greater familiarity with the disease (*7*).

The 3 persons with the prior cases of imported *M. ulcerans* disease in the United States had traveled to western Africa; the case-patient described herein had returned from Australia. Most *M. ulcerans* cases in Australia are linked to temperate, coastal Victoria and tropical, northern Queensland (*8*). Persons with cases imported to other *M. ulcerans*–nonendemic countries mostly traveled to Africa (1 traveled to South America), where the disease is present but uncommon (*9*–*12*).

In regions where *M. ulcerans* disease is endemic, it is readily recognized on the basis of lesion appearance and chronicity. In areas of Australia where Buruli ulcer disease is nonendemic, diagnosis is delayed (*13*). A hallmark of imported cases is the difficulty in arriving at a diagnosis due to nonfamiliarity with the disease. For the 4 imported US cases, the median time to empiric antimycobacterial therapy was 20 weeks, and the median time to definitive diagnosis was 8 months. The differential diagnosis for *M. ulcerans* disease is broad, spanning infectious and noninfectious etiologies, including filariasis, phycomycosis, resolving furuncle, and pyoderma gangrenosum. Samples from the case-patients were uniformly AFB smear-positive, and for 1 case in which the culture failed to grow *M. ulcerans,* diagnosis was made by clinical and epidemiologic history and presence of AFB in tissue. Because the organism is slow growing, prolonged culture (>3 months) at low temperatures is required. A hallmark of *M. ulcerans* histopathology is the absence of granulomas and presence of extensive necrosis caused by the organism's

Table. Characteristics of persons with *Mycobacterium ulcerans* infection diagnosed and treated in the United States but acquired in a different country*

Patient no., age, y	Location of ulcer	Risk factor	Travel history	Time, wk, to first drug therapy	Final treatment regimen	Time, mo, to diagnosis	Length, mo, of drug therapy	Surgical management	Outcome
1, 20	Left foot	None	Nigeria	20	Lamprene, clofazimine	–	–	Amputation below knee	Amputation
2, 34	Right elbow	None	Nigeria	–	Surgery alone	7	–	Debridement and split-thickness skin grafting	Cure
3, 36	Left calf	Fresh Water	Northern, western, and Central Africa	17	Clarithromycin and ciprofloxacin	8	18	Debridement and split-thickness skin grafting	Cure
4, 63	Right calf and left foot	Hiking in Daintree Rainforest in sandals	QLD, Australia	36	Rifampin, clarithromycin, moxifloxacin	9	15	Debridement and split-thickness skin grafting	Cure

*All patients were male. Except for negative smear results for patient 1, culture and smear results for all patients were positive. QLD, Queensland; –, Information not available.

secretion of mycolactone toxin, which suppresses the host's immune response (*7*). The absence of granulomas on hematoxylin and eosin staining may result in tissue not being stained for AFB unless clinicians have a high index of suspicion. Furthermore, depending on the bacterial load and focal distribution, bacteria may not be detected by AFB staining. Molecular analysis of tissue is the most sensitive and specific method for rapid and confirmatory diagnosis of *M. ulcerans* and should be pursued when disease is suspected (*14*).

Treatment of *M. ulcerans* disease has changed markedly over the last several decades. Prior to the early 2000s, availability of effective drugs was limited, so surgery was the primary treatment. However, combination therapy with streptomycin, rifampin, and surgery (including skin grafting) has been shown to be effective (*1*), and some cases can be managed with medical therapy alone. Standard antimicrobial treatment, according to World Health Organization guidelines, consists of administering rifampin (10 mg/kg body weight daily by mouth) for 8 weeks and streptomycin (15 mg/kg body weight daily by intramuscular injection) for 8 weeks (*1*). Extensive clinical experience has also shown a combination of oral antimicrobial drugs (rifampin with clarithromycin or moxifloxacin) to be effective against Buruli ulcer disease (*15*). Of the 4 reported US cases, 1 required surgery alone and 3 required drug treatment and surgery. Response to therapy has been favorable: 2 US cases were cured by treatment with oral antimicrobial drugs.

Because awareness of Buruli ulcer disease is limited in regions where *M. ulcerans* is nonendemic, the potential for delayed diagnosis in such areas is increased. For persons with recent travel from *M. ulcerans*–endemic regions, Buruli ulcer disease should be considered in the differential diagnoses of unusual chronic cutaneous ulcers and skin ulcers nonresponsive to conventional therapy.

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Severe Fever with Thrombocytopenia Syndrome Virus, South Korea, 2013

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During 2013, severe fever with thrombocytopenia syndrome was diagnosed in 35 persons in South Korea. Environmental temperature probably affected the monthly and regional distribution of case-patients within the country. Phylogenetic analysis indicated that the isolates from Korea were closely related to isolates from China and Japan.

Severe fever with thrombocytopenia syndrome (SFTS) is a newly emerging infectious disease. Symptoms and laboratory abnormalities are fever, thrombocytopenia, leukocytopenia, and elevated serum enzyme levels. Multiorgan failure occurs in severe cases, and 6%–30% of case-patients die. The syndrome is caused by the SFTS virus (SFTSV) (genus *Phlebovirus*, family *Bunyaviridae*). SFTS case-patients were first reported in China (*1*) and more recently were reported in Japan (*2*) and South Korea (*3*). Two case-patients with symptoms consistent with a similar virus, Heartland virus, were reported in the United States (*4*).

Ixodid tick species are implicated as vectors of SFTSV (*1*,*5*,*6*). One study described a SFTSV prevalence in *Haemaphysalis longicornis* ticks, a major vector of SFTSV, of 0.46% minimum infection rate in South Korea (*7*); in another study, SFTSV was detected in ticks that had bitten humans (*6*). From these studies, we realized that SFTSV was common throughout the country. We aimed to evaluate the prevalence of SFTS in South Korea and isolate the SFTSV to analyze its phylogenetic properties.

The Study

In March 2013, we established molecular diagnostic methods to detect SFTSV. During April–December 2013, from 125 hospitals throughout the country, we collected 301 serum samples from hospitalized persons who had SFTS signs and symptoms, such as high fever (temperatures ≥38°C), vomiting, diarrhea, and/or fatigue and showed laboratory parameters consistent with thrombocytopenia and/or leukocytopenia. We conducted reverse transcription PCR (RT-PCR) to detect the SFTSV medium (M) segment gene from acute-phase serum specimens with a previously described method (*6*). We also detected the SFTSV small (S) segment gene by RT-PCR with specific primers (SF3, 5′-GGGTCCCTGAAGGAGTTG-TAAA-3′; SR1, 5′-TGGTGAGCAGCAGCTCAATT-3′). The RT-PCR conditions were as follows: an initial step of 30 min at 50°C for reverse transcription and 15 min at 95°C for denaturation, followed by 35 cycles of 20 s at 95°C, 40 s at 58°C (for M segment) or 55°C (for S segment), and 30 s at 72°C and a final extension step of 5 min at 72°C.

From the 301 samples, we detected M and S segment genes from 34 and 29 samples, respectively. The nucleotide sequences were assembled by the SeqMan program implemented in DNASTAR software (version 5.06; Madison, WI, USA) to determine the consensus sequences. The nucleotide sequences of the Korea isolates showed 93%–98% homology to the China and Japan isolates.

To isolate SFTSV, we inoculated subconfluent monolayers of Vero E6 cells with the RT-PCR–positive serum. After the monolayers underwent 3 blind passages in new monolayers of Vero E6 cells (*8*), we examined the Vero E6 cells for SFTSV by RT-PCR. We considered the virus to be isolated when the specific genes were amplified by RT-PCR. The viruses did not cause cytopathic effects in Vero E6 cells during isolation. Isolation of SFTSV also was confirmed by indirect immunofluorescent assay (IFA) (Figure 1, panels A,B) and electron microscopy (Figure 1, panel C). For IFA, Vero E6 cells infected with SFTSV were incubated at 37°C in a CO_2 incubator. Cells were harvested, inoculated, and fixed with acetone on Teflon-coated well slides. IFA was conducted by using a monoclonal SFTSV nucleocapsid protein (N) antibody (manufactured in our laboratory) as the primary antibody. N proteins of SFTSV were distributed throughout the cytoplasm (Figure 1, panels A,B). By electron microscopy, Vero E6 cells infected with the SFTSV Korea isolate KAJJH showed bunyavirus-like particles, 80–100 nm in diameter, located in cytoplasmic vacuoles, presumably in the Golgi apparatus (Figure 1, panel C).

The amplified DNA products from the isolates were sequenced and compared with the sequences of other GenBank-registered SFTSV isolates. The sequences of partial M and S segments of the 26 Korea isolates (GenBank accession nos. KF282701, KF282702, and KJ739543–KJ739592) were closely related to those of the SFTSV isolates from China and Japan with 92%–100% identity. A phylogenetic tree was constructed by the neighbor-joining method on the basis of the partial M (Figure 2, panel A, http://wwwnccdc.gov/EID/article/20/11/14-0888-F2.htm) and S segment (Figure 2, panel B) sequences of the Korea SFTSVs in the study and 15 SFTSVs from China and

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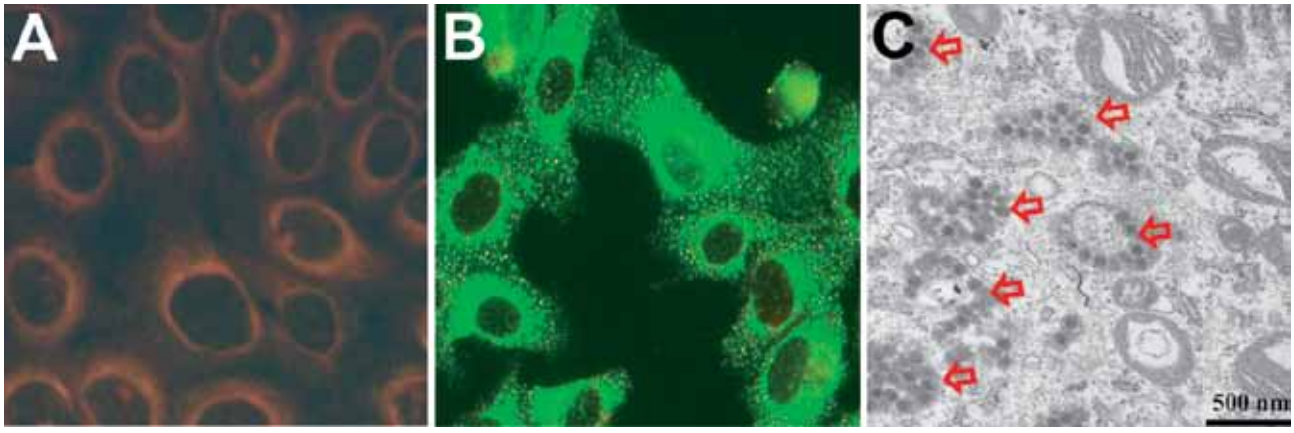

Figure 1. Isolation of severe fever with thrombocytopenia syndrome virus (SFTSV) from case-patients, South Korea, 2013. A, B) Indirect immunofluorescent features of Vero E6 cells primed with SFTSV N protein monoclonal antibody and reacted with fluoresce in isothiocyanate-conjugated anti-mouse IgG. B) Transmission electron microscopy image of Vero E6 cells infected with SFTSV. Scale bar indicates 500 nm.

Japan registered in GenBank. SFTSV isolates formed 2 major clusters in M and S segment sequences, and 1 other small group comprising only Korea isolates, KAGNH2 and KAUSH, was formed in M-segment sequences. Many Korea isolates formed the first cluster with the Japan isolates. Some Korea isolates clustered with the major group of China isolates, forming the second group.

Conclusions

We confirmed the SFTS in several localities around South Korea. We also isolated several SFTSVs from case-patient serum and analyzed the phylogenetic properties of the isolates. A total of 36 SFTS case-patients were reported in South Korea. The first SFTS case was identified in a retrospective study from 2012 (*3*). Subsequently, SFTS was diagnosed in 35 additional case-patients in South Korea. Another group diagnosed the first of the 35 cases in the country; we diagnosed the other 34 cases, from which we isolated the 26 SFTSVs. The major signs and symptoms of the 35 case-patients, including fever (100%), gastrointestinal symptoms (74%), fatigue (74%), thrombocytopenia (100%), and leukocytopenia (100%), were similar to those of case-patients in China and Japan (*9*).

The case–fatality rate for SFTS in South Korea was 47.2% (17/36), higher than that of the recent China cases (≈8.7%) (*10*). The low sensitivity of the detection method, the conventional 1-step RT-PCR, and the absence of a serologic diagnosis may have contributed to the relatively high case–fatality rate. Most cases occurred in older persons; ≈80% of patientswere >50 years of age. Approximately 70% were farmers, including persons who cultivated vegetable gardens (*9*). In many case-patients, the disease evolved during a relatively warm time of year, from late spring to early autumn (Figure 3, panel A). The geographic distribution of SFTS case-patients also indicated that environmental temperature affected the SFTS prevalence because many (86%, 30/35) SFTS cases evolved in relatively warm southern provinces and cities south of Chungcheongbuk, Chungcheongnam, and Gangwon Provinces (Figure 3, panel B). We have also observed that the tick density is high during May–August, a generally warm season in South Korea (*7*). SFTSV was also mainly detected during this season. These results indicate that the virus infection in humans is closely related to a high tick density and SFTSV infection in ticks in a warm climate.

As described in another report, Japan isolates formed an independent cluster from the China isolates (*2*). In our current study, SFTSV isolates formed 2 major clusters. Most of the Korea isolates formed a cluster with the Japan isolates, although some Korea and China isolates were included in the other group, perhaps not surprising given the geographic location of South Korea between China and Japan.

Acknowledgments

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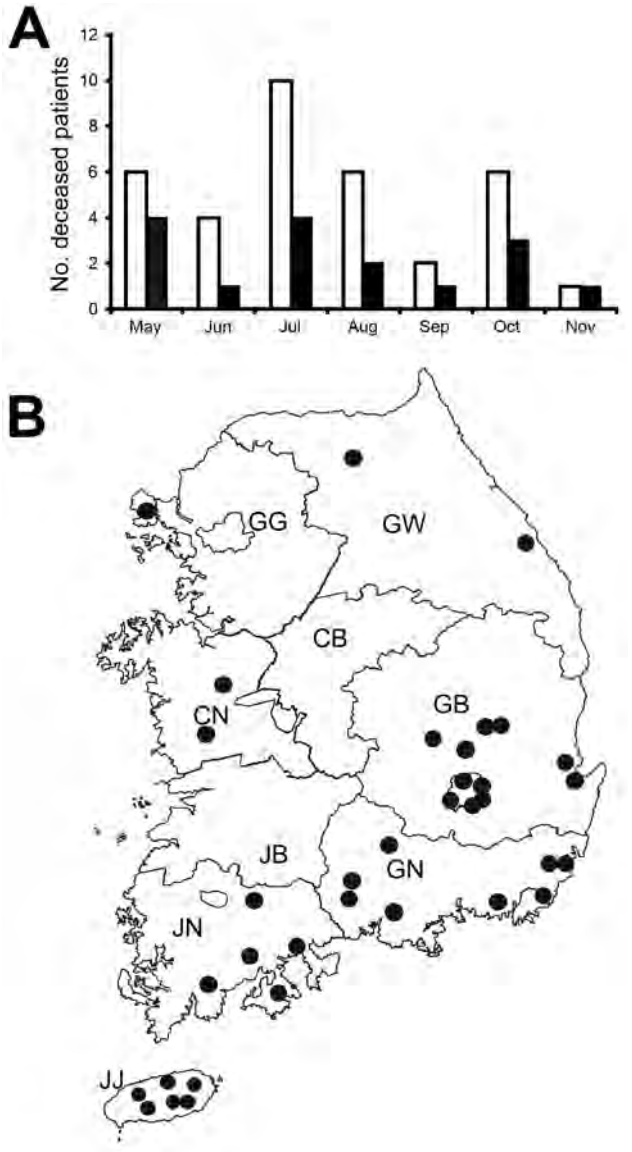

Figure 3. Seasonal (A) and geographic (B) distribution of case-patients with severe fever with thrombocytopenia syndrome (SFTS), South Korea, 2013. A) White and black bars indicate the numbers of total and deceased SFTS patients, respectively, in the indicated months. B) Black circles indicate the approximate residential regions of 35 SFTS case-patients in 2013 in South Korea. GG, Gyeonggi Province; GW, Gangwon Province; CB, Chungcheongbuk Province; CN, Chungcheongnam Province; GB, Gyeongsangbuk Province; GN, Gyeongsangnam Province; JB, Jeollabuk Province; JN, Jeollanam Province; JJ, Jeju special autonomous Province.

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Human Herpes Simplex Virus Type 1 in **Confiscated Gorilla**

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In 2007, we detected human herpes simplex virus type 1, which caused stomatitis, in a juvenile confiscated eastern lowland gorilla (*Gorilla beringei graueri*) that had a high degree of direct contact with human caretakers. Our findings confirm that pathogens can transfer between nonhuman primate hosts and humans.

Most emerging infectious diseases of humans are of wildlife origin (*1*). The close genetic relatedness of humans and nonhuman primates (NHPs) makes each uniquely susceptible to pathogens of the other (*2*). Spillover of NHP pathogens into humans can lead to human disease pandemics (e.g., HIV-1 from the chimpanzee strain of simian immunodeficiency virus [*3*]), and some NHP viruses are potentially acutely lethal to humans (e.g., herpes B virus of Asian macaques [*4*]). The opposite transmission event—human to NHP—is less frequently reported. Although of concern from a wildlife conservation standpoint (*5*), human-to-NHP transmission events substantiate the fact that pathogen sharing is bidirectional (*6*). To provide further evidence for the potential for disease emergence at the human–NHP interface, we report a human herpes simplex virus type 1 (HSV-1) infection in an eastern lowland gorilla (Grauer's gorilla, *Gorilla beringei graueri*).

The Study

A 2-year-old female Grauer's gorilla, confiscated from poachers in Goma, Democratic Republic of the Congo, in November 2005, was group-housed in a facility with 6 other juvenile Grauer's gorillas and 1 juvenile mountain gorilla (*G.b. beringei*). All gorillas had daily direct contact with one another and with human caretakers who provided hands-on care. In March 2007, 5 of the Grauer's gorillas exhibited bouts of lethargy and anorexia and oral lesions. Three of the affected gorillas, including the gorilla from Goma, were chemically immobilized for examination. All 3 gorillas exhibited multiple clear fluid-filled vesicles up to 2 cm in diameter that affected the mucosa of the lips and gingiva (Figure 1, panel A). Biopsy and swab samples were obtained from the lesions of the gorilla from Goma. The biopsy sample was fixed in 10% buffered formalin; the swab samples were stored frozen at –80°C, and these specimens were shipped to the United States for diagnostic testing.

Histologic examination of the biopsy sample showed variably thickened, ulcerated, nonkeratinizing, stratified squamous oral mucosae. Margins of ulcers showed marked epidermal swelling (intracellular edema, ballooning degeneration) and large numbers of epithelial syncytial cells, often with marginated chromatin and smudgy intranuclear inclusion bodies filling the nucleus (Figure 1, panel B.). Inclusion bodies were present in individual cells in the stratum basalis and stratum spinosum adjacent to the ulcers. Using transmission electron microscopy, we observed virions consistent with herpesvirus in shape and size within the nucleus of syncytial cells and budding through the nuclear membrane (Figure 1, panel C.). Histopathology and transmission electron microscopy findings were essentially identical to findings from human HSV-1 infections (*7*).

DNA was extracted from swab samples from this animal (*8*) and amplified by PCR using pan-herpesvirus–specific primers designed to the DNA polymerase gene (*UL30)* (*9*). A cloned and sequenced 731-nt amplicon (GenBank accession no. KJ396198) showed 99% identities with the DNA and protein sequences of HSV-1 *UL30*, and 94% and 95% identities (DNA and protein, respectively) to *UL30* of HSV-2 (Table). Because sequences from gorilla-specific herpesviruses have not been annotated, we performed a phylogenetic analysis of the amplicon from the gorilla swab sample using the *UL30* genes from broadly representative isolates from the 3 herpesvirus subfamilies. The gorilla-derived DNA and protein (Figure 2) exhibited the strongest phylogenetic relationship with HSV-1. Alignments of human and NHP alphaherpesvirus representatives show that the region amplified with the consensus herpesvirus primers is a highly conserved region of *UL30* (Figure 2). The sequence alignments strongly suggested that the herpesvirus DNA in the oral lesion was HSV-1, although the gorilla-derived amplicon was an outlier in comparison with other HSV-1 isolates (data not shown).

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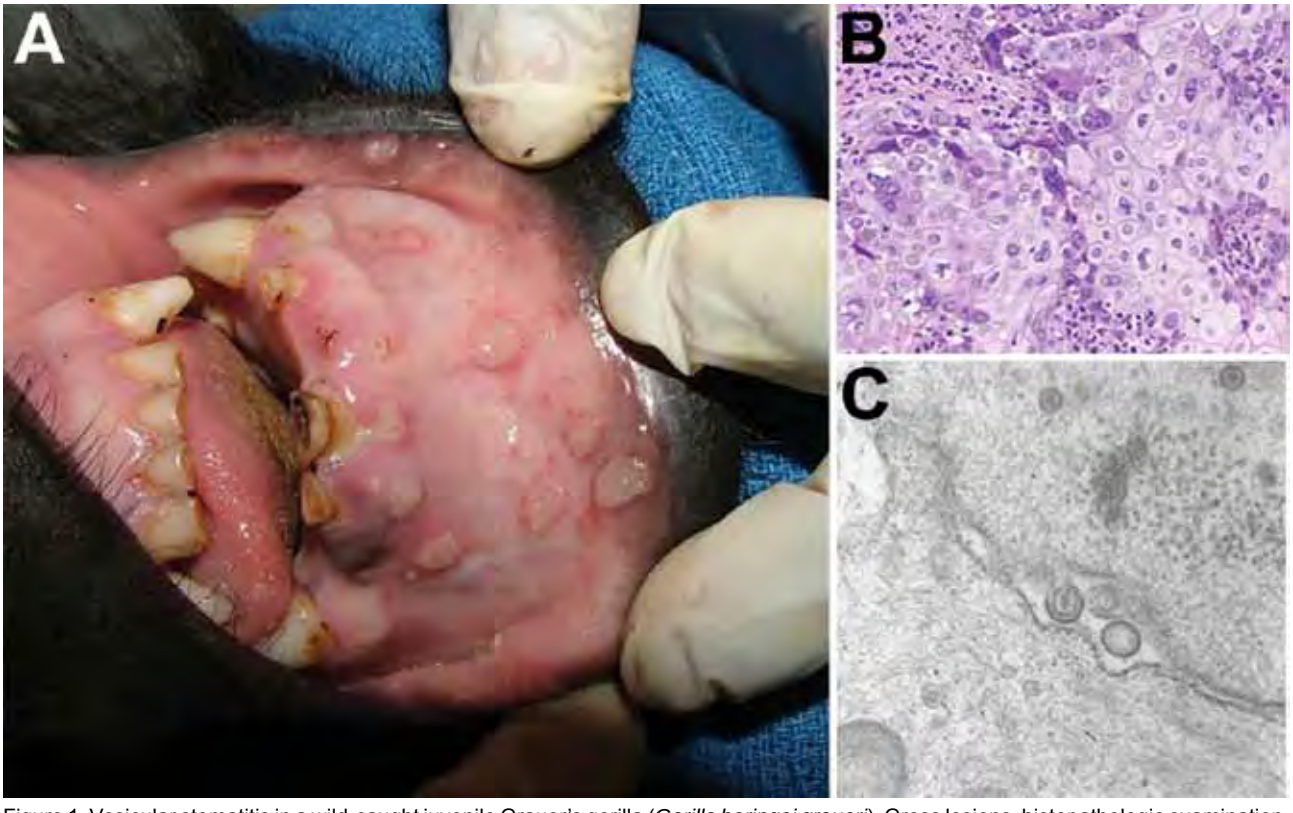

Figure 1. Vesicular stomatitis in a wild-caught juvenile Grauer's gorilla (*Gorilla beringei graueri*). Gross lesions, histopathologic examination, transmission electron microscopy, and molecular screening confirmed human herpesvirus type 1 (HSV-1) as the etiologic agent. A) Human HSV-1 lip lesions in a wild-caught juvenile Grauer's gorilla. B) Section of oral mucosa adjacent to a vesiculo-ulcerative lesion exhibits epithelial cell necrosis, cytoplasmic swelling, nuclear chromatin margination (sometimes with discrete Cowdry type A inclusions), and multinucleated syncytia typical of herpesviral cytopathic effects (hematoxylin and eosin stain). Original magnification ×200. C) Electron micrograph of the same lesion demonstrates intranuclear, unenveloped virions ≈100 nm in diameter that are budding through the nuclear membrane to form enveloped virions ≈140 nm in diameter; morphologic features of both are compatible with a herpesvirus. Original magnification ×60,000.

The relative divergence of the amplicon was due primarily to nucleotide changes encoding a 3-aa motif not found in any other annotated sequence (Figure 2). Whether adaptation of the putative HSV-1 in the gorilla host represents a compensatory change to replication in the ectopic gorilla host is not known.

To confirm our conclusion that the lesion was associated with HSV-1 infection, we used HSV-1–specific primers to amplify a region of *UL30* (aa 134–246) outside of the amplicon defined by the pan-herpesvirus–specific primers. Amplification was positive for the oral swab sample DNA with this second primer pair and for 3 human-isolated HSV-1 strains. Sequence analysis of the amplicon demonstrated 100% and 89% sequence identity with HSV-1 and HSV-2, respectively (data not shown).

Conclusion

On the basis of the results of the histopathologic features and molecular screening of the virus, we concluded that the stomatitis in this juvenile female Grauer's gorilla confiscated from poachers in Goma, Democratic Republic of the Congo, was caused by HSV-1. Alphaherpesvirus

Table. Comparison of gorilla amplicon to other herpesviruses*

Gorilla amplicon identity	HSV-1	HSV-2	MaHV-1	PaHV-2	HHV-3	HHV-4	HHV-5	HHV-6	HHV-8	EHV-1
DNA, %	99.3	93.6	85.3	86.0	53.9	61.2	49.3	43.3	48.6	65.1
Protein, %	99.2	94.6	90.1	90.1	65.7	48.8	39.9	45.9	46.0	68.6

*Pairwise alignment of the gorilla amplicon DNA sequence (GenBank accession no. KJ396198) and predicted amino acid sequence of the corresponding region of HSV-1 (HHV-1; AFI98948), HSV-2 (HHV-2; AGI44412), MaHV-1 (AAT67222); PaHV-2 (YP_443877), HHV-3 (varicella zoster virus; ABF21820), HHV-4 (Epstein-Barr virus; YP_401712), HHV-5 (human cytomegalovirus; AAP37469), HHV6 (BAF93477), HHV-8 (Kaposi sarcoma virus; ACY00400), and EHV-1 (ADI96155). DNA–DNA alignments were performed by using the Wilbur-Lipman Method of the MegAlign (DNAStar, Madison, WI, USA) sequence alignment program (Ktuple = 3; gap penalty = 3; window = 20). Protein–protein alignments were performed by using the Lipman-Pearson method (Ktuple = 2; gap penalty = 4; gap length penalty = 12). EHV, equid herpesvirus; HSV, herpes simplex virus; HHV, human herpesvirus; MaHV, Macacine herpesvirus; PaHV, Papiine herpesvirus.

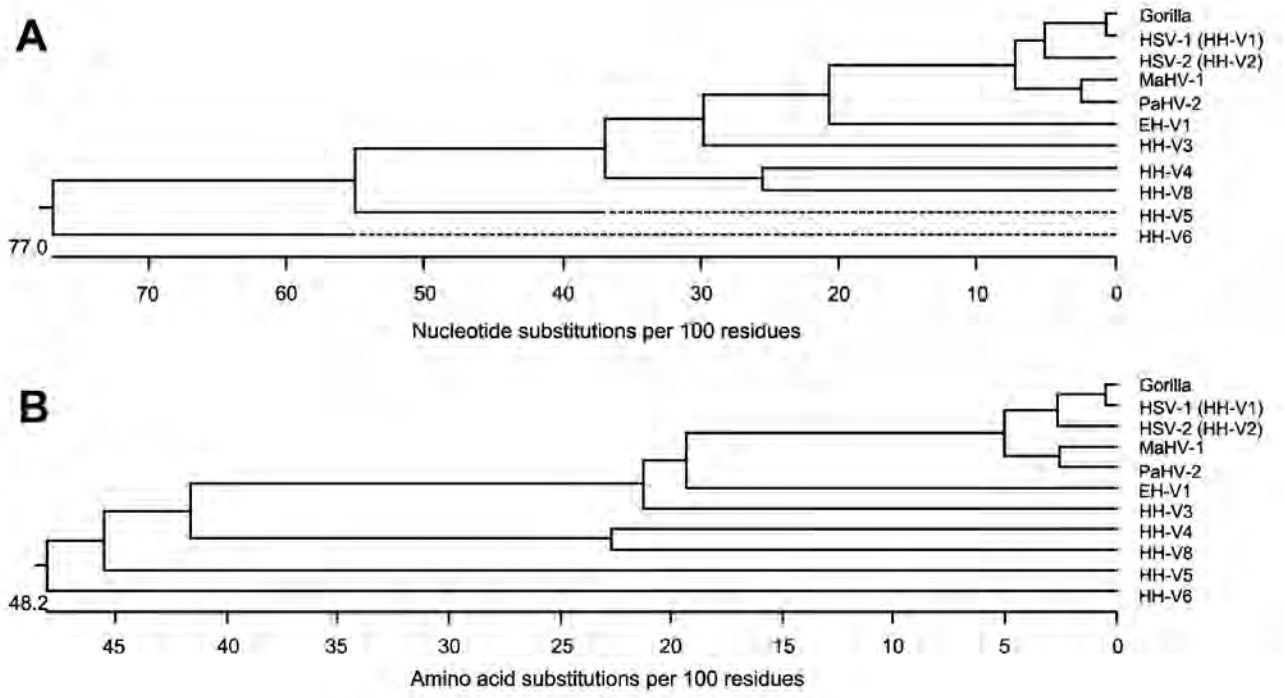

Figure 2. Phylogenetic analysis of the nucleotide sequence (A) and predicted amino acid sequence (B) from the swab sample amplicon from the gorilla with the corresponding regions of HSV-1 (HHV-1; GenBank accession no. AFI98948); HSV-2 (HHV-2; AGI44412); MaHV-1 (AAT67222); PaHV-2 (YP_443877); HHV-3 (varicella zoster virus; ABF21820); HHV-4 (Epstein-Barr virus; YP_401712); HHV-5 (human cytomegalovirus; AAP37469); HHV-6 (BAF93477); HHV-8 (Kaposi sarcoma virus; ACY00400), and EHV-1 ADI96155). Sequences were aligned by the Clustal W method (http://www.clustal.org) by using the MegAlign (DNAStar, Madison, WI, USA) sequence alignment program (multiple alignment parameters: gap penalty = 15.00; gap length penalty = 6.66; delay divergent seqs (%) = 30; DNA transition weight = 0.50. Pairwise alignment parameters: slow-accurate; gap penalty = 15.00; gap length = 6.66). EHV, equid herpesvirus; HSV, herpes simplex virus; HHV, human herpesvirus; MaHV, Macacine herpesvirus; PaHV, Papiine herpesvirus.

exposure in wild gorillas in this region has been reported (*10*), but to our knowledge, human HSV infection in wild-born captive gorillas has not been detected with molecular techniques. Fatal HSV-1 infection in a captive-born, nursery-reared western lowland gorilla (*G. gorilla gorilla*) (*11*) has been reported. Serologic tests for HSV-1 on samples collected before the outbreak from other gorillas in the confiscated gorilla's group showed that 2 clinically unaffected gorillas were seropositive for HSV-1, suggesting previous exposure to an alphaherpesvirus (*12*). However, serologic tests for HSV-1 and -2 exposure in the 5 clinically affected gorillas during this outbreak, including the gorilla from Goma, were negative, which suggests these animals were experiencing their first HSV infections, and antibodies were not yet detectable. Although the HSV infection status of the poachers who captured and initially held the gorilla from Goma was unknown, as was the human HSV infection status of the caretakers (from whom samples were not collected) who had daily contact with this gorilla and her captive cohort, we can safely assume most of these persons were representative of the general human population and therefore infected because HSV-1 seropositivity has been documented at a prevalence of >90% in the region (*13–15*), and HSV infections are lifelong.

Our findings have major ramifications for the possible reintroduction of these confiscated gorillas into the wild (all remain in captivity) because such a management action might introduce a novel (human) pathogen to a naïve population. Screening wild Grauer's gorillas and other gorilla populations for human HSV and other human pathogens will be critical to adequately assess the risk for reintroducing captive gorillas to wild populations of these endangered great apes. From a global health standpoint, our finding of human HSV-1 in a gorilla proves once again that pathogens transfer among human and NHP hosts.

Acknowledgments

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Vaccine-Induced Waning of *Haemophilus influenzae* Empyema and Meningitis, Angola

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In Angola during 2003–2012, we detected *Haemophilus influenzae* in 18% of 2,634 and 26% of 2,996 bacteriologically positive pleural or cerebrospinal fluid samples, respectively, from children. After vaccination launch in 2006, *H. influenzae* empyema declined by 83% and meningitis by 86%. Severe *H. influenzae* pneumonia and meningitis are preventable by vaccination.

In sub-Saharan Africa, most children with severe community-acquired pneumonia are discharged from care with the causative agent unidentified. In the few diagnosed cases, diagnosis is usually based on positive blood culture and, to a growing extent, positive urine antigen test result for *Streptococcus pneumoniae* (*1*). For meningitis diagnosis, cerebrospinal fluid (CSF) (and sometimes blood) samples are cultured routinely.

Acute pneumonia can result in fluid collection in the pleural space, a parapneumonic effusion visible on chest radiographs. Effusions tend to become purulent. Fluid removal (often >100 mL) by thoracentesis, along with appropriate antimicrobial drug therapy, relieves dyspnea, speeds healing, and sometimes saves lives. Identification of the causative agents gives a hint of the etiology of nontrivial forms of pneumonia. Following this rationale, we analyzed a large series of empyema and bacterial meningitis cases in children in Angola. We sought to discover to what extent *Haemophilus influenzae* type b (Hib) vaccination, started in June 2006, influenced occurrence of these potentially life-threatening diseases.

The Study

Our prospectively collected data came from 23,134 children 2 months to 13 years of age during 2003–2012. All had attended Angola's largest pediatric center, the 300-bed David Bernardino Children's Hospital in Luanda, which has been the core of our treatment trials (*2*), all approved by the local ethics committee. From these children, 6,030 pleural fluid samples and 17,104 CSF samples were examined by microscopy, gram-stained, and cultured. Any child with symptoms and signs of pleural effusion and a large, not blade-like, opacity on chest radiographs underwent thoracentesis, with or without chest tube insertion, under local anesthesia. Some 200 children a year fall into this category (*3*), and the procedure has become routine among attending pediatricians or surgeons.

In 2002, in collaboration with the National Health Institute of Lisbon, Portugal, the hospital established a basic bacteriology laboratory and provided staff training (*4*,*5*). Microscopy and gram-staining of each pleural or CSF sample were performed 24 hours a day (Ziehl-Neelsen staining on demand), whereas bacterial culture on blood and chocolate agar plates was conducted only during working hours (specimens maintained at room temperature). Culture techniques were standard. Lack of resources limited searches to aerobic pathogens.

Routine serotyping of *H. influenzae* and *S. pneumoniae* isolates was also beyond the reach of this institution. However, as routine hospital procedure, several hundred pleural fluid and CSF samples were sent to bacteriologists in Lisbon for further investigation (serotyping, PCR, some culture and susceptibility confirmation). Neither antigen-detection methods in Luanda nor PCR in Lisbon were used routinely, nor was HIV testing routinely performed in Luanda; however, in an earlier meningitis trial (*2*), we found that 8% of children in the study were HIV positive, in accordance with findings of a local epidemiologic survey (*6*). For patients with mixed bacterial infections, all agents were taken into account for analysis. Likely skin contaminants (5%) were excluded by a microbiologist (L.M.). No parasitologic or fungal investigations were conducted.

For most patients, the pleural fluid was purulent. After thoracentesis, pneumothorax or subcutaneous emphysema developed in a few patients but resolved spontaneously for all. The use of pretreatment antimicrobial drugs could not be determined, but because 271 (40%) of 679 children in our prospective meningitis study (*2*) were receiving antimicrobial drugs when they arrived at the hospital, pretreatment was likely.

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In all, 2,634 (44%) of 6,030 pleural fluid samples yielded bacteria. As is characteristic of empyema patients (*3*), most were 7–23 months of age. *H. influenzae* was the third most common isolate (486 [18%]), exceeded only by *S. pneumoniae* (1,044 [40%]) and *Staphylococcus aureus* (690 [26%]). Thereafter came *Escherichia coli* (95 [4%]), streptococci other than pneumococcus (88 [3%]), *Proteus* spp. (87 [3%]), *Klebsiella* spp. (48 [2%]), and other gram-negative bacteria (96 [4%]). No *Mycobacterium tuberculosis* was detected.

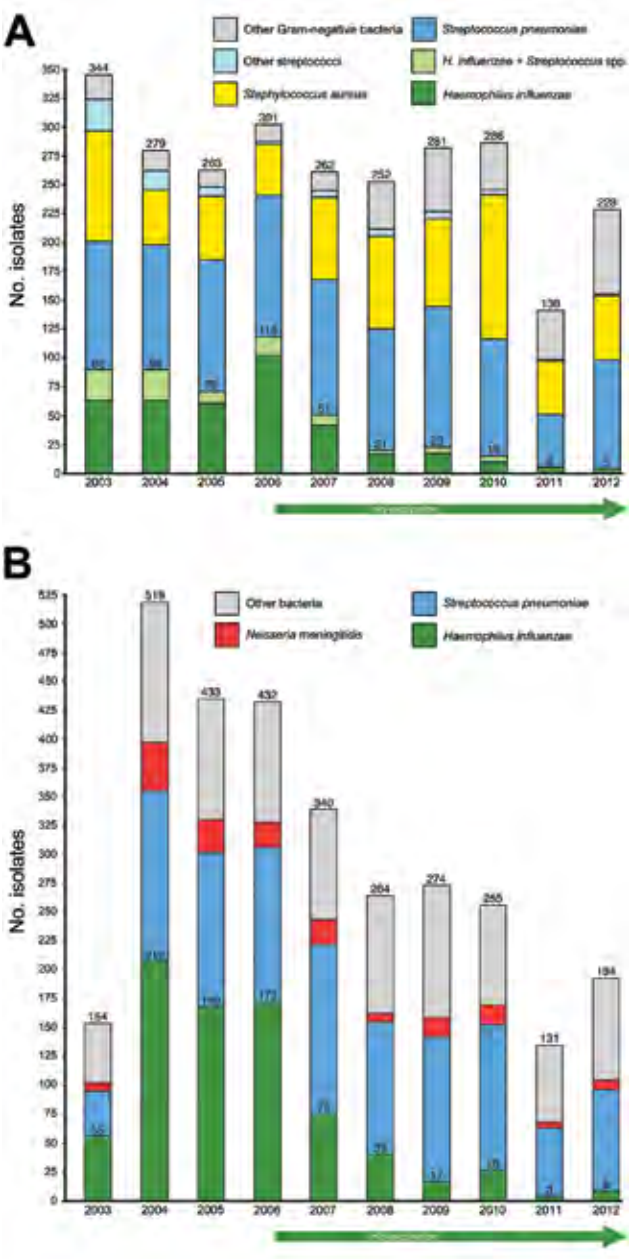

Figure 1. Distribution of 2,634 pleural fluid (empyema) (A) and 2,996 cerebrospinal fluid (CSF) isolates (B) from children who received treatment at David Bernardino Children's Hospital, Luanda, Angola, during 2003–2012. Numbers above the light green bars in the upper panel comprise the total Haemophilus influenzae isolates found alone or with Streptococcus spp. (mostly S. pneumoniae). The numbers of mixed infections were as follows: 24, 24, 9, 15, 6, 2, 4, 6, 0, and 0, respectively. Hib, H. influenzae type b.

After vaccination launch in 2006, the number of *H. influenzae* isolations from patients with empyema clearly decreased (Figure 1). Although *H. influenzae*, sometimes concomitantly with pneumococci, was isolated 415 times during 2003–2007, it was isolated only 71 times during 2008–2012, an 83% decline. Serotyping was performed for 29 isolates, and all except 1 were Hib. Only 8 isolates of *S. pneumoniae* from patients with empyema were serotyped; distribution was 6A/B/C (n = 3), 1 (n = 2), 14 (n = 2), and 23F/A/B (n = 1).

After vaccination launch in 2006, the number of *H. influenzae* isolates from patients with meningitis also decreased (Figure 1). Of 17,104 CSF specimens, 2,996 yielded bacteria, of which 1,109 (37%) were *S. pneumoniae;* of 45 serotyped strains, the following serotypes were found: 6 (n = 14), 23 (n = 11), 1 (n = 6), 19F/A/B/C (n = 5), 14 (n = 3), 4 (n = 2), 18F/A/BC (n = 2), 3 (n = 1), and 5 (n = 1). *H. influenzae* (776 [26%]) was the second most common bacterial agent isolated. From the first 5-year period (683 cases) to the second period (93 cases), *H. influenzae* isolations decreased by 86%. All 55 serotyped *H. influenzae* strains were type b. No similar decreasing trend was found for *Streptococcus agalactiae,* 196 (7%); *Neisseria meningitidis,* 175 (6%); or gram-negative rods such as *E. coli,* 164 (5%), *Klebsiella* spp., 152 (5%), or *Salmonella* spp., 51 (2%).

Figure 2 depicts organisms isolated for cases of empyema and meningitis combined. All major changes occurred only for the empyema and meningitis caused by Hib, not for diseases caused by *S. pneumoniae* or other bacteria.

Conclusions

Although empyema represents only a fraction of all cases of pneumonia among children in Angola, and only a few *H. influenzae* or *S. pneumoniae* isolates from pleural fluid and CSF were serotyped, the potential for decreasing pneumonia and bacterial meningitis by use of conjugate vaccines was demonstrated. The institution's policy of performing thoracentesis for patients with empyema enabled us to examine the effects of Hib vaccination because the etiologic agent was determined for specimens obtained directly from the infection focus. A method much simpler and safer than chest tube insertion would have been transthoracic needle aspiration, for which risks are much exaggerated (*7*,*8*); regrettably, this reliable method is rarely used (*9*). When performed in the right place by the right clinicians on the right patients, it is a great tool for clinicians, patients, and epidemiologists.

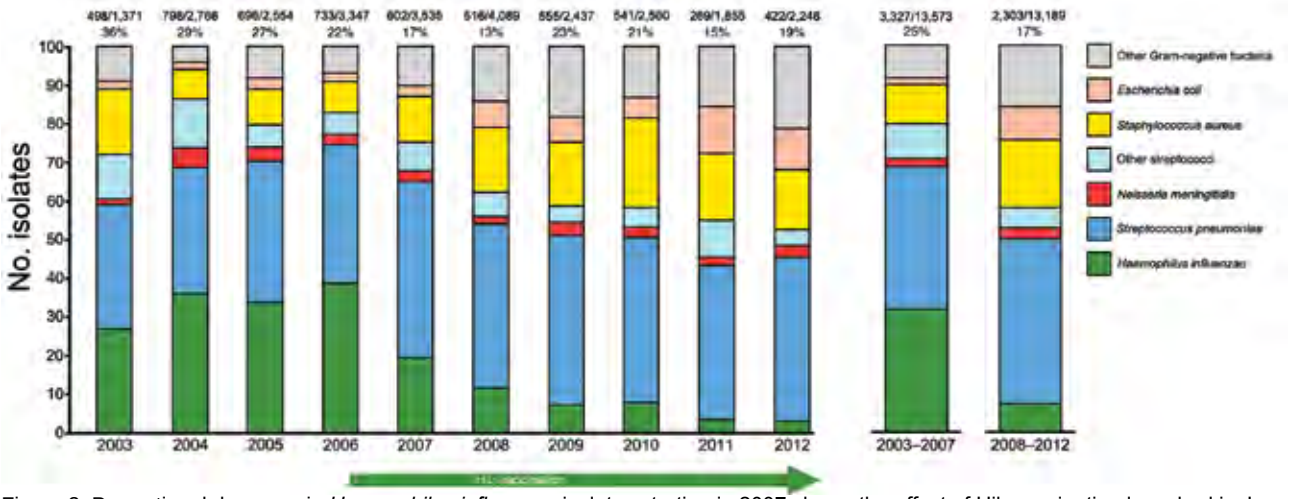

Figure 2. Proportional decrease in *Haemophilus influenzae* isolates starting in 2007 shows the effect of Hib vaccination launched in June 2006. Numbers above the bars indicate no. positive samples/no. cultured cultured samples (%) for all empyema and meningitis cases combined. Hib, *H. influenzae* type b.

That the bacterium most commonly isolated from pleural fluid was *S. pneumoniae* was not unexpected, nor was the large role of *S. aureus*, as documented in Africa, unexpected (*10*,*11*). The lesson learned was the high prevalence of Hib among patients with empyema. Because 79% (11/14) of cases of *H. influenzae* pneumonia in The Gambia were caused by Hib (*12*), and because a vaccination study in the same country provided strong evidence that Hib causes 21% of radiologically proven cases of pneumonia (*13*), the dramatic decline in Hib pneumonia and meningitis in Luanda fits the general picture well. Because by 2012, already 91% of vaccinees had received 3 doses, the vaccine effect was undeniable. A serologic study from China also showed Hib to be a major cause of childhood pneumonia (*14*). Obviously, not only empyema but also many other forms of pneumonia are preventable by Hib vaccination (*15*).

We are aware of study shortcomings. Because of economic constraints, only a fraction of the strains could be typed, and no *M. tuberculosis*, parasites, or viruses were sought. Still, our data showed that, besides meningitis, Hib is a major cause of severe pneumonia in Luanda. This vaccine-preventable disease is almost certainly not a problem specific to Angola; therefore, efforts should be made to implement Hib vaccination throughout Angola and elsewhere in sub-Saharan Africa.

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Poultry Market Closures and Human Infection with Influenza A(H7N9) Virus, China, 2013–14

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Closure of live poultry markets was implemented in areas affected by the influenza virus A(H7N9) outbreak in China during winter, 2013–14. Our analysis showed that closing live poultry markets in the most affected cities of Guangdong and Zhejiang provinces was highly effective in reducing the risk for H7N9 infection in humans.

A novel avian influenza A(H7N9) virus was first identified in China during March 2013, and by March 25, 2014, it had caused 390 laboratory-confirmed human cases in mainland China. The majority of patients with laboratory-confirmed cases reported recent exposure to live poultry markets (LPMs) in urban areas (*1*,*2*), and the H7N9 virus has been identified in LPMs in affected areas (*3*–*6*). Temporary closure of LPMs in Shanghai, Nangjing, Hanghzou, and Huzhou during the 2013 spring outbreak of influenza was associated with immediate and substantial reductions in incidence of confirmed H7N9 infection in those cities (*7*).

Although few confirmed human H7N9 infections were identified in the summer and autumn of 2013, the virus reemerged during the winter of 2013–14, and 251 confirmed cases were reported during December 1, 2013–March 25, 2014, mostly from cities in Guangdong Province in southern China and cities in Zhejiang Province in eastern China. In response to the identification of H7N9 in humans, poultry, or the environment, local authorities of affected cities implemented various control measures, the highest profile of which was closure of LPMs. The objective of our study was to estimate the effect of closure of LPMs in reducing incidence of human infections with H7N9 in the most affected cities of Guangdong and Zhejiang provinces during the 2013–14 winter outbreak.

The Study

During December 1–March 25, 2014, a total of 93 and 89 confirmed human H7N9 cases were reported in Guangdong and Zhejiang provinces, respectively. In response to local cases, many urban areas introduced LPM closures for varying durations. To estimate the effect of these interventions, we used the same methods applied to estimate the effect of LPM closures in cities in eastern China in April 2013 (*7*). We selected 9 specific areas where LPMs were closed for ≥7 consecutive days, ≥3 H7N9 cases were reported in that local urban area during the study period of December 1, 2013–March 7, 2014, and ≥1 case was confirmed before the local LPM closure (Table). The dates of these interventions in relation to the dates of onset of illness of the 69 confirmed human cases in these 9 areas are shown in Figure 1 (http://wwwnc.cdc.gov/EID/article/20/11/14-0556-F1.htm).

In our analysis we assumed the following: 1) the incidence rate of human infection with H7N9 was constant for the 2 weeks before the implementation of LPM closure in each area; 2) the incidence rate of human infection with H7N9 during the LPM closure period had a different rate from the pre-closure period and was also constant, so the ratio of incidence rates during closure versus before closure indicates the effect of LPM closure, and an incidence rate <1 indicated a reduction in incidence; and 3) illness onset in each human case-patient occurred after an incubation period based on a stochastic incubation period distribution. We further assumed that the incubation periods of cases among humans in all cities followed the same lognormal distribution. The start date of the study time horizon for a given area was either 14 days before the start date of LPM closure or the onset date of the first confirmed local

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Table. Summary of closures of live poultry markets in the urban areas of Guangdong and Zhejiang Provinces, China, during the 2013–14 winter outbreak of influenza A(H7N9)

Area	No. urban cases	Start date of LPM closure, 2014	End date of LPM closure, 2014
Guangdong Province			
Shenzhen	19	Jan 31	Feb 13
Guangzhou	19	Feb 15	Feb 28
Huaiji County, Zhaoqing	3	Jan 31 or Feb 5*	Feb 18
Nanhai District, Foshan	6	Jan 7 or Jan 13†	Jan 29
Zhejiang Province			
Hangzhou	5	Jan 24‡	Mar 7
Downtown§	3	Jan 21	Mar 7
Xiaoshan	4	Jan 23	Mar 7
Yuhang			
Ningbo downtown (4 districts)¶	6	Jan 26	Feb 18
Shaoxing	4	Jan 22	Feb 11

*Only the live poultry markets (LPMs) epidemiologically linked to cases were closed Jan 31–Feb 2, 2014. All LPMs in the county were closed Feb 5–18.
†2 LPMs epidemiologically linked to cases were closed during Jan 7–9. All LPMs in the district were closed during Jan 13–29.
‡End dates of LPM closure in these districts/cities are later than the end date of the study time horizon.
§Hangzhou includes 6 districts in the downtown area (Gongshu, Shangcheng, Xiacheng, Jianggan, Xihu and Binjiang), 2 suburban districts (Xiaoshan and Yuhang), 3 cities (Fuyang, Jiande and Linan), and 2 counties (Tonglu and Chunan).
¶Ningbo includes 6 districts in the downtown area (Haishu, Jiangdong, Jiangbei, Beilun, Zhenhai , Yinzhou), 3 cities (Yuyao, Cixi , Fenghua), and 2 counties (Xiangshan, Ninghai). Four districts included here are Haishu, Jiangdong, Jiangbei, and Yinzhou.

H7N9 case in 2014, whichever was later. The end date of the study time horizon was either the last day of local LPM closure or March 7, 2014, whichever was earlier, to allow for a possible delay of case notification for 2–3 weeks.

Our model therefore included these parameters: the incidence rates before and after closure in each city and the parameters of the incubation period distribution. We estimated these parameters using Markov chain Monte Carlo method (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/11/14-0556-Techapp1.pdf), using flat priors for the logarithms of the incidence rate before closure and the incidence rate ratio, a lognormal distribution with mean of 3.3 days and 97.5th percentile 5.7 days for the incubation period, and a lognormal distribution with mean of 0.76 and 97.5th percentile 5.2 for the coefficient of variation of the incubation period corresponding to the posteriors from our previous analysis (*7*). After estimating separate effects of LPM closure in each urban area, we fitted an overall model, assuming the incidence rate ratio was the same across all areas.

Point estimates of the effectiveness of LPM closure varied from 61% to 89% in the 4 areas in Guangdong Province, and from 70% to 89% in the 5 areas in Zhejiang Province with generally wide CIs, and the effectiveness was estimated to be 97% (95% CI 89%–100%) in the overall model, assuming the same incidence rate ratio associated with LPM closure in each area (Figure 2). In the latter model, the incubation period distribution had a mean of 3.4 days (95% CI 2.2–5.0) and 95th percentile of 4.8 days.

As in our previous analysis of LPM closures in April 2013 (*7*), here we found that LPM closures were effective in reducing human risk for H7N9 infection in cities in Guangdong and Zhejiang provinces during the 2013–14 winter outbreak. The relatively short closure periods and the small number of cases in each city prohibited area-specific estimates of effectiveness that had narrow credibility intervals (Figure 2). In addition, the effect of LPM closure estimated here may incorporate other contemporaneous interventions as well as potential reductions in poultry consumption and population exposure to live poultry associated with the H7N9 outbreak (*8*), seasonal variation of avian influenza cases as observed in H5N1 infections, decline in media coverage, decline in seeking of health care by possible patients, and decreased laboratory testing output related to staff fatigue during the second wave of the epidemic.

Although our results support the effectiveness of LPM closure in protecting human health, closure of LPMs is a temporary and drastic measure that may be associated with substantial costs to society and the poultry industry (*9*). More sustainable interventions are needed. In the special administrative region of Hong Kong, LPM rest days, on which stalls are cleared of unsold poultry and disinfected, have been used since 2001 to reduce the amplification of avian influenza viruses in LPMs (*10*,*11*). Ideally, improved surveillance of avian viruses in poultry would enhance identification and closure of contaminated markets and uncontaminated markets could remain open.

Our findings are limited by the ecologic nature of our analysis. It is possible that the incidence rate of human infection with H7N9 in a city appeared to decline substantially at the same time LPMs were closed in that city for reasons other than the closure of LPMs or by chance. Information about the prevalence of H7N9 virus in poultry in different markets and the distribution network of poultry farms and markets would support construction of more complex models of the underlying transmission dynamics. Another limitation was that we could not include interventions used in locations where LPMs were not closed. Our analysis was designed to be self-controlled by comparing

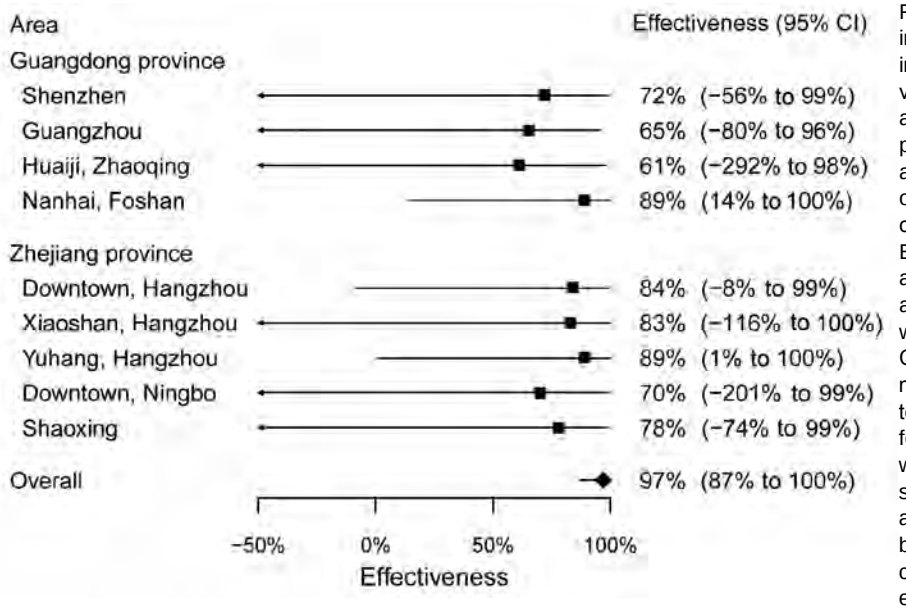

Figure 2. Estimates of the effect of interventions in reducing human risk for infection with avian influenza A(H7N9) virus in urban areas of Guangdong and Zhejiang provinces. Estimates are presented as effectiveness, calculated as 1 minus the ratio of incidence rates of infection after closure versus before closure, within 95% confidence intervals. Estimates are shown for each urban area, and a single summary measure is also shown assuming the effectiveness was the same across all areas. For Huaiji County and Nanhai District, live poultry markets (LPMs) epidemiologically linked to confirmed H7N9 cases were closed a few days before all the other local LPMs were closed. To account for differential start dates of LPM closure within these areas, we performed sensitivity analysis by setting the overall start date of LPM closure in a given area to be either the earliest or the last day on which local LPMs began to close. Results in the 2 scenarios were similar.

incidence of human cases before and after market closures. Finally, we could not examine the effect of LPM closures in other locations that had few cases during winter 2013–14. In general, our findings apply to urban areas, where live poultry purchases mainly occur in LPMs.

Conclusions

Closure of live poultry markets was highly effective in reducing human risk for H7N9 infection during winter months of the 2013–14 influenza season. However, preventive actions such as enhanced surveillance of poultry and scheduling regular rest days could prevent the necessity of using this costly intervention.

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Raw Pig Blood Consumption and Potential Risk for *Streptococcus suis* Infection, Vietnam

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We assessed consumption of raw pig blood, which is a risk factor for *Streptococcus suis* infection in Vietnam, by using a mix-method design. Factors associated with consumption included rural residency, age, sex, occupation, income, and marital status. We identified risk groups and practices and perceptions that should be targeted by communication programs.

Consumption of undercooked animal products is a well-established risk factor for acquiring many infectious diseases (*1*–*5*). In Vietnam, raw blood of pigs or other animals is consumed in a dish known as *tiet canh*. The main ingredients of porcine *tiet canh* include coagulated, fresh, uncooked blood mixed with chopped cooked pork tissues (Figure). A recipe is shown in online Technical Appendix Table 1 (http://wwwnc.cdc.gov/EID/article/20/11/14-0915-Techapp1.pdf). Consumption of raw pig products is associated with trichinellosis and *Streptococcus suis* meningitis in humans in Vietnam (*6*–*8*).

S. suis is a common gram-positive bacterium found in pigs, which can cause severe infections in humans; ≈90% of human cases are reported from Asia (*9*,*10*). Case-fatality rates range from 3% to 7% but may reach ≈60% among patients with severe sepsis, as observed in a large outbreak in Sichuan, China, in 2005 (*11*). Studies have identified occupational exposure to pigs and consumption of specific traditional pork dishes as key risk factors for contracting *S. suis* infection (*10*). Effective control of diseases transmitted through consumption of undercooked pig products requires a thorough understanding of this food practice. Therefore, we investigated consumption of porcine *tiet canh* in northern Vietnam and explored community perceptions regarding associated disease risks.

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The Study

The study was conducted in 2 health care and demographic surveillance sites in Hanoi Province, Vietnam: Ba Vi District (rural) and Dong Da District (urban). Each site contained ≈11,000 households that were selected by cluster sampling to represent the district population (*12*). This study was approved by ethical committees at the University of Oxford and Hanoi Medical University.

A quantitative survey on *tiet canh* consumption was administered to household members at health care and demographic surveillance sites (Ba Vi: May–June 2012; Dong Da: December 2012–January 2013). Field surveyors visited households as part of their routine survey schedules and interviewed 1 member per household individually. A total of 6,993 participants in Ba Vi and 3,991 participants in Dong Da were interviewed (no households refused). After persons for whom no data were available regarding age and sex were excluded, 6,943 (99.3%) persons in Ba Vi and 3,921 (98.2%) in Dong Da were included in the analysis (mean age [range]: 47.0 [8–97] years in Ba Vi and 48.3 [9–102] years in Dong Da).

Rural and urban respondents differed significantly by sex (24.6% vs. 34.5% male participants, respectively), education (21.9% vs. 74.3% with ≥10 years of education), and occupation (2.4% vs. 29.6% office workers). Subsequently, 10 focus groups that involved 81 participants in the 2 districts were formed (April–June 2013). Participants in focus groups were selected on the basis of reported consumption of *tiet canh* in the previous survey and were stratified by district, sex, and consumption status. For each district, 1 focus group was also conducted for local government workers. Details on data collection, characteristics of participants, and data analysis are described in the online Technical Appendix.

A total of 35% (95% CI 33.8%–36.1%) of persons in the rural area vs. 8.6% (95% CI 7.7%–9.5%) in the urban area reported eating porcine *tiet canh* in the past year. Duck blood was the second most common source of *tiet canh* (online Technical Appendix Table 3). Subsequent analyses were restricted to porcine *tiet canh*. Sex, age, level of education, occupation, economic status, and marital status were associated with consumption patterns by univariate analysis (Table 1). However, level of education was not associated by multivariable regression (Table 2).

More men than women reported consumption, and this difference was greater in the urban setting than the rural setting. Given that more women than men participated in the survey, the estimated frequency of persons consuming *tiet canh* will likely be higher than reported in this study.

Figure. Traditional dish (*tiet canh*) containing raw pig blood, Vietnam.

The practice was more common in persons 40–49 years of age than in other groups. Persons who reported highest consumption included farmers, manual laborers, persons working in service and sales. In the urban district, household economic status was negatively associated with consumption levels (odds ratio >2.0 for 2 lowest quintiles compared with the highest quintile). This finding was further confirmed in focus groups because *tiet canh* is relatively inexpensive and available in most markets. Therefore low-income workers are more likely to eat this dish (online Technical Appendix Table 5).

Conclusions

Consumption of *tiet canh* is closely linked with traditional family celebrations, particularly weddings. These traditions are a source of pride and social bonding among community members. Pigs are frequently slaughtered at homes of families hosting celebrations. Several male participants expressed pride and fond memories of their experience in participating in slaughtering events. *Tiet canh* is sometimes served at family celebrations expressly to demonstrate that slaughtered pigs are healthy. Cultural contributions of *tiet canh* must be understood to develop effective communication messages to reduce health risks associated with this practice.

Participants articulated strong confidence in the safety of raw pig products when the source of the pig was known to the consumer and the pig appeared healthy. Sources of pigs considered relatively safe were home-raised pigs, wild boars, or pig breeds locally known as *'lợn mán* and *lợn mường* (typically free-range, scavenging

Table 1. Factors associated with consumption of raw pig blood among respondents in 2 districts of Hanoi, Vietnam*

	Ba Vi District (rural)			Dong Da District (urban)		
Factor	Consumption, no. (%)	No consumption, no. (%)	OR (95% CI)	Consumption, no. (%)	No consumption, no. (%)	OR (95% CI)
Sex						
M	900 (52.6)	810 (47.4)	**3.0 (2.7–3.4)**	250 (18.5)	1,103 (81.5)	**6.4 (4.9–8.2)**
F	1,527 (29.2)	3,706 (70.3)	1	88 (3.4)	2,480 (96.6)	1
Age, y						
<20	17 (13.8)	106 (86.2)	**0.4 (0.2–0.7)**	2 (4.1)	47 (95.9)	0.8 (0.2–3.7)
20–29	209 (28.8)	516 (71.2)	**1.5 (1.2–1.8)**	37 (8.3)	411 (91.7)	**1.7 (1.1–2.7)**
30–39	535 (38.3)	863 (61.7)	**2.3 (2.0–2.8)**	68 (8.7)	713 (91.3)	**2.2 (1.5–3.4)**
40–49	759 (42.3)	1,037 (57.7)	**2.6 (2.2–3.0)**	85 (11.3)	668 (88.7)	**2.8 (1.9–4.1)**
50–59	593 (36.2)	1,046 (63.8)	**1.9 (1.6–2.2)**	104 (11.2)	823 (88.8)	**2.6 (1.8–3.8)**
≥60	314 (24.9)	948 (75.1)	1	42 (4.4)	921 (95.6)	1
Education, y						
≤5	303 (27.3)	807 (72.7)	1.5 (1.0–2.1)	10 (6.8)	138 (93.2)	2.2 (1.0–4.5)
6–9	1,637 (38.0)	2,673 (62.0)	**1.7 (1.2–2.5)**	97 (11.4)	755 (88.6)	**2.0 (1.5–2.8)**
10–12	441 (32.6)	910 (67.4)	1.3 (0.9–1.9)	124 (7.9)	1,450 (92.1)	1.1 (0.9–1.5)
>12	44 (26.7)	121 (73.3)	1	103 (7.8)	1,218 (92.2)	1
Occupation						
Office worker	41 (24.4)	127 (75.6)	1	88 (7.6)	1,067 (92.4)	1
Manual laborer†	277 (41.6)	389 (58.4)	**1.5 (1.0–2.3)**	55 (16.2)	284 (83.8)	**1.9 (1.3–2.8)**
Services and sales	195 (34.8)	366 (65.2)	**1.6 (1.0–2.3)**	113 (12.2)	810 (87.8)	**1.9 (1.4–2.5)**
Farmer	1,649 (36.9)	2,825 (63.1)	**2.0 (1.4–2.9)**	0	2 (100)	–
Other	98 (37.7)	162 (62.3)	1.4 (0.9–2.3)	12 (21.4)	44 (78.6)	2.1 (1.1–4.3)
Not working‡	156 (19.8)	630 (80.2)	1.1 (0.7–1.6)	69 (4.8)	1,355 (95.2)	1.2 (0.8–1.9)
HES quintiles						
Lowest	346 (30.4)	794 (69.6)	0.9 (0.7–1.0)	71 (11.0)	574 (89.0)	**2.1 (1.4–3.0)**
Second	472 (32.0)	1,002 (68.0)	0.9 (0.7–1.0)	100 (12.5)	697 (87.5)	**2.2 (1.5–3.1)**
Third	617 (37.9)	1,011 (62.1)	1.1 (0.9–1.3)	58 (6.9)	779 (93.1)	1.1 (0.8–1.7)
Fourth	561 (36.9)	960 (63.1)	1.0 (0.9–1.2)	54 (6.5)	773 (93.5)	1.1 (0.7–1.6)
Highest	416 (36.2)	732 (63.8)	1	55 (6.8)	751 (93.2)	1
Marital status						
Married	2,186 (38.1)	3,559 (61.9)	**1.6 (1.4–1.9)**	260 (8.9)	2,663 (91.1)	0.8 (0.6–1.1)
Single	241 (20.1)	957 (79.9)	1	78 (7.8)	920 (92.2)	1

*Values in bold are significant (p<0.05). OR, odds ratio; HES, household economic status. OR was adjusted for sex and age.
†Includes construction, factory work, casual manual work on call, handicraft work, and mining.
‡Includes children, housewives, elderly persons, and retired persons.

Table 2. Variables in models predicting consumption of raw pig blood , Vietnam*

Group	Variables in final model†	Nagelkerke R‡
Rural persons	Sex, age, occupation, marital status, HES	0.123
Rural farmers	Sex, age, marital status, HES	0.086
Rural non-farmers	Sex age, occupation, marital status	0.168
Urban persons	Sex, age, occupation, HES	0.185
Rural and urban persons	Sex, age, occupation, marital status, location (rural vs. urban)	0.242

*HES, household economic status.
†For model selection, all variables were forced into logistic regression. Each variable that was not significant (p ≥0.10) was removed step by step until all remaining variables were significant (p<0.10) in the model.
‡Higher values indicate a stronger model.

pigs raised by ethnic minorities). These perceptions contrast with findings of prevalence studies that showed high carriage rates of *S. suis*, even in apparently healthy pigs and pig products (*13*), and with reports of transmission of neurocystercercosis (*14*) and trichinellosis (*6*,*15*), which suggested increased transmission risks associated with scavenging pigs.

Beliefs about potential health benefits of eating *tiet canh*, such as preventing anemia or a general cooling effect, were widespread. However, participants did not fully understand the health risks posed by infectious agents or contaminants, and risks were dismissed or overlooked. Although concerns regarding the risk for diseases associated with *tiet canh* were raised in all focus groups, few participants knew what specific diseases are transmissible to humans through *tiet canh* consumption. In contrast, risk underestimation through optimistic bias was common, and fatalistic attitudes were shared in the group setting (online Technical Appendix).

The Agriculture Ministry of Vietnam had issued an official letter (no. 18 BNN/CĐ, May 21, 2009) that requested coordinated actions in controlling transportation, slaughtering, selling, and consumption of animals and animal products in response to recent disease reemergence. This letter also recommended a ban on selling of *tiet canh*. However, this proposed ban was considered to be unenforceable and ineffective among participants in all focus groups. The profit from selling *tiet canh* and consumer demand were considered key features that will perpetuate this traditional dish. Furthermore, trade in raw pig products is too widespread and decentralized, and the food chain from pig producers to pork consumers is too complex to enable regulation or enforcement of trade bans.

This study showed that consumption of *tiet canh* was more common among adult working-age men, outdoor workers, low-income urban inhabitants, and married persons in rural areas. Children rarely eat *tiet canh*, which may partly explain why *S. suis* meningitis is mainly a disease of adults and more common in men. Disease surveillance and reporting should be improved to better estimate the incidence of *S. suis* infections and clarify the relative role of the foodborne transmission route.

Given the traditions of consumption of *tiet canh* during family celebrations, interventions such bans on consumption or simple education messages on health risks without accounting for associated cultural values are unlikely to be effective. However, changes in education, urbanization, and increasing income levels will affect social and behavioral attitudes toward consumption of *tiet canh* in the future. Food safety research could benefit consumers by exploring methods of preparation of *tiet canh* designed to reduce infectivity of any pathogens in raw blood and preserve desired texture or taste characteristics of this traditional cuisine.

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Beijing Lineage of MDR *Mycobacterium tuberculosis* in Bulgaria, 2007–2011

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To assess the spread of the *Mycobacterium tuberculosis* Beijing genotype among patients with multidrug-resistant and extensively resistant tuberculosis in Bulgaria, we genotyped 188 (72%) of 261 microbiologically confirmed resistant isolates obtained during 2007–2011. The estimated prevalence of the Beijing genotype among these patients was 3.2%.

In 1995, the Beijing genotypic family was identified in Beijing, China, where it accounted for at least 86% of *Mycobacterium tuberculosis* isolates (*1*). Genotyping of 2,092 multidrug resistant (MDR) and extensively drug-resistant (XDR) strains, isolated in 24 European countries during 2003–2011, indicated that 470 (22.5%) strains belonged to the Beijing lineage, many of which originated from eastern European countries (*2*). Strains of this genotypic family account for at least 13% of *M. tuberculosis* strains worldwide, and the percentage seems to be increasing (*3*,*4*).

Bulgaria is 1 of 27 countries that the World Health Organization describes as having a high burden of MDR tuberculosis (TB). TB case notification rates in Bulgaria were 39.6 cases/100,000 population (3,038 cases) in 2007, 41.2 cases/100,000 population (3,150 cases) in 2008, 38.3 cases/100,000 population (2,910 cases) in 2009, 35 cases/100,000 population (2,649 cases) in 2010, and 32.1 cases/100,000 population (2,407 cases) in 2011. During this same period, the MDR/XDR TB notification rate was 7.5% (76 cases) in 2007, 3.3% (31 cases) in 2008, 5.1% (43 cases) in 2009, 5.8% (56 cases) in 2010, and 7.5% (55 cases) in 2011 (*5*).

In a nationwide study of 197 drug-susceptible isolates obtained during 2003–2004, no isolates of the Beijing genotype were identified in Bulgaria (*6*). A subsequent study also did not identify the Beijing genotype among 133 drug-resistant and drug-susceptible *M. tuberculosis* isolates obtained during 2004–2006 (*7*). Since then, Beijing strains of *M. tuberculosis* have been identified during routine screening of MDR/XDR TB isolates in Bulgaria. In this study, after receiving ethics approval, we assessed the spread of the *M. tuberculosis* Beijing genotype among patients with MDR and XDR TB in Bulgaria.

The Study

In Bulgaria during 2007–2011, a total of 188 MDR/XDR *M. tuberculosis* isolates were characterized by drug-susceptibility testing and spoligotyping (31 in 2007, 31 in 2008, 39 in 2009, 47 in 2010, and 40 in 2011) and represent 72% of the 261 MDR/XDR *M. tuberculosis* isolates identified during that period. The MDR/XDR strains were isolated from sputum of 181 (96.2%) patients, gastric lavage fluid from 3 (1.5%), bronchoalveolar lavage fluid from 2 (1%), pleural fluid from 1 (0.5%), and fistula swab sample from 1 (0.5%). The first MDR/XDR *M. tuberculosis* strain isolated per patient was analyzed for this study.

Drug susceptibility of these strains was confirmed by the National Reference TB Laboratory, which used liquid culture at concentrations of 0.1 μg/mL for isoniazid, 1 μg/mL for rifampin, 5 μg/mL for ethambutol, 1 μg/mL for streptomycin, 2.5 μg/mL for capreomycin, 1 μg/mL for amikacin, 5 μg/mL for kanamycin, and 2 μg/mL for ofloxacin. Of the MDR/XDR strains, 77 (41%) were resistant to all first-line anti-TB drugs, 51 (27%) were resistant to isoniazid and rifampin; 38 (20%) to isoniazid, rifampin, and streptomycin; and 22 (12%) to isoniazid, rifampin, and ethambutol. Second-line drug-susceptibility testing was performed for 174 (81%) of the MDR strains. Of these, 140 (80%) were sensitive to all second-line anti-TB drugs and 20 (12%) were resistant to ofloxacin. Five percent (n = 9) of XDR strains had combined resistance to ofloxacin, amikacin, kanamycin, and capreomycin.

To detect and genotype the Beijing strains, we used a spoligotyping kit (Isogen Bioscience BV, Maarssen, the Netherlands), and we performed single-nucleotide polymorphism typing by bead-based multiplex ligation-dependent probe amplification (MLPA) (*8*,*9*). We also screened for the presence of the Beijing genotype in 117 drug-sensitive strains collected from across the country in 2011, representing a convenience sample of ≈10%. Both methods identified 1 drug-sensitive *M. tuberculosis* strain

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Table 1. Phenotypic characteristics of the Mycobacterium tuberculosis Beijing genotype strains identified in Bulgaria, 2007–2011*

Strain	Patient origin	Patient age, y/sex	Case status	Drug susceptibility	First-line drugs				Second-line drugs			
					STR	INH	RIF	EMB	OFL	AMK	KAN	CAP
BG_07_09	Dobrich	35/M	PT	MDR	R	R	R	R	S	S	S	S
BG_35_09	Sofia	42/M	New	MDR	R	R	R	R	S	S	S	S
BG_104_10	Sofia	25/F	New	XDR	R	R	R	R	R	R	R	R
BG_85_10	Varna	32/M	New	MDR	R	R	R	R	S	S	S	S
BG_95_10	Varna	59/M	New	MDR	R	R	R	R	S	S	S	S
BG_112_11	Sofia	32/M	New	Susceptible	S	S	S	S	NA	NA	NA	NA
BG_54_11	Sofia	48/M	PT	MDR	R	R	R	R	S	S	S	S

*AMK, amikacin; CAP, capreomycin; EMB, ethambutol; INH, isoniazid; KAN, kanamycin; NA, not applicable; OFL, ofloxacin; PT, previously treated; R, drug resistant; RIF, rifampin; S, drug sensitive; STR, streptomycin.

of the Beijing genotype (BG_112_11, Table 1). The MLPA test assigned this isolate to the Beijing K1 sublineage (*10*).

Among the 188 MDR/XDR strains collected during 2007–2011, a total of 6 isolates with the Beijing genotype were identified (prevalence 3.2%, 95% CI 0.7%–5.7%): 2 in 2009 (Beijing SA+/CHIN+/V+), 3 in 2010 (Beijing SA+/CHIN+/V+ and Beijing K1), and 1 in 2011 (Beijing SA+/CHIN+/V+). These 6 drug-resistant strains were isolated from 5 male and 1 female patients. Drug-susceptibility testing and molecular drug-resistance markers confirmed that 1 strain was XDR. A total of 4 strains were Spoligo International Type 1 (SIT1) and 2 strains were SIT265 (Tables 1 and 2). In the neighboring countries of Albania, Greece, Romania, and Turkey, no MDR *M. tuberculosis* Beijing strains were isolated or prevalence was 1%–3%, mostly of imported origin (*11–14*). The origins of the MDR/XDR strains and the drug-sensitive strain identified in Bulgaria are described below.

The first XDR Beijing strain identified (BG_104_10) was from a patient who arrived in Bulgaria from Moldova in 2009 and in whom TB developed a few months later. Before coming to Bulgaria, the patient had had contact with a TB patient in Ukraine. The first *M. tuberculosis* isolate recovered from this Moldovan patient was confirmed as primary XDR *M. tuberculosis*. The patient received treatment with second-line TB drugs in 2010, underwent surgery, and in 2011 was considered cured.

The 2 SIT265 Beijing genotype clinical isolates (BG–85-10 and BG–95–10) originated from 2 neighbors. One patient was an alcohol-dependent sailor in whom TB developed after a ship voyage. TB developed in his neighbor (who had diabetes) a few months later. *M. tuberculosis* transmission most likely occurred between them. The epidemiologic link was supported by identical drug-susceptibility testing results, MLPA, spoligotyping, and variable number–tandem repeat typing profiles with no similarities to other genotypes identified in Bulgaria. Most of the 50 SIT265 Beijing genotype strains, reported in the SITVITWEB database (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE), originated from the United States, Russia, Israel, and Spain (*4*). Both patients were receiving treatment as of 2012.

The other 3 MDR Beijing isolates had different VNTR patterns but shared the same SIT1 spoligotype and the same MLPA lineage type (SA+/V+/CHIN+) (Table 2). Of these 3 patients, 2 lived in the capital city of Sofia (with strains BG_54_11 and BG_112_11) and the third (with strain BG_07_09) lived in Dobrich, in the northeastern part of the country. The patient was alcoholic, antisocial, and homeless and refused treatment. The MDR TB was diagnosed in 2007, but the strain was lost. In 2009, the patient was hospitalized, and Beijing genotype MDR *M. tuberculosis* was identified. The patient died that same year.

Table 2. Genotyping characteristics of the *Mycobacterium tuberculosis* Beijing genotype strains identified in Bulgaria, 2007–2011*

Strain	Spoligotype pattern	SIT	24 MIRU-VNTR†	MLPA lineage type‡
BG_07_09	□□□□□□□□□□□□□□□□□□□□□□□□□□□□ □□□□□□■■■■■■■■■	1	244233352634425153353823	SA+/V+/ CHIN+
BG_35_09	□□□□□□□□□□□□□□□□□□□□□□□□□□□□ □□□□□□■■■■■■■■■	1	244233352644425173343723	SA+/V+/ CHIN+
BG_104_10	□□□□□□□□□□□□□□□□□□□□□□□□□□□□ □□□□□□■■■■■■■■■	1	244233352644425153353823	SA+/V+/ CHIN+
BG_85_10	□□□□□□□□□□□□□□□□□□□□□□□□□□□□ □□□□□□■■□■■■■■■	265	244233352644425153353823	K1
BG_95_10	□□□□□□□□□□□□□□□□□□□□□□□□□□□□ □□□□□□■■□■■■■■■	265	244233352644425153353823	K1
BG_112_11	□□□□□□□□□□□□□□□□□□□□□□□□□□□□ □□□□□□■■■■■■■■■	1	NA	K1
BG_54_11	□□□□□□□□□□□□□□□□□□□□□□□□□□□□ □□□□□□■■■■■■■■■	1	244233352634425153353823	SA+/V+/ CHIN+

*MIRU, mycobacterial interspersed repetitive unit; NA, not applicable; SIT, Spoligo International Type second to SITVITWEB database classification; VNTR, variable number–tandem repeat.
†24 loci MIRU-VNTR order: 154, 424, 577, 580, 802, 960, 1644, 1955, 2059, 2163b, 2165, 2347, 2401, 2461, 2531, 2687, 2996, 3007, 3171, 3192, 3690, 4052, 4156, 4348.
‡SA+, South Africa; V+, Vietnam; CHIN+, China; K1, Uzbekistan (*9*,*10*).

Another Beijing strain was identified in 2009 from a patient who lived in Sofia (BG_35_09, Tables 1 and 2). The patient's parents were from Armenia, where the prevalence of the Beijing genotype is no less than 41% (*4*) and prevalence of MDR TB in 2009 was 22.9% (*5*). The patient had not been to Armenia, but the possibility of casual contact with migrating Armenians was not excluded. This patient died in 2010.

Conclusions

Among MDR/XDR *M. tuberculosis* isolates in Bulgaria, prevalence of the Beijing genotype is low. Not all detected cases of TB caused by the Beijing genotype were a result of human migration; MDR/XDR TB transmission within the country was also observed. The *M. tuberculosis* Beijing genotype strains are considered to be large drivers of international TB transmission and are associated with the emergence and spread of MDR/XDR TB (*2*). This finding demands organization of wider surveillance in Bulgaria that includes monitoring genotypes of drug-susceptible and drug-resistant *M. tuberculosis* strains.

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Another Dimension

Emerging Infectious Diseases accepts 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.

Human Influenza A(H7N9) Virus Infection Associated with Poultry Farm, Northeastern China

Ming Fan, Biao Huang, Ao Wang, Liquan Deng, Donglin Wu, Xinrong Lu, Qinglong Zhao, Shuang Xu, Fiona Havers, Yanhui Wang, Jing Wu, Yuan Yin, Bingxin Sun, Jianyi Yao, and Nijuan Xiang

We report on a case of human infection with influenza A(H7N9) virus in Jilin Province in northeastern China. This case was associated with a poultry farm rather than a live bird market, which may point to a new focus for public health surveillance and interventions in this evolving outbreak.

Since it was first reported in spring 2013 (*1*), influenza A(H7N9) virus has caused 436 confirmed human infections, resulting in 167 deaths as of July 8, 2014 (Chinese Center for Disease Control and Prevention [China CDC], unpub. data). Most cases have occurred in eastern and southern China and have been associated with exposure to poultry at live poultry markets (LPMs). We report a case of human H7N9 virus infection in Jilin Province in northeastern China, an area not contiguous with provinces in which human cases have been previously reported (Figure 1). Furthermore, this case was associated with a small-scale poultry farm, not an LPM.

The Case

On February 15, 2014, a 50-year-old man, owner of a small farm in Changchun, Jilin Province, experienced an isolated fever (axillary temperature 38.3°C). He had no history of underlying medical conditions. The man sought medical care that day at Jilin University Third Hospital and returned to the hospital on February 16, 17, and 19 with ongoing fever. Radiographic imaging showed evidence of pneumonia; he received intravenous administration of azithromycin and xiyanping, a traditional Chinese medicine. On February 19, the man sought care at Jilin University First Hospital, where throat swab samples were taken. The same day, testing of the samples at the local and provincial Centers for Disease Control and Prevention yielded positive results for influenza A(H7N9) virus by real-time reverse transcription PCR (RT-PCR); China CDC confirmed results the next day. Treatment with oseltamivir (150 mg 2′/d) and methylprednisolone (80 mg 2′/d) was initiated, and oxygen was administered by nasal cannula. The man recovered and was discharged from the hospital on March 7.

The man denied exposure to poultry other than on his farm, including to poultry on other farms or in LPMs. He reported no contact with persons who had similar symptoms before onset of his illness. As part of the case investigation, 68 close contacts of the case-patient were monitored for 7 days; 1 contact had influenza-like illness. Throat swab specimens were collected from this person on days 2 and 3 after symptom onset and tested for influenza A(H7N9) by using real-time RT-PCR; results were negative.

The virus from the case-patient's specimens was isolated in egg culture and designated A/Jilin/10117/2014 (H7N9) (full sequence available from GISAID, accession no. EPI_ISL_161665). The isolate's 8 genes were similar to those of the virus A/Anhui/ 02/2013 (H7N9) (GISAID accession no. EPI_ISL_141190); nucleotide/amino acid homology was 99.5%/99.6% for hemagglutinin, 99.2%/98.9% for neuraminidase, 99.3%/99.7% for polybasic 1, 96.4%/98.9% for polybasic 2, 97.9%/99.6% for polymerase acidic, 99.7%/99.6% for nucleoprotein, 97.7%/100.0% for matrix protein, and 99.5%/99.1% for nonstructural protein.

During August 2013–February 2014, the case-patient had introduced 7 groups of birds to his farm, totaling ≈1,100 birds from 8 source farms (Figure 2). The birds were turkeys, guinea fowl (*Numida meleagris*), black-bone silkie chickens (*Gallus gallus domesticus*), local chickens, and a goose. None of the birds received avian influenza vaccines on the case-patient's farm; previous vaccination histories were unknown. Birds commingled in an egg production warehouse (Figure 3, http://wwwnc.cdc.gov/EID/article/20/11/14-0608-F3.htm) maintained by the case-patient and by 2 farmers hired in early January 2014. The warehouse was seldom cleaned and never disinfected. The case-patient cared for the birds daily and did not use personal protective equipment.

In October 2013, a group of 280 silkie chickens and guinea fowl was introduced to the warehouse and

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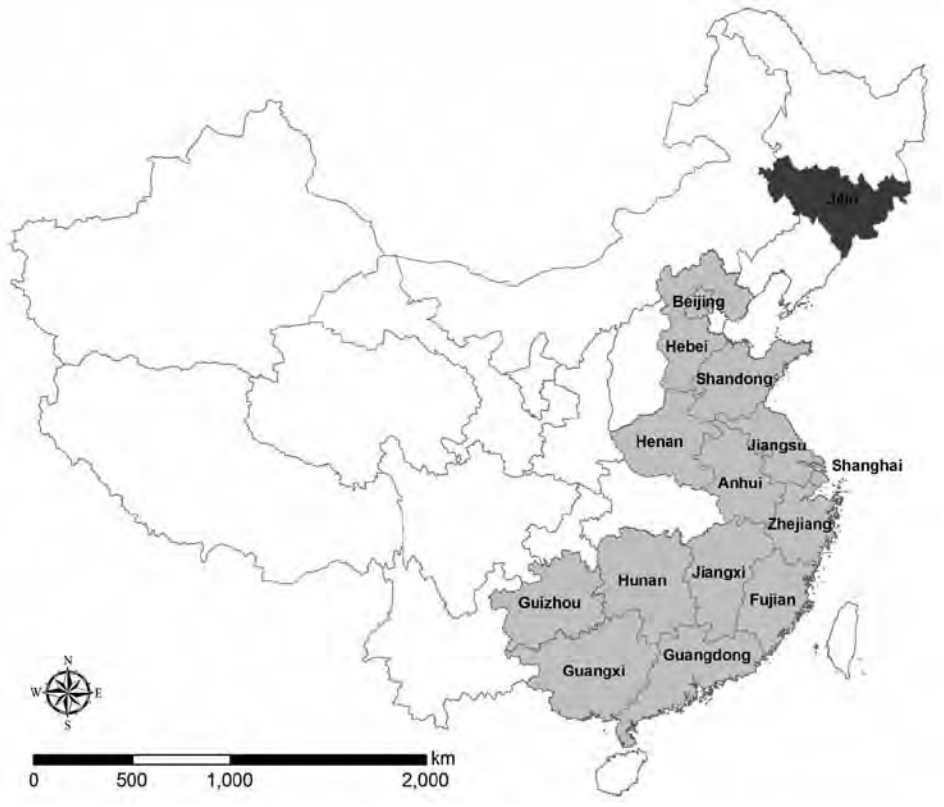

Figure 1. Provinces in China in which human cases of infection with influenza A(H7N9) virus have been confirmed (gray shading). Jilin Province (dark shading), where the case described in this article occurred, shares borders with the Russian Federation and the Democratic People's Republic of Korea (North Korea).

subsequently exhibited signs of illness; many of these birds died during the next 3 months. A veterinarian reportedly diagnosed air sacculitis and enteritis; testing for influenza A was not performed. By January 2014, all but 4 silkie chickens and 40 guinea fowl had died. More birds were added on February 3, but no further illness was noted until 2 additional flocks, totaling 290 birds, arrived on February 10 and 12. Widespread illness then occurred among the birds, including respiratory and eye secretions, wheezing, and death; >100 birds had died by February 15, when the case-patient's symptoms began. The Changchun Prefectural Bureau of Agriculture performed necropsies and attributed these deaths to a mixed infection of fowlpox and colibacillosis.

Investigation and sampling at the case-patient's farm and related suppliers were conducted by local, provincial, and national CDCs. Public health laboratories tested 84 poultry and environmental specimens collected from the farm (Table); 19 samples were positive by real-time RT-PCR for both influenza A(H7N9) and A(H9N2), 1 was positive only for H7N9, and 3 were positive only for H9N2. A total of 374 samples were collected from the 8 source farms, the farms and transport vehicles of the 2 distributors who delivered chickens to the case-patient's farm, and 56

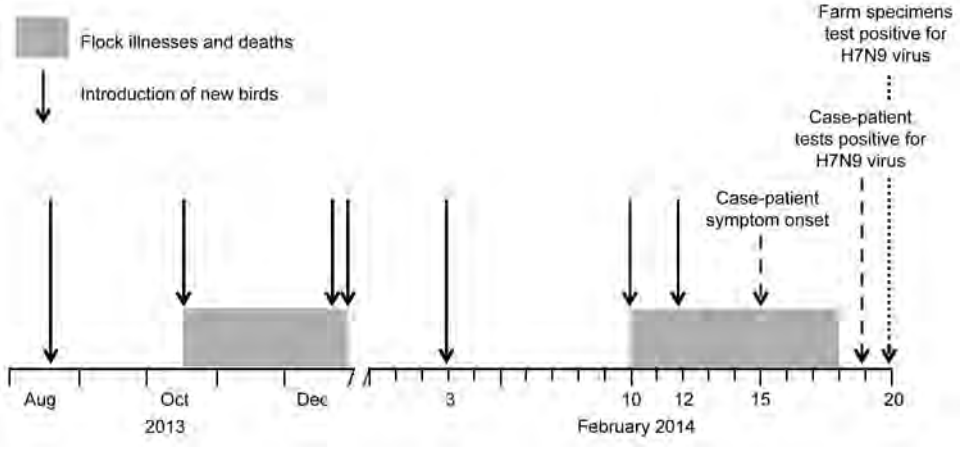

Figure 2. Timeline of introduction of new birds to the farm of the case-patient with influenza A(H7N9) virus infection in Jilin Province, China, 2013–2014. Dates of illnesses and deaths among bird flock on farm, the case-patient's symptom onset, and confirmed testing results are indicated.

Table. Specimen collection and results of real-time reverse transcription PCR testing for epidemiologic investigation into source of human influenza A(H7N9) virus infection, Jilin Province, China

Source and specimen type*	Total no. specimens	No. (%) positive results†		
		H7N9 and H9	H7N9 only	H9 only
Case-patient poultry farm				
Poultry feces	25	1 (4)	0	0
Sewage	8	3 (38)	0	0
Environmental swab samples (chicken troughs)	13	7 (54)	0	0
Oropharyngeal samples	20	7 (35)	1 (5)	3 (15)
Cloacal samples	17	0	0	0
Cloacal and oropharyngeal samples	1	1 (100)	0	0
Environmental samples from distributors' farms and transport vehicles‡	13	0	0	4 (31)
Source farms and other area farms§				
Poultry feces	148	0	0	0
Sewage	32	0	0	0
Environmental swab samples (chicken troughs)	150	0	0	0
Oropharyngeal samples	12	0	0	0
Cloacal samples	19	0	0	0

*One oropharyngeal swab sample from a goose and 1 oropharyngeal swab from a turkey were included in the specimens from case-patient poultry farm; both samples were positive for influenza A(H7N9) and A(H9N2). All other cloacal and/or oropharyngeal swab specimens were from local chickens.
†H9-positive samples from the case-patient poultry farm were influenza A(H9N2). H9-positive samples from other sources were not further characterized by subtype.
‡Distributors bought poultry from companies or local farmers (source farms) and then sold the poultry to others, including the case-patient. Poultry was kept at distributors' farms before resale. Samples were taken from transport vehicles and farms of 2 distributors who transported the chickens to the case-patient's farm. All positive specimens were from a single distributor: 3 from his transport vehicle and 1 from his farm.
§Eight farms that supplied the birds to the case-patient (source farms) and 56 other farms located in the villages of the case-patient and source farms.

other local farms. Four specimens from 1 distributor tested positive for influenza A H9 by RT-PCR; neuraminidase subtyping was not performed on these specimens.

Conclusions

We describe a human case of influenza A(H7N9) virus infection epidemiologically linked to a poultry farm from which samples tested positive for H7N9 and H9N2 viruses. Previous reports of human subtype H7N9 virus infections in mainland China identified a poultry-related exposure for 84% of case-patients, 77% of which were linked to LPMs, but none were linked to farms (China CDC, unpub. data). According to China's Ministry of Agriculture, almost all positive samples have been from LPMs; H7N9 virus was initially isolated from farms in Guangdong Province in southern China in mid-March 2014 (*2*). Samples collected from a farm in Zhejiang Province were also positive for influenza A(H7N9) virus by real-time RT-PCR, but human cases were not epidemiologically linked to this farm (*3*) .

Unlike avian influenza A(H5N1) virus, which frequently causes severe disease and death in poultry, influenza A(H7N9) virus is a low pathogenicity virus and has not been observed to cause substantial deaths in avian species. Under experimental conditions, birds exhibited no clinical disease after H7N9 virus challenge, although chickens and quail shed high levels of virus (*4*). The poultry on this case-patient's farm were co-infected with other pathogens, and many died in the week preceding the case-patient's illness. The introduction of even a low pathogenicity virus into a flock already challenged with other pathogens likely contributed to the rates of illness and deaths.

This case report underscores the importance of highly sensitive assays in detecting H7N9 virus in poultry and their surrounding environments. The virus was detected in a substantial proportion of specimens on the case-patient's farm (20/84, 24%); in addition, we found that 95% (19/20) of specimens positive for H7N9 virus were also positive for H9N2 virus. The H7N9 virus possesses internal gene cassettes from poultry H9N2 virus, another low pathogenicity virus that circulates widely in poultry in Asia (*3*,*5*,*6*). Co-circulation of H7N9 and H9N2 viruses was reported in LBMs in Hangzhou but not on farms (*3*).

Public health measures taken to contain the outbreak of H7N9 virus infection have thus far focused on LPMs (*7*–*14*), but our findings suggest that small-scale farms may be another source. Inadequate cleaning and disinfecting, a lack of personal protective equipment, and the comingling of bird species found on this farm are common in small-scale poultry farms. These factors enable evolution of novel avian influenza viruses and spread of H7N9 virus. Improved surveillance and biosecurity on farms in China is crucial to containing this outbreak.

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Detection of Rare G3P[19] Group A Rotavirus in Human Patient, Italy

Giovanni Ianiro, Roberto Delogu, Rosalia Graffeo, Maurizio Sanguinetti, Lucia Fiore, and Franco M. Ruggeri

Infection with a rare G3P[19] rotavirus A strain was identified in an immunosuppressed patient in Italy. The strain showed a P[19] viral protein 4 gene and a complete AU-1–like genomic constellation. Phylogenetic analyses showed high nucleotide identity between this strain and G3P[19] rotavirus A strains from Asia, indicating possible reassortment events.

Group A rotavirus (RVA) is the leading cause of acute gastroenteritis in children <5 years of age worldwide, causing ≈450,000 deaths annually. The RVA genome is composed of 11 double-stranded RNA segments, encoding 6 structural viral (VP) and 5 nonstructural (NS) proteins (*1*). The outer capsid proteins, VP7 and VP4, elicit neutralizing antibodies. The genes encoding these proteins specify at least 27 G and 37 P genotypes, which are used for RVA binary classification.

Most RVA human infections worldwide are related to 5 major genotypes: G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] (*2*). Genome segment reassortment between human strains or human and animal strains during co-infections can generate viruses with novel genotype combinations, possibly influencing the virus phenotype (*2*). Some human and animal RVA strains possess unusual genotype combinations (*3*,*4*), and some strains might partially escape vaccine-induced immune protection (*5*).

Since 2007, the RVA surveillance network RotaNet-Italy has confirmed circulation of common RVA genotypes among children in Italy, despite sporadic uncommon, exotic, or zoonotic genotypes (*6*,*7*). We describe infection with a rare G3P[19] RVA strain in an immunosuppressed adult patient in Italy who had severe diarrhea.

The Study

In 2012, a 35-year-old woman who was hospitalized in the Hematology Unit of Rome University Hospital "Agostino Gemelli" in Rome, Italy; she experienced acute gastroenteritis after a bone marrow allotransplant. Stool samples were collected and tested for classic bacterial, viral, and parasitic enteropathogens. The study was performed in compliance with informed consent guidelines in Italy.

Viral RNA was extracted by using the Viral RNeasy MiniKit (QIAGEN/Westburg, Milan, Italy) and stored at –80°C until use. Rotavirus G- and P-genotyping were performed by reverse transcription nested PCR by using VP7 or VP4 primer mixtures described previously (*8*,*9*). Nucleotide sequencing was performed by Macrogen, Inc. (Seoul, South Korea) by using the PCR primers. After analysis in Chromas Pro 2.23 (http://www.technelysium.com.au), consensus sequences were obtained by using SeqMan II (http://www.dnastar.com/t-seqmanpro.aspx). Multiple sequence alignments were carried out, and phylogenetic trees were created by using MEGA5 software (http://www.megasoftware.net) (*10*), using the maximum-likelihood method and Kimura 2- (NS 4–5) or Tamura 3- (all other genes) parameter tests. Strain sequences from this study were deposited in GenBank (accession nos. KF729023–729032).

The patient had Down syndrome, acute lymphatic leukemia, and blood type A Rh+ CCDeekk phenotype; a transcranial Doppler scane did not show any abnormalities. She had received a stem cell allotransplant, followed by immunosuppressive treatment. Acute gastroenteritis began 2 days after immunosuppression, on day 10 after admission to the Hematology Unit. Diarrhea was nonbloody and watery, not accompanied by vomiting and fever, and lasted 3 days, during which rehydration therapy was administered. The patient was released from the hospital in stable condition; she died of systemic complications 3 months later.

Stool samples were collected at diarrhea onset and tested for bacterial and viral enteric pathogens. Results were negative for *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, *Escherichia coli*, staphylococci, *Giardia*, norovirus, and adenovirus. Only rotavirus and *Klebsiella pneumoniae* were detected; because the patient did not exhibit chronic/bloody diarrhea or other systemic pathologies typically related to *K. pneumoniae* infection, this pathogen was not investigated further.

The rotavirus strain, RVA/human-wt/ITA/ROMA116/2012/G3P[19] (ROMA116), was characterized by analyzing its 11 genomic RNA segment sequences in RotaC Tool (http://rotac.regatools.be/). The strain showed the genotype constellation of G3-P[19]-I3-R3-C3-M3-A3-N3-T3-E3-H3. Phylogenetic analyses confirmed a full AU-1–like genomic constellation, associated with the P[19] VP4 gene (Figures 1, 2). The strain clustered strictly with RVA/human-tc/CHN/L621/2006/G3P[9] from China (*11*), sharing 98%–99% nucleotide identities for most genes except VP1 (identity 91%), and the VP4 and NS5 genes, which belonged to different genotypes (Figures 1, 2). ROMA116

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also showed high nucleotide identities (98%–99%) in VP2, VP6–7, and NS1–4 genes with strain RVA/human-wt/THA/CU365-KK/2008/G3P[9] from Thailand (*12*).

The VP7 tree (Figure 1, panel D) revealed strict clustering of ROMA116 with G3 strains from China, Thailand, and Hong Kong, all associated with P[9] VP4. However,

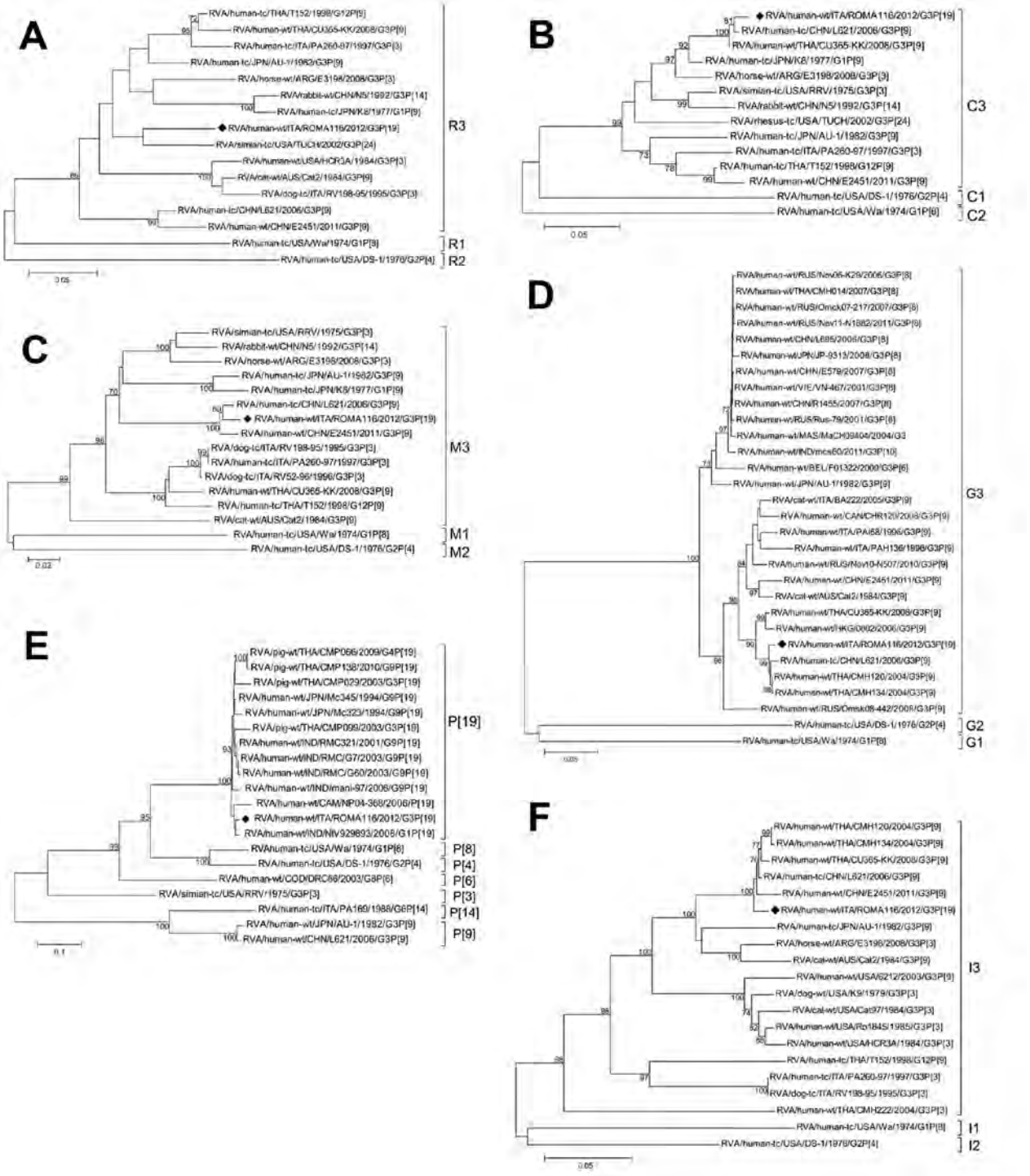

Figure 1. Phylogenetic trees of rotavirus A (RVA) isolates based on the open reading frames of genes coding for the viral protein (VP) regions. A) VP1 (nt 73–390); B) VP2 (nt 1–425); C) VP3 (nt 44–880); D) VP7 (nt 48–1029); E) VP4 (nt 36–817); F) VP6 (nt 44–1326). The G3P[19] strain ROMA116 identified from the patient in Italy described in this article is highlighted with a black diamond. Trees were built with the maximum likelihood method and bootstrapped with 1,000 repetitions; bootstrap values <70 are not shown. Scale bars indicate nucleotide substitutions per site. Letters and numbers on the right indicate the specific gene genotype (http://rotac.regatools.be/classificationinfo.html).

other G3 RVA strains from Italy reported in humans or cats grouped in the same cluster. The VP4 tree (Figure 1, panel E) shows the correlation of the ROMA116 P[19] sequence with P[19] sequences detected in human and swine strains from 1994–2010, suggesting possible human-pig reassortment at the origin of ROMA116 VP4. Further evidence of reassortment resulted from both VP1 and NS5 tree analyses. In VP1, ROMA116 showed the highest nucleotide

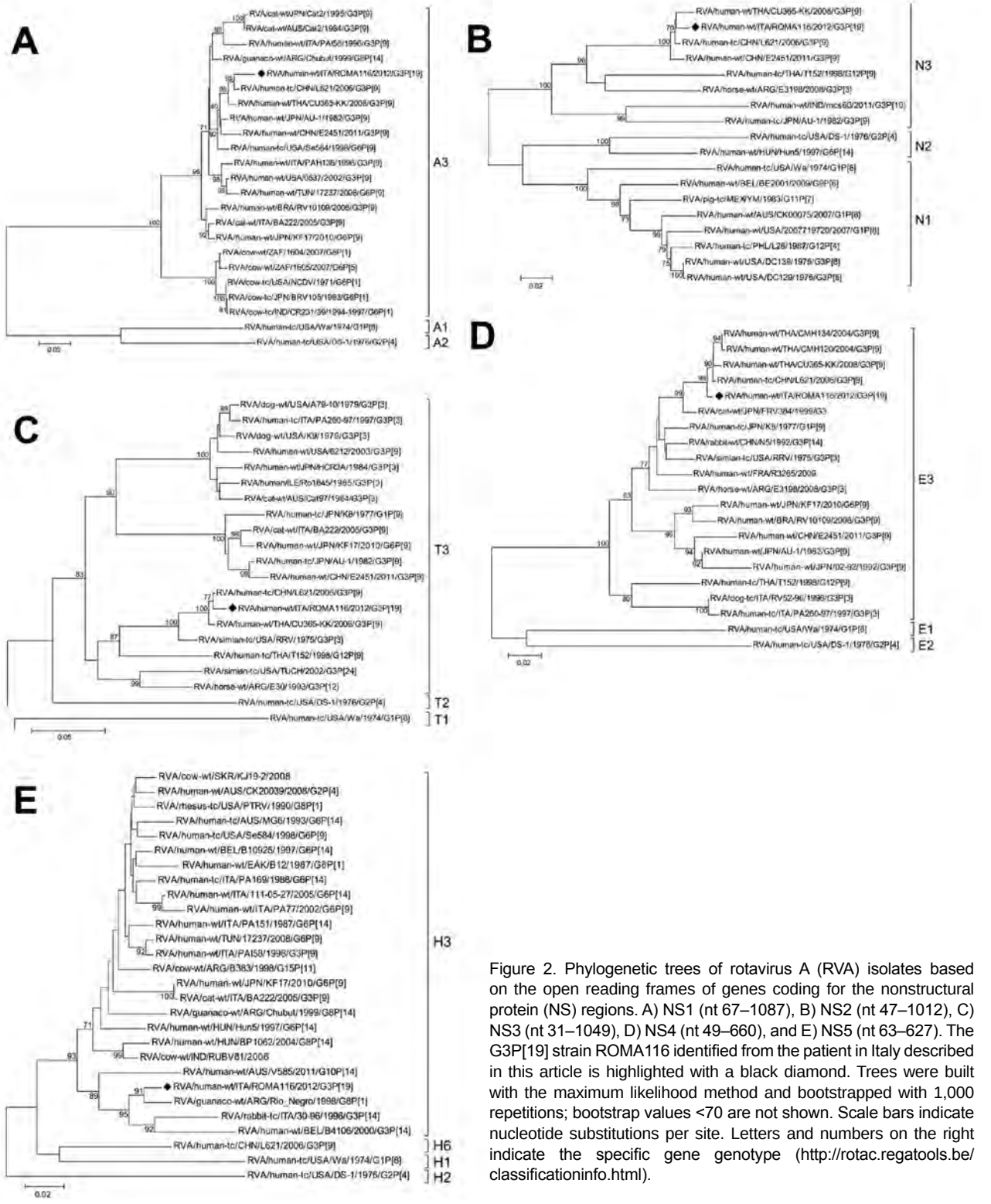

Figure 2. Phylogenetic trees of rotavirus A (RVA) isolates based on the open reading frames of genes coding for the nonstructural protein (NS) regions. A) NS1 (nt 67–1087), B) NS2 (nt 47–1012), C) NS3 (nt 31–1049), D) NS4 (nt 49–660), and E) NS5 (nt 63–627). The G3P[19] strain ROMA116 identified from the patient in Italy described in this article is highlighted with a black diamond. Trees were built with the maximum likelihood method and bootstrapped with 1,000 repetitions; bootstrap values <70 are not shown. Scale bars indicate nucleotide substitutions per site. Letters and numbers on the right indicate the specific gene genotype (http://rotac.regatools.be/classificationinfo.html).

identity (95%) with simian strain TUCH (Figure 1, panel A); in NS5, the uncommon H3 genotype of ROMA116 clustered with strains detected in or derived from animals (Figure 2, panel E).

The phylogenetic trees show the divergence of ROMA116 from the constellation 3 putative ancestor AU-1 (*13*), characterized during the 1980s. ROMA116 shared relatively high nucleotide sequence conservation of only the NS1 gene with AU-1, but all other genes analyzed clustered more closely with RVA strains detected in Asia. This mixed genomic pattern probably was generated by previous reassortment events between strains circulating in that area. Analysis of the VP1, VP4, and NS5 gene trees together indicates that ROMA116 may have evolved through multiple reassortment events involving RVA strains of different animal origins.

Conclusions

The G3P[19] RVA strain we identified represents a single sporadic detection among >7,000 human RVA strains investigated in Italy during a 7-year period, which suggests either a recent introduction or a low ability of this strain to spread among humans. However, the phylogenetic analysis shows that the overall genome of ROMA116 is more similar to those reported for human strains than for animal strains, suggesting that the strain has a lower fitness for replicating in animal hosts than in humans. A study in Thailand (*14*) reported an outbreak of diarrhea in piglets caused by G3P[19] RVA, but no information was available for the other genes of that strain.

The possible importation of an apparently exotic rotavirus strain such as ROMA116 into Italy is not surprising; the country's geographic position favors massive migratory flows of persons from developing countries. Although rare, similar events have been suggested previously (*7*). The source of this infection was not identified; no additional case was reported among hospital ward patients and personnel or in the patient's family. The patient's parents had been cleared to assist their daughter daily after the transplant, but strict control measures for opportunistic infectious agents were otherwise enforced. The patient's family lived in a rural area where swine, bovine, and ovine farming activities occur in close proximity to human residential settlements, which may favor the circulation and zoonotic transmission of viruses from domestic animals to a higher extent than is possible inside urban settings such as Rome. The G3P[19] RVA strain may have been transmitted by an asymptomatic but infected relative, or the patient may have been harboring the strain in the gut before hospital admission, with active viral replication and disease occurring after immunosuppressive treatment.

Because no other enteropathogens were detected among the large panel of bacteria, viruses, and parasites investigated, it is likely that rotavirus was directly involved in causing illness in the patient, whose clinical symptoms were compatible with acute watery rotavirus diarrhea. It is possible that this RVA genotype may not cause disease in immunocompetent persons and that the compromised immune status of this patient played a critical role. Even if G3P[19] RVA, as with other uncommon viral strains, does not present a direct risk for public health in Italy, it could nonetheless be a donor of atypical RVA genes that might reassort into novel epidemic strains that could escape existing herd immunity in humans. In this view, RVA surveillance of both farmed and pet animals could be of valuable support to human surveillance of severe cases in hospitals (*15*), particularly in the postvaccine globalized world.

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New Parvovirus in Child with Unexplained Diarrhea, Tunisia

Tung G. Phan, Khira Sdiri-Loulizi, Mahjoub Aouni, Katia Ambert-Balay, Pierre Pothier, Xutao Deng, and Eric Delwart

A divergent parvovirus genome was the only eukaryotic viral sequence detected in feces of a Tunisian child with unexplained diarrhea. Tusavirus 1 shared 44% and 39% identity with the nonstructural protein 1 and viral protein 1, respectively, of the closest genome, Kilham rat parvovirus, indicating presence of a new human viral species in the *Protoparvovirus* genus.

Parvoviruses are small icosahedral viruses with linear single-stranded DNA genomes of ≈5 kb that are associated with a wide spectrum of illnesses in humans and animals. The subfamily *Parvovirinae*, which infects vertebrates, is currently classified into 8 genera, 5 of which contain human parvoviruses (*Dependoparvovirus*, *Erythroparvovirus*, *Bocaparvovirus*, *Tetraparvovirus*, and *Protoparvovirus*) (*1*). In 2012, bufaviruses 1 and 2 were sequenced from the feces of children with diarrhea from Burkina Faso and a child with nonpolio acute flaccid paralysis from Tunisia (*2*) and classified as founding members of the *primate protoparvovirus 1* species (*1*). Bufavirus DNA also was detected in diarrheal samples from adults in Finland and Holland (*3*,*4*) and a child from Bhutan (*5*). A related protoparvovirus was recently found in feces and serum from rhesus monkeys with simian AIDS in primate research centers (*6*). Here we describe the genome of a new parvovirus that we propose as prototype for a new species, *primate protoparvovirus 2*.

The Study

Using metagenomic deep sequencing, we analyzed fecal samples from 180 infants and children ages 7 days–96 months (mean 18.7 months) in Tunisia who had unexplained diarrhea that tested negative for rotavirus, norovirus, astrovirus, sapovirus, adenovirus types 40 and 41, and Aichi virus by reverse transcription PCR (*7*). The University of California at San Francisco committee on human research approved the study.

The fecal supernatants were filtered through a 0.45-μm filter (Millipore, Darmstadt, Germany) to remove bacterium-sized particles, and the filtrates were digested with a mixture of DNases (Turbo DNase from Ambion, Carlsbad, CA, USA; Baseline-ZERO from Epicenter, Madison, WI, USA; and Benzonase from Novagen, San Diego, CA, USA) and RNase (Fermentas, Pittsburgh, PA, USA) to digest unprotected nucleic acids. Enriched viral nucleic acids (RNA and DNA) were then extracted and amplified by using ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicenter) and analyzed in pools of 10 specimens in 2 Illumina MiSeq run of 250-bp end reads, yielding 20,693,619 unique sequences. We compared the Illumina sequences with the GenBank nonredundant protein databases using BLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Using a BLASTx E-score cutoff of 10^{-5}, we identified, in decreasing frequency, sequences related to the mammalian viruses: sapovirus (120,177 reads), anelloviridae (14,841 reads), parechovirus (10,557 reads), norovirus (4,551 reads), enterovirus (3,857 reads), circoviridae (2,127 reads), group A rotavirus (839 reads), adeno-associated virus (812 reads), picobirnavirus (274 reads), bufavirus (168 reads), WU polyomavirus (136 reads), bocavirus (62 reads), adenovirus (58 reads), papillomavirus (22 reads), cosavirus (20 reads), group C rotavirus (17 reads), human astrovirus 1 (14 reads), salivirus (4 reads), and Aichi virus (2 reads). One pool showed a single read encoding a parvovirus-like protein segment with high levels of genetic similarity (BLASTx E-score of 5×10^{-8}) to the nonstructural protein (NS) 1 of rat parvovirus (GenBank accession no. AFV67813).

The individual fecal sample within the pool containing this sequence was then identified by using PCR and underwent further deep sequencing as above, generating 11 more parvovirus sequences. No other eukaryotic viral sequences were identified from 260,000 unique sequence reads from this patient. The near complete parvovirus genome was then acquired by filling genome gaps by PCR and amplifying 5′ and 3′ extremities using RACE (Rapid Amplification of cDNA Ends, Life Technologies). Amplicons were directly sequenced by primer walking. We named this virus Tusavirus 1 for Tunisian stool-associated parvovirus.

A nearly complete 4,424-bp genome (Tusavirus 1, GenBank accession no. KJ495710) was successfully acquired with partial 5′ untranslated region (243 bp), complete NS1 open reading frame (625 aa), complete viral protein (VP) 1 open reading frame (715 aa), and a partial 3′ untranslated region (68 bp). Tusavirus has a potential upstream start codon MSS in a weaker Kozak consensus

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sequence than MAQ (Figure, panel A), which we selected as the start codon. The Walker loop ^{396}GPATTGKS^{403} [GXXXXGK(T/S)], which is an ATP- or GTP-binding motif, was found in the NS1. We also identified 2 conserved replication initiator motifs ^{127}EFHIHVLLW^{135} and ^{187}VLQYKHSQTR^{196}. Potential splicing signals to express VP1 were identified on the basis of alignments to other protoparvoviruses and classic RNA splicing motifs (Figure,

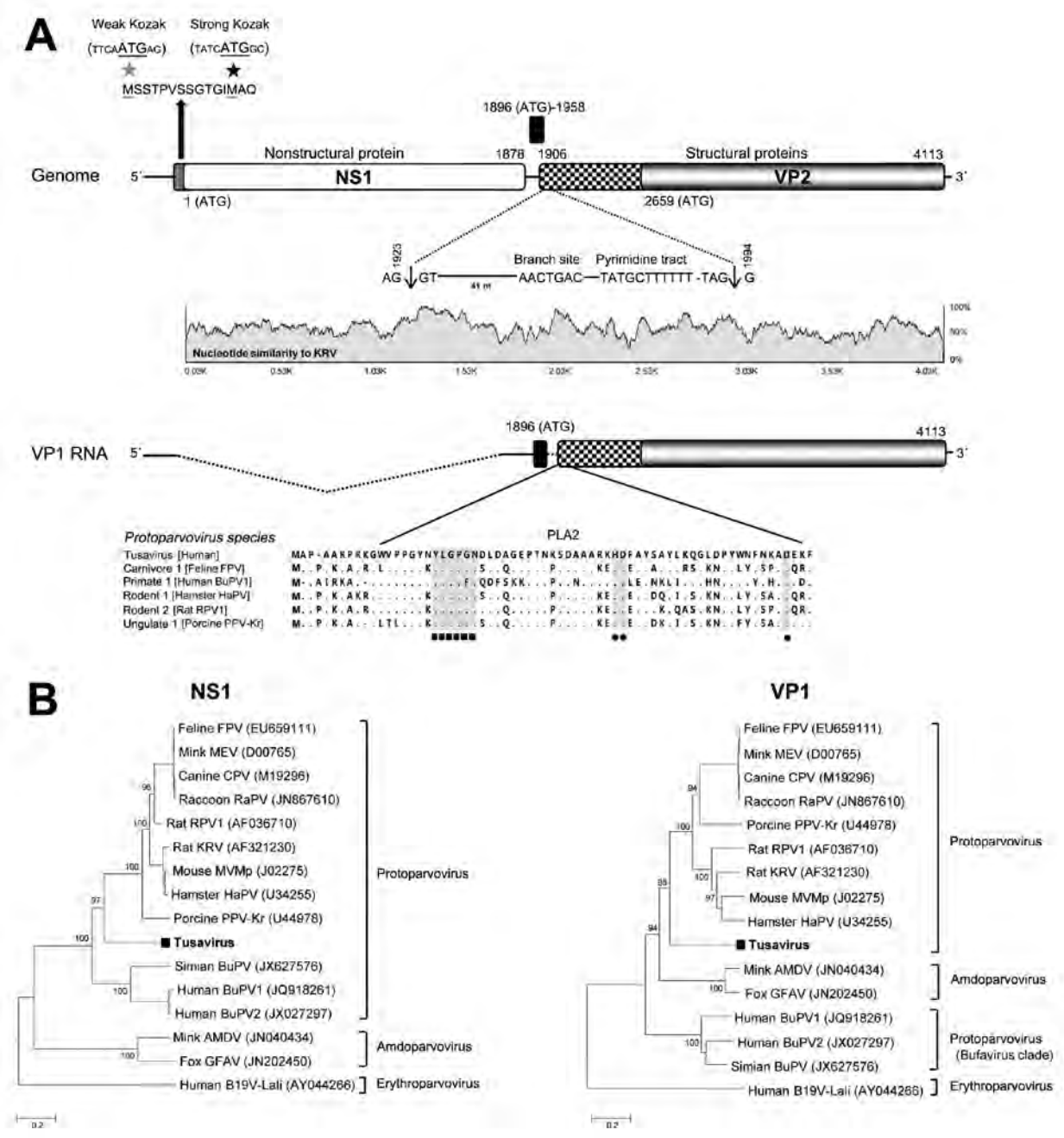

Figure. New parvovirus genome and phylogeny. A) Organization of the Tusavirus genome. Theoretical splicing for expression of viral protein (VP) 1 is shown. The alignment of the PLA2 regions of representatives of 5 protoparvovirus species show the calcium-binding region and catalytic residues in Tusavirus. Pairwise sliding window of percentage nucleotide similarity of Tusavirus aligns with the genetically closest Kilham rat parvovirus. B) Phylogenetic trees generated with nonstructural protein (NS) 1 and VP1 of Tusavirus and of the 5 International Committee on Taxonomy of Viruses–designated species in the *Protoparvovirus* genus. Scale bar indicatesamino acid substitutions per site. FPV, feline parvovirus; MEV, mink enteritis virus; CPV, canine parvovirus; RaPV, raccoon parvovirus; RPV1, rat parvovirus 1; MVMp, minute virus of mice, prototype; HaPV, hamster parvovirus; PPV-Kr, porcine parvovirus Kresse; Simian BuPV, Simian bufavirus; BuPV1, bufavirus 1; BuPV2, bufavirus 2; AMDV, Aleutian mink disease virus; GFAV, gray fox amdovirus; B19V-Lali, human parvovirus B19-Lali. Bootstrap values (based on 100 replicates) for each node are given if >70%.

panel A). The phospholipase A_2 (PLA2) motif was identified in VP1 N-termini with expected calcium-binding site and catalytic residues. The methionine codon of VP2 was located upstream of glycine-rich sequence (GGGARAGGVG). An unusual serine-rich sequence (SSSDSGPSSS) was also seen near VP1 N-termini.

Protein sequence alignments were made by using ClustalX version 2.0.3 (http://www.clustal.org) with the default settings; a phylogenetic tree with 100 bootstrap resamples of the alignment datasets was generated by using the neighbor-joining method based on the Jones-Taylor-Thornton matrix-based model in MEGA5 (http://www.megasoftware.net). Bootstrap values for each node are given if >70%. Resulting trees were examined for consistency with published phylogenetic trees.

Phylogenetic analysis showed that Tusavirus 1 was distinct from known members of the *Protoparvovirus* genus (Figure, panel B). NS1 shared the highest identity to the Kilham rat parvovirus (44%). VP1 and VP2 shared identities of 39% and 37%, respectively, to those of Kilham rat parvovirus. According to the International Committee on Taxonomy of Viruses, the members of the same parvovirus genus should share >30% and members of the same species >85% aa identity in NS1. The *Protoparvovirus* genus currently comprises species infecting carnivores, rodents, pigs, and humans (*1*). Tusavirus 1 is proposed as prototype for *primate protoparvovirus 2* species that would join bufaviruses as human viruses in this genus.

We used a nested PCR targeting NS1 to determine the prevalence of this virus in the 180 Tunisia diarrhea samples. Primers Tusa-F1 (5′-GAAGAAGCTGGAAACTGTGGTCA-3′) and Tusa-R1 (5′-CTCGTCTTTCTCCCAGGCATCT-3′) were used for the first round of PCR, and primers Tusa-F2 (5′-ATTGCTCCAACACCAGTCATCA-3′) and Tusa-R2 (5′-TCTGGTCTGGTCCAATCTTCTTC-3′) for the second round of PCR. The PCR conditions were 95°C for 5 min, 35 cycles 95°C for 30 s, 52°C or 51°C (for the first or second round, respectively) for 30 s, and 72°C for 1 min, a final extension at 72°C for 10 min. No samples except the one initially detected by deep sequencing were PCR positive, yielding a low prevalence of 0.56% (1/180) in this Tunisian population.

Conclusions

We detected fecal shedding of a previously uncharacterized parvovirus in a child with unexplained diarrhea. The 18-month-old girl showed twice daily liquid and greenish feces over 3 days but no fever (37.4°C), vomiting, or dehydration. To identify other viral infections in this patient, the Tusavirus-positive sample was individually analyzed by the same metagenomics method. No other mammalian virus was detected, suggesting a possible role for Tusavirus 1 in this patient's gastrointestinal illness, although the lack of testing for pathogenic bacteria and parasites does not enable us to exclude these alternative explanations. Wider geographic sampling of human samples, including case–control PCR studies of unexplained diarrhea and serologic tests, are needed to define the prevalence and disease association of this new parvovirus species in different age groups and populations.

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Hepatitis E Virus Infections in Blood Donors, France

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We screened plasma samples (minipools of 96 samples, corresponding to 53,234 blood donations) from France that had been processed with solvent–detergent for hepatitis E virus RNA. The detection rate was 1 HEV-positive sample/2,218 blood donations. Most samples (22/24) from viremic donors were negative for IgG and IgM against HEV.

Hepatitis E virus (HEV), family *Hepeviridae*, genus *Hepevirus*, is a small nonenveloped RNA virus that has an icosahedral capsid and is transmitted by the fecal–oral route (*1*). Transfusion-transmitted HEV infections have been documented in several countries in Europe and Asia (*2*–*4*). HEV has also been detected in human blood products (*5*–*12*). Despite considerable geographic variation in HEV seroprevalence in Europe, southern France seems to be an area to which this virus is hyperendemic (*13*).

We conducted a prospective study in France of minipools of plasma from blood donations that were processed with solvent–detergent and used standardized molecular assays for detection of HEV RNA. Data obtained were used to estimate the detection rate of HEV infections in blood donors in France and the risk for HEV transmission by blood transfusion.

The Study

Since November 27, 2012, systematic nucleic acid amplification screening for HEV has been used on blood donations (pools of 96 samples) in France for a 70-L plasma pool that was processed with solvent–detergent. A mixture of 96 plasma samples (50 μL from each sample) was prepared by using a distributor (Tecan, Männedorf, Switzerland) to give a final volume of 4.8 mL. Simultaneously, a 96-well archive microplate was used for serologic testing and to identify individual HEV RNA–positive samples. Plasma samples were collected at 13/14 regional blood transfusion establishments of the French Blood Service in continental France (Figure 1).

HEV RNA was extracted by using the Nuclisens Easy MAG Instrument (bioMérieux, Marcy-l'Etoile, France). Amplification was performed by using the single-round RealStar Reverse Transcription PCR (Altona Diagnostics, Courtaboeuf, France), which is specific for the open reading frame 2 (ORF2)–ORF3 overlapping region (*12*); the 95% detection limit was 23 IU/mL. HEV RNA quantification was performed as described (*14*); the limit of quantification was 60 IU/mL. HEV genotyping was performed by sequencing a 305-nt fragment within the ORF2 region and performing phylogenetic analysis.

IgG and IgM against HEV were detected by using an enzyme immunoassay (Wantai Biologic Pharmacy Enterprise, Beijing, China) according to the manufacturer's instructions (*13*). World Health Organization reference material for IgG against HEV was used to determine concentrations of IgG against HEV (*15*). The limit of detection was 0.25 World Health Organization U/mL.

We screened 558 pools (53,234 samples) for HEV RNA. Of these pools, 22 (3.94%) showed positive results (Table 1). We detected 1 HEV RNA–positive sample in each of 20 pools and 2 HEV RNA–positive samples in the remaining 2 pools. The estimated detection rate of HEV RNA in plasma donations was 0.045% (95% CI 0.043%–0.047%).

The geographic distribution of HEV-positive samples included all but 2 blood transfusion establishments (in Centre Atlantique and Ile de France) (Figure 1). The frequency of HEV RNA–positive samples was 0.072% (95% CI 0.067%–0.077%) in 3 blood transfusion establishments in southern France and 0.037% (95% CI 0.035%–0.037%,) in establishments in northern France (odds ratio [OR] 1.98, 95% CI 0.86–4.53, p = 0.14) (Figure 2). The frequency of HEV RNA–positive samples was 0.049% (95% CI 0.047%–0.051%) in men and 0.018% (95% CI 0.014%–0.022%) in women (OR 2.7, 95% CI 0.4–111.1, p = 0.51). The frequency of HEV RNA–positive samples was 0.048% (95% CI 0.046%–0.052%) for persons ≥45 years of age and 0.041% (95% CI 0.040%–0.042%) for persons <45 years of age (OR 1.2, 95% CI 0.5–2.9, p = 0.83).

HEV RNA concentrations in 11 pools were below the limit of quantification, and the plasma pool with the highest virus load contained 29,796 IU/mL (Table 1). The range of virus load in individual plasma samples was 468

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Figure 1. Prevalence (no. samples positive/no. tested) of hepatitis E virus RNA in plasma collected during November 27, 2012–December 1, 2013, at regional establishments of the French Blood Agency, France. Southern France: AL, Aquitaine-Limousin; PM, Pyrénées-Méditerranée; AM, Alpes-Méditerranée (including Corsica). Northern France: NDF, Nord de France; NO, Normandie; IDF, Ile de France; LC, Lorraine-Champagne; ALS, Alsace; BR, Bretagne; PDL, Pays de Loire; CA, Centre Atlantique; BFC, Bourgogne-France Comté; AULO, Auvergne-Loire; RA, Rhône-Alphes.

IU/mL–5,155 800 IU/mL. All strains that were available for sequence analysis were assigned to HEV genotype 3 (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/11/14-0516-Techapp1.pdf). A total of 59% of the strains were subgenotype 3f and 36% were subgenotype 3c (Table 2).

Of the 183 pools tested for IgG against HEV, 175 had positive results and 8 eight had indeterminate results. Of these 175 IgG-positive pools, 173 were negative for IgM against HEV; the remaining 2 pools had indeterminate results. The range of IgG titers against HEV was 0.3 U/mL–10.6 U/mL.

All HEV RNA–positive pools were negative for IgM against HEV and positive for IgG against HEV, except for 1 pool that was negative for IgG against HEV (Table 1). IgG titers against HEV in viremic pools ranged from 0.3 U/mL to 2.7 U/mL. The prevalence of IgG against HEV for 861 blood donors (whose samples were included in the first 9 HEV RNA–positive pools) was 23.6%. IgG-positive pools included 13.5%–30.2% of plasma collected from blood donors who were positive for IgG against HEV. Pools negative for IgG included 10% of IgG-positive donations. Most (22/24, 91.7%) viremic blood donors were negative for IgM and IgG against HEV at the time of their donation, which indicated recent infection. Two donors (D6 and D21) were positive for IgG and IgM against HEV (Table 2).

Conclusions

We conducted a prospective study with a sensitive method to screen plasma pools for HEV RNA and found that the frequency of HEV infections in donated blood in France was relatively high (1/2,218). This is 1 of the highest frequencies reported for Europe. Frequencies in Europe were 1/14,520 in Scotland (*7*), 1/7,040 in England (*10*), 1/4,525 and 1/1,240 in Germany (*5*,*12*) and 1/2,700 in the Netherlands (*11*).

Our study used plasma pools that contained 96 samples. Therefore, the true frequency of viremic samples could have been underestimated because of a dilution effect. The limit of detection of the PCR was low (<23 IU/mL). However, some pools could have been missed, although 5 positive individual donations having HEV RNA concentrations of 468 IU/mL–2,293 IU/mL were detected (Table 2).

The detection rate for viremic blood donations in southern France was 2-fold greater than that for the rest of France. This finding is consistent with a previously reported high seroprevalence (52%) of IgG against HEV in blood donors (*13*) and the high incidence (3.2%) of infection documented by molecular techniques in local transplantation patients (*15*). However, HEV-positive blood donations were detected in all but 2 of the regions of continental France tested. All strains were genotype 3 and the

Table 1. Characteristics of 22 HEV RNA–positive plasma pools, France, November 27, 2012–December 1, 2013*

Pool	HEV RNA, IU/mL	IgG titer against HEV, U/mL	IgM against HEV
P1	<60	+, <0.30	–
P2	578	+, 0.80	–
P3	3,978	+, 0.30	–
P4	415	+, 2.70	–
P5	200	+, 1.00	–
P6	<60	+, 1.10	–
P7	1,458	+, 1.60	–
P8	<60	+, 0.32	–
P9	<60	NT	NT
P10	5,374	+, NT	–
P11	29,796	+, 0.56	–
P12	<60	+, 1.93	–
P13	<60	+, 0.42	–
P14	<60	+, NT	–
P15	2,948	+, 0.49	–
P16	171	+, 1.44	–
P17	2,137	+, 0.44	–
P18	<60	–	–
P19	<60	+, 0.84	IND
P20	3,705	+, 0.26	–
P21	<60	+, 0.81	–
P22	<60	+, 2.61	–

*HEV, hepatitis E virus; +, positive; –, negative; NT, not tested because of insufficient material; IND, indeterminate.

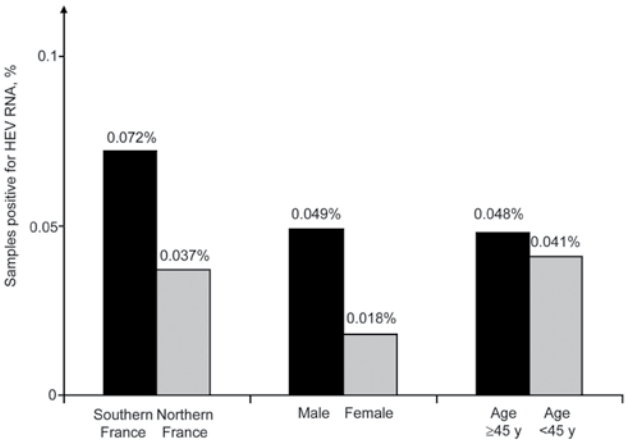

Figure 2. Frequency of blood donations positive for hepatitis E virus (HEV) RNA, by location and donor age and sex, France, November 27, 2012–December 1, 2013. Comparisons between groups by using χ^2 and Fisher exact tests showed no significant differences.

proportions of subgenotypes 3f and 3c strains was similar to those observed in HEV-infected pigs in France, which suggested a zoonotic origin of the 24 HEV infections as reported by Kamar et al. (*1*).

Infections were detected at an early stage because serologic markers were absent for >90% of the cases. Since November 2012, testing for HEV RNA has reduced the risk for HEV transmission in patients in France who are given plasma processed with solvent–detergent. This testing helps overcome the situation that this treatment does not inactivate HEV, a nonenveloped virus. In January 2015, nucleic acid amplification testing for HEV will begin in Europe for plasma processed with solvent–detergent.

We detected IgG against HEV in nearly all plasma pools, and IgG concentrations ranged from 0.3 U/mL to 10.6 U/mL. The potential of antibodies against HEV to neutralize virus infectivity if the unit is transfused is unknown, but a recent study indicated that IgG against HEV at a concentration of <10 U/mL cannot protect immunocompromised patients against reinfection (*15*).

Our findings should be useful for determining the best safety measures to prevent HEV transmission by blood transfusion. The implementation of nucleic acid amplification testing relies on full assessment of transfusion risk in exposed patients and the cost-effectiveness of different strategies.

Table 2. Characteristics of 24 blood donors with HEV RNA–positive archived blood samples, France, November 27, 2912–December 1, 2013*

Donor/age, y/sex	HEV RNA, IU/mL	Genotype	IgG against HEV	IgM against HEV
1/32/M	1,903	3f	–	–
2/37/M	128,700	3f	–	–
3/60/M	1,404,100	3f	–	–
4/52/F	179,400	3c	–	–
5/55/M	102,960	3c	–	–
6/59/M	19,344	3f	+	+
7/41/M	1,333	3f	–	–
8/61/M	391,560	3c	–	–
9/28/M	2,293	3f	–	–
10/40/M	8,502	3	–	–
11/57/M	803,400	3f	–	–
12/37/M	5,155,800	3c	–	–
13/37/M	1,981	3f	–	–
14/58/M	6,208	3c	–	–
15/53/M	13,728	3c	–	–
16/59/M	111,696	3f	–	–
17/44/M	61,932	3c	–	–
18/54/M	258,180	3c	–	–
19/34/M	4,852	3f	–	–
20/41/M	3,994	3f	–	–
21/48/M	468	NT	+	+
22/55/M	306,540	3f	–	–
23/25/M	4,118	3f	–	–
24/57/M	2,355	NT	–	–

*HEV, hepatitis E virus; –, negative; +, positive; NT, not tested because of insufficient material.

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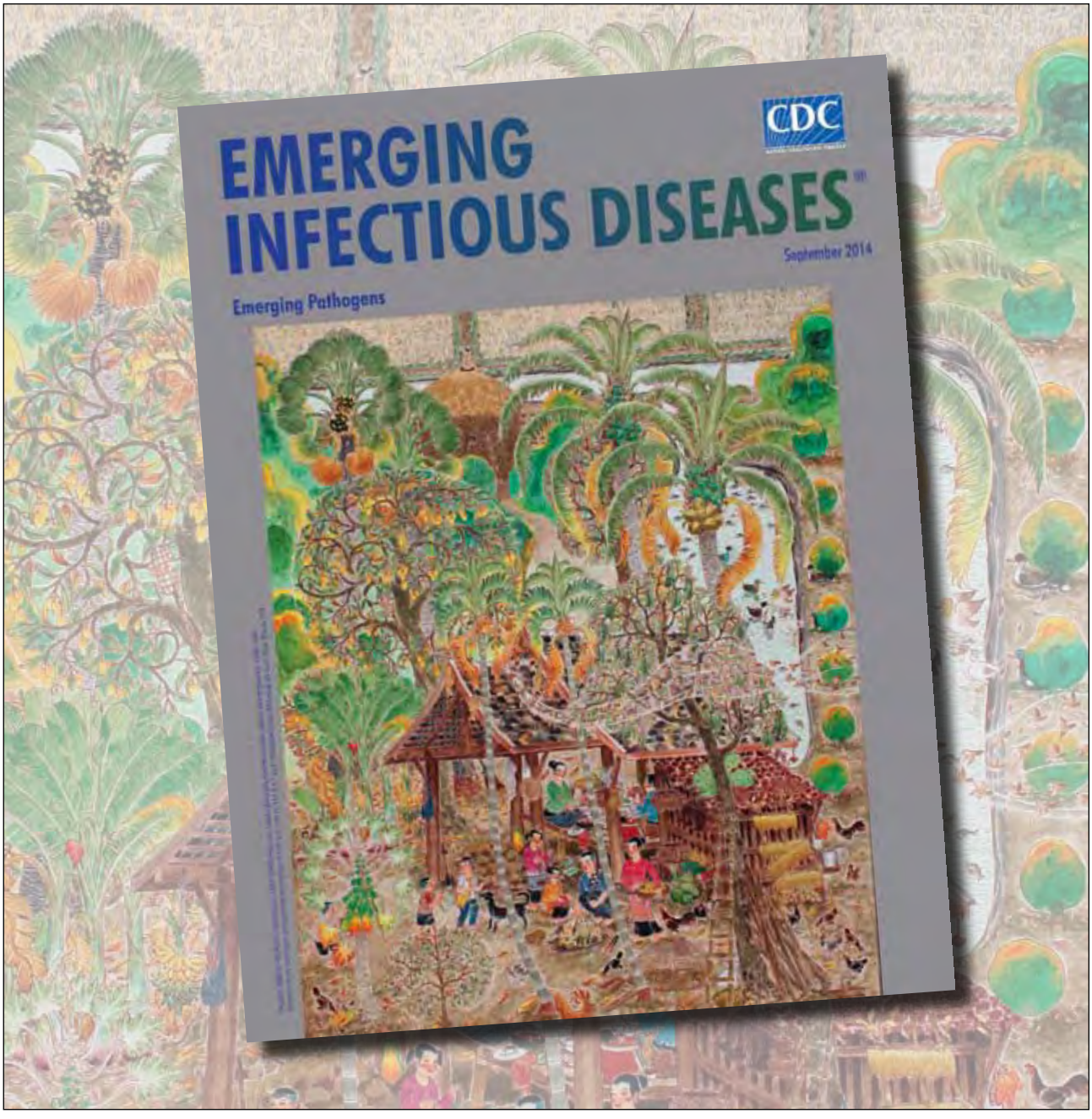

ESBL-Producing *Salmonella enterica* Serovar Typhi in Traveler Returning from **Guatemala** to Spain

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We report a case of typhoid fever in a traveler returning to Spain from Guatemala that was caused by *Salmonella enterica* serovar Typhi which produced an extended-spectrum β-lactamase (ESBL). This finding demonstrates the presence of ESBL-producing *S. enterica* ser. Typhi strains in the Americas. Enhanced surveillance is necessary to prevent further spread.

Salmonella enterica serovar Typhi is the causative agent of typhoid fever, an enteric bacterial infection that results in systemic febrile illness. *S. enterica* ser. Typhi is strictly adapted to humans; its transmission occurs through the fecal-oral route, person-to-person contact, or contaminated water or food. An estimated 22 million new cases of typhoid fever occur each year worldwide, resulting in 200,000 deaths (*1*). *S. enterica* ser. Typhi infection is uncommon in industrialized countries, where infections occur sporadically and mainly in travelers returning from disease-endemic areas and in newly arrived immigrants.

Chloramphenicol was used successfully as the first-line agent for the treatment of typhoid fever from the 1950s through the 1970s. Strains resistant to this compound emerged in 1972, associated with self-transferable *Inc*HI plasmids. Trimethoprim-sulfamethoxazole and ampicillin were then employed, but strains resistant to all 3 drugs arose rapidly during the 1980s and 1990s in southern and Southeast Asia, the Middle East, and Africa (*2*,*3*). To overcome these new resistances, fluoroquinolones were proposed as the drug of choice. However, during the past decade, ciprofloxacin-resistant strains have been reported in the Asian subcontinent (*2*).This situation has resulted in the use of ceftriaxone or cefotaxime as alternatives for treatment of enteric fever (*3*).

Until now, extended-spectrum β-lactamase (ESBL)–producing *S. enterica* ser. Typhi strains have been uncommon and have been described only in a few patients of Asian origin and in travelers returning from that region (*4*–*7*). We report the detection and molecular characterization of an ESBL-producing *S. enterica* ser. Typhi strain isolated in Barcelona, Spain, from a patient with typhoid fever who had traveled to Guatemala.

The Study

In September 2013, a 41-year-old man residing in Barcelona visited the emergency room of the Vall d'Hebron Hospital, reporting 10 days of fever and pain in the right upper abdominal quadrant, preceded by 3 days of diarrhea. At the time of his hospital visit, the patient had been back in Spain for 4 days after a 5-month stay in rural Guatemala. Physical examination revealed a temperature of 37.8°C and unremarkable blood pressure and heart rate results. Abdominal examination showed tenderness of the right upper and lower quadrants. General biochemistry values and blood cell counts were within reference ranges, but C-reactive protein level and erythrocyte sedimentation rate were elevated. Results of initial blood and stool culture testing were negative. Because of the patient's persistent abdominal pain and newly documented fever, computed tomographic scan of the abdomen was performed; results showed thickening of the terminal ileum, adjacent fat tissue stranding, and regional lymphadenopathy. Antimicrobial drug treatment with amoxicillin-clavulanate acid was begun. New blood cultures, performed because of the patient's persistent fever, yielded *S. enterica* ser. Typhi, and the strain produced an ESBL. Subsequently, intravenous ertapenem (1 g/d) was administered for 14 days; the patient experienced complete clinical recovery, and subsequent blood and stool culture results were negative.

The isolate was identified by using the VITEK2 system (bioMérieux, Marcy l'Etoile, France). Serotyping by slide agglutination by using commercial antisera according to the Kauffmann-White scheme yielded the antigenic formula 9,12,[Vi]:d:-. Multilocus sequence typing (MLST), which was done using primers described elsewhere (*8*) with allele sequences and allelic profiles verified in the MLST database (http://mlst.ucc.ie/mlst/dbs/Senterica), showed that the strain belonged to sequence type (ST) 2. This ST is one of the most prevalent among *S. enterica* ser. Typhi; isolates of this ST have been detected in Asia, Africa, and South America (*9*).

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Antimicrobial susceptibility to β-lactams was assessed by disk diffusion following Clinical Laboratory Standards Institute recommendations (http://www.clsi.org). Suggestive evidence of ESBL production was observed as synergy between amoxicillin/clavulanate and ≥1 of the following: cefotaxime, ceftazidime, aztreonam, and cefepime. In addition, MICs of β-lactams, quinolones, trimethoprim/sulfamethoxazole, chloramphenicol, and azithromycin were determined by E-test (bioMérieux) (Table). According to Clinical Laboratory Standards Institute interpretative criteria, the isolate was resistant to all β-lactams evaluated except amoxicillin/clavulanate, piperacillin/tazobactam, cefoxitin, and carbapenems and was susceptible to chloramphenicol, trimethoprim/sulfamethoxazole, nalidixic acid, ciprofloxacin, and gentamicin. The MIC of azithromycin was 4 μg/mL; according to the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org), isolates with an MIC ≤16 μg/mL of this drug should be considered wild-type organisms that are expected to respond to treatment (*10*).

To screen for TEM and SHV β-lactamases, CTX-M ESBL genes, and genes encoding resistance to quinolones (*qnrA*, *qnrB*, *qnrS*, *qepA*, *oqxAB*, and *acc(6')-Ib-cr*), we used PCR as described previously (*11*,*12*). Mutations in *aac(6')-Ib* that confer quinolone resistance and in the quinolone resistance–determining regions of *gyrA*, *gyrB*, *parC*, and *parE*, were studied by sequencing (*12*,*13*). These experiments showed that the *S. enterica* ser. Typhi isolate possessed $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$, and *acc(6')-Ib* but none of the studied quinolone resistance genes or detectable quinolone resistance–determining region mutations.

IS*Ecp1*, IS*26*, and *orf*477 are elements that previously have been identified in the genetic environment surrounding $bla_{\text{CTX-M-15}}$. The presence of such elements was investigated by PCR mapping and sequencing in combination with $bla_{\text{CTX-M-15}}$–specific primers, as reported previously (*14*).These results showed that the ESBL gene was situated between a nontruncated IS*Ecp1* and *orf*477, as noted previously in other *Enterobacteriaceae*.

Identification and characterization of the location of $bla_{\text{CTX-M-15}}$ was carried out by conjugation, using a nalidixic acid–resistant derivative of *Escherichia coli* HB101 as recipient, PFGE of total DNA of donor and transconjugant digested with S1-nuclease, Southern hybridization with specific probes, and PCR-based replicon typing, as described previously (*14*). These studies showed that the *S. enterica* ser. Typhi $bla_{\text{CTX-M-15}}$ was located in an IncL/M self-transferrable plasmid of 65 kb that also carried the $bla_{\text{TEM-1}}$ and *acc(6')-Ib* genes. MICs to antimicrobial agents for donor, recipient, and transconjugant are shown in the Table.

Conclusions

We describe an ESBL-producing *S. enterica* ser. Typhi strain isolated from a man in Spain who had traveled to Guatemala. It is well documented that ESBL-producing *Enterobacteriaceae* are spreading worldwide. CTX-M-15 is one of the most commonly identified ESBLs; its high prevalence has been driven mainly by the pandemic spread and expansion of the ST131 *E. coli* clonal group. Extended-spectrum cephalosporin resistance has increased during the past few years in nontyphoidal *S. enterica*, principally in developing countries, where such resistance appears to be endemic in some areas (*15*).

Table. Susceptibility profiles of the CTX-M-15–producing *Salmonella enterica* serovar Typhi strain from a patient in Spain who had traveled to Guatemala in 2013, compared with profiles of the *Escherichia coli* recipient strain and the transconjugant strain

	MIC, μg/mL		
Antimicrobial agent(s)	*S. enterica* ser. Typhi 301812 (donor)	*E. coli* HB101-Nal (recipient)	*E. coli* HB101-Nal TC-301812 (transconjugant)
Ampicillin	>256	1.5	>256
Amoxicillin-clavulanate	16	3	16
Piperacillin-tazobactam	3	1	1.5
Cefoxitin	12	3	3
Ceftazidime	>256	0.064	8
Cefotaxime	>256	0.016	>256
Cefotaxime-clavulanate	0.19	0.016	0.016
Cefepime	>256	0.032	24
Aztreonam	>256	<0.016	32
Imipenem	0.25	0.38	0.38
Meropenem	0.064	0.023	0.023
Ertapenem	0.064	0.002	0.002
Chloramphenicol	2	2	2
Trimethoprim-sulfamethoxazole	0.047	0.25	0.25
Nalidixic acid	3	>256	>256
Ciprofloxacin	0.023	0.125	0.125
Azithromycin	4	3	3
Gentamicin	1.5	0.75	3
Tobramycin	24	1	>256
Amikacin	32	2	>256

To our knowledge, 5 cases of *S. enterica* ser. Typhi resistant to β-lactams by ESBL production have been reported, all with Asian origins (Bangladesh, the Philippines, Iraq, and India) (*4*–*7*). For the isolate originating in the Philippines, SHV-12 was the enzyme responsible for ESBL resistance (*7*); for the other 4 isolates, a CTX-M enzyme was detected, and in 3 of those, the CTX-M-15 variant was identified (*4*–*6*). In our case, however, we found a different genetic environment of $bla_{\text{CTX-M-15}}$. Specifically, we found a nontruncated IS*Ecp1* upstream of $bla_{\text{CTX-M-15}}$, whereas in the previously reported Iraq-origin strain, IS*Ecp1* was truncated by IS*26* (*5*). Genetic environments of $bla_{\text{CTX-M-15}}$–producing *S. enterica* ser. Typhi isolates from India are not clear, but according to the methods used by the authors (*4*), a truncated IS*Ecp1* or a different structure may have been involved. Our results also confirm that the plasmid harboring $bla_{\text{CTX-M-15}}$, which also carries $bla_{\text{TEM-1}}$ and *acc(6')-Ib*, carries an IncL/M replicon.

In summary, we report a case of typhoid fever caused by an ESBL-producing *S. enterica* ser. Typhi isolate from a traveler returning to Spain from Guatemala. This case represents the acquisition of an ESBL-producing *S. enterica* ser. Typhi strains in the Americas. Because typhoid fever is a serious public health issue, meticulous microbiological and epidemiologic investigation of strains of this sort are necessary to prevent further spread of this disease.

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Nosocomial Neonatal Legionellosis Associated with Water in Infant Formula, Taiwan

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We report 2 cases of neonatal *Legionella* infection associated with aspiration of contaminated water used in hospitals to make infant formula. The molecular profiles of *Legionella* strains isolated from samples from the infants and from water dispensers were indistinguishable. Our report highlights the need to consider nosocomial legionellosis among neonates who have respiratory symptoms.

Legionella infection was first reported in adults at an American Legion convention in Philadelphia in 1976 (*1*). The disease has a variety of clinical manifestations, ranging from mild respiratory tract illness to fatal pneumonia, especially among immunocompromised persons (*2*).

Legionella infection in neonates occurs rarely among both healthy and immunocompromised patients (*3–8*). Water birth and use of cold-mist humidification have been associated with neonatal legionellosis (*9–11*), but investigations of transmission modes are limited. We report 2 cases of neonatal *Legionella* infection associated with contaminated water used in infant formula in a hospital setting.

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The Cases

Case 1 involved a male neonate delivered by cesarean section in obstetric hospital A in Taiwan in April 2013, after an uneventful pregnancy of 38 weeks. He was fed infant formula in the hospital's nursery. On postpartum day 7, he had a fever of 39°C and tachypnea and was taken to a tertiary hospital. Chest radiograph showed a ground-glass opacity in the middle lobe of his right lung, which subsequently developed into cavities. Microbiological testing of sputum and blood specimens yielded no definitive results. His pulmonary condition deteriorated during the following days. The case was reported to the Unknown Pathogen Discovery/Investigation Group at the Taiwan Centers for Disease Control for extensive etiologic survey. Multiplex real-time reverse transcription PCR showed sputum and blood specimens to be negative for adenovirus, respiratory syncytial virus, coronaviruses (229E, OC43, NL63, HKU1, and middle east respiratory syndrome coronavirus), metapneumovirus, influenza virus, parainfluenza viruses 1–4, herpes simplex viruses 1 and 2, varicella-zoster virus, Epstein-Barr virus, cytomegalovirus, human herpesviruses 6 and 7, bocavirus, parvovirus, enterovirus, and rhinovirus. However, *Legionella pneumophila* serogroup 5 was isolated from the sputum specimen. The patient was treated with antibacterial drugs and was discharged on postpartum day 17.

Water specimens from all sources with which the patient had contact were tested for *Legionella* by culture. *L. pneumophila* serogroup 4 was isolated from 3 tap water sources, and *L. pneumophila* serogroup 5 was isolated from the cold water source of the hot and cold water dispenser from which water for making infant formula was collected (Table). The water dispenser was located in the room next to the nursery. The hot water faucet provided boiled water at 95°–100°C, and the cold water faucet provided unboiled water that was treated by a built-in reverse osmosis device. The pulsed-field gel electrophoresis (PFGE) profiles of the *L. pneumophila* serogroup 5 strains isolated from a sputum sample from the infant and those from a water sample from the water dispenser were indistinguishable (Figure). Both strains were sequence type 1032 (*12*).

Parents of the 175 neonates that were delivered ≤3 months before the patient's birth in hospital A were interviewed by telephone; 3 of the 175 infants had fever within the first month of life. Serologic testing 3 weeks after their fever episodes was negative for IgM or IgG antibodies against *L. pneumophila* serogroups 1–6.

The water dispenser in hospital A was replaced with one that provided only hot water. The point-of-use filters were mounted on the water faucets in the nursery

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Table. Results of *Legionella* culture in environmental water specimens associated with nosocomial infection of neonates, Taiwan*

Site of sampling	Strain (sequence type); concentration, CFU/L‡	
	First sampling†	Second sampling†
Case 1		
Tap water source 1 for bathing infants in nursery	*L. pneumophila* serogroup 4 (ST1712); 2.5×10^3	*L. pneumophila* serogroup 4 (ST1712); TMTC
Tap water source 2 in nursery	*L. pneumophila* serogroup 4 (ST1712); 2×10^3	*L. pneumophila* serogroup 4 (ST1712); TMTC
Drainage from air conditioner in nursery	ND	Negative
Tap water source in room adjacent to nursery	ND	*L. pneumophila* serogroup 4 (ST1764); TMTC
Cold water source of the hot/cold water dispenser§	*L. pneumophila* serogroup 5 (ST1032)¶; 2.2×10^4	ND
Tap water source in maternity ward	ND	Negative
Case 2		
Tap water source for rinsing infants in nursery	Negative	Negative
Tap water source for reverse osmosis water in nursery	Negative	Negative
Cold water source of the hot/cold water dispenser§	*L. pneumophila* serogroup 1 (ST1)¶; 2×10^2	*L. pneumophila* serogroup 1 (ST1)¶; TMTC
Hot water source of the hot/cold water dispenser	ND	Negative
Tap water source 1 in maternity ward	Negative	Negative
Tap water source 2 in maternity ward	ND	Negative
Tap water source 3 in maternity ward	Negative	Negative
Tap water source 4 in maternity ward	ND	Negative
Tap water source for bathing in infant's house	ND	Negative
Tap water source for drinking water in infant's home	ND	Negative

*Both case-patients were delivered by cesarean section and were not breast fed or placed in incubators. All potential infection sources were tested and listed. No humidifier was used in either nursery. ST, sequence type; TMTC, too many to count; ND, testing not done.
†The first samples from the environments of case-patients 1 and 2 were collected 33 d and 23 d after birth, respectively. The second samples were collected 34 d and 26 d after birth, respectively.
‡Concentration of *Legionella* spp. detected in water specimens.
§Used in formula for neonates.
¶The pulsed-field gel electrophoresis profile of the strain was indistinguishable from that of the strain isolated from the patient's sputum. The ST of the strain was the same as that of the strain isolated from the patient's sputum.

(Pall-AquaSafe Water Filter, Portsmouth, UK). All subsequent tests of water samples were negative for *Legionella* spp. No *Legionella* infection was identified in any neonate who was born after the patient's birth in hospital A during the following 8 months.

Case 2 involved an asymptomatic male neonate delivered by cesarean section in obstetric hospital B in November 2013 after an uneventful pregnancy of 38 weeks. An optional screening test requested by the parents for severe combined immunodeficiency was negative. He remained asymptomatic and was fed infant formula throughout his 8-day stay in the hospital. At home on the day of discharge, he had a fever of 38.5°C and poor appetite. He had tachypnea and cyanosis of the lips during the following days, and on day 10 after birth, he was taken to the same tertiary hospital as case-patient 1. Bilateral pulmonary infiltrates and mild right pneumothorax were identified. The patient's urine and sputum specimens tested positive for *L. pneumophila* serogroup 1. Azithromycin was administered on the day of admission. His clinical condition improved gradually. However, pulmonary fibrosis and pneumatoceles were identified later. He was discharged on day 55 after birth.

Testing of related environmental water specimens for *Legionella* spp. by culture was negative for all specimens except that from the cold water source of the hot and cold water dispenser in hospital B, which was positive for *L. pneumophila* serogroup 1 (Table). The water dispenser in the nursery, which consisted of a tank containing boiled, hot water and another tank containing cool water pipelined from the boiled water tank, was used for making infant formula. The PFGE profiles of the strains from the patient and those from the water dispenser were indistinguishable (Figure). Both strains were sequence type 1.

The parents of the 79 neonates born ≤3 months before the patient's birth in hospital B were interviewed by telephone. None of the neonates had fever during the first month of life. The water dispenser in hospital B was replaced with one that provided only hot water. All following water tests showed negative results. No *Legionella* infection was identified in any neonate who was born after the patient's birth in hospital B during the following 3 months.

Conclusions

We report 2 cases of neonatal legionellosis associated with infant formula prepared with contaminated water. Only the cold water from both hospitals' hot and cold water dispensers used for making infant formula was positive for *L. pneumophila*, with indistinguishable PFGE profiles and the same sequence types as those isolated from the neonates. Some environmental specimens were tested twice to

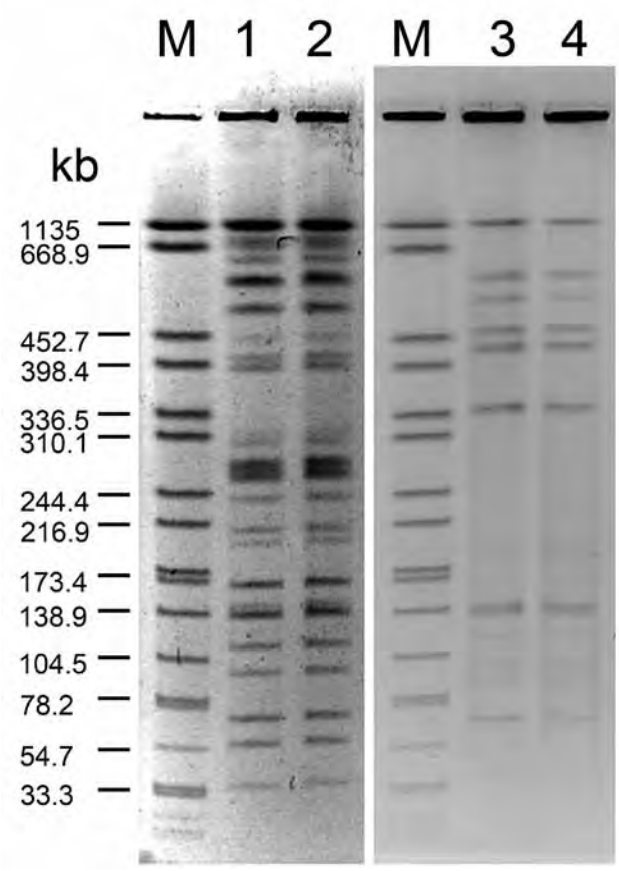

Figure. Pulsed-field gel electrophoresis patterns for *Legionella pneumophila* isolates from neonates with *Legionella* infection and hospital water sources. Genomic DNA was digested with *Sfi*I and separated in 1% agarose gel by Bio-Rad CHEF MAPPER. Lane M, reference size maker (*Xba*I-digested genomic DNA fragments of *Salmonella enterica* ser. Braenderup H9812); lanes 1 and 2, clinical and environmental isolates of *L. pneumophila* serogroup 5 from case-patient 1; lanes 3 and 4, clinical and environmental isolates of *L. pneumophila* serogroup 1 for case-patient 2.

compensate for inadequate sensitivity of the culture method and yielded the same results. No humidifier was used in either case. In the hospital where the first case-patient was identified, the water dispenser was not located in the nursery, which likely reduced the risk of transmitting *Legionella* spp. through contaminated aerosol droplets generated by the water dispenser.

Aspiration is a major transmission mode in hospital-acquired *Legionella* infection among adults (*13*,*14*). Neonatal *Legionella* infection through aspiration of contaminated water during water birth has been reported (*9*,*10*). Our results also indicate the *Legionella* infection described here was likely transmitted by aspiration of contaminated infant formula during the first days of life.

Both water dispensers were colonized with *Legionella* spp. Although the pervasiveness of these 2 problematic types of water dispenser is not known, hot and cold water dispensers are used in many hospitals in Taiwan. We speculate the complicated pipeline system and inappropriate maintenance of water dispensers might increase the risk for *Legionella* colonization, thus facilitating *Legionella* infection. Our report highlights the potential risk for *Legionella* transmission posed by contaminated water dispensers.

Few cases of neonatal legionellosis have been reported in the literature (*3*–*5*). Most cases were hospital acquired, and the patients had severe pneumonia that was associated with a high mortality rate (*8*,*15*). The manifestation of *Legionella* infection is not pathognomonic (*15*), therefore, underdiagnoses or delayed diagnoses combined with inappropriate treatment likely contribute substantially to mortality rates and severity of neonatal legionellosis. The 2 neonates described here survived after prompt diagnosis and treatment enabled by an extensive etiologic investigation and high suspicion of *Legionella* infection. Our report underscores the importance of suspecting and testing for *Legionella* infection when neonates have respiratory symptoms.

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Frequent Hepatitis E Virus Contamination in Food Containing Raw Pork Liver, France

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Food products containing raw pork liver are suspected to be vehicles for transmission of hepatitis E virus. Four categories of food products, comprising 394 samples, were analyzed to determine hepatitis E virus prevalence. Virus was detected in 3%–30% of the different categories. Phylogenetic analysis showed high identity with human and swine sequences.

In humans, hepatitis E virus (HEV) is responsible for an acute, entero-transmissible form of hepatitis, similar to that caused by hepatitis A. In most cases, it is a self-limited infection with rapid viral clearance, but it can evolve into more severe forms, including fatal fulminant hepatitis. Chronic hepatitis E also has occurred in solid-organ transplant recipients and has progressed to more serious conditions, such as fibrosis or liver cirrhosis and liver failure (*1*).

HEV is the only hepatitis virus that can infect species other than primates. HEV infects many animal species, especially pigs, in which a very high prevalence has been described (*2*). Infections acquired in Western countries involve strains that are genetically similar to local swine strains, suggesting an autochthonous origin. Although water is the main vector of contamination in countries to which HEV is endemic, the origin of sporadic cases in other areas is more likely zoonotic. Direct contact with infected animals and consumption of infected meat are possible transmission pathways (*2*).

In France, the annual number of autochthonous cases appears to have increased, from 9 cases in 2002 to nearly 800 in 2012 (http://www.cnrvha-vhe.org/wp-content/uploads/2012/03/2012-Rapport-VHA-VHE.pdf). In a national survey in 2009, the presence of HEV in the swine reservoir was characterized and 65% of pig farms were found to have infected animals; 4% of pork livers entering the food chain were contaminated by the virus (*3*). Molecular analysis of HEV sequences in humans and pigs has shown high identity between the 2 populations (*4*). Food products containing pork liver have repeatedly been suspected of causing indigenous cases of HEV infection (*5*,*6*) and might be responsible for nearly 40% of the autochthonous HEV cases (*12*). Recent studies have confirmed the presence of HEV in the pork food chain, as well as in sausages (*7*,*8*). The objective of this study was to determine the apparent prevalence of HEV contamination in food products containing raw pork liver that were not marketed to be eaten without cooking.

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The Study

In 2011, four different categories of food products in France that were marketed by the food industry were identified as containing raw pork liver but sold to consumers to be eaten after cooking. These 4 categories were 1) figatellu and fitone, 2) dried salted liver, 3) quenelle and quenelle paste, and 4) dried or fresh liver sausages. HEV can be heat-inactivated by thorough cooking at 71°C for 20 min (*9*); however, consumers might not apply such precise thermal treatment. Thus, these food products might be able to transmit HEV. All 4 categories were local regional culinary specialties from eastern or southeastern France. The samples were collected, then frozen directly at the production step after packaging, just before distribution for commercial sale. The frozen samples were sent to the French Agency for Food, Environmental and Occupational Health and Safety laboratory and kept at –80°C until analysis. For each sample, HEV detection was performed on 20 g of product, which had been manually defatted and homogenized in 25 mL phosphate-buffered saline by using a blender. To avoid cross-contamination, each blender was autoclaved between samples. RNA extraction was performed on 500 μL suspension by using the RNeasy lipid Tissue Midi kit (QIAGEN, Hilden, Germany). Presence of inhibitors was assessed by addition of synthetic HEV RNA to the extracts (*9*).

Forty producers were randomly selected, and their relative contributions to total production within each food category were obtained. For each producer, 10 products were randomly selected. Six products were not included in the final analysis because the food category was uncertain. HEV RNA was detected by using real-time reverse transcription PCR as previously described (*9*). Of the 394 food samples analyzed, 68 were found positive (29 figatelli, 1 dried salted liver, 10 quenelle and quenelle paste, and 28 dried or fresh liver sausages). The HEV RNA quantification obtained was 10^2–10^6 copies of HEV RNA/g of food (Table). The prevalence for each food category was estimated by using the relative production weight for each food category, and the 95% CI was estimated by bootstrap. Statistical estimates were performed by using R 2.13.1

Table. Quantification and prevalence of HEV RNA in food containing raw pork liver, France, 2011

Food category	No. samples analyzed, N = 394	No. copies HEV RNA/g, range*	Prevalence (95% CI)
Figatelli and fitone	140	1.7×10^2 to 6.9×10^5	0.3 (0.23–0.38)
Dried salted liver	30	6.9×10^5	0.03 (0–0.10)
Quenelle and quenelle paste	55	2.6 v 10^2 to 2.83×10^5	0.25 (0.15–0.37)
Dried or fresh liver sausages	169	1×10^2 to 2.3×10^6	0.29 (0.22–0.36)

*Minimum and maximum numbers in each food product per category. Detection limit of the method used is 1×10^2 copies of HEV RNA/g. HEV, hepatitis E virus.

software (http://www.r-project.org). HEV RNA prevalence was high in figatelli (30% [95% CI 23%–38%]), liver sausages (29% [95% CI 22%–36%]), and quenelles (25% [95% CI 15%–37%]). The prevalence of HEV RNA was lower in dried salted liver: 3% (95% CI 0%–10%) (Table).

Four HEV RNA–positive food samples collected in the present study were tested for infectious virus in collaboration with 2 laboratories (Animal Health and Veterinary Laboratories Agency, Weybridge, UK, and Wageningen University and Research Centre Central Veterinary Institute, Lelystad, the Netherlands). Virus growth from 1 HEV-positive sample was observed in a 3-dimensional culture system developed by these laboratories (*10*). This analysis thus confirms that live viruses can be present in food products.

In preparation of these food products, a large quantity of liver (up to 750 livers per batch) was mixed with fat and spices. Therefore, even if only 4% of raw livers are infected, as shown in a previous study (*3*), the entire batch becomes contaminated; consequently, HEV prevalence is high in the food products. Because high quantities of virus can be present in liver (up to 10^8 copies of HEV RNA/g) (*9*), the dilution within a large batch will be limited and will not substantially reduce the risk for contamination. The oral infectious dose of HEV is still unknown. In contrast, dried liver is made from only 1 liver; thus the prevalence observed agrees with the prevalence of HEV in liver at the slaughterhouse (i.e., 4%) (*3*).

For further evaluating the link between HEV RNA in pork liver sausages and human autochthonous cases, 68 partial open reading frame 2 sequences were amplified (*3*). This sequence, although short (≈290 nt), reflects the diversity of the HEV full-length genome and is frequently used in phylogenic studies (*11*). All sequences obtained (GenBank accession nos. KJ558436–KJ558503) were of genotype 3, which is the major HEV genotype circulating in autochthonous cases in France and the rest of Europe. The overall mean distance of the sequences from the 68 food products was estimated to be 0.16 nt. To screen for high sequence identity between food products and human or swine HEV, each sequence was analyzed by using BLAST (http://blast.ncbi.nlm.gov/Blast.cgi) to identify the closest sequences. Thirty-three sequences had ≥98% nt identity with human and/or swine sequences. The human sequences with the highest identity originated in France, except for 2 sequences from the United Kingdom and 1 from Spain (Figure). This result confirms that most autochthonous cases might have a foodborne origin. Two food sequences had >99% nt identity with swine sequences previously described (Figure). Therefore, swine are also sources of autochthonous cases through foodborne transmission.

Conclusions

Our findings clearly demonstrate that some food products that contain raw pork liver and are marketed to be

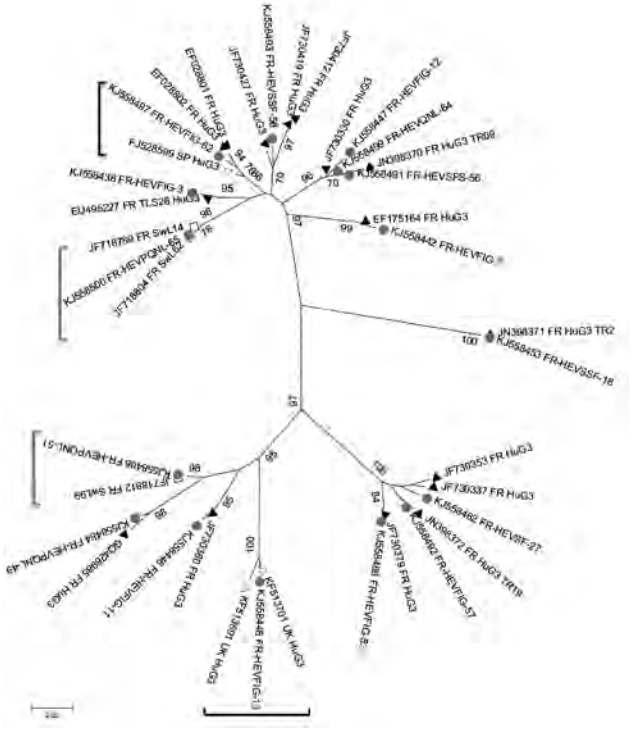

Figure. Phylogenetic tree of hepatitis E virus (HEV) sequences identified in food samples, France, 2011. Phylogenetic tree including 16 HEV sequences detected in food samples (gray circles) and the closest human (black triangles, French origin; white triangles, British or Spanish origin) or swine (white squares) sequences was constructed by using the neighbor-joining method with a bootstrap of 1,000 replicates based on the ClustalW alignment (MEGA4, http://www.megasoftware.net) on 290 nt sequences from open reading frame 2. HEV sequences retrieved from GenBank with ≥98% nt identity are indicated with their accession numbers. Bootstrap values of >70% are indicated on respective branches. Scale bar indicates nucleotide substitutions per site. Similar human and food sequences are shown in black brackets, similar swine and food sequences are shown in gray brackets.

cooked by the consumer can harbor HEV. The close sequence identity observed strongly suggests that foodborne transmission of HEV occurs frequently. Considering this high prevalence, consumers at risk for developing severe forms of HEV (e.g., solid-organ transplant recipients, person having underlying liver conditions, or pregnant women) should be informed about the HEV risk and should avoid eating such pork liver food products without thoroughly cooking them.

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Global Incidence of Carbapenemase-Producing *Escherichia coli* ST131

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We characterized *Escherichia coli* ST131 isolates among 116 carbapenemase-producing strains. Of isolates from 16 countries collected during 2008–2013, 35% belonged to ST131 and were associated with bla_{KPC}, *H*30 lineage, and virotype C. This study documents worldwide incidents of resistance to "last resort" antimicrobial drugs among a common pathogen in a successful sequence type.

Escherichia coli sequence type 131 (ST131) was identified as pathogenic to humans in 2008; retrospective research suggests that its isolates have been present since at least 2003. The group has spread extensively and has been linked to the rapid global increase in the prevalence of antimicrobial resistance among *E. coli* strains (*1*). The intercontinental dissemination of this sequence type has contributed immensely to the worldwide emergence of fluoroquinolone-resistant and CTX-M–producing *E. coli* (*1*,*2*). Recent surveillance studies have shown that its overall prevalence ranges from 12.5% to 30% of all *E. coli* clinical isolates, from 70% to 80% of fluoroquinolone-resistant isolates, and from 50% to 60% of extended spectrum beta-lactamase-producing isolates (*3*).

The development of resistance to carbapenems among *E. coli* is of particular concern because these agents are often the last line of effective therapy available for the treatment of persons with serious infections (*4*). New Delhi metallo-β-lactamase (NDM) and carbapenem-hydrolyzing oxacillinase-48 (OXA-48) are the most common carbapenemases among *E. coli* worldwide (*5*).

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The Study

This study describes the characteristics of ST131 isolates among carbapenemase-producing *E. coli* strains collected globally by 2 research groups during 2008–2013. The Merck Study for Monitoring Antimicrobial Resistance Trends (SMART) (http://www.merck.com/about/featured-stories/infectious_disease.html) started in 2002 and AstraZeneca's global surveillance study of antimicrobial resistance (unpublished data) began in 2012, to monitor global antimicrobial resistance trends among gram-negative bacteria (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/11/14-1388-Techapp1.pdf). Antimicrobial susceptibilities of different antimicrobial agents (Table 1, wwwnc.cdc.gov/EID/article/20/11/14-1388-T1.htm) were determined by using frozen broth microdilution panels according to 2013 Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing guidelines (*6*). Established PCR and sequencing methods were used to identify β-lactamase genes (*7*,*8*) and define O25b:H4, O16:H5 ST131, *fimH*30 lineage, *H*30-Rx sublineage (*9*–*11*), and virotypes (*12*).

Overall, 47,843 *E. coli* isolates were collected and tested for susceptibility; 407 were found to be nonsusceptible to ertapenem or imipenem and were molecularly characterized for β-lactamase genes. A total of 116 of the 407 isolates were positive for NDM, KPC, OXA-48-like, VIM, and IMP types of carbapenemases. Various gene types were identified: 44 (38%) were positive for bla_{NDM}, 38 (33%) for bla_{KPC}, 30 (26%) for $bla_{OXA\text{-}48\text{-}like}$, 2 (2%) for $bla_{VIM\text{-}1}$ and 2 (2%) were positive for bla_{IMP} (Table 1).

The countries from which the *E. coli* isolates were obtained are shown in Table 2. The isolates were cultured from intraabdominal specimens (37%), peritoneal fluid (16%), biliary fluid (10%), urine (30%), and from miscellaneous sources such as sputum and tissue (9%).

PCR testing for O25b:H4, O16:H5, and MLST showed that 41/116 (35%) belonged to the sequence type ST131. Antimicrobial susceptibilities, types of β-lactamases, the presence of the *fimH*30 lineage, and virotypes are shown in Table 1. ST131strains were more likely than non-ST131 strains to be nonsusceptible to ciprofloxacin and to be positive for bla_{KPC}, the *H*30 lineage, and virotype C; non-ST131 isolates were more likely to be positive for bla_{NDM}.

The majority, i.e., 24 (58%), of ST131strains were positive for bla_{KPC}, 13 (32%) for $bla_{OXA\text{-}48\text{-}like}$, 3 (7%) for $bla_{NDM\text{-}1}$, and 1 (2%) for $bla_{IMP\text{-}14}$. ST131 was present in 16 countries spanning 5 continents (Table 2). The distribution of ST131 during 2008–2013 is shown in Table 3.

Various *fimH* alleles were identified among ST131 isolates: 24 *H*30 (58%), 3 H41 (7%), 3 *H*54 (7%), 2 *H*22 (5%), 2 *H*27 (5%), and 2 *H*191 (5%); and 1 each (2%) belonging to *H*24, *H*32, *H*65, and the new fim*H* alleles

*H*434 and *H*435. Of the 24 *H*30 ST131 strains, 19 (79%) belonged to the *H*30-R sublineage and 5(21%) to the *H*30-Rx sublineage.

Conclusions

NDM variants were the most common carbapenemase identified and were especially prevalent in *E. coli* strains from India and Vietnam (Table 2). KPCs, which were the second most common carbapenemase identified, were distributed globally, i.e., in South America, Central America, North America, Europe, the Middle East, and Asia (Table 2). This was unexpected because KPCs have been relatively rarely reported among *E. coli* (*5*).

Because of the unprecedented global success of ST131, the presence of carbapenemases had been carefully monitored by molecular epidemiologists but has been limited to case reports from several countries (*1*). The largest collections of ST131 with carbapenemases were reported from Hangzhou, Zhejiang Province, China (*13*) and Pittsburgh, Pennsylvania, USA (*14*). Of note, 24/38 (63%) of *E. coli* strains with bla_{KPC} belonged to ST131, as opposed to 3/44 (7%) for NDMs and 13/30 (43%) for OXA-48-like strains. Our results suggest that ST131 is most likely responsible for the global distribution of *E. coli* with bla_{KPC}.

The expansion of the *H*30 lineage and its *H*30-R and *H*30-Rx sublineages have contributed substantially to the spread of ST131 *E. coli* (*11*,*15*). In our study, *H*30-R, which belongs to virotype C, was the most common lineage among ST131 strains (i.e., 58%); it was associated with bla_{KPC} and was especially prominent during 2012–2013. The increase of the ST131 *H*30 lineage with bla_{KPC} during 2012–13 is cause for concern.

E. coli ST131 has received comparatively less attention than other antimicrobial-resistant pathogens. Retrospective molecular surveillance studies have shown that ST131 with $bla_{CTX\text{-}M\text{-}15}$ was rare during the early 2000s, but that an explosive global increase followed during the mid-to-late 2000s (*1*). The results of this study show a similar scenario with *E. coli* ST131 and bla_{KPC}; a low prevalence combined with a global distribution. This study is of special concern because we documented resistance to "last resort" antimicrobial drugs (i.e., carbapenems) in most regions of the world, in a common community and hospital pathogen (i.e., *E. coli*) among a very successful sequence type (i.e., ST131). We urgently need well-designed epidemiologic and molecular studies to clarify the dynamics of transmission, risk factors, and reservoirs for ST131.

The medical community can ill afford to ignore *E. coli* ST131strains with carbapenemases. This sequence type poses a major threat to public health because of its worldwide distribution and association with the dominant *H*30 lineage. This sequence type among *E. coli* has the potential to cause widespread resistance to carbapenems.

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Table 2. *Escherichia coli* with carbapenemases from combined Merck Study for Monitoring Antimicrobial Resistance Trends and AstraZeneca surveillance programs*

Carbapenemase (no.)	Total: country (no.)	ST131: country (no.)†
NDM (44)		
NDM-1 (39)	India (25), Vietnam (10), Serbia (1), Philippines (1), Thailand (1), China (1)	Philippines (1), India (1), Thailand (1)
NDM-4 (2)	India (2)	None
NDM-5 (n2)	Saudi Arabia (1), Kuwait (1)	None
NDM-6 (n1)	India (1)	None
KPC (38)		
KPC-2 (32)	Argentina (1), Brazil (2), Colombia (9), China (5), Ecuador (2), Italy (1), Jordan (1), Panama (1), Puerto Rico (5), USA (2), Vietnam (3)	Argentina (1), Colombia (5), China (4), Ecuador (1), Italy (1), Panama (1), Puerto Rico (4), USA (2), Vietnam (2)
KPC-3 (6)	Puerto Rico (1), Israel (1), USA (4)	USA (3)
OXA-48-like (30)		
OXA-48 (28)	Egypt (1), Jordan (1), Lebanon, (3), Morocco (2), Turkey (18), Vietnam (3), UAE (1)	Jordan (1), Morocco (1), Turkey (10), UAE(1)
OXA-163 (1)	Argentina (1)	None
OXA-244 (1)	Tunisia (1)	None
IMP (2)		None
IMP-1 (1)	India (1)	None
IMP-14 (1)	Thailand (1)	Thailand (1)
VIM-1 (2)	Italy (1), Greece (1)	None
Total	116	41

*NDM, New Delhi metallo-β-lactamase-1; KPC, *Klebsiella pneumoniae* carbapenemase; USA, United States of America; OXA, oxacillinase; UAE, United Arab Emirates; IMP, imipenemase; VIM, Verona integron–encoded metallo-β-lactamase.
†PCR-based screening of *E. coli* ST131 may infrequently identify isolates that belong to the 131 Clonal Complex as ST131 and rarely misidentifies non-ST131 *E. coli* as ST131.

Table 3. Temporal distribution of *Escherichia coli* ST131 in 2 global studies, 2008–2013*

Year	Total no. *E. coli*	No. carbapenem-nonsusceptible *E. coli*	No. (%) carbapenemase-producing *E. coli*	Type of carbapenemases (no.)	No. ST131	fim*H*30	Type of carbapenemases among ST131 (no.)
2008	3,739	45	10 (0.3)	NDM-1 (9), IMP-1 (1)	0	0	0
2009	5,913	63	21 (0.4)	NDM-1(16), NDM-4 (2), NDM-6 (1), OXA-48 (2)	1	0	NDM-1 (1)
2010†	8,951	71	17 (0.2)	KPC-2 (7), OXA-48 (10)	17	10	KPC-2 (7), OXA-48 (10)
2011	10,009	81	21 (0.2)	KPC-2 (9), KPC-3 (1), NDM-1 (5), OXA-48 (5), OXA-163 (1)	8	2	KPC-2 (6), KPC-3 (1), NDM-1 (1)
2012‡	14,275	97	35 (0.2)	KPC-2 (n12), KPC-3 (2), NDM-1 (7), NDM-5 (1), OXA-48 (11), OXA-244 (1), IMP-14 (1)	9	7	KPC-2 (5), OXA-48 (3), IMP-14 (1)
2013	4,956	50	12 (0.2)	KPC-2 (4), KPC-3 (3), NDM-1 (2), NDM-5 (1), VIM-1 (2)	6	5	KPC-2 (3), KPC-3 (2), NDM-1 (1)
Total	47,843	407	116	NDM-1 (39), NDM-4 (2), NDM-5 (2), NDM-6 (1), KPC-2 (32), KPC-3 (6), OXA-48 (28), OXA-163 (1), OXA-244 (1), IMP-1 (1), IMP-14 (1), VIM-1 (2)	41	24	KPC-2 (21), KPC-3 (3), NDM-1 (3), OXA-48 (13), IMP-14 (1)

*The 2 studies were the Merck Study for Monitoring Antimicrobial Resistance Trends (SMART) and the AstraZeneca global antimicrobial drug surveillance program. Isolates from SMART were not available for analysis in 2013: during 2008–2009, 1/32 (3%) *E. coli* isolates with carbapenemases from SMART were ST131 as opposed to 13/44 (30%) during 2011–2012. The limitation of the current study is that it uses a convenience set of isolates and differences over time could be related to differences in sampling rather than true increases in prevalence. Isolates from India were only obtained during 2008–10 while isolates from China were submitted in 2008, 2012 and 2013. NDM, New Delhi metallo-β-lactamase-1; IMP, imipenemase; OXA, oxacillinase, KPC, Klebsiella pneumonia carbapenemase; VIM, Verona integron–encoded metallo-β-lactamase.
†ST131 from 2010 should be interpreted with caution because 9 of the 17 isolates were submitted from a single hospital within Turkey. These isolates were positive for bla_{OXA-48}, $bla_{CTXM-15}$, and belonged to the *H*30-R sublineage. It is likely that this institution housed an outbreak during that time. If the 2010 isolates are removed from consideration, there was a substantial increase in ST131 toward the latter part of this study.
‡The AstraZeneca global surveillance program was initiated in 2012.

Dr Peirano is a research associate at Calgary Laboratory Services and the University of Calgary. Her main research interests are related to the detection and molecular epidemiology of antimicrobial drug resistance mechanisms among Gram-negative bacteria.

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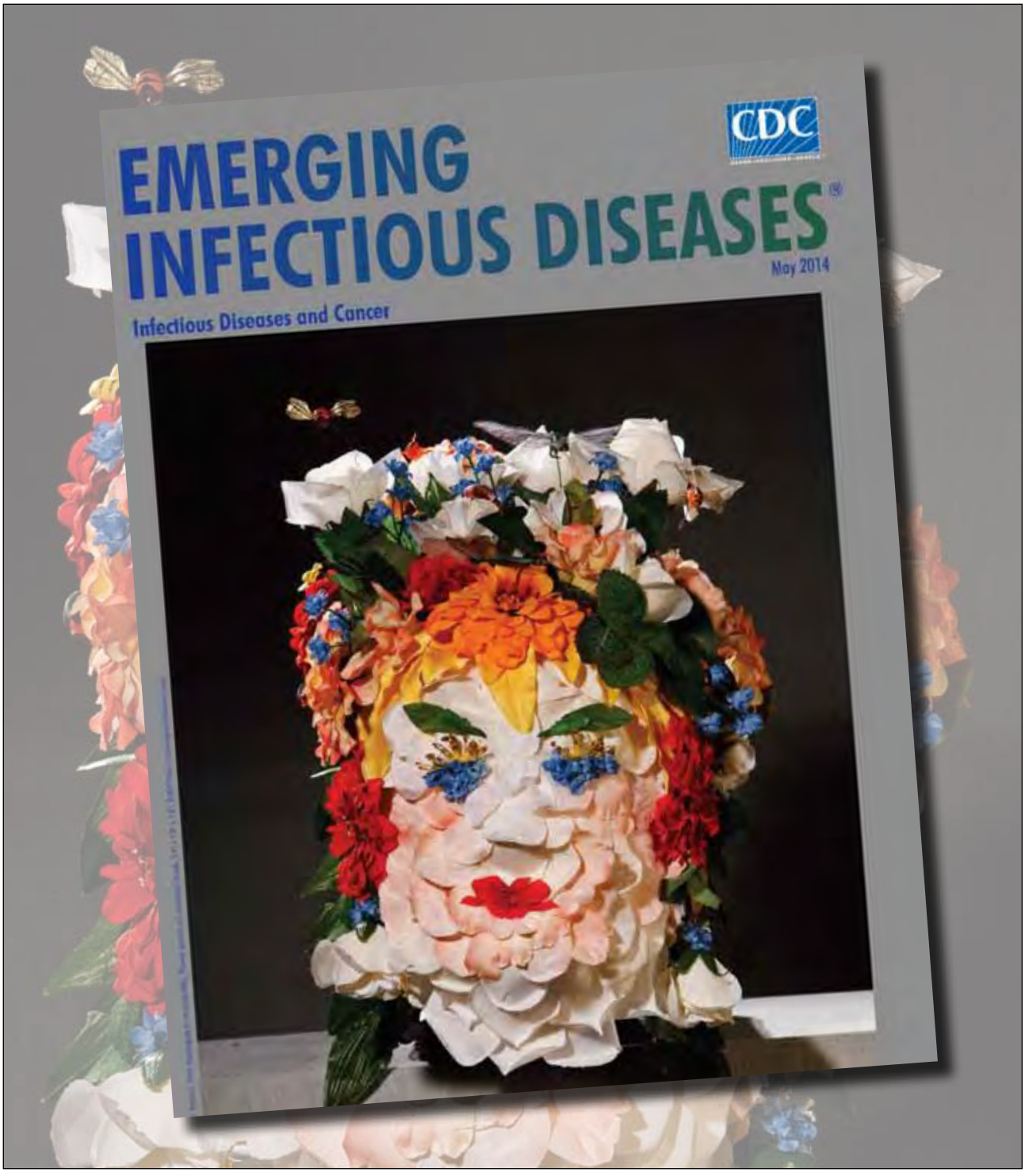

Mycobacterium tuberculosis Beijing Genotype Resistance to Transient Rifampin Exposure

To the Editor: We read with interest the discussion between de Steenwinkel et al. and Werngren (*1*) regarding the high mutation frequencies de Steenwinkel et al. reported for some *Mycobacterium tuberculosis* strains (*2*). We investigated the effect of rifampin exposure on the growth of these strains in detail.

We share Werngren's surprise at the extraordinary mutation frequencies observed for the Beijing strains. We previously estimated the mutation rate of 1 of the strains tested at ≈1×10^{-7} (*3*), which is within the range typically reported for acquisition of rifampin resistance by strains from Beijing and other lineages of *M. tuberculosis* (*4*,*5*). Several explanations were proposed for the detection of a high frequency of mutants on rifampin exposure, mainly related to methods or experimental variation (*1*). We propose an alternative interpretation of these findings on the basis of new experimental data (Figure).

These data show that some *M. tuberculosis* strains persist longer than others after transient exposure to low concentrations of rifampin, which could create a wider window for the (possibly stimulated) generation and selection of resistant mutants. Thus, the frequency of 10^{-3} resistant CFUs selected on rifampin may represent not the frequency of preexisting mutants but rather frequency of mutants generated by a population of persisting (stressed) cells during exposure to low levels of antimicrobial drugs. We investigated the effect of transient exposure to rifampin on colony growth rate and post–antimicrobial drug outgrowth on a panel of Beijing and non-Beijing *M. tuberculosis* strains, including some studied in the work of Steenwinkel et al. (*2*). We used a culture method developed on the basis of den Hertog et al. (*6*) but with higher throughput and improved imaging and analysis, in which individual colonies are monitored over time and growing colonies can be moved from 1 solid medium to another.

We injected ≈5×10^4 CFUs of *M. tuberculosis* per cm^2, consisting mostly of single cells (*6*), on porous aluminum oxide supports (PAOs) on MB7H11 agar + OADC (Becton Dickinson, Sparks, MD, USA). After 8 days' incubation at 36°C, the PAOs containing microcolonies consisting of ≈200 cells were moved onto medium containing 0.5–2 μg/mL of rifampin for 4 h, after which the PAOs were returned to nonselective medium. Filters were monitored for colony growth by using a MuScan microscopic system (LumiByte BV, Nuenen, the Netherlands) at 5× magnification; a ≈0.44-cm^2 area was imaged at specific intervals before and after exposure to rifampin. We used Fiji (http://fiji.sc/Fiji) and in-house software (*6*) to extract the surface areas of all identified colonies at all available time points; individual growth rates could thus be calculated for all colonies. In total, 36,408 colonies were defined as objects with a growth rate of >20% from days 6 and 8 after inoculation with >0.5 circularity.

As shown in the Figure, the growth of non-Beijing strains studied (East African/Indian strains and strain H37Ra) was almost completely inhibited during the first 24 hours after rifampin exposure. In contrast, all the Beijing genotype strains studied showed residual growth in most colonies in the first 24 hours after rifampin exposure. At 1–5 days postexposure, the pattern remained the same; the Beijing strain colonies were more capable of persisting and exhibited slow but sustained growth after exposure

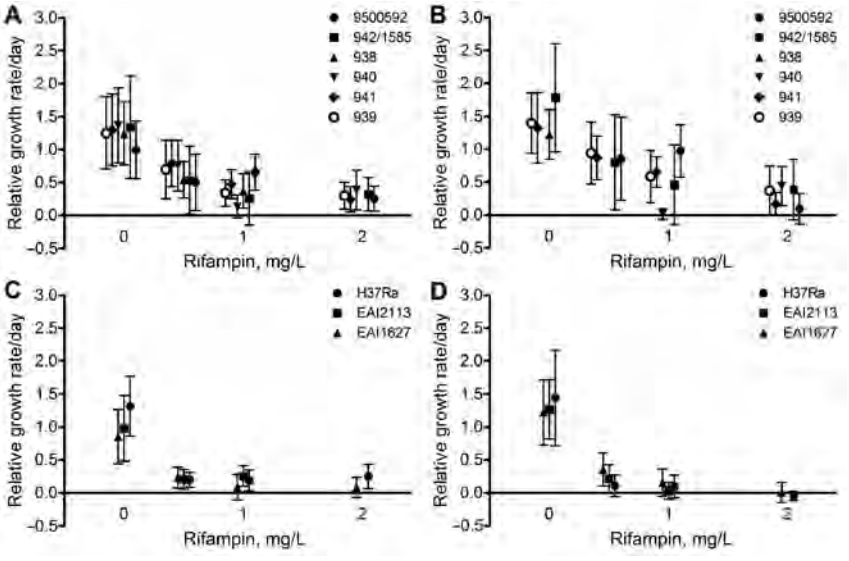

Figure. Eight-day-old microcolonies (≈10^2 cells per colony) of a panel of *Mycobacterium tuberculosis* Beijing strains (A, B) and East African Indian strains and strain H37Ra (C, D). Growth of the strains was monitored after 4 hours of exposure to different concentrations of rifampin. For all colonies, the growth rate relative to preexposure growth rate was calculated at 0–1 days after exposure for a median of 889.5 (interquartile range 478.75–1611.25) colonies (left panels) and 1–5 days after exposure for a median of 363 (interquartile range 241.25–843.00) colonies (right panels) per strain and per condition. Plots are averages ±SD (indicated by error bars). The experimental conditions failed to totally inhibit growth of most Beijing colonies even at 5 days postexposure, whereas for the non-Beijing strains, virtually no growth was detectable at 5 days postexposure.

to low concentrations of rifampin. To confirm that this effect was a result of persistence rather than generation of resistant mutants, we transferred the colonies growing after transient rifampin exposure of Beijing strain 1585 to a medium containing 8 mg/L rifampin. Their growth was completely inhibited, and molecular analysis did not detect any of the most prevalent rifampin resistance–associated mutants (data not shown).

We believe that these results provide a possible explanation for the otherwise unrealistically high (apparent) mutation frequency reported by de Steenwinkel et al. (*2*). If these strains are capable of persisting at low concentrations of rifampin, this extended period would provide a window for the generation of mutants during or after exposure. Stress may also play a role; *rpoB* gene mutants have shown to exhibit a stringent-like response (*7*), and defective *rpoB* activity as a result of low-level rifampin exposure could induce a similar response. If rifampin induces a stress response, the situation may be analogous to the high mutation rates seen after quinolone exposure (*8*).

In summary, our data show that the high apparent *M. tuberculosis* strain mutation frequency reported by de Steenwinkel et al. (*2*) may be a result of the higher tolerance to rifampin of some Beijing strains. This tolerance likely results in a specific window of rifampin concentrations that, possibly combined with subsequent error-prone replication/outgrowth, enables the generation and selection of new mutants, rather than the selection of preexisting mutants. When interpreted in the light of our observations, the unexpected results of de Steenwinkel et al. could help explain the association of Beijing genotype strains with drug resistance and relapse (*9*,*10*). Drug levels achieved during treatment may be much more critical in preventing the accumulation of rifampin-resistant mutants for these strains than for other genotypes.

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Bacteria in Dairy Products in Baggage of Incoming Travelers, Brazil

To the Editor: International air travel can lead to the rapid global dissemination of infectious agents. Unlike products and byproducts of animal origin imported between countries under agreements that legally establish sanitary standards, products introduced into a country illegally or irregularly do not follow specific standards and can come from any source, thereby posing a risk to the health

status of a country. Animal products transported clandestinely in baggage can contain infectious agents harmful to animal and human health (*1–4*). We investigated *Brucella* spp., *Mycobacterium bovis*, and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in dairy products seized from baggage of passengers on flights at the 2 main international airports (Guarulhos Airport, São Paulo, and Galeão Airport, Rio de Janeiro) in Brazil.

During 2010–2011, 12 missions were instigated by the International Agriculture Surveillance (VIGIAGRO/MAPA) in airports to detect and seize unauthorized dairy products carried by passengers; 195 products were collected from multiple flights from different destinations. Baggage was scanned by using an x-ray machine and, on detection of a product, was opened by the owner in the presence of a federal agriculture inspector. To avoid contamination, the products were not opened and were sent to the designated Ministry of Agriculture, Livestock and Food Supply Laboratory in their original packaging. All seized products were packed according to the International Air Transport Association standards (*5*) and transported by commercial aviation with official monitoring to the laboratory.

After completing real-time quantitative PCR (Promega, Madison, WI, USA) using TaqMan technology (Life Technologies, Darmstadt, Germany), we extracted DNA directly from the sample (*6*,*7*). The technique for the detection of MAP and eryD *Brucella* (except strain 19 *Brucella abortus*) and also using the region RD4 to detect *M. bovis* were proposed by Irange et al. (*8*). To detect *M. bovis*, we used the primers *M. bovis*-88-F (5′-CGC.CTT.CCT.AAC.CAG.AAT.TG-3′), *M. bovis*-88-R (5′-GGA.GAG.CGC.CGT.TGT.AGG-3′) and to detect *Brucella*, we used Bru-Eri-Taq-92-F (5′-GCC.ACA.CTT.TCT.GCA.ATC.TG-3′) and Bru-Eri-Taq-92-F (5′-GCG.GTG.GAT.AAT.GAA.ATC.TGC-3′).

We analyzed 35 containers of dulce de leche, a caramelized milk paste confection, from Argentina (n = 30), Angola (n = 1), and Uruguay (n = 4). We tested all specimens for *Brucella* spp. and MAP, and 32 for *M. bovis*. We detected MAP in 1 specimen from Argentina and 1 from Uruguay, *Brucella* spp. in 3 specimens from Argentina and 1 from Uruguay, and *M. bovis* in 1 specimen from Argentina.

Three containers of liquid milk from the United States were collected and analyzed for the presence of MAP; 2 were analyzed for *M. bovis* and *Brucella. Brucella* was detected in 1 specimen. Five containers of powdered milk were seized: 2 from Chile, 2 from Angola, and 1 from Portugal. *Brucella* was detected in 1 container from Chile; *Brucella* and *M. bovis* were found in 1 container from Angola. Four containers of yogurt were seized, 1 each from the United States, China, Angola, and South Africa. MAP was detected in those from Angola and South Africa, and the yogurt from South Africa also showed *Brucella*.

We analyzed samples from 147 cheeses that were confiscated from baggage owned by travelers from 21 countries, mainly from Italy (24.5%), Portugal (22.4%), and France (14.3%). *M. bovis* was identified in 18 (17.5%) cheeses collected from Italy, Portugal, Spain, the United States, the Netherlands, Lebanon, Morocco, and Norway. MAP was amplified in specimens from 13 cheeses from Spain, United States, Iraq, Israel, Norway, Peru, France, and Portugal, the last 2 countries showed the highest occurrence. *Brucella* was detected in 62 of the cheeses analyzed, which originated in Bolivia, Chile, Iraq, Lebanon, and Morocco (n = 1 in each country), Netherlands, Israel, and Norway (n = 2 in each country), Turkey and Spain (n = 3 in each country), United States, France and England (n = 4 in each country), Portugal (n=10), and Italy (n = 23).

Both *M. bovis* and *Brucella* were detected in 13 (8.8%) cheeses (1 each from Spain, Netherlands, Morocco, and Norway; 4 from Portugal, and 5 from Italy); *Brucella* and MAP were detected in 4 (2.7%) cheeses (Spain, France, Portugal, and Iraq). Co-amplification of the 3 genes (*Brucella* + MAP + *M. bovis*) occurred in 3 (2%) cheeses (United States, Norway, and Portugal). Among the cheeses analyzed, 84 (57.1%) contained isolates that amplified >1 of the genes for the 3 bacteria examined.

Of the 166 dairy products analyzed, *Brucella* was detected in 70 (42.1%). Cheeses were the most seized products (n = 121) and had the highest number of *Brucella*-positive results (62/121[51.2%]). *Brucella* was detected in dairy products that originated in Argentina, Spain, France, Iraq, Israel, Italy, Lebanon, Portugal, and Turkey; it was detected in 4 (21%) of the 19 cheeses from France and in 3 of the 4 (75%) cheeses that originated in Spain. *M. bovis* was detected in dulce de leche from Argentina, powdered milk from Chile, and in cheeses from Spain, Netherlands, Italy, Lebanon, Morocco, Norway, and Portugal.

Bacteria can be introduced into a country through contaminated animal products that are brought across borders illegally. The risk may be even greater when these products are carried in passengers' baggage on international flights because of the growing number of international travelers and the wide range of origins of these passengers. Greater attention should be given to agricultural surveillance at airports to mitigate the risk for introduction of these products.

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Evidence of Evolving Extraintestinal Enteroaggregative *Escherichia coli* ST38 Clone

To the Editor: Several clones of extended-spectrum β-lactamase (ESBL)–producing extraintestinal pathogenic *Escherichia coli* (ExPEC) have globally expanded their distribution, including multilocus sequence types (MLSTs) ST38, ST131, ST405, and ST648 (*1*). ExPEC infections often originate from the patient's own intestinal flora, although the degree of overlap between diarrheagenic *E. coli* and ExPEC pathotypes is unclear. Relatively little is known about antimicrobial drug resistance in the most common diarrheagenic *E. coli* groups, including enteroaggregative *E. coli* (EAEC), and bacterial gastroenteritis is generally managed without use of antimicrobial drugs.

The ability of diarrheagenic *E. coli* to cause extraintestinal infections has been shown in previous studies: a study among children in Nigeria linked EAEC to uropathogenic clonal group A (*2*), and a study in Brazil showed that EAEC markers were present in 7.1% of the *E. coli* isolates from urinary tract infections (*3*). Neither of these studies identified clonal lineages of EAEC specifically associated with extraintestinal infections.

We conducted this study to establish the presence and characteristics of ESBL-producing EAEC in a well-defined collection of ESBL-producing isolates (*4*). The isolates were from human and animal sources in Germany, the Netherlands, and the United Kingdom. The study was conducted at Public Health England during January–April 2013.

DNA from 359 ESBL isolates (*4*) was screened for the presence of the EAEC transport regulator gene (*aggR*), located on the EAEC plasmid, by using a real-time PCR assay and the following primers and probe: AggR_F 5′-CCATTTATCGCAATCAGATTAA-3′ AggR_R 5′-CAAGCATCTACTTTTGATATTCC-3′, AggR_P Cy5-CAGCGATACATTAAGACGCCTAAAGGA-BHQ. The amplification parameters were 50°C for 2 min, 95°C for 2 min, and 40 cycles at 95°C for 10 s and at 60°C for 20 s. Isolates positive for *aggR* were confirmed to be *E. coli* by using the Omnilog GenIII MicroPlate (Biolog, Hayward, CA, USA). Serotyping was done by using standard methods (*5*).

The phylogroup was determined for each isolate, and isolates were then assigned to 1 of the 4 major *E. coli* groups: A, B1, B2, and D (*6*). A microarray was used to detect ESBL genes, such as $bla_{\text{CTX-M}}$, at the group level, as previously described (*4*). The antimicrobial drug susceptibilities of EAEC isolates were determined by using the agar incorporation method, as described in the British Society for Antimicrobial Chemotherapy guidelines (*7*).

Virulence factors associated with intestinal and extraintestinal infection

Table. Characteristics of human-derived ESBL-producing enteroaggregative *Escherichia coli* isolates from sources in Germany, the Netherlands, and the United Kingdom*

Isolate	Serotype†	ST	Cplx‡	Country	Source	Phylotype	*aggR*§	Plasmidic ESBL
ESBL-723	OR:H30	38	38	UK	Urine	D	+	CTX-M-15
ESBL-746	O125ac:H30	38	38	UK	Urine	D	+	CTX-M-15
ESBL-884	O19a:H30	38	38	UK	Urine	D	+	CTX-M-14
ESBL-831	O19a:H30	38	38	UK	Urine	D	+	CTX-M-14
ESBL-815	O19a:H30	38	38	UK	Blood	D	+	CTX-M-15
ESBL-26	O153:H30	38	38	Netherlands	Urine	D	+	CTX-M-51
ESBL-221	O92:H33	34	10	Germany	Feces	A	+	CTX-M-3
ESBL-45	O?:H26	58	155	Netherlands	Urine	B1	+/–	CTX-M-14
ESBL-46	O?:H-	694	None	Netherlands	Urine	A	+/–	CTX-M-15
ESBL-48	O15:H1	545	None	Netherlands	Urine	D	+/–	CTX-M-1
ESBL-64	O?:H23	224	None	Netherlands	Urine	B1	+/–	CTX-M-1

*All isolates were collected in 2009 (*4*). ESBL, extended-spectrum β-lactamase. ST, sequence type.
†H- not motile; O?, O unidentifiable; R, rough reaction.
‡Cplx–ST complex comprising single-locus variants.
§*aggR*, enteroaggregative *E. coli* regulatory gene; +, positive in screen and isolates; –, negative in screen and isolates; +/–, positive in screen but negative in isolates, indicating unstable plasmid.

(*8*) and with EAEC were investigated as previously described (*9*). We assigned a virulence score (total number of virulence factor genes detected; maximum possible score 22) and a resistance score (total number of drug classes; maximum score 11) to each isolate.

We isolated 11 EAEC from humans. Eight of the EAEC were isolated from urine specimens, and 1 was isolated from a blood culture; 63% belonged to phylogroup D (Table). EAEC ST38, the most common (55%) ST, was significantly associated with extraintestinal sites in the subset of 140 human isolates (Fisher exact test, $p<0.0001$).

In this study, we identified multidrug-resistant EAEC isolates belonging to ST38; the isolates had various somatic antigens and $bla_{\text{CTX-M}}$ genes (Table). The multiple somatic antigens, variety of antimicrobial drug–resistance scores, and variety of gene complements in this successful ST indicate multiple acquisitions of virulence markers, rather than clonal expansion from a single source (Table; online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/20/11/13-1845-Techapp1.pdf).

In the MLST public database, which contained 5,143 *E. coli* entries in June 2013, ST38 is predominantly associated with urinary tract infections, but in-house MLST studies at the Gastrointestinal Bacteria Reference Unit, Public Health England, have shown that ST38 is a successful EAEC group. The presence of EAEC virulence factors, such as aggregative adherence fimbria AAFI and *aggR*, can mediate adherence of *E. coli* to bladder epithelial cells, but the virulence factors do not impart uropathogenic properties to all EAEC isolates (*10*). The ST38 strain described here probably originated from the gut and independently acquired the 2 phenotypes (uropathogenic *E. coli* [UPEC] and EAEC), which would suggest the emergence of a UPEC/EAEC hybrid strain. It seems likely that an ST38 *E. coli* strain adapted to EAEC plasmid carriage (a change that would help survival in the gut through increased adherence) has acquired UPEC virulence factors, facilitating the exploitation of an extraintestinal niche, the urinary tract.

Despite the characterization of numerous virulence factors, no single genetic feature currently defines EAEC or UPEC isolates. Because the EAEC ST38 strain had 4–7 ExPEC-associated virulence factors, we suggest that, on the basis of epidemiologic, microbiological, and molecular characteristics, the EAEC ST38 described in this study should be considered an ExPEC associated with uropathogenic infections. It is possible that the multidrug-resistant EAEC ExPEC group has expanded globally but is currently underreported. We therefore urge testing for the EAEC genotype in all clinical studies of *E. coli* pathotypes.

Our findings show the potential for EAEC, previously considered a gut pathogen, to cause extraintestinal infection. We suggest that the UPEC/EAEC pathotype may be an evolving clonal group. In particular, a single sequence type, ST38, was associated with multidrug resistance and with urinary tract infection in humans.

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Resolution Threshold of Current Molecular Epidemiology of Diphtheria

"The fox who longed for grapes, beholds with pain

The tempting clusters were too high to gain;

Grieved in his heart he forced a careless smile,

And cried, 'They're sharp and hardly worth my while.'"

(Aphra Behn, 1687, after Aesop's *The Fox and the Grapes*)

To the Editor: Diphtheria is an extremely rare disease in Europe but remains a major health issue in developing countries (*1*–*3*). In recent years, steady progress has been made toward understanding the factors of pathogenicity of its causative agent (*Corynebacterium diphtheriae*). In contrast, remarkable advances in its basic genomics have not been sufficiently translated into the molecular epidemiology of diphtheria. A recent report by Zasada (*4*) offers an apt opportunity to take a new look at this issue.

The current genotyping repertoire of *C. diphtheriae* includes several methods but those most frequently used are classical ribotyping and pulsed-field gel electrophoresis (PFGE). More recently, a multilocus sequence typing (MLST) scheme for *C. diphtheriae* was developed (*5*). Compared with ribotyping, PFGE, and other methods based on analysis of banding profiles, MLST results are digital, unambiguous, and portable. MLST discrimination of 150 isolates from 18 countries and spanning 50 years was "in accordance with previous ribotyping data, and clonal complexes associated with disease outbreaks were clearly identified by MLST" (*5*).

In the report by Zasada (*4*), all 3 recommended methods (PFGE, MLST, and ribotyping) were used to genotype 25 nontoxigenic *C. diphtheriae* isolates from Poland. The author concluded that these isolates "represent a single clone despite isolation … in different part of the country over a 9-year period" and raised the question of whether a single clone of *C. diphtheriae* is circulating in Poland (*4*). These isolates are related genetically, but do they represent a truly single clone or might they be further discriminated? Their circulation in Poland may be caused by their high pathogenicity, but also (or instead) it might reflect their endemic, historical prevalence in this country. I believe that these questions are unlikely to be answered by the internationally agreed-upon methods for *C. diphtheriae* typing because of their insufficient resolution: the discriminatory power of MLST does not exceed that of ribotyping (*5*).

Two in silico–inspired approaches have recently been pursued toward more precise molecular genetics and epidemiology of diphtheria. The first approach is based on whole-genome sequencing (WGS). After years of stagnation, the number of complete *C. diphtheriae* genomes has finally started to increase: currently 13 complete and 3 draft genomes are available in GenBank (as of April 14, 2014). The second approach is locus oriented and makes use of the repetitive DNA sequences, namely, variable number tandem repeats (VNTR) and clustered, regularly interspaced short palindromic repeats (CRISPR) loci.

A study in Poland showed a discriminatory capacity of some of the VNTR loci in *C. diphtheriae* (*6*), although preliminary results were not compared with those from other typing methods. CRISPR loci in *C. diphtheriae* have been studied in more detail and CRISPR-based spoligotyping showed a high level of discrimination for an epidemic clone in Russia and Belarus (*7*,*8*). In particular, 156 isolates from Russia of the epidemic clone (classical ribotypes Sankt-Petersburg and Rossija) were subdivided into 45 spoligotypes (*7*).

Further studies underlined the limitations of CRISPR-based typing: the 3 described CRISPR loci are not present simultaneously in all isolates; and most strains have unique spacers at the leader part of the array, which indicates their independent evolution after they diverged from a common ancestor (*9*). Accordingly, Sangal et al. suggested that CRISPR-based typing might not necessarily provide information on evolutionary relationships between different strains, but it might offer a high level of discrimination to study local epidemiology (*9*).

Recent advances in *C. diphtheriae* genomics concern an increasing number of complete genomes in GenBank, development of new ideas (e.g., revisiting biochemical subdivision into biovars) (*10*), and development of new typing schemes (MLST, VNTR, and spoligotyping). Nevertheless, in spite of lack of genetic support, biochemical classification into biovars is still uncritically used. In spite of other demonstrated or potential tools (e.g., CRISPR, VNTR), classical or new methods (all with limited resolution) are still used in many studies, both global and local. In spite of many available complete genomes, this wealth of information has not been translated into a WGS-informed high-resolution typing scheme. It might be sufficient to sequence all 3 CRISPR/*cas* loci in all strains studied by Zasada (*4*) to gain some insight into their relatedness and possible spatiotemporal evolution. A phylogenetically more robust, albeit more expensive, solution would be using WGS analysis to achieve the same objective.

In conclusion, molecular epidemiology of diphtheria would definitely benefit from implementation of more precise molecular genetics. First, WGS (or at least core genome) analysis might offer a broader range of possible general solutions from global tracing of large clonal clusters (current threshold) toward fine-tuned strain discrimination. At the same time, a multicenter evaluation of recently developed inexpensive and discriminatory VNTR and CRISPR methods is warranted to see if and how they could complement regional surveillance.

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Klebsiella pneumoniae–Induced Liver Abscesses, Germany

To The Editor: Monomicrobial liver abscesses caused by *Klebsiella pneumoniae* are an emerging problem in Asia. Among 77 capsular types of *Klebsiella* spp., K1 and K2 are the most virulent (*1*–*3*). In Asia, isolated strains are mainly serotype K1, followed by K2. In Taiwan, about 63.4% of liver abscesses caused by *K. pneumoniae* are associated with K1 strains and 14.2% by K2 (*4*); no comparable epidemioogic data for Western countries are available (*5*,*6*). Cases of *K. pneumoniae* liver abscesses have been also reported from North America and Europe (*6*–*9*); patients outside of Asia predominantly have a history of traveling to high-prevalence countries and/or Asian ethnicity (*10*).

We describe 2 cases of *K. pneumoniae* liver abscesses in patients in Germany. Both cases were associated with life-threatening metastatic spread of *K. pneumoniae* in previously healthy white patients with no travel history, no Asian ethnicity, and no contact with persons in high-risk groups, including patients with *K. pneumoniae* infection.

Patient 1 was a 48-year-old white male medical doctor with no history of serious medical conditions, who suddenly experienced fever up to 40°C, abdominal pain, mild diarrhea, and fatigue. Blood analysis revealed signs of inflammation: C-reactive protein level of 26 mg/dL. Erythrocyte and leukocyte counts were within reference ranges, but thrombocytes were decreased (43,000 cells/mL) and liver enzymes were increased (glutamate pyruvate transaminase 112 U/L, aspartate aminotransferase 75 U/L, gamma glutamyl transferase 332 U/L). An initial chest radiograph and abdominal ultrasonograms were unremarkable. *K. pneumoniae* was isolated from blood and urine and was later characterized as K1 capsular type (no further typing techniques could be performed because no sample was stored). Ceftriaxone was administered, in accordance with an antibiogram, but the patient's condition worsened and he required mechanical ventilation and treatment with a catecholamine in an intensive care unit. A computed tomographic (CT) scan showed 2 liver abscesses and infiltrates of both lungs with pleural effusion (Figure). Under CT guidance, the liver abscesses were punctured and drained percutaneously by using pigtail catheters. After this intervention, the patient recovered gradually. The drain was removed after 10 days, and ceftriaxone treatment was continued for a total of 21 days. The patient recovered and was transferred to a rehabilitation hospital.

Patient 2 was a 71-year-old white woman with type-2 diabetes, who was hospitalized for epigastric pain and fatigue. Laboratory results showed an increased level of C-reactive protein (13 mg/dL), blood count and liver enzymes within reference range, and a urinary tract infection positive for nitrite and leukocytes (500/mL). Abdominal ultrasonography revealed a 3 × 4-cm subcapsular lesion in the left lobe of the liver, highly suspect for metastatic spread of an unknown tumor. Chest radiographs, taken in search of the primary lesion, revealed a lesion in the right upper lung; on follow-up CT images, this lesion was suspect for malignancy (Figure). For diagnostic purposes, the liver lesion was punctured and a sample was obtained. Histologic analysis revealed pus, which was in accordance with an abscess. A pigtail drain was placed, and *K. pneumoniae* was cultured from the liver punctate as well as from the urine. The isolate proved to be serotype K2. Multilocus sequencing of this strain confirmed the presence of the *wzx2* and *wzy2* genes. In accordance with susceptibility test results, therapy with ceftriaxone and ciprofloxacin was initiated. Dislocation of the pigtail catheter resulted in an abscess of the abdominal wall, which required additional surgical treatment. However, the patient recovered within 2 weeks. Follow-up CT images showed resolution of the thoracic lesion and only a residual scar on the liver lobe (online Technical Appendix http://wwwnc.cdc.gov/EID/article/20/11/14-0149-Techapp1.pdf).

These 2 cases of community-acquired *K. pneumoniae* serotype K1 and K2 liver abscesses with metastatic spread to the lung and urinary systems

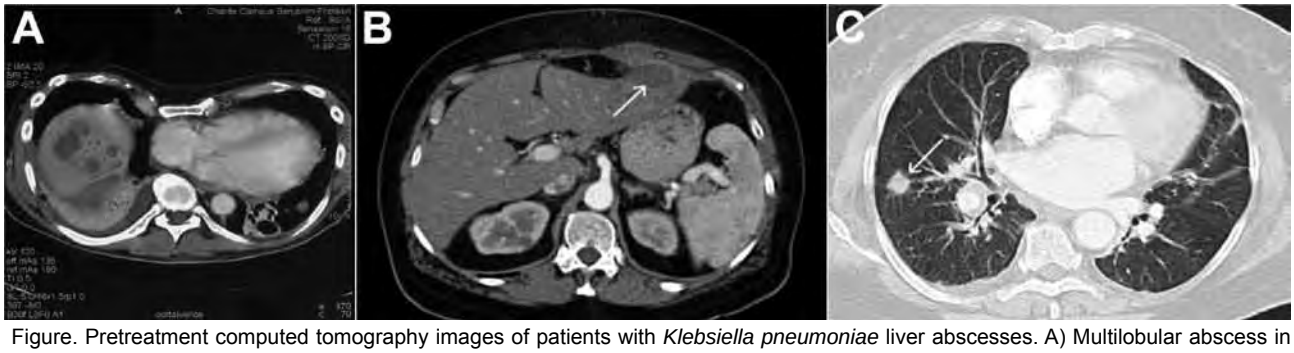

Figure. Pretreatment computed tomography images of patients with *Klebsiella pneumoniae* liver abscesses. A) Multilobular abscess in segment 8 of the liver and pleural effusion in 48-year-old white man (patient 1). B) Liver abscess (arrow) in 71-year-old white woman with type-2 diabetes (patient 2). C) Lung lesion (arrow) in patient 2.

in previously healthy white patients from Germany differ from previously published cases. These 2 patients were not of Asian ethnicity and had no travel history, no contact with persons in a high-risk group (*10*), and no common risk factors such as malignancy (*8*); however, 1 patient had type-2 diabetes. *K. pneumoniae* liver abscesses might be an emerging problem with global spread. Although initial radiographic findings might more commonly indicate metastasis than abscesses, differential diagnosis of liver lesions should include *K. pneumoniae*–induced abscesses.

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Hepatitis E Virus and Implications for Blood Supply Safety, Australia

To the Editor: Hepatitis E virus (HEV) is an emerging public health concern for industrialized countries (*1*). Although HEV infection has been associated with travel to countries where the virus is endemic, cases of autochthonous HEV are increasing (*2*). Detection of HEV RNA in blood donations in the United Kingdom, Germany, the Netherlands, Japan, and China and accumulating reports of HEV transmitted through blood transfusion highlight the potential risk this virus poses to blood supply safety (*1*–*4*).

In Australia, where most HEV infections are associated with travel (*5*), an average of 25 HEV cases occurred each year during 1999–2013 (http://www9.health.gov.au/cda/source/rpt_3.cfm). HEV infection is nationally notifiable in Australia, but the presence of subclinical infections and the lack of recent seroprevalence studies have prevented the accurate estimation of HEV incidence and population exposure. Thus, we examined HEV seroprevalence in a cohort of Australian blood donors, assessed risk factors for exposure, and used the data to examine the effectiveness of current blood safety strategies for the management of HEV in Australia.

Plasma samples (n = 3,237) were collected from donors during August–September 2013. Information on age, sex, state of residence, new/repeat donor status, and overseas travel disclosure was obtained. Details of any relevant blood donation deferral (malaria, diarrhea) applied on previous donation attempts were also collected. Application of a specific malaria deferral code is routine for donors disclosing travel to a malaria-endemic country, and a diarrhea deferral applies when a donor reports having had diarrhea (of viral or unknown cause) 1 week before any attempted donation.

All samples were tested for HEV IgG by using the Wantai HEV-IgG ELISA (Beijing Wantai Biologic Pharmacy Enterprise Co., Ltd, Beijing, China). Positive samples were tested for HEV IgM by using the Wantai HEV-IgM ELISA and for HEV RNA by using a prototype transcription-mediated amplification assay (Hologic Inc., San Diego, CA, USA).

Of 3,237 samples, 194 (5.99%) were positive for HEV IgG (95% CI 5.18–6.81). Compared with estimates

from previous studies that used the Wantai ELISA (*6–9*), our estimate is comparable to those reported from Scotland (4.7%) and New Zealand (4.2%) but lower than those from the United States (18.8%) and southwestern France (52.5%). Considerable debate exists regarding the sensitivity and specificity of HEV detection methods (*2,10*); however, on the basis of studies in France and the United Kingdom (*9,10*), we believe that the measured seroprevalence in our study is accurate.

Our findings showed an increased seroprevalence of HEV associated with previous malaria deferral, diarrhea deferral, and age (multivariate logistic regression) (Table), the latter of which is consistent with previous findings (*9*). IgG seropositivity was also higher (7.73%) in donors who had traveled to a malaria-endemic country. HEV is often endemic to malaria-endemic countries (http://wwwnc.cdc.gov/travel/yellowbook/2014); however, the HEV exposure status of travelers is unknown before departure, so the exact place of exposure cannot be determined. Furthermore, 3.37% of donors in our study had evidence of previous HEV exposure; these donors had not reported travel outside Australia, so they may have acquired HEV locally. Because subclinical HEV infection is possible, persons infected locally may not be identified by the current donor screening questionnaire and thus pose a potential risk to blood supply safety.

Detection of HEV IgM in 4 (2.06%) of the 194 samples from IgG-positive donors indicates the donors had been recently exposed to HEV (95% CI 0.06–4.06). All 4 donors had traveled overseas; 3 reported travel to malaria-endemic countries. HEV RNA was not detected in any of the HEV IgG–positive samples. Although it is encouraging that HEV nucleic acid was not detected, the sample size is insufficient to accurately determine the true rate of HEV RNA carriage among donors in this study; a larger study is planned.

Management strategies to safeguard the Australian blood supply against transfusion-transmitted HEV are based on donor selection guidelines. To identify donors with possible bacteremia/viremia, including HEV, blood donation staff members ask donors several medical, behavioral, and travel-based questions before donation. These include questions relating to general wellness, sex practices, gastric upset, diarrhea, abdominal pain, and vomiting within the previous week. In addition, for 4 months after a donor's return from travel to a malaria-endemic country, donations are

Table. HEV (IgG) seroprevalence, and risk factors for exposure, in Australian blood donors*

Risk factor	No. tested	HEV IgG seropositive		Univariate analysis		Multivariate analysis	
		No.	% (95% CI)	OR (95% CI)	p value	OR (95% CI)	p value
Sex							
F	1,453	78	5.37 (4.21–6.53)	†	†	–	–
M	1,784	116	6.50 (5.36–7.65)	1.23 (0.91–1.65)	0.177	–	–
Age, y					0.000		0.000
<25	564	14	2.48 (1.20–3.77)	†	†	†	†
25–34	569	13	2.28 (1.06–3.51)	0.92 (0.43–1.98)	0.827	0.82 (0.38–1.77)	0.61
35–44	510	22	4.31 (2.55–6.08)	1.77 (0.89–3.5)	0.100	1.72 (0.87–3.42)	0.118
45–54	666	40	6.01 (4.20–7.81)	2.51 (1.35–4.66)	0.004	2.43 (1.30–4.52)	0.005
55–64	673	68	10.10 (7.83–12.38)	4.41 (2.46–7.94)	0.000	4.18 (2.32–7.54)	0.000
≥65	255	37	14.51 (10.19–18.83)	6.67 (3.54–12.58)	0.000	6.09 (3.21–11.55)	0.000
State of residence					0.580	–	–
ACT	406	25	6.16 (3.82–8.50)	†	†	–	–
NSW	405	23	5.68 (3.42–7.93)	0.92 (0.51–1.64)	0.773	–	–
NT	407	26	6.39 (4.01–8.76)	1.04 (0.59–1.83)	0.892	–	–
QLD	402	18	4.48 (2.46–6.50)	0.71 (0.38–1.33)	0.289	–	–
SA	404	32	7.92 (5.29–10.55)	1.31 (0.76–2.25)	0.328	–	–
TAS	401	20	4.99 (2.86–7.12)	0.80 (0.43–1.46)	0.800	–	–
VIC	411	23	5.60 (3.37–7.82)	0.90 (0.50–1.62)	0.733	–	–
WA	401	27	6.73 (4.28–9.19)	1.10 (0.63–1.93)	0.739	–	–
Overseas travel							
No	416	14	3.37 (1.63–5.10)	†	†	†	†
Yes	2,821	180	6.38 (5.48–7.28)	1.96 (1.12–3.40)	0.017	1.24 (0.69–2.25)	0.471
Previous malaria deferral							
No	1,684	74	4.39 (3.42–5.37)	†	†	†	†
Yes	1,553	120	7.73 (6.40–9.06)	1.82 (1.35–2.45)	0.000	1.80 (1.31 –2.47)	0.000
Previous diarrhea deferral							
No	3,179	185	5.82 (5.01–6.63)	†	†	†	†
Yes	58	9	15.52 (6.20–24.84)	2.97 (1.44 – 6.14)	0.003	2.55 (1.22–5.33)	0.013
Donor status							
New	307	13	4.23 (1.98–6.49)	†	†	–	–
Repeat	2,930	181	6.18 (5.31–7.05)	1.49 (0.84–2.65)	0.175	–	–

*ACT, Australian Capital Territory; HEV, hepatitis E virus; NSW, New South Wales; NT, Northern Territory; OR, odds ratio; QLD, Queensland; SA, South Australia; TAS, Tasmania; VIC, Victoria; WA, Western Australia; –, indicates factor was not included in multivariate analyses.
†Reference group used in respective analyses.

restricted to plasma for fractionation. Some protection against blood donations from HEV-infected persons may occur because HEV and malaria are coendemic to many countries. Our findings showed a higher HEV seroprevalence among donors with prior malaria or diarrhea deferrals; thus, malaria- and diarrhea-related screening questions may reduce contributions from donors with travel-associated HEV infection.

Our findings showed HEV exposure in travelers and nontravelers, suggesting the possibility of imported and locally acquired HEV in Australia. Prior HEV exposure was higher in donors who were temporarily excluded from donating blood on previous donation attempts, suggesting the current management strategy in Australia is partially effective in minimizing any risk of HEV transmission through blood transfusion. However, the presence of HEV IgG in donors who reported no overseas travel and/or no prior related deferrals, coupled with the knowledge that asymptomatic infection is possible, suggests that additional safety precautions may be warranted.

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Helicobacter cinaedi Infection of Abdominal Aortic Aneurysm, Japan

To the Editor: Infected abdominal aortic aneurysm (IAAA) is uncommon, but life-threatening; the mortality rate ranges from 25% to 30% (*1*,*2*). Identification of the pathogen is essential for diagnosis and treatment. Previous studies have shown that species of the genera *Salmonella*, *Staphylococcus*, and *Streptococcus* are the most common pathogens associated with IAAA, but a causative organism is not identified in 14%–40% of patients (*1*,*2*). *Helicobacter cinaedi* has mainly been isolated from immunocompromised patients with bacteremia, cellulitis, and septic arthritis (*3*,*4*). Here, we report 3 cases of IAAA caused by *H. cinaedi* detected by 16S ribosomal RNA (16S rRNA) gene analysis.

The 3 patients (case-patients 1–3) were referred to Tohoku University

Hospital, Sendai, Japan, for surgical treatment of IAAA in 2013. None had a history of disease known to cause immunodeficiency. Because their abdominal aneurysms enlarged rapidly, all 3 patients underwent resection of the aneurysm and extensive local debridement and irrigation. Histopathologic examination of the surgical specimens revealed severe atherosclerosis and inflammation, consistent with a diagnosis of IAAA. For each case-patient, blood culture (BacT/ALERT; bioMérieux Industry, Tokyo, Japan) was negative, as was culture of surgically removed tissue on HK semisolid agar (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) at 35°C under aerobic conditions for 7 days for enrichment of microorganisms, and on chocolate agar at 35°C under 5% CO_2 for 48 h. We then used 16S rRNA gene analysis to identify a pathogen. We extracted DNA from resected tissues using a QIAamp DNA Mini kit (QIAGEN K.K., Tokyo, Japan), amplified it using PCR, and sequenced it using universal primers for 16S rRNA (*5*). We used the EzTaxon-e Database for sequence analysis (http://eztaxon-e.ezbiocloud.net/), which revealed that the 16S rRNA gene sequence of bacteria in the aneurysmal tissues was identical to that of *H. cinaedi*.

For case-patient 3, we cultured microaerophilic tissue at 35°C using Trypticase Soy Agar II with 5% sheep blood (Kyokuto Pharmaceutical Industrial Co.) and an Anaero Pouch-MicroAero (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) to detect *H. cinaedi*. We observed bacterial colonies, after Gram staining, which showed gram-negative spiral rods. By 16S rRNA gene analysis, we confirmed that the isolate was *H. cinaedi*.

For each of the 3 case-patients, species identification was further confirmed by sequence analysis of 23S ribosomal RNA (23S rRNA) (DNA Data Bank of Japan: http://blast.ddbj.nig.ac.jp/blastn?lang = ja) and amplification of the *gyrB* gene region that is specific to *H. cinaedi* (*6*,*7*). In samples from the 3 patients, there were mutations of the 23S rRNA gene and amino acid substitutions in GyrA related to macrolide and fluoroquinolone resistance, respectively (*6*,*8*). After identifying the pathogen, we selected antimicrobial agents based on the reported drug susceptibility profile of *H. cinaedi* (*6*,*8*). The patients survived and are being followed up as outpatients. Clinical and molecular characteristics of the 3 cases of IAAA with *H. cinaedi* infection are shown in the Table.

Although the high negative culture rate for pathogens causing IAAA had been explained by prolonged preoperative antimicrobial drug therapy (*2*), another possibility is that *H. cinaedi* may be a causative organism. Earlier research has suggested that *H. cinaedi* infections can remain undiagnosed or be incorrectly diagnosed because of difficulty in isolating this microorganism (*9*). *H. cinaedi* grows slowly under microaerophilic conditions, but no current standard laboratory methods result in a diagnosis of this pathogen (*6*,*7*,*9*). We isolated *H. cinaedi* from surgically removed tissue from case-patient 3 by microaerophilic culture after taking this pathogen into consideration. For diagnosis of *H. cinaedi* infections, methods leading to accurate identification by clinical microbiological laboratories are needed. Currently, *H. cinaedi* is identified by molecular analysis of the 16S rRNA gene (*6*,*7*,*10*). In addition, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (*10*), may become a useful tool for this purpose.

Standard breakpoints of antimicrobial drugs for *H. cinaedi* have not been defined, but all isolates in this study had mutations that indicated resistance to macrolides and fluoroquinolones. For adequate treatment for *H. cinaedi* infections, guidelines for selection of antimicrobial drugs and surveillance of its antimicrobial susceptibility profile are required.

During November 2012–November 2013, 8 patients underwent their first operation for IAAA at the university hospital. We used 16S rRNA gene analysis of surgical tissues and culture of blood and tissue specimens to detect pathogens (data not shown). Identification of *H. cinaedi* in 3 of 8 patients suggests that it could be a prevalent pathogen related to IAAA. Taking such information into consideration could affect the prognosis of many patients. Accordingly, tissue should be cultured while considering *H. cinaedi* infection in patients with IAAA. *H. cinaedi* colonizes the gastrointestinal tract, and bacterial translocation may lead to bacteremia associated with mucosal damage (*4*). However, the route of transmission and reason most *H. cinaedi* infections have been reported in Japan are unclear. To clarify the relationship between *H. cinaedi* and IAAA, further clinical and epidemiologic studies are needed. Meanwhile, we recommend clinical consideration of *H. cinaedi* infection, use of appropriate laboratory procedures to identify cases, and development of treatment guidelines.

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Table. Clinical characteristics of 3 patients with *Helicobacter cinaedi* infected abdominal aortic aneurysms and molecular characteristics of isolates, Japan*

Characteristic	Case-patient 1	Case-patient 2	Case-patient 3
Age, y/sex	64/M	59/M	62/M
Underlying diseases	Hypertension, hyperlipidemia	None	History of myocardial infarction
Risk factors for infection	None	None	None
Clinical signs and symptoms before surgery	Fever, back pain	Fever, abdominal pain	Low back pain
CT results			
Site of aneurysm	Infrarenal abdominal, bilateral common iliac, internal iliac, L femoral, aortic arch†	Infrarenal abdominal, bilateral common iliac	Infrarenal abdominal
Inflammatory findings around aneurysms	+	+	+
Maximum leukocyte count/μL)/C-reactive protein, mg/dL before operation	10,600/25.3	9,100/6.05	7,050/ 5.29
Surgical management	In situ grafting	In situ grafting	In situ grafting
Microbiological diagnosis			
Blood culture	–	–	–
Tissue culture	–	–	+‡
rRNA gene sequence similarity, %§			
16S	99.8	99.6	99.6
23S	99.8	99.8	99.8
Amplification of *gyrB* specific to *H. cinaedi*	+	+	+
Aneurysms in which *H. cinaedi* was identified	Infrarenal abdominal, L common iliac, R internal iliac, L femoral	Infrarenal abdominal	Infrarenal abdominal
MLST	ST15 (CC7)	ST10 (CC9)	ST10 (CC9)
Mutation of 23S rRNA gene and amino acid substitutions in GyrA	2018 A→G and T84I D88G	2018 A→G and T84I	2018 A→G and T84I
Antimicrobial therapy dosage and duration			
Before admission	Ceftriaxone, 2 g/d, and levofloxacin, 500 mg/d, for 2 d	Piperacillin/tazobactam, 4.5 g/d for 12 d; faropenem sodium hydrate, 600 mg/d for 10 d	Oral antimicrobial agent, 4 d
After admission	Doripenem, 1.5 g/d for 22 d, and vancomycin, 3.0 g/d, for 14 d	Piperacillin/tazobactam, 4.5 g/d for 28 d	Doripenem, 1.5 g/d for 28 d
After identification of pathogen	Sulbactam/ampicillin, 3.0 g/d, and minocycline, 100 mg/d for 25 d	Continuation of piperacillin/tazobactam	Continuation of doripenem
At discharge	Oral amoxicillin, 1,500 mg/d, and minocycline, 200 mg/d, until follow-up visit	Oral amoxicillin, 1,500 mg/d, and minocycline, 200 mg/d, until follow-up visit	Oral amoxicillin, 1,500 mg/d, and minocycline, 200 mg/d, until follow-up visit
Postoperative complications	None	None	None
Outcome	Survived	Survived	Survived

*CT, computed tomography; +, positive; –, negative; L, left; R, right; MLST, multilocus sequence typing; ST, sequence type; CC, clonal complex; A, adenine; G, guanine; T, threonine; I, isoleucine; D, aspartic acid; G, glycine.
†Aortic arch was replaced 5 weeks after the abdominal operation.
‡Species unidentifiable under microaerophilic conditions.
§Compared with the type strain of *H. cinaedi* (CCUG 18818).

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Foodborne Transmission of Hepatitis E Virus from Raw Pork Liver Sausage, France

To the Editor: The number of sporadic autochthonous cases of acute hepatitis E is increasing in many industrialized countries (*1*). These cases involve hepatitis E virus (HEV) genotypes 3 and 4, which are zoonotic. Although risk for foodborne transmission from pork is now recognized, we report here direct HEV transmission through ingestion of raw pig liver sausages (figatellu [plural: figatelli]) in southeastern France.

The index case-patient was a 45-year-old woman from Hyères (southeastern France) who had no underlying medical condition. She visited her general practitioner on December 17, 2013, reporting 3 days of weakness. Acute hepatitis was diagnosed 2 days later on the basis of elevated liver enzymes (alanine aminotransferase 1,265 IU/L [reference <35 IU/L]) and bilirubin (65 μmol/L [reference <17 μmol/L]). Serum markers for acute hepatitis A, B, and C; cytomegalovirus; and Epstein-Barr virus were negative. Jaundice appeared on December 19, and the patient was referred to the Medical Unit of Hyères for additional investigations. A serum sample collected on December 20 tested positive for HEV RNA; viral load was 3.3 $\log_{10}$ IU/mL (Ceeram, La Chapelle sur Erdre, France), and IgM and IgG against HEV were found (Wantai, Beijing, China), which led to the diagnosis of acute hepatitis E. The HEV genotype was 3f, as determined from the phylogenetic analysis of a portion of the open reading frame (ORF) 2 (*2*). The index case-patient recovered by the end of January; HEV viremia was undetectable on January 17, 2014.

The index case-patient and her family regularly ate figatelli (raw pork liver sausages) made in Corsica. The patient had most recently eaten figatelli at a lunch with 8 family members on October 28, 2013, seven weeks before illness onset. After receiving informed consent, we conducted laboratory investigations of samples from the other family members; tests included HEV serology and HEV RNA detection in serum and fecal samples. Samples were obtained from family members during January 8–21, 2014 (41–54 days after the lunch). Positive HEV IgM and detectable HEV RNA were found in the serum of the index case-patient's daughter, who was asymptomatic. Because the sample was tested 10 weeks after the family lunch, the daughter's HEV viral load was too low to enable sequence characterization and clustering of HEV strains. Three other family members were IgG positive for HEV, indicating previous HEV infection. Leftover sausages had been kept frozen and were available for HEV testing.

HEV RNA was detectable from the leftover sausages, and HEV sequences were amplified in 2 different genomic regions (ORF1: RNA-dependent RNA polymerase and ORF2), as described previously (*2*). Comparison with the index case-patient's sequences showed 100% nt identity for both regions (Figure). Samples of food and samples from the index case-patient were analyzed in 2 independent laboratories to avoid any cross-contamination. The level of contamination of the figatellu was ≈4.8 10^4 copies of HEV RNA/g of sausage (*3*).

Figatellu, a dried sausage, contains 30% pork liver and no heating step occurs during its manufacture. Usually deep cooking is recommended on the package, but consumers might not follow the cooking recommendation; also, figatelli can be sold in small local shops with no label. In the instance reported here, the figatellu was sold without any warning label and was eaten raw.

That HEV was transmitted through ingestion of contaminated food is supported by the following evidence. First, 3 case reports have provided direct evidence of HEV transmission through ingestion of contaminated animal food products with identical or near identical sequences between the patients and the contaminated food they ate. Two cases occurred in the early 2000s in Japan through consumption of grilled wild boar (*4*) or sashimi of Sika deer (*5*); the third, reported recently in Spain, was transmitted through ingestion of pig meat (*6*). Second, HEV widely infects domestic pigs and wild boar (*7*). Third, swine and human HEV strains have genetic similarities and, in

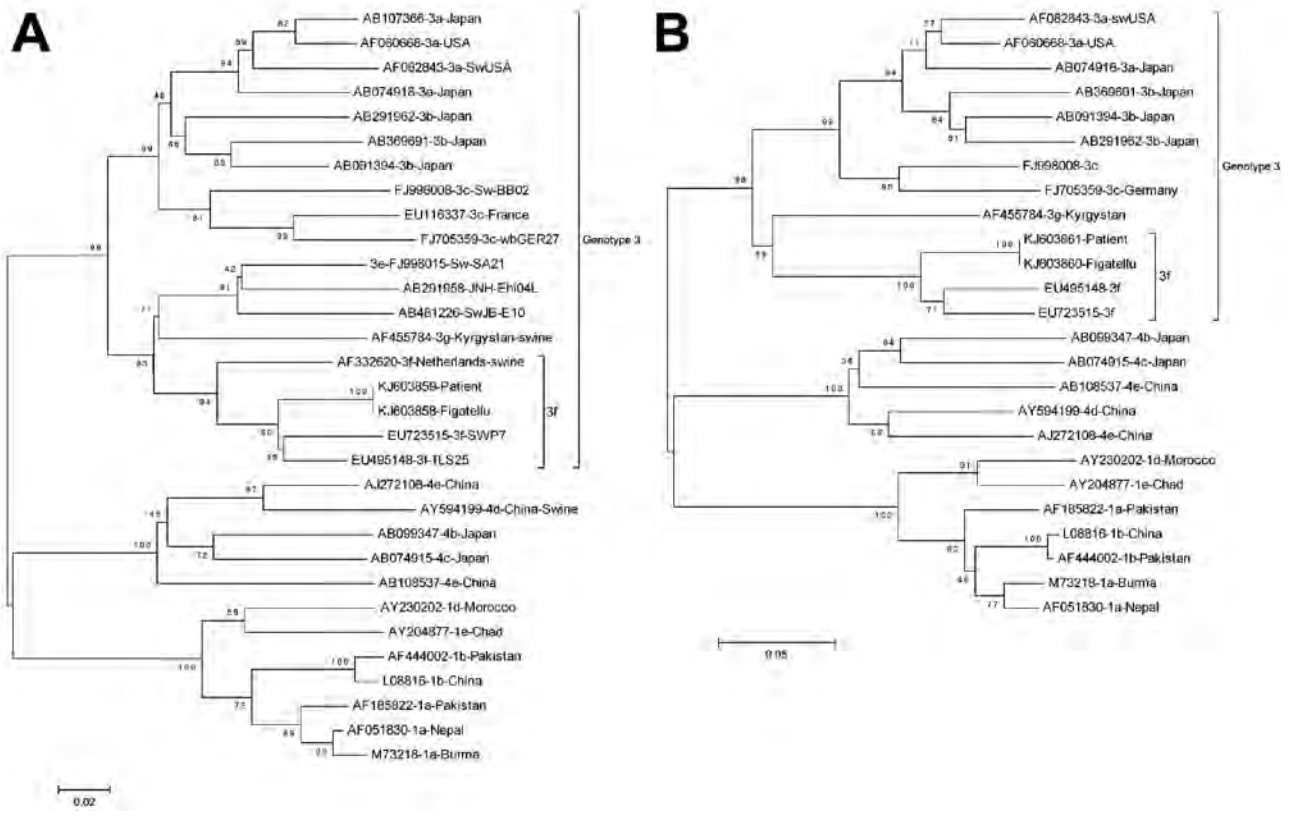

Figure. Phylogenetic analysis of partial open reading frame (ORF) 2 and ORF1 sequences of hepatitis E virus (HEV). Phylogenetic trees were constructed in MEGA6 software (http://www.megasoftware.net) by using the neighbor-joining method from a Kimura 2-parameter distance matrix based on partial nucleotide sequences of ORF2 (A) and ORF1 (B). Bootstrap values obtained from 500 resamplings are shown. Sequences were retrieved from the serum of a 45-year-old woman in France in whom hepatitis E was diagnosed in December 2013 and from frozen leftovers of the figatellu she had eaten in October 2013. Sequences obtained from the figatellu (GenBank accession nos. KJ603858 and KJ603860) were 100% nt identical to sequences obtained from the index case-patient (GenBank accession nos. KJ603859 and KJ603861). Reference sequences are indicated by their GenBank accession numbers. Scale bars indicate nucleotide substitutions per site.

some cases, are indistinguishable (*1*). Fourth, in Marseille, France, a case–control study identified ingestion of figatellu as a risk factor for HEV infection. Genetic similarities were found between sequences isolated from patients with autochthonous hepatitis E and nonrelated figatelli purchased in the same region (*8*). Finally, infectious virus, replicating in a 3-dimensional culture system, was identified in a HEV RNA-positive figatellu (*9*).

In the present study, the homology between sequences recovered from the index case-patient and those recovered from leftovers of figatellu provides additional proof of HEV foodborne transmission in a Western country. In France, information about the risk for HEV transmission through the ingestion of such delicatessen was published by French authorities in 2010 (*10*), but the present case demonstrates that public education and warning, or larger and more explicit labels on the package, must be improved to reduce the risk for HEV exposure.

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Burkholderia pseudomallei in Water Supplies, Southern Thailand

To the Editor: Melioidosis is an infectious disease caused by the environmental gram-negative bacillus *Burkholderia pseudomallei*, which is present in northern Australia and across much of Asia (*1*,*2*). In Thailand, melioidosis is highly endemic to the northeast, where most infected persons are agricultural farmers with repeated environmental exposure (*3*). Melioidosis is infrequently reported from southern Thailand, although a cluster of 6 cases occurred in Phangnga Province after the December 2004 tsunami (*4*). Given the infrequency of reported cases, a cluster of 11 persons with melioidosis on Koh Phangan (an island in the Gulf of Thailand) during January–March 2012 (*5*) led to an investigation. Three case-patients were foreign tourists; 8 Thai case-patients were from 7 different villages throughout the island, and and none were agricultural workers (*5*). Three cases were fatal; water inhalation was suspected as a route of infection in a fatal case in a neonate who was born in a birthing pool outside of a hospital (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/20/11/14-0832-Techapp1.pdf). The lack of history for environmental exposure, such as farming, led to the hypothesis that water was the source of infection. After a request by Koh Phangan Hospital and the Thai Ministry of Public Health, an environmental survey was conducted for *B. pseudomallei* in water supplies on the island.

In March 2012, we randomly collected water from accessible water supplies in local residences and hotels from all 14 villages on Koh Phangan. A total of 190 samples were collected (range 10–18 samples per village, Figure) for culture, genotyping, and analysis (online Technical Appendix). Isolates from 3 persons who died (a single bacterial colony saved from each person) from Koh Phangan were also available for genotyping and analysis. 26 (14%) of 190 samples were culture positive for *B. pseudomallei*. The positivity rate did not differ by source of the water sample: spring (5 [28%] of 18 samples), well (17 [13%] of 127), and tap water (4 [9%] of 45; $p = 0.16$, Fisher exact test). Of the 26 samples, 16 (62%), 9 (34%), and 1 (4%) were from local residences, hotels, and an ice cream shop, respectively. Positive water samples were distributed across the island (Figure). The median quantitative *B. pseudomallei* count was 30 CFU/L (range <10–11,300 CFU/L). The quantitative count did not differ by sample source ($p = 0.16$, Kruskal-Wallis test), and the sample with the highest quantitative count (11,300 CFU/L) was from well water. Of the 26 samples, only 1 was from a source that was consumed as drinking water.

We identified 12 multilocus sequence types (STs): 10 STs from water samples and 2 different STs from 3 clinical isolates (online Technical Appendix). The most frequent ST (ST1117, 10 isolates) was widely distributed across the island (Figure; online Technical Appendix Table 2). Phylogenetic analysis showed 12 genetically diverse STs identified on Koh Phangan and separate clusters of the clinical and environmental isolates (online Technical Appendix Figure).

Public tap water contaminated with *B. pseudomallei* has been reported previously in northeastern Thailand (*6*). The country's National Tap Water Quality Assurance Program does not include *B. pseudomallei* (*7*), a situation that warrants review. A combination of filtration and chlorination is recommended for treatment of village tap water systems in Thailand, but recent studies report that the quality of village tap water is suboptimal (*8*). Chlorination with sufficient contact time and free available chlorine can kill *B. pseudomallei* (*9*,*10*).

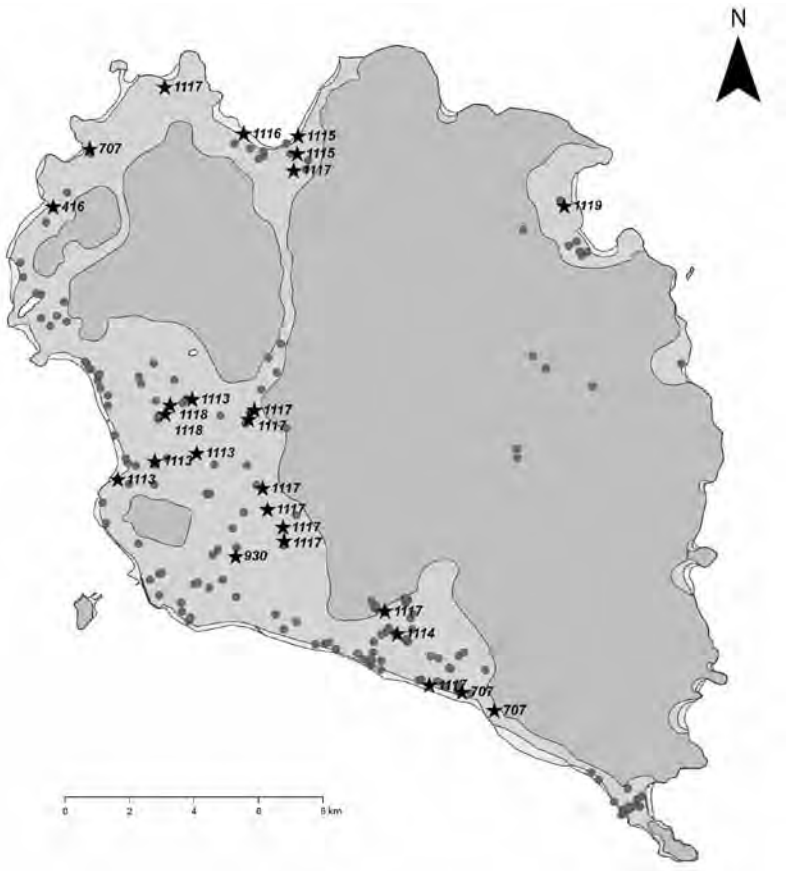

Figure. Location and multilocus sequence types of *Burkholderia pseudomallei* from water supplies on Koh Phangan, Thailand, 2012. A total of 190 water samples are indicated on the map. Twenty-six samples that were culture positive for *B. pseudomallei* are shown by black stars together with the sequence type, and 164 samples that were culture negative for *B. pseudomallei* are indicated as gray dots.

We reported our findings to Koh Phangan Hospital, Koh Phangan Public Health Office, and the Thai Ministry of Public Health. Our findings led to advice being provided by Thai Ministry of Public Health to every water treatment plant, household, and hotel on Koh Phangan in April 2012 to appropriately chlorinate water before general consumption. We recommend that residents and tourists to this island drink bottled or boiled water to prevent melioidosis and other waterborne infectious diseases.

Our finding that drinking water contained *B. pseudomallei* provides evidence for ingestion as a route of infection. Other routes include skin inoculation and inhalation, but we have no evidence from the clinical history to support this, other than possible inhalation in the case of the neonate born in a birthing pool. We did not find matching genotypes in water supplies and human samples. Possible explanations include the following: the considerable genetic diversity of *B. pseudomallei* found in water in this study and elsewhere (*6*), the small sample size, and that fact that we genotyped a single colony per sample when the sample could contain multiple genotypes.

Culture-positive water samples originated from different water sources and were distributed across the island; the genotyping results were consistent with endemic infection and ruled out a single outbreak. Soil sampling and a case–control study on Koh Phangan might provide a more extensive analysis of activities associated with development of melioidosis in this setting.

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Antimicrobial Drug–Resistant Bacteria Isolated from Syrian War–Injured Patients, August 2011–March 2013

To the Editor: Soft-tissue injuries sustained during wars are subject to environmental contamination and, thus, to a high risk for infection. Efforts to describe the epidemiology of war-associated infections are complicated by difficult access to patients, limited availability of microbiology support, and widespread empirical antimicrobial drug use. Nevertheless, identifying the relevant pathogens is critical because war-associated injuries commonly become infected and antimicrobial drug–resistant bacteria are well-described in these injuries, including those in the Middle East (*1*–*3*).

The Médecins Sans Frontières (MSF) surgical project in Amman, Jordan, was initially developed for war-injured Iraqis needing surgical reconstruction or management of chronic osteomyelitis. Infection management is based on organism-directed antimicrobial agents and wide surgical resection of involved tissue. The proximity of this project to the Syrian conflict provided an opportunity to describe microbiologic features of infections caused by war-associated injuries in Syrians, who may be at increased risk for infection-associated complications because of exclusion from care in official health systems. We describe a cross-sectional series of 61 Syrian orthopedic patients who had suspected infections, as determined on the basis of surgical samples obtained intraoperatively.

Syrian patients admitted to the MSF clinic underwent initial surgical exploration of wounds; if infection was suspected, ≥3 intraoperative samples (bone, fibrous tissue, fluid) were obtained for culture and transported (at 4°–8°C) within 2 h to the laboratory at Ibn al-Haytham Hospital in Amman. Patients who were treated with antimicrobial drugs within 2 weeks before admission were excluded from analysis.

We retrospectively reviewed data for patients admitted during August 1, 2011–March 31, 2013. Data were collected from databases and individual charts in Amman and analyzed by using Stata 12 (http://www.stata.com/stata12/). This study was deemed exempt from additional ethical approval by the MSF review board because it involved routinely collected data.

We defined a multidrug-resistant (MDR) isolate as 1) extended-spectrum β-lactamase–expressing *Enterobacteriaceae*; 2) *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates resistant to at least 1 agent in 3 antimicrobial categories typically used for treatment; or 3) methicillin-resistant *Staphylococcus aureus* (MRSA). Pathogen identification was conducted by using conventional methods and the API system (bio-Mérieux, Durham, NC, USA). Antimicrobial drug susceptibility testing was conducted by using the MicroScan Walk-Away System (Dade Behring, West Sacramento, CA, USA).

During the study period, 870 patient consultations were conducted, of which 345 (40%) were for patients from Syria. At the initial operating room evaluation, infection was suspected in 61 (18%) Syrians. These patients had a median age of 26 years (interquartile range 22–34); 98% were male. The median time from injury to admission was 5 months (interquartile range 1.2–8.1), but for 27 (44%) patients, the time from injury to admission was >6 months. The 2 most common injuries were gunshot wounds (32 patients [52%]) and wounds from explosions (20 patients [33%]). The dominant injury was located in an upper extremity in 14 (23%) patients and a lower extremity in 47 (77%) patients.

For the 61 patients, a total of 67 bacterial isolates were identified from cultures of surgical specimens. Overall, 45 (74%) patients had at least 1 positive culture, and 6 (13%) patients had polymicrobial results. Gram-negative organisms represented 24 (56%) of 43 isolates; 10 (23%) were *P. aeruginosa*, 8 (19%) were *E. coli*, and 6 (14%) were *A. baumannii*. Gram-positive bacteria, including MRSA, represented 19 (44%) of 43 isolates (Table). Overall, 31 (69%) of 45 patients with confirmed infection were positive for MDR organisms. Within this group, MRSA represented 8 (42%) of 19 staphylococcal isolates.

Patients who had experienced delayed definitive management were frequently positive for MDR organisms, especially gram-negative pathogens and MRSA. For a humanitarian surgical project, infection with MDR organisms leads to formidable diagnostic, treatment, and control challenges. For example, treatment of MDR infections requires ongoing access to high-quality clinical microbiology support; late-generation antimicrobial drugs, which are typically given parenterally for up to 6 weeks; trained personnel; and sufficient hospital space to isolate patients with resistant strains. Our findings support the previously reported linkage between war-associated injuries and infection with antimicrobial drug–resistant organisms (*1*–*4*) and the implications for patient management.

The source of antimicrobial drug–resistant organisms in war-associated injuries remains uncertain; possibilities include nosocomial transmission (*5*), particularly through prior contact with severely compromised health systems (*6*). Another possibility is fecal colonization with extended-spectrum β-lactamase–producing gram-negative bacteria. (*7*,*8*). Another likely contributor in Syria is the wide availability of antimicrobial drugs without a prescription (*9*).

This study has limitations. Although measures were taken to ensure that positive cultures represented clinical infection rather than colonization, we cannot exclude colonization as a possible source of some recovered organisms. In neglected war-associated injuries, multiple pathogens are potentially present, but every strain is not necessarily clinically relevant (*10*). Furthermore, complete patient histories are difficult to obtain in crisis settings, limiting our ability to describe all prior interventions. Study strengths included partnership with a high-quality culture laboratory, which is uncommon in programs treating war injuries; systemic sampling of patients with suspected infection; and use of intraoperative samples for culture. Further research needed in this neglected area includes prospective studies to determine the effect of MDR isolates on patient outcomes and randomized clinical trials of antimicrobial drug strategies to inform treatment protocols.

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Table. Antimicrobial drug resistance among frequently isolated bacterial isolates from Syrian patients with war-associated wound infections, August 2011–March 2013*

	No. MDR-resistant isolates/no. total (%)			
Antimicrobial drug	*Staphylococcus aureus*, n = 19	*Pseudomonas aeruginosa*, n = 10	*Escherichia coli*, n = 8	*Acinetobacter baumannii*, n = 6
Amikacin		1/ 11 (9)	1/7 (14)	6/6 (100)
Ampicillin			5/5 (100)	
Amoxicillin/clavulanic acid			6/6 (100)	
Cefotaxime			6/8 (75)	
Ceftriaxone			5/8 (62)	
Ceftazidime		3/9 (33)	5/8 (62)	4/4 (100)
Cefepime			5/8 (62)	5/5 (100)
Cefixime			5/8 (62)	5/5 (100)
Ciprofloxacin	7/17 (41)	5/8 (62)	2/7 (28)	5/5 (100)
Colistin		NA	NA	0/5
Trimethoprim/sulfamethoxazole	3/14 (21)		3/5 (60)	
Gentamicin	10/18 (55)	4/9 (44)	4/8 (50)	6/6 (100)
Piperacillin/tazobactam		2/9 (22)	3/7 (42)	NA
Imipenem		0/9	1/7 (14)	4/5 (80)
Penicillin	9/10 (90)			
Oxacillin	7/17 (41)			
Clindamycin	9/17 (52)			
Rifampin	6/15 (40)			
Fusidic acid	10/15 (66)			

*Blank cells indicate that testing was not done.

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Multidrug-Resistant IncA/C Plasmid in *Vibrio cholerae* from Haiti

To the Editor: The agents of epidemic cholera are *Vibrio cholerae* toxigenic serogroups O1 and O139. Cholera symptoms include watery diarrhea and severe dehydration, which can rapidly result in death unless rehydration therapy is prompt (*1*). Antimicrobial agents may reduce the severity and duration of disease (*1*); commonly used are tetracyclines, fluoroquinolones, macrolides, and trimethoprim/sulfamethoxazole (*1*). However, *V. cholerae* resistance to antimicrobial drugs is increasing because of the accumulation of genetic mutations and the acquisition of resistance genes, which are usually transferred on mobile genetic elements such as integrating conjugative elements (ICEs) (*1*).

As of March 12, 2014, the ongoing cholera outbreak that began in Haiti in October 2010 had caused 700,796 cases and 8,548 deaths (*2*). To characterize infections, the National Public Health Laboratory in Haiti and the US Centers for Disease Control and Prevention (CDC) collaborated to perform standard microbiological and antimicrobial-drug susceptibility testing on isolates from case-patients. Since October 2010, the National Public Health Laboratory has identified 465 isolates, which were then forwarded to CDC for determination of MICs for 15 antimicrobial agents by broth microdilution (Sensititer; Trek Diagnostics Systems, Cleveland, OH, USA) according to manufacturer's recommendations (Table). Resistance was defined by the Clinical and Laboratory Standards Institute interpretive standards, when available (*3*). The typical outbreak strain (2010EL-1786) displayed resistance to streptomycin, sulfisoxazole, trimethoprim/sulfamethoxazole, and nalidixic acid, and decreased susceptibility to ciprofloxacin and chloramphenicol (*4*). Resistance was caused by mutations in the QRDR regions of the *gyrA* and *parC* genes and presence of ICE*Vch*Hai1 containing the *dfrA1*, *floR*, *strAB*, and *sul2* resistance genes (*4*).

In April of 2012, the 2 agencies began sentinel laboratory-based surveillance for acute diarrheal disease at 4 hospitals in Haiti (*5*). As part of this surveillance, fecal specimens were sent to the National Public Health Laboratory for organism isolation, identification, antimicrobial-drug testing, and subsequently to CDC for expanded antimicrobial-drug testing and molecular characterization. One isolate, 2012EL-2176, showed the typical resistance phenotype of the outbreak strain but additional resistance to ampicillin, amoxicillin/clavulanic acid, cefoxitin, ceftriaxone, ceftiofur; the tetracycline MIC was intermediate (Table).

Analysis of this isolate by serotype, pulsed-field gel electrophoresis, multilocus variable number–tandem repeat analysis, and whole-genome sequencing confirmed that the isolate was similar to outbreak isolates (data not shown) (*6*). PCR and whole-genome sequencing analysis by use of ResFinder (http://www.genomicepidemiology.org/) identified the original outbreak resistance determinants and additional determinants (*aac(3)-IIa*, bla_{CMY-2}, $bla_{CTX-M-2}$, bla_{TEM-1}, *dfrA15*,

Table. Susceptibility, resistance genes, and plasmids associated with Haiti *Vibrio cholerae* outbreak isolates

Isolate	Resistance profiles [MIC mg/L]*	Resistance genes/mutations	Plasmids
V. cholerae 2010EL-1786	STR [>64], FIS [>256], TMP/SXT [>4], CIP(I) [0.5], NAL [>32]	*strAB sul2, dfrA1, floR, gyrA*(S83I)/*parC*(S85L)	None
V. cholerae 2012EL-2176	AMP [>32], AMC [>32], CRO [>64], FOX [32], TIO [>8], CHL(I) [16], GEN [>8], STR [>64], FIS [>256], TMP/SXT [>4], TET(I) [8], CIP(I) [0.5], NAL [>32]	bla_{CMY-2}, $bla_{CTX-M-2}$, bla_{TEM-1}, *floR, aac(3)-IIa ,strAB, sul2, dfrA1, dfrA27, tetA, mphA, gyrA*(S83I)/*parC*(S85L)	IncA/C2
Escherichia coli DH10B p2012EL-2176	AMP [>32], AMC [>32], CRO [>64], FOX [>32], TIO [>8], AZM [>16], CHL [>32], GEN [>8], STR [>64],† FIS [>256], TMP/SXT [>4], TET [32]	bla_{CMY-2}, $bla_{CTX-M-2}$, bla_{TEM-1}, *mphA, floR, aac(3)-IIa. strAB, sul2, dfrA27, tetA*	IncA/C2

*Drugs tested were AMP, ampicillin; AMC, amoxicillin/clavulanic acid; AZM, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; CRO, ceftriaxone; FIS, sulfisoxazole; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline; TIO, ceftiofur; and TMP/SXT, trimethoprim/sulfamethoxazole; S83I, serine-to-isoleucine change at amino acid position 83; S85L, serine-to-leucine change at amino acid position 85. Drugs that yielded intermediate results are followed by (I). Clinical Laboratory Standards Institute break points are not established for AZM. The AZM break points used in this study (>16 mg/L) were established by the National Antimicrobial Resistance Monitoring System and should not be used to predict clinical efficacy.
†*E. coli* DH10B is intrinsically resistant to STR.

mphA, *sul1*, *and tetA*) (*7*). Plasmid transfer by electroporation into *Escherichia coli* (DH10B) confirmed that the resistance determinants were plasmid encoded. PCR-based replicon testing identified an IncA/C2 plasmid, and PCR and whole-genome sequencing confirmed that the plasmid encoded a unique set of resistance determinants (*aac(3)-IIa*, bla_{CMY-2}, $bla_{CTX-M-2}$, bla_{TEM-1}, *dfrA15*, *mphA*, *sul1*, and *tetA*) and a second copy of the resistance genes *floR*, *strAB*, and *sul2* identical to those located in ICE*Vch*Hai1 (*8*). Antimicrobial-drug susceptibility testing of the transformant demonstrated transfer of the resistance profile and additional resistance to chloramphenicol, tetracycline, and decreased susceptibility to azithromycin (Table). The lack of association between the presence of the resistance determinants *floR* and *tetA* and the lack of resistance in *V. cholerae* has been observed previously, possibly because of lower gene expression (*9*). The plasmid was mobilizable by conjugation (conjugation efficiency = $1.3–1.4 \times 10^{-2}$) when *E. coli* J53 was used as the recipient.

IncA/C plasmids are widespread in *Enterobacteriaceae* and commonly confer multidrug resistance. BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) comparison of the completed plasmid p2012EL-2176 sequence with the National Center for Biotechnology Information nucleotide collection showed similarities to other IncA/C-bla_{CMY} plasmids; most similarity (total score = 3.009×10^5) was to pAR060302, found in an *E. coli* isolate from a dairy calf (*10*). Most IncA/C-bla_{CMY} plasmids have 3 resistance regions: *sul2* region (*floR-tetA-strAB-sul2*), *cmy-2* insertion region, and Tn21-like region (*aad-aac*). Plasmid p2012EL-2176 contains the *sul2* and *cmy-2* insertion regions and a putative *arr3-drfA27-aadA16-sul1* resistance gene cassette at the Tn-21 location. This plasmid has additional resistance gene insertions: a putative cassette containing a bla_{TEM-1} and *aac(3)-IIa* gene upstream of the *sul2* region and insertions of $bla_{CTX-M-2}$, *sul1*, and *mphA* genes downstream of the *arr3-drfA27-aadA16-sul1* cassette (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/11/14-0889-Techapp1.pdf).

Since discovery of isolate 2012EL-2176, sentinel surveillance has not detected increased antimicrobial-drug resistance among *V. cholerae* in Haiti. The ability of IncA/C plasmids to acquire novel resistance cassettes from multiple sources makes it difficult to hypothesize as to the origins of plasmid p2012EL-2176. Although this plasmid was most closely related to a plasmid found in *E. coli*, it was also closely related to plasmids in *Salmonella*, *Klebsiella*, and *Providencia*. *Enterobacteriaceae* are found in the environment and/or in the host gut; therefore, the isolate could have acquired the plasmid in the environment or within the host. The latter scenario would limit the possible spread of this plasmid and could explain its rarity. The original Haiti outbreak isolate has been shown to be poorly naturally transformable, accounting for the lack of acquired chromosomal genes and nearly homologous genomic content among outbreak isolates (*6*). Therefore, the acquisition of plasmids, and their resistance genes, may represent the major source of future variability among *V. cholerae* involved in the Haiti outbreak.

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Human Co-Infection with Avian and **Seasonal Influenza** Viruses, China

To the Editor: In April 2013, a case of co-infection with avian-origin influenza A(H7N9) virus and seasonal influenza A(H3N2) virus was reported in Jiangsu Province, China (*1*). This case raised concern over the possible occurrence of new reassortants with enhanced transmissibility among humans. Because of the nature of the dynamic reassortment of A(H7N9) virus with A(H9N2) virus in the environment and in poultry (*2*,*3*), close surveillance for possible new reassortment in human patients with A(H7N9) infection is needed. We report co-infection in 2 patients in Hangzhou, the capital Zhejiang Province, China, in January 2014. The co-infections involved influenza A(H7N9) virus and a seasonal A(H1N1)pdm09 virus (1 patient) or a seasonal influenza B virus (1 patient).

Of 60 patients with laboratory-confirmed influenza A(H7N9) infections in Hangzhou in April 2013 and in January–February 2014, testing of pharyngeal swab samples indicated that 2 patients were also positive for seasonal influenza virus. The pharyngeal samples were tested by real-time reverse transcription PCR according to protocols provided by the Chinese National Influenza Center. Informed consent for this study was provided by each patient's spouse.

On January 6, 2014, patient 1 (male, 58 years of age), a resident of Xiaoshan District, had a high fever (39.6°C) and a cough; at a hospital, he received a diagnosis of severe acute interstitial pneumonia. The patient had a history of chronic myelogenous leukemia; his history of exposure to live poultry was not clear. On January 13, infection with influenza A(H7N9) virus was laboratory confirmed; viral RNA from a pharyngeal swab sample collected before oseltamivir treatment was positive for the following: influenza A virus (cycle threshold [C_t] = 26), H7 (C_t = 27), N9 (C_t = 26), influenza A(H1N1)pdm09 virus H1 (C_t = 30), and N1 (C_t = 30). The 2 viruses were named A/Hangzhou/10–1/2014(H7N9) and A/Hangzhou/10–2/2014(H1N1)pdm09. The patient received oseltamivir while in the hospital but died on January 18.

On January 5, patient 2 (male, 54 years of age), also from Xiaoshan District, had fever and a cough; at a hospital, he received a diagnosis of severe acute pneumonia. He had a history of aplastic anemia and had been exposed to live poultry 1 week before symptom onset. On January 18, infection with influenza A(H7N9) virus was laboratory confirmed. Viral RNA from a pharyngeal swab sample collected before oseltamivir treatment was positive for the following: influenza A virus (C_t = 22), H7 (C_t = 23), N9 (C_t = 22), and influenza B virus (C_t = 22). The viruses were named A/Hangzhou/17–1/2014(H7N9) and B/Hangzhou/17–2/2014. This patient received oseltamivir but died on January 22.

The hemagglutinin (HA) and neuraminidase (NA) sequences of viruses from these 2 patients were determined by Sanger sequencing. The specific primers used are listed in online Technical Appendix Table 1 (http://wwwnc.cdc.gov/EID/article/20/11/14-0897-Techapp1.pdf). The accession numbers of these sequences and the reference sequences for phylogenetic analyses are

listed in online Technical Appendix Table 2. Phylogenetic analyses (*4*) revealed that these 2 influenza A(H7N9) viruses were clustered into the clade of A/Shanghai/2/2013(H7N9)-like viruses (Figure). Some amino acid features within the HA and NA of these 2 viruses were the same as those in the A/Shanghai/2/2013(H7N9) strain: L226 and G228 in HA, believed to control host receptor specificity; the cleavage site in HA, relevant for virulence; a deletion in NA stalk (position 69–73), associated with the adaption to gallinaceous hosts; and R294 in NA, related to virus sensitivity to oseltamivir (5). The HA and NA sequences of A/Hangzhou/10–2/2014(H1N1) pdm09 and B/Hangzhou/17–2/2014 were very close to those of A(H1N1) pdm09 virus and B/Yamagata-lineage viruses that had recently circulated in China (*6*,*7*).

Co-infection with A(H7N9) virus and seasonal influenza viruses is probably associated with the overlap of A(H7N9) virus and seasonal virus circulation in both time and space and with increased prevalence of influenza virus infections within the population. From November 2012 through March 2014, outbreaks of A(H7N9) infection (in April 2013 and in January–February 2014) were concurrent with increases in seasonal influenza

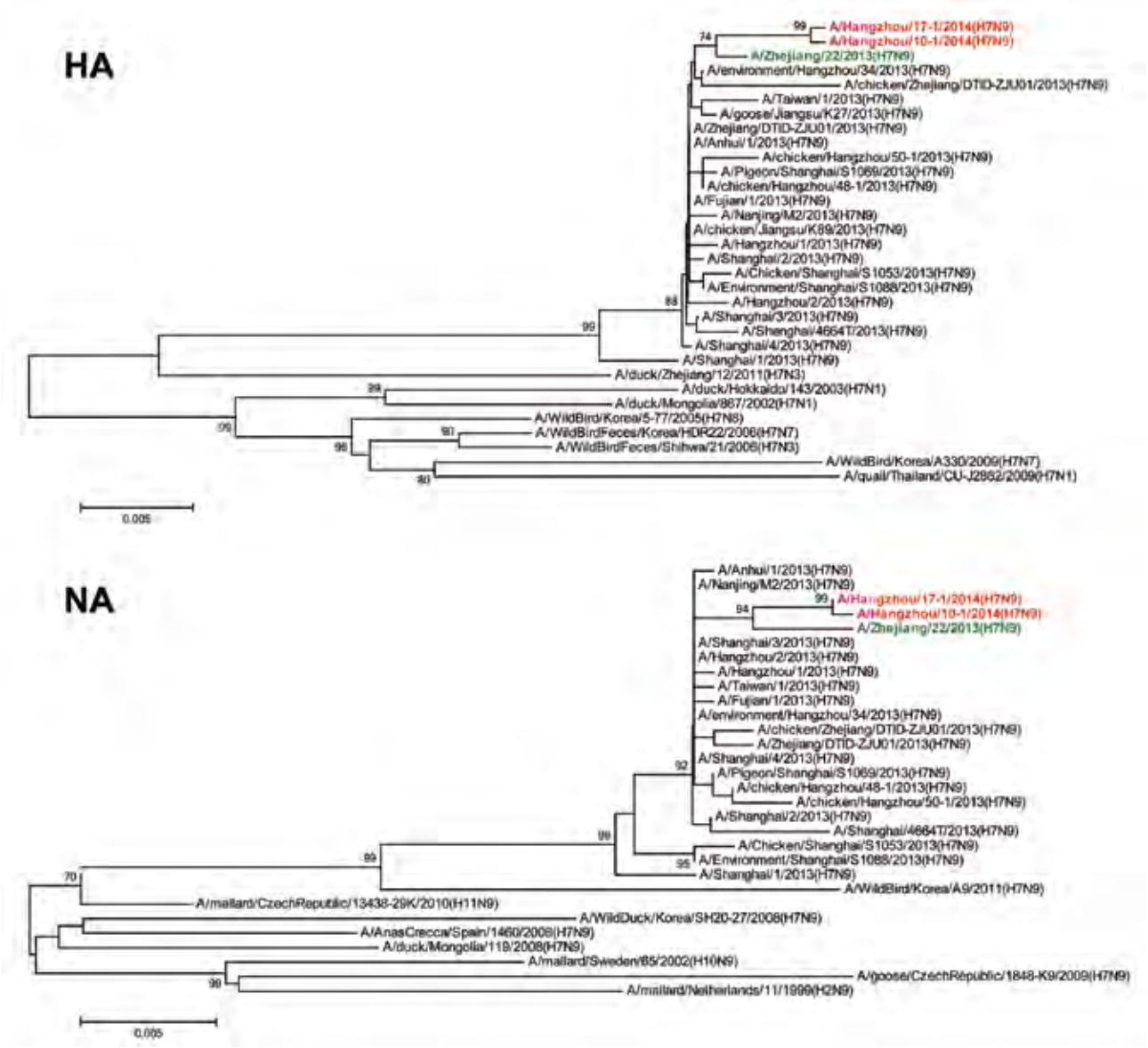

Figure. Phylogenetic analyses of hemagglutinin (A) and neuraminidase (B) of influenza A(H7N9) viruses. The trees were constructed by using the neighbor-joining method with bootstrap analysis (n = 1,000) in the MEGA5.0 program (*4*). Red indicates the 2 viruses isolated from co-infected patients in Hangzhou, China, and green indicates the first strain isolated during the second wave of the influenza A(H7N9) outbreak in China, which started in October 2013. Scale bars indicate nucleotide substitutions per site.

virus infections in Hangzhou (online Technical Appendix Figure). Prompt control of A(H7N9) infection outbreaks and vaccination against seasonal influenza viruses could reduce the potential for co-infections with A(H7N9) virus and seasonal viruses.

Taken together with the previous finding of human co-infection with A(H7N9) virus and A(H3N2) virus (*1*), our results show that human co-infection with A(H7N9) virus and each of the 3 seasonal influenza viruses currently circulating worldwide can occur. Avian influenza viruses, including A(H7N9), preferentially replicate in the lower respiratory tract of humans (*8*,*9*). In contrast, seasonal influenza viruses preferentially infect the upper respiratory tract of humans (*10*). Coexistence of A(H7N9) virus with either A(H1N1)pdm09 virus or influenza B virus in the pharyngeal swab samples from 2 patients suggests that the upper respiratory tract could provide a location for the A(H7N9) virus to reassort with other influenza viruses. The possibility that seasonal influenza viruses might provide some gene segments that increase the human-to-human transmissibility of possible new reassortants is cause for concern. For detection of such new influenza virus reassortants, extensive surveillance to identify influenza virus co-infections is necessary.

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Misidentification of *Diphyllobothrium* Species Related to Global Fish Trade, Europe

To the Editor: Diphyllobothriosis, infection by tapeworms of the genus *Diphyllobothrium* (Cestoda: Diphyllobothriidea) (*1*), is a well-known disease of humans. In Europe, infections caused by 3 species of *Diphyllobothrium* have recently been reported in humans: *D. latum* is considered to be the principal species infecting persons in Europe (*1*); 4 cases of *D. dendriticum* infection and 6 cases of *D. nihonkaiense* infection have also been reported (*2*,*3*). Except for those caused by *D. latum*, which is autochthonous in northeastern Europe and subalpine lakes, most of the cases in Europe have been imported or caused by consumption of fish imported from areas to which the parasites are endemic (*1*,*3*,*4*).

Diphyllobothriosis is not endemic to Spain, but 7 cases of *D. latum*

infection have been reported there (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/20/11/14-0996-Techapp1.htm). Most recently, Pastor-Valle et al. confirmed, using molecular tools, an imported case of infestation by *Diplogonoporus balaenopterae* and 3 imported cases of diphyllobothriosis caused by *D. pacificum* (*5*), a tapeworm endemic to the Pacific coast of South America (*1*,*4*).

Specific identification of most human-infecting *Diphyllobothrium* tapeworms based on clinical material is virtually impossible (*1*,*3*); the only exception is identifying the Pacific broad tapeworm, *D. pacificum*. This tapeworm can be easily distinguished from other human-infecting diphyllobothriideans by the presence of pits alongside the median line on the ventral surface of its proglottids; smaller, more spherical, eggs; and the almost equatorial position of the genital pore, a feature that is markedly pre-equatorial in other species (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/20/11/14-0996-Techapp1.htm). Several hundred cases of infection by this species have been reported from Peru, and a few reports have been made from Ecuador, Chile, and Japan (*1*). The life cycle of *D. pacificum* is not completely known, but several species of marine fish have been identified as sources of human infection in Peru (*4*).

We critically examined all recent records of diphyllobothriosis in Spain to clarify species identification because published morphologic data indicated misdiagnosis (online Technical Appendix Table). Tapeworms detected in 2 recent human cases reported by Colomina et al. (*6*) and Esteban et al. (*7*), described as *D. latum*, resembled those of *D. pacificum* because of the morphology of proglottids and eggs (*6*,*7*). Therefore, we requested material of these cestodes for scrutiny. Morphologic and molecular evaluation (partial lsrDNA and *cox1* gene sequences; multiplex PCR testing by Wicht et al. (*8*), (Figure, online Technical Appendix Figures 1, 2) actually confirmed that *D. pacificum* was misidentified as *D. latum* in both cases, despite the molecular identification through multiplex PCR.

No voucher specimens for re-identification were available for another 2 alleged cases of *D. latum* infection (online Technical Appendix Table). However, the eggs reported in the study by Gil-Setas et al. were more similar in shape and size to those of *D. nihonkaiense* or *Diplogonoporus balaenoterae* than to those of *D. latum* (*9*).

D. latum is the principal causative agent of human diphyllobothriosis; its fish intermediate hosts are perch, pike, burbot, and ruff in Europe (*1*,*4*). Other fish, such as salmonids and marine fish, cannot transmit this parasite and serve as intermediate hosts of other species of *Diphyllobothrium* and *Diplogonoporus* (*4*).

The information on the spectrum of fish intermediate hosts of *D. pacificum* is limited. From very scarce anamnestic data about individual case-patients infected with *D. pacificum* in Spain, it is not possible to unravel the actual source of their infection. However, it is obvious that the recent emergence of diphyllobothriosis caused by nonendemic species such as *D. pacificum*, *D. dendriticum* (*3*), *D. nihonkaiense* (*2*), and *D. balanopterae* (*5*) is related to the global importation of fish that have not been frozen. If the fish are merely chilled, plerocercoids of diphyllobothriids may survive for several days (*10*).

Spain is the third largest importer of fish and seafood in the world; the value of fish products imported from >104 countries reached $7 billion (US) in 2011 and increases continuously. More than 200,000 tons of fresh or chilled fish, which may serve as source of human fishborne diseases if eaten raw or undercooked, are imported to Spain every year. The fourth largest importer is Ecuador, the sixth is Chile, and the seventh is Peru; *D. pacificum* is endemic to each of these countries (*4*).

In the present study, we confirmed human infections with the Pacific broad tapeworm, *D. pacificum*, in Europe, but it is highly probable that this species can be introduced anywhere through the importation of fresh or chilled fish from the Pacific coast of South America. This has implications

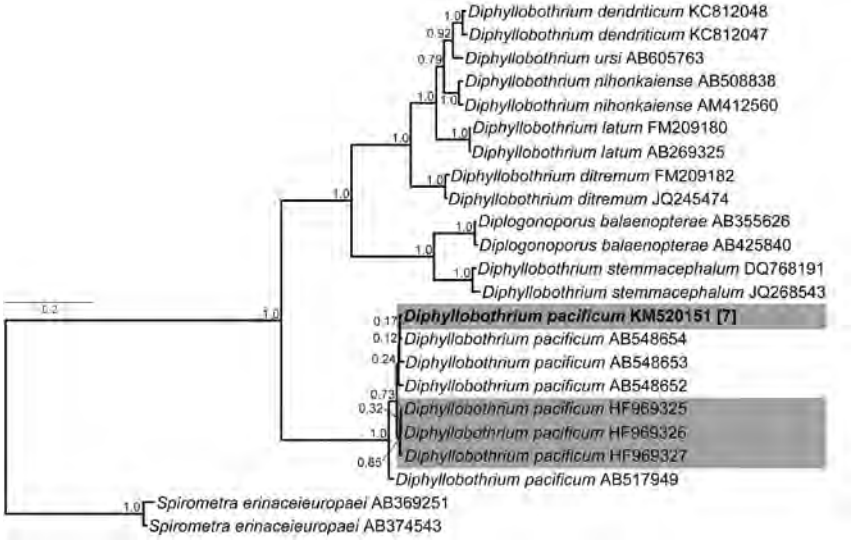

Figure. Bayesian inference phylogenetic analysis of selected human diphyllobothrideans based on cox1 gene analyzed as 3 independent data parts according to the nucleotide coding positions by using (GTR+G)(HKY)(GTR+G) evolutionary model setup in MrBayes (mrbayes.sourceforge.net). Topologies sampled every 1,000th generation over 4 runs and 20,000,000 generations, burn-in 25%. *Diphyllobothrium pacificum* identified in Spain marked in gray; new sequence is in bold type. Scale bar indicates nucleotide substitutions per site.

for food safety rules and human health risk measures taken by national health and veterinary agencies. Regarding adequate processing of clinical samples and their preservation for morphologic and genetic evaluation, we strongly recommend fixation of positive fecal samples with eggs or segments (proglottids) immediately with 96%–99% molecular grade (i.e., not denatured) ethanol for future molecular diagnosis (*1*,*4*,*8*).

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Drug Resistance in *Salmonella enterica* ser. Typhimurium Bloodstream Infection, Malawi

To the Editor: *Salmonella enterica* serotype Typhimurium is one of the most common causes of bloodstream infection in sub-Saharan Africa (*1*). Among adults, the principal risk factor for invasive nontyphoidal *Salmonella* (iNTS) disease is advanced HIV infection; up to 44% of HIV-infected patients experience bacteremic recurrence through recrudescence of the original infection (*2*,*3*). Epidemics of iNTS disease in sub-Saharan Africa have been associated with a novel genotype of *S. enterica* ser. Typhimurium of multilocus sequence type (ST) 313 that is rarely seen outside the region and is associated with multidrug resistance (MDR) to chloramphenicol, cotrimoxazole, and ampicillin (*4*,*5*). As a consequence, ceftriaxone has become a key agent in the empirical management of nonfocal sepsis in Malawi (*6*).

In March 2009, a 40-year-old HIV-infected and antiretroviral therapy–naïve woman sought care in Blantyre, Malawi, with an MDR *S. enterica* ser. Typhimurium bloodstream infection. She was treated with ceftriaxone (2 g intravenously once daily) and discharged with oral ciprofloxacin (500 mg twice daily) for 10 days. She was readmitted 1 month later with recurrent fever. At this time, she had an MDR *S. enterica* ser. Typhimurium bloodstream infection with additional resistance to ceftriaxone and ciprofloxacin. In the absence of a locally available effective antimicrobial drug, she was treated with ceftriaxone, gentamicin, and high-dose ciprofloxacin but died shortly thereafter.

To help clarify how this extended MDR *S. enterica* ser. Typhimurium emerged, we determined the molecular mechanisms underpinning this disturbing pattern of antimicrobial resistance

(online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/11/14-1175-Techapp1.pdf)). We conducted phenotypic drug susceptibility testing by disk diffusion on *S. enterica* ser. Typhimurium strains A54285 (initial presentation) and A54560 (recurrence); both isolates were resistant to ampicillin, chloramphenicol, and cotrimoxazole, but A54560 exhibited additional resistance to ceftriaxone, ciprofloxacin, and tetracycline.

Paired-end sequencing of isolates A54285 (European Nucleotide Archive [ENA] accession number ERS035867) and A54560 (ENA accession no. ERS035866) that were cultured 1 month apart showed no differences between the conserved regions of these genomes (Figure). The similarity of these *S. enterica* ser. Typhimurium genomes strongly suggests that this recrudescence occurred after incomplete clearance of the first infection; although re-infection from the same source is unlikely, it cannot be excluded. Comparison of the accessory genomes, however, showed an additional 300 kb DNA in A54560.

Plasmid extraction and gel electrophoresis of genomic DNA identified a plasmid migrating in the gel to a position approximately equivalent to 120 kb, the size of ST313 virulence plasmid pSLT-BT in both strains, but no 300-kb plasmid was visualized in the ceftriaxone- and ciprofloxacin-resistant strain (A54560, data not shown), possibly because of the difficulty large plasmids have entering standard 1% agarose gels. However, ceftriaxone resistance was mobilized to *Escherichia coli* by conjugation at a frequency 6.5×10^{-2} transconjugants per donor at 26°C. This frequency dropped dramatically to $\approx 1 \times 10^{-7}$ transconjugants per donor when conjugation was performed at 37°C. The presence of an IncHI2 plasmid in the transconjugants was confirmed by PCR for the IncHI2 region (*7*), and drug susceptibility testing confirmed that transconjugant clones acquired resistance to ceftriaxone, ciprofloxacin, and tetracycline.

These data confirm the presence of an extended-spectrum b-lactamase (ESBL)–producing IncHI2 plasmid in strain A54560 that is capable of conjugative transfer and suggest that the plasmid might have been acquired by residual index strain within the patient by transfer from an unknown donor

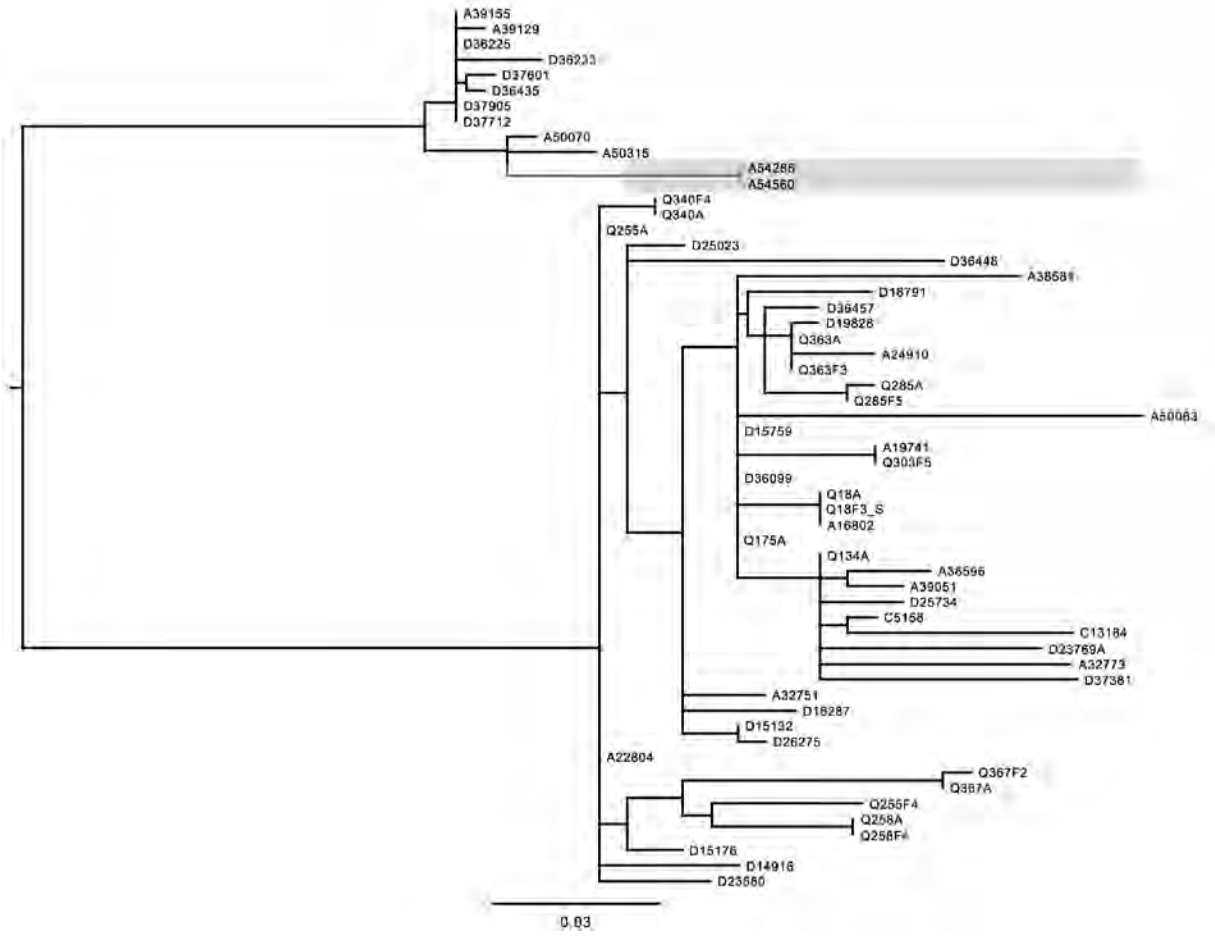

Figure. Midpoint-rooted phylogenetic tree of published whole-genome sequence data from D23580-like *Salmonella enterica* serotype Typhimurium sequence type 313s from Malawi based on 204 informative single-nucleotide polymorphisms. A54285 and A54560, highlighted in gray, are indistinguishable. Scale bar indicates nucleotide substitutions per site.

bacterium. Partial decolonization of the patient's gastrointestinal tract by ceftriaxone and fluoroquinolone antimicrobial therapy might have rendered it receptive to colonization by ESBL-producing bacteria, which we hypothesize donated the plasmid to the residual index strain.

The transconjugant plasmid DNA was sequenced by using the PacBio RSII platform (Pacific Biosciences, Menlo Park, CA, USA; http://www.pacificbiosciences.com), which assembled as a single contiguous sequence of 309,406 bp, designated pSTm-BTCR (online Technical Appendix Figure, ENA accession no. LK056646). We identified 331 predicted coding sequences, including 109 genes required for replication and transfer and 61 genes predicted to be associated with metabolism, membranes, virulence, antimicrobial resistance, and a toxin/antitoxin addiction system. We found an additional 160 predicted, hypothetical genes. Fifteen putative antimicrobial resistance genes were identified, predicted to encode resistance to; tetracycline (*tetA*(C), *tetR*(C)), b-lactams ($bla_{CTX-M15}$, bla_{TEM-1b}, bla_{OXA-30}), chloramphenicol (*catB3*, *catA1*), aminoglycosides (*strA*, *strB*, *aadA1*, *aacA4*, *aacC3*), ciprofloxacin (*qnrB1*), ulfonamiides (*sul2*), and trimethoprim (*dfrA14*).

In our experience, ESBL and fluoroquinolone-resistant iNTS remain extremely uncommon in Blantyre, Malawi. This is surprising because diverse ESBL genotypes were observed in other members of *Enterobacteriaceae* in Blantyre within a year after ceftriaxone came into common use locally (*8*). That IncHI2 plasmids transfer most efficiently at temperatures <30°C (*9*), a lower temperature than in the human gastrointestinal tract, might explain why the acquisition of ESBL-producing enzymes through IncHI2 plasmids has not been commonly observed within patients with recurrent iNTS disease in this setting. However, rates of transfer might differ when bacteria are growing in the intestine.

The spread of mobile genetic elements that confer antimicrobial resistance among gram-negative organisms is of considerable concern. Wide dissemination of this strain or the IncHI2 (pSTm-BTCR) plasmid among other salmonellae in sub-Saharan Africa would rapidly render iNTS effectively untreatable with currently available antibacterial drugs.

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Zika Virus, French Polynesia, South Pacific, 2013

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DOI: http://dx.doi.org/10.3201/eid2011.141253

To the Editor: We wish to clarify an inaccuracy in a letter in Emerging Infectious Diseases by Cao-Lormeau et al. (*1*). The authors state "In 2007, the first ZIKV outbreak reported outside Africa and Asia was retrospectively documented from biological samples from patients on Yap Island, Federated States of Micronesia, North Pacific, who had received an incorrect diagnosis of dengue virus (DENV)." Although the first outbreak of Zika virus (ZIKV) infection reported outside Africa or Asia was in Yap, it was not retrospectively identified from serum samples incorrectly diagnosed as positive for dengue virus. The outbreak was first identified by the Yap State Department of Health Services, and an investigation to determine the etiologic agent was initiated.

Although dengue was initially part of the differential diagnosis, and a few patients had evidence of IgM against dengue virus by a rapid diagnostic test, clinicians in Yap believed that the clinical syndrome was not consistent with dengue. Thus, assistance was requested from the US Centers for Disease Control and Prevention and the World Health Organization to strengthen the epidemiologic investigation and provide confirmatory laboratory testing.

Serum samples collected during the active investigation were sent to the Arboviral Diseases Diagnostic Laboratory at the Centers for Disease Control and Prevention where testing determined that the cause of the infections was ZIKV (*2*). This discovery of ZIKV as the etiologic agent was not achieved through retrospective testing of serum from patients incorrectly diagnosed as having dengue, but rather the result of an active, coordinated investigation by the Yap State Department of Health Services with instrumental assistance from international partners.

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2 Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS. Zika outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009;360:2536–43. http://dx.doi.org/10.1056/NEJMoa0805715

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In Response: I want to respond to the letter by Hancock et al. (*1*) regarding the previously published letter, Zika Virus, French Polynesia, South Pacific, 2013 (*2*). My comment aims to clarify an inaccuracy in the following sentence. "In 2007, the first Zika outbreak ever reported outside Africa and Asia was retrospectively documented from biological samples of patients on Yap Island, Federated States of Micronesia, North Pacific, who had received an incorrect diagnosis of dengue virus (DENV)" (*2*).

I recognize that this sentence does not provide an accurate description of the efforts in Yap State to investigate the outbreak and further confirm that it was caused by Zika virus (ZIKV). As specified in the article by Lanciotti et al. (*3*), outbreak investigations continued although initial laboratory testing suggested dengue virus as the causative agent: "In April 2007, an epidemic of rash, conjunctivitis, and arthralgia was noted by physicians in Yap State, Federated States of Micronesia. Laboratory testing with a rapid assay suggested that a dengue virus (DENV) was the causative agent. In June 2007, samples were sent for confirmatory testing to the Arbovirus Diagnostic Laboratory at the Centers for Disease Control and Prevention (CDC, Fort Collins, CO, USA)."

I apologize to the Yap Epinet Team for this inaccuracy, and I encourage the reader to consult the articles by Lanciotti et al. (*3*) and Duffy et al. (*4*) to get a complete description of the clinical and laboratory investigations conducted during the ZIKV outbreak in Yap State. If data and laboratory protocols (reverse transcription PCR) related to this first ZIKV outbreak in the Pacific had not been available to the scientific community, identification of ZIKV as the cause of an outbreak in French Polynesia in 2013 would have been greatly delayed.

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Missing Microbes: How the Overuse of Antibiotics Is Fueling Our Modern Plagues

Martin J. Blaser

Henry Holt and Company, LLC, New York, NY, USA, 2014
ISBN: 978-0-8050-9810-5
Pages: 288; Price: US $28.00

This engaging book on the importance of the microbiome in human health weaves the personal and professional experiences of its author, Dr Martin Blaser, into a thought-provoking commentary on the perils of inappropriate antimicrobial drug use. In 16 chapters, Blaser, director of the New York University Human Microbiome Program, builds a case for recognizing the importance of commensal organisms, describes the effects of overuse of antibiotics on human ecology, and cites both personal and professional experiences to support his concerns.

Blaser proposes that perturbations to the human microbiome have led to an increasing incidence of obesity, juvenile diabetes, and asthma, which he terms "modern plagues." He uses a discussion of his work with *Helicobacter pylori* to illustrate the concept of amphibiosis, a phenomenon in which an organism may be friend or foe, depending on the environment of the host. Blaser proposes that the eradication of *H. pylori* and the inflammatory gastritis that was associated with its presence resulted in a replacement of ulcers and gastric cancer with heartburn and esophageal adenocarcinoma. He also posits that celiac and inflammatory bowel diseases may be the consequence of alterations in bowel flora resulting from multiple courses of antimicrobial drugs. He cites the example of his daughter to support this theory, tying her clinical diagnosis of celiac disease to the multiple courses of amoxicillin she received as a child for otitis media and courses of metronidazole she received for traveler's diarrhea during international travel while she worked in resource-challenged settings.

Blaser draws on his experiences as an infectious disease specialist, chair of medicine, advisor, and epidemiologist at the Centers for Disease Control and Prevention to compare and contrast the alterations in our microbiome with the consequences of climate change. He describes the individual and societal implications of these events and warns against an "antibiotic winter" in which antimicrobial resistance and disease prevail because of the destruction of our internal and external ecosystems.

In the final chapter and epilogue, Blaser proposes solutions that may be implemented at the individual and community levels. As initial steps, he proposes avoiding antimicrobial drugs as growth promoters in animals; minimizing the use of these drugs for unclear indications in infants, whose gut flora are still being established; and considering how each drug course will affect the individual and the community.

Despite his emphasis on missing microbes, however, Blaser is wary of using probiotics, prebiotics, and synbiotics to restore microbial balance. He reasonably cites the challenges arising from the unsubstantiated claims of manufacturers and the paucity of well-designed trials to evaluate probiotics. What he neglects to mention is that the requirements imposed by government agencies in North America and Western Europe make probiotic studies infeasible, if not impossible, in the current regulatory climate.

Overall, this book is an important and worthwhile read for members of the general public and the scientific community. Blaser's appealing and personable prose is captivating, easy to read, and convincing and reminds us that health care advances also have consequences.

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Correction: Vol. 19, No. 3

The affiliations of author Amal Bassili were listed incorrectly in the article Multidrug-Resistant Tuberculosis, Somalia, 2010–2011 (I. Sindani et al.). She was affiliated with the World Health Organization, Cairo, Egypt, and the Medical Research Institute, Alexandria University, Alexandria, Egypt. The article has been corrected online (http://wwwnc.cdc.gov/eid/article/19/3/12-1287.htm).

Correction: Vol. 20, No. 3

In the review of Lifting the Impenetrable Veil: From Yellow Fever to Ebola Hemorrhagic Fever and SARS (S. Bloom), the affiliation for Charlie Calisher was misstated. He is professor emeritus at Colorado State University. The article has been corrected online (http://wwwnc.cdc.gov/eid/article/20/3/13-1889.htm).

Correction: Vol. 20, No. 6

Animal testing data were described incorrectly in the article Schmallenberg Virus Circulation in High Mountain Ecosystem, Spain (X. Fernández-Aguilar et al.). Testing showed Schmallenberg virus in 105 (86.8% [95% CI 80.7%–92.8%]) of 121 animals from cow herds and 16 (41% [95% CI 25.6%–56.5%]) of 39 animals from mixed sheep–goat herds. The article has been corrected online (http://wwwnc.cdc.gov/eid/article/20/6/13-0961.htm).

Paul Cézanne 1839–1906, *Still Life with Apples* (detail). 1893–1894, Oil on canvas, 65.4 x 81.6 cm (25 3/4 x 32 1/8 in.) Digital image courtesy of the Getty's Open Content Program, The J. Paul Getty Museum, Los Angeles, CA.

Unique Perspectives

Jared Friedberg

Paul Cezanne was a visionary who bridged 19th century impressionism with 20th century cubism and other modern styles. Born in Aix-en-Provence, France, into the family of a successful banker, Cezanne had a comfortable childhood, culminating in studies at the College Bourbon. There he met lifelong friends with Emile Zola and Baptistin Baille, who would become prominent scholars in writing and acoustics, respectively.

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Cezanne enrolled in the Free Municipal School of Drawing in Aix-en-Provence, but his father pressured him to enter law school to focus on more "practical" pursuits. Cezanne continued taking drawing lessons. Defying his father's wishes, he moved to Paris in 1861 to join Zola and pursue becoming an artist. His father eventually softened to Cezanne's career choice and granted him a large inheritance that freed Cezanne from financial uncertainty. Cezanne focused his early art on the whimsical landscapes of his childhood. As his style matured, he became captivated with defining paintings by simple shapes, exceptional

lighting, and uncommon perspectives. By the 1890s, he had achieved artistic and financial success in Paris but preferred to return to his native Provence where he preferred to paint.

Still Life with Apples (1893–1894) is a deconstruction of the impressionist art of the era. In this still life, the displayed apples monopolize the imagery, but their position on the table draws the gaze to the right. Cezanne overlaps and blends the vases and bottle, in a style that is in contrast to the full and complete forms championed by impressionists. The haphazard table cloths invoke the chaotic turbulence of ocean water, adding motion to the stillness of the painting. The red, green, and yellow hues of the apples emphasize their prominence against the softer colors of the table and vases. The perspective of the painting is also unique to Cezanne's style: each piece of fruit, the bottle, the pots, and even the plate, retains an individual presence, giving each an independence from each one another.

Cezanne's interest in still life paintings was about the form, shade, and color of the presented objects. Cezanne stresses the geometry of the apples over their functionality and appeal as a source for nourishment. The emphasis on shape allowed Cezanne to subvert the tropes of classical impressionism by demonstrating a unique viewpoint on how the apple's form occupies its given space.

Cezanne asks people to look at the apples of his painting from a different perspective, and this may serve as a lesson for those who work to promote food safety. The food we eat may be touched by many people and rests on many surfaces before it comes to our plates. Our food may contact a multitude of microbes during its journey from farm or field to table. The capacity to detect, investigate, and prevent food-borne diseases varies across the world. Adapting surveillance processes and prevention techniques may require new approaches and unique perspectives, in much the way Cezanne implemented innovations to impressionism when it grew too limiting.

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EMERGING INFECTIOUS DISEASES®

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

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

Why an "Emerging" Infectious Diseases journal?

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

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

What are the goals of Emerging Infectious Diseases?

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

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

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

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

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Residual Infestation and Recolonization during an Urban *Triatoma infestans* Bug Control Campaign

Circulation of Reassortant Influenza A(H7N9) Viruses in Poultry and Humans, Guangdong Province, China, 2013

Seroconversion for Infectious Diseases among UK Military Personnel Deployed to Afghanistan, 2008–2011

Molecular Evolution of Peste des Petits Ruminants Virus

Replication and Shedding of MERS-CoV in Upper Respiratory Tract of Inoculated Dromedary Camels

Accuracy of Herdsmen Reports versus Serologic Testing for Estimating Foot and Mouth Disease Prevalence

Gouleako and Herbert Viruses in Pigs, South Korea, 2013

Equine Influenza A(H3N8) Virus Isolated from Bactrian Camel, Mongolia

Human Hantavirus Infections in the Netherlands

Putative New West Nile Virus Lineage in *Uranotaenia unguiculata* Mosquitoes, Austria, 2013

Human Infection with Influenza Virus A(H10N8) from Live Poultry Markets, China, 2014

Two Outbreaks of *Listeria monocytogenes* Infection, Northern Spain

Avian Bornavirus in Free-Ranging Psittacine Birds, Brazil

Mycobacterium Species Related to *M. leprae* and *M. lepromatosis* from Bovine Nodular Thelitis

Novel Porcine Epidemic Diarrhea Virus Variant with Large Genomic Deletion, South Korea

MERS Coronavirus Neutralizing Antibodies in Camels, Eastern Africa, 1983–1997

Reemergence of Foot-and-Mouth Disease, South Korea, 2000–2011

Equine Influenza A(H3N8) Virus Infection in Cats

Echinococcus ortleppi Infections in Humans and Cattle, France

Evaluation of Commercially Available Serologic Diagnostic Tests for Chikungunya Virus

Novel Bluetongue Virus in Goats, Corsica, France

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

Upcoming Infectious Disease Activities

2014

November 2–6, 2014
ASTMH
American Society of Tropical Medicine and Hygiene
63rd Annual Meeting
New Orleans, LA, USA
http://www.astmh.org/Home.htm

November 15–19, 2014
APHA 142nd Annual Meeting & Expo
New Orleans, LA, USA
http://www.apha.org/meetings/AnnualMeeting

November 30–December 4, 2014
ASLM2014 International Conference
Cape Town International Convention Centre, South Africa
http://www.aslm2014.org/

2015

March 8–11, 2015
ICEID
International Conference on Emerging Infectious Diseases
Atlanta, GA, USA

2016

March 2–5, 2016
ISID
17th International Congress on Infectious Diseases
Hyderabad, India

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to http://www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Legionnaires' Disease Incidence and Risk Factors, New York, New York, USA, 2002–2011

CME Questions

1. You are seeing a 60-year-old woman with a 2-week history of cough, severe malaise, hemoptysis, and fever. You consider whether this patient has Legionnaires' disease (LD). Which of the following statements regarding the epidemiology of LD is most accurate?

A. The prevalence of LD has been decreasing steadily nationwide for the past decade
B. The incidence of LD increased by more than 200% between 2002 and 2009 in the current study
C. The greatest change in the prevalence of LD in the current study was among young adults
D. Nearly all cases of LD in the current study occurred during the winter

2. As you evaluate this patient, which of the following choices should you consider as a demographic variable most significantly associated with the risk for LD in the current study?

A. Female gender
B. Age between 60 and 79 years
C. Non-Hispanic black race
D. Recent stay in a skilled nursing facility

3. Which of the following statements regarding the clinical profile and prognosis of patients with LD in the current study is most accurate?

A. The mortality rate was approximately 13%
B. Community-acquired and healthcare-associated infections carried similar risks for mortality
C. Only 30% of patients had an established risk factor for LD
D. HIV infection was the most common medical risk factor associated with LD in the current study

4. Which of the following occupations was associated with a higher risk for LD in the current study?

A. Plumbing
B. Firefighting
C. Nursing
D. Transportation

Activity Evaluation

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

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to http://www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Blastomycosis Mortality Rates, United States, 1990–2010

CME Questions

1. Your patient is a 55-year-old man with pulmonary symptoms who is suspected of having blastomycosis, According to the epidemiologic study report by Khuu and colleagues, which of the following statements about the clinical features of blastomycosis is correct?

A. Infection occurs primarily through inhalation of Blastomyces dermatitidis spores into the lungs where they undergo transition to the invasive yeast phase
B. Extrapulmonary disease has not been reported
C. The incubation period for blastomycosis is 10 to 30 days
D. Treatment is limited to supportive care

2. According to the epidemiologic study by Khuu and colleagues, which of the following variables is most likely a risk factor for blastomycosis-related mortality?

A. Ages 55 to 64 years
B. Female gender
C. White race
D. Native American ancestry

3. According to the epidemiologic study by Khuu and colleagues, which of the following statements about geographic differences in blastomycosis-related mortality would most likely be correct?

A. Risk for blastomycosis-related death was higher in the West than in the South
B. Risk for blastomycosis-related death was higher in the West than in the Midwest
C. Mortality rate in the Midwest increased during the study period
D. Mortality rate in the Southern region increased during 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

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

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

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

Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

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

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

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

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

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

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.