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Antimicrobial resistance, a major public health concern, largely arises from excess use of antibiotic and antifungal drugs. Lack of routine diagnostic testing for fungal diseases exacerbates the problem of antimicrobial drug empiricism, both antibiotic and antifungal. In support of this contention, we cite 4 common clinical situations that illustrate this problem: 1) inaccurate diagnosis of fungal sepsis in hospitals and intensive care units, resulting in inappropriate use of broad-spectrum antibacterial drugs in patients with invasive candidiasis; 2) failure to diagnose chronic pulmonary aspergillosis in patients with smear-negative pulmonary tuberculosis; 3) misdiagnosis of fungal asthma, resulting in unnecessary treatment with antibacterial drugs instead of antifungal drugs and missed diagnoses of life-threatening invasive aspergillosis in patients with chronic obstructive pulmonary disease; and 4) overtreatment and undertreatment of Pneumocystis pneumonia in HIV-positive patients. All communities should have access to nonculture fungal diagnostics, which can substantially benefit clinical outcome, antimicrobial stewardship, and control of antimicrobial resistance.

Antimicrobial resistance (AMR) is a major public health concern and a major threat to modern medicine (1). In the United States, it is estimated that antibiotic-resistant infections are associated with 23,000 deaths per year (2) and excess healthcare-associated costs of approximately US $20–25 billion (3). Minimizing AMR has been the focus of accelerating efforts with multipronged approaches tailored to individual countries and healthcare settings. Even if the difficult task of developing new antimicrobial drugs is successful, current efforts aimed at reducing the development of resistance will need to be maintained to protect these novel compounds.

A central tenet of controlling AMR is antibiotic drug stewardship, which seeks to limit inappropriate antibiotic drug usage by avoiding unnecessary prescribing, including discontinuing antibiotic therapy if it is not required. Within the context of stewardship programs, inadequate attention has been paid to fungal infection as the cause of antibacterial treatment failure. Furthermore, the importance of the accurate and timely diagnosis of fungal infections in defeating AMR has been starkly absent from policy discussions (4). Accurate diagnosis or exclusion of fungal infection will have a substantial effect on antimicrobial drug usage and on our ability to limit AMR to bacteria.

Few infections are microbiologically diagnosed in real time, so, in general, physicians empirically prescribe most antimicrobial drugs. Furthermore, most infections are never microbiologically confirmed, which matters little if the patient improves and infection resolves, but if the patient’s condition deteriorates, additional empiric antimicrobial drugs are usually given (5). Fungal infections, a frequent fatal complication (superinfection) of numerous diseases that contribute to inappropriate antibiotic drug usage (e.g., cancer; liver, respiratory, and renal failure; sepsis; AIDS), are often undiagnosed and untreated.

This inappropriate use of antibiotic drugs must stop. We provide 4 examples of specific clinical situations that require greater application of existing fungal diagnostics and improved overall fungal diagnostic capability and are in line with the 95–95 by 2025 Roadmap from the Global Action Fund for Fungal Infections (6).

Accurate Diagnosis of Fungal Sepsis in Hospitals
Hospitalized patients, especially those in intensive care units (ICUs), are often inappropriately placed on broad-spectrum antibiotic drugs because fungal diseases involving
Candida spp. are not routinely diagnosed. Bloodstream infection and invasive candidiasis are substantially more common than realized and probably result from multiple factors, including unrestrained antibiotic drug use, indwelling devices, increasing populations of immunocompromised patients, and increased renal support. Multiple studies have shown the incidence of bloodstream infections with Candida spp. to be 1.2–26 cases/100,000 population, and the highest rates are in middle- and high-income countries, notably the United States (7,8). Healthcare-associated infections account for 93% of these infections (≈80% are among hospital inpatients), and the other 7% are community-acquired (9). In Brazil, which has a population of 194 million persons, Candida bloodstream infections are seen in 14.9 persons/100,000 population, which translates to 29,000 infected persons each year (10), based on prospective data obtained 10 years ago from 11 medical centers (11). A 2015 prospective study from 27 ICUs in India showed a mean incidence of 6.51 cases of ICU-acquired candidemia per 1,000 ICU admissions and a death rate of 35%–75% (12). An estimated 14.3 million patients are admitted to ICUs in India each year. Undoubtedly, this high rate of ICU-acquired candidemia is an underestimate of the problem because blood culture is only ≈40% sensitive for invasive candidiasis (including intraabdominal candidiasis), and use of fluconazole and echinocandins substantially reduces the yield from blood culture (13–15). Therefore, it is probable that the actual number of cases in ICUs in India exceeds 200,000, resulting in ≈100,000 deaths. If, as is found in other countries, bloodstream infection caused by Candida spp. (and presumably invasive candidiasis) outside ICUs in India are at least twice as common as in ICUs, then >600,000 persons each year in India are estimated to have invasive candidiasis. Assuming these patients are treated, ≈300,000 die each year, but many more die if patients are not treated.

In addition, a high prevalence of candidemia has been reported in children, including neonates, in India and Latin America. Central nervous system involvement is common in premature infants, leading to a high rate of neurologic sequelae (16).

Although invasive candidiasis is strongly associated with prior bacterial infection and antibiotic therapy, inappropriate escalation and combination antibacterial therapy will typically have been administered to patients with invasive candidiasis. In a study of 444 patients with Candida spp. bloodstream infections, 81% were exposed to multiple antibacterial drugs, either concomitantly or sequentially (17), and in an ICU study from India, 95% of patients were receiving antibiotic drugs (usually ≥2) (12). Early therapy of Candida spp. bloodstream infection greatly improves patient outcomes and the outcome is even better if correct therapy is given immediately (18).

Once Candida-associated sepsis is confirmed, antibacterial agents can usually be stopped and, if Candida sepsis is ruled out, empiric antifungal therapy can be stopped. Inflammation without infection requires no antimicrobial therapy. Three well-validated diagnostic tools, 2 of which are configured for ruling out a diagnosis of invasive candidiasis, are now available: the 1,3β-D-glucan assay (19); the Candida albicans germ tube antibody test, which is used with serum samples (20); and a nonculture-based molecular assay (newly approved by the Food and Drug Administration) that is used with EDTA blood and is substantially more sensitive than blood culture for making a diagnosis of Candida spp. infection (21). Among patients without invasive candidiasis, antimicrobial stewardship programs based on such diagnostics have successfully curtailed the use of antifungal therapy in the ICU without worsening patient outcomes (18,22). The economics of these methods depend on the cost of diagnostic reagents and testing, antifungal drug costs, and incidence of infection (23). Overuse of antifungal agents is costly, can promote antifungal resistance, and has the potential for causing toxicity and various detrimental drug interactions in patients (18). Modeling will be required to determine the magnitude of the effect of these diagnostic tools on antibacterial prescribing. What is not in dispute is that outcomes for patients with fungal infection will improve. Antimicrobial stewardship programs will be even more effective if these diagnostic tools, with their rapid turnaround times, are readily available (24). Widespread implementation of rapid nonculture diagnostics for Candida spp. will greatly improve prescribing practices for hospitalized patients with multiple concurrent conditions and poorly functioning organs and using multiple medications.

Misdiagnosis of Smear-Negative Pulmonary Tuberculosis as Tuberculosis

Smear-negative pulmonary tuberculosis (TB) is a problematic area for clinicians and policymakers. Post-TB sequelae are common, are poorly studied, and may be mistaken for active, recurrent TB (25). An apparent underrecognized issue for patients with smear-negative TB is chronic pulmonary aspergillosis (CPA), which can mimic the signs and symptoms of TB. In 544 patients in the United Kingdom who had previously received treatment for TB with a residual cavity, precipitating antibodies to Aspergillus fumigatus developed in 24.6% at 2 years and in 34.0% at 5 years. Within 2 years, aspergilloma, a late stage of CPA, developed in 78 (58%) of the 134 patients with precipitating antibody to A. fumigatus (26). Few prospective studies have been conducted on CPA after treatment for TB, so the incidence of such cases cannot be stated with certainty; conservatively, however, a rate of ≈10% among survivors of pulmonary TB is likely and a global prevalence of ≈1.2 million cases is probable (26).
Culture for *Mycobacterium tuberculosis* in samples from smear-negative patients is slow, and results may be falsely negative. The use of new, highly sensitive, DNA detection assays (e.g., Xpert MTB/RIF) directly on respiratory specimens has transformed the rapidity of detecting positive samples, but there remain millions of unwell, smear-negative, PCR-negative patients. Some of these patients have relapsed after anti-TB therapy, and CPA has developed subsequent to cured TB. Among HIV-positive persons, those with smear-negative TB test results have a higher death rate than those with smear-positive results (27), probably because many do not have TB at all. It is increasingly recognized that many of these patients are chronically infected with *Aspergillus* spp., resulting in CPA that is largely undiagnosed and untreated.

Weight loss, worsening cough, chest pains, dyspnea, and fatigue are common manifestations of TB and CPA, and abnormalities seen on chest radiographs are similar for the 2 diseases (Figure 1). In studies from the United Kingdom, Brazil, South Korea, Iran, and India, the frequency of elevated serum levels of *Aspergillus* antibody after TB varied upwards from 20% (28,29). *Aspergillus* antibody detection is the key diagnostic test for CPA; the test has 96%–97% sensitivity and 92%–98% specificity (30,31). Given that CPA is a common sequela to TB and has a 5-year death rate of 75%–80%, it needs to be sought actively by *Aspergillus* antibody testing in symptomatic patients who have completed antituberculous therapy (32). A study from Iran showed that serum samples from almost all patients thought to have recurrent TB were positive for *Aspergillus* antibody (33). In Brazil and Uganda, Pneumocystis jirovecii DNA was identified in the sputum of up to 7.0% and 6.8% of patients, respectively, diagnosed with smear-negative TB. This finding and the recognition of patients with pulmonary histoplasmosis (Figure 2) or coccidiodomycosis (5) indicate that other, potentially treatable diseases, not smear-negative TB, may be responsible for illness attributed to TB.

Empirc anti-TB therapy is unnecessary in patients with CPA or other fungal infections because it is ineffective and exposes the patient to potential toxicity. Yet, this therapy remains the default approach for most patients with clinical features and radiologic findings partially consistent with TB. When such treatment fails, as it usually does, there is a risk that patients are assumed to have multidrug-resistant TB, and the inappropriate substitution of second- and third-line anti-TB agents adds to potential toxicities and healthcare costs. The size of this problem is substantial; the World Health Organization reported a total of 2,755,870 patients with smear-negative TB in 2013 (34). *Aspergillus* antibody testing should be made widely available and integrated into TB control programs, and emphasis should be placed on the possibility of CPA as a post-TB sequela. All patients with symptoms consistent with recurrent TB should be screened for antibody to *Aspergillus* spp.

### Fungal Exacerbation of Asthma and Chronic Obstructive Pulmonary Disease

It is common practice to treat asthma and exacerbations of chronic obstructive pulmonary disease (COPD) with antibiotic drugs and corticosteroids, although guidelines caution against their unnecessary use, especially in patients with COPD (35). Most patients respond, even if the exacerbation is virus-induced. Patients with moderate and severe COPD are frequently hospitalized and have an in-hospital death rate of 6%. More than 80% of patients are treated with antibiotic drugs (36), although multiple guidelines advise against this in the absence of purulent sputum and pulmonary infiltrates. It is now well established that colonization (or infection) of the Airways with *Aspergillus* spp. is strongly associated with exacerbations (37). A study in Spain found that 1.3% of hospitalized patients with COPD had invasive aspergillosis, and 65% died (38); in southern China, the frequency rate was 3.9%, and 43% died (39). These rates are probably underestimates because they were based on the results of *Aspergillus* sputum cultures, which are insensitive. Most patients do not receive treatment for invasive aspergillosis, but they are treated unnecessarily with antibiotic drugs. The scale of this problem is large. In China, an estimated 11,858,000 COPD patients >40 years of age (87 persons/10,000 population) were hospitalized in

![Figure 1. Chest radiograph showing bilateral upper lobe chronic pulmonary aspergillosis, which can be easily mistaken for pulmonary tuberculosis. White arrows indicate areas of abnormality (some pleural thickening and opacification) in both apices, which are similar, although slightly more obvious, to findings in pulmonary tuberculosis. Black arrow indicates the trachea pulled to one side by the contraction and fibrosis on that side. Image used with permission of David Denning (©2016, all rights reserved).](image-url)
Failure to properly diagnose and treat patients with asthma and COPD who are colonized with *Aspergillus* spp. continues to increase the inappropriate use of antibiotic drugs and corticosteroids among these patients. Recognition of fungal infection and allergy and treatment with direct-ed antifungal therapy would greatly reduce exacerbations, medical consultations, and hospital admissions. It is critical that fungal culture and nonculture diagnostics (i.e., *A. fumigatus* IgE, IgG, and antigen testing and PCR) for COPD and asthma exacerbations be evaluated and implemented and that fungal asthma be properly diagnosed and treated.

**Making and Excluding the Diagnosis of *Pneumocystis* Pneumonia in AIDS**

*Pneumocystis* pneumonia (PCP) in AIDS is often diagnosed empirically based on a subacute onset of cough; breathlessness out of proportion to abnormalities seen on chest radiographs; and subtle, bilateral changes seen on chest radiographs, in the context of a low CD4 cell count (Figure 3). Co-trimoxazole (trimethoprim/sulfamethoxazole, Bactrim, Septrin) is the most effective agent for prevention and therapy of PCP. A low dose is effective for prophylaxis, but a 3-week course of high and potentially toxic doses is required for effective therapy. The differential diagnosis of PCP is broader in children because bacterial pneumonia is more common among them. If a precise diagnosis could be achieved in most cases of PCP, much of the inappropriate use of co-trimoxazole could be prevented.

Rates of PCP among newly hospitalized adults with advanced HIV infection are highly variable, ranging from <1% to 60%, and rates rise as gross domestic product increases (43). Without the availability of adequate diagnostics, many persons will unnecessarily receive high-dose co-trimoxazole, with or without corticosteroids, for 3 weeks. If the actual number of PCP cases in patients with AIDS is 400,000, then hundreds of thousands of hospitalized HIV patients may be given co-trimoxazole unnecessarily, and toxicity rates among them could be as high as 90% (5,44). Early detection and diagnosis of PCP can help prevent unnecessary hospitalizations and reduce adverse events and healthcare costs.

Currently, bronchoscopy and microscope examination of bronchoalveolar lavage fluid is the most common definitive means of establishing a diagnosis of PCP; this method has a sensitivity of 75%–90%, depending on the microscopy technique (45). *P. jiroveci* fungus is nonculturable in routine laboratories; in Europe, it is commonly molecularly detected using PCR, which has a sensitivity of 95%–99% (46). *Pneumocystis* PCR performed on expectorated sputum is also effective for detecting *P. jiroveci* fungus (47–49), but this method is infrequently used. For children who are breathless, PCR of nasopharyngeal aspirates is currently the only realistic means of establishing a diagnosis. 1,3 β-D-glucan is detectable in the serum of
Antimicrobial Resistance and Fungal Diagnostics

nearly all patients with PCP (19); if a sample is negative, infection is effectively ruled out.

Assuming that 25% of PCP cases are mild, immediate diagnosis and use of oral therapy will potentially avoid 100,000 hospital admissions each year and even more if the diagnosis is ruled out and patients are not admitted for unnecessary PCP therapy (5). Mild PCP responds well to treatment and prevents progression to moderate or severe infection.

Provision of rapid diagnostics for Pneumocystis pneumonia will enable early diagnosis and discontinuation of broad-spectrum antibiotic drugs if test results are positive and discontinuation of high-dose co-trimoxazole and corticosteroids if results are negative. Furthermore, PCP diagnoses that are missed because of concurrent bacterial infection will be minimized. Pneumocystis PCR should be used to test all respiratory samples in laboratories serving large immunocompromised populations, and 1,3 β-D-glucan testing should be used on serum in high-volume laboratories. These diagnostics will definitively improve the outcome for immunocompromised patients without AIDS for the same reasons they will improve the outcome for patients with AIDS.

Other Clinical Scenarios
We have not addressed multiple other clinical situations in which a precise fungal diagnosis could reduce the inappropriate prescribing of antimicrobial drugs (i.e., overtreatment or incorrect treatment). Among those situations are cases of cryptococcal meningitis (Figure 4); Candida infection or colonization of the respiratory or urinary tract; febrile neutropenia in leukemia; Aspergillus bronchitis in bronchiectasis; and allergic, chronic, and invasive fungal sinusitis and PCP in HIV-negative patients. In all these clinical situations, inappropriate antibacterial or antifungal prescribing is common because of the lack of adequate fungal diagnostic testing. We also have not addressed rapid detection of antifungal drug-resistant fungi (i.e., A. terreus, C. krusei) or the use of diagnostics to prevent superinfection with fungi that are commonly resistant to antifungal drugs, such as C. glabrata or Rhizopus oryzae. Antifungal drug resistance is problematic in some settings and demands informed prescribing, therapeutic drug monitoring, and development of new antifungal agents (50).

Conclusions
The lack of availability and underuse of nonculture fungal diagnostics results in overprescribing, prescription of unduly long courses of antibacterial agents, and excess empirical use of antifungal agents and leaves many millions of patients with undiagnosed fungal infections. This lack and

Figure 3. Chest radiograph showing early, subtle Pneumocystis pneumonia–associated abnormalities in both lower lungs of a patient newly diagnosed with AIDS; this diagnosis was unsuspected in the patient, a 63-year-old married man. Magnified images on right show normal lung (top image) and infiltrates adjacent to and behind the heart and overlain by rib (bottom image). Similar differences between the upper and lower lobes are seen in the radiograph on the left. Image used with permission of David Denning (©2016, all rights reserved).

Figure 4. An ulcerative skin lesion that was positive for Cryptococcus neoformans fungus on biopsy. For several weeks before being correctly diagnosed, the lesion was misdiagnosed as a bacterial infection. Image used with permission of Arnaldo Colombo (©2016, all rights reserved).
underuse of proper diagnostics squanders resources. The large scale of the problem, even in many of the world’s most advanced medical centers, compromises AMR control. In many countries, the government and private healthcare providers should be actively promoting diagnosis of fungal infections to minimize deaths and illness from fungal disease; such efforts will probably also have a positive benefit on inappropriate antibacterial drug usage and support stewardship programs. Public health authorities must embrace acute and chronic fungal disease as areas of considerable need and seize the opportunity to improve health and preserve what remains of the antimicrobial drug toolbox.

Dr. Denning, an infectious diseases clinician who is heavily involved in postgraduate teaching and lectures worldwide, leads LIFE (Leading International Fungal Education) and is president of the Global Action Fund for Fungal Infections. His primary research interests are chronic and allergic pulmonary fungal disease, the global burden of fungal infection, and azole resistance in *Aspergillus*.

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Antimicrobial Resistance and Fungal Diagnostics
Brucellosis is a bacterial zoonosis caused by Brucella spp., which can be transmitted from animal reservoirs, such as cattle, sheep, goats, and pigs, to humans through direct contact with infected animals or ingestion of unpasteurized animal products (1–3). The global epidemiology of brucellosis has drastically changed over the past decades, particularly in industrialized countries where the disease was previously endemic but is now mainly associated with returning travelers. However, brucellosis remains a serious concern in low- and middle-income countries, which have most new human cases globally (estimated ≈500,000 cases annually) and major economic losses in animal production resulting from the adverse effects of infection on livestock reproduction (2,4,5). Additionally, human brucellosis is of particular concern because of high initial treatment failure, substantial residual disability of infected patients, and relapse rates (1,6). Moreover, Brucella spp. are highly infectious through the aerosol route, making them a potential agent of biological weapons and bioterrorism (7).

In China, brucellosis was first recorded as Malta fever for 2 foreigners in Shanghai in 1905, but several patients in China who had similar clinical symptoms had been observed in the 10 years before this report (8). After this report, 3 cases were reported from Chongqing in 1906 (9). The first person with a definite diagnosis of Brucella infection by serologic tests was reported from Fujian in 1916 (10). Subsequently, Brucella sp. was isolated from a foreigner and his goats who traveled from Punjab, India, to Henan Province, China, in 1925 (11), and human infection in a laboratory setting was reported in Beijing in 1936 (12). Thus, human brucellosis was seen in China before 1950, especially in the northern provinces (13).

Since 1950, activities for prevention and control of brucellosis have been gradually introduced in mainland China (14,15). During 1950–1963, the reporting for human brucellosis was established nationwide, and some surveys were conducted. Vaccination for animals and humans was implemented as the main control measure during 1964–1976 in regions with severe epidemics, such as Inner Mongolia, Xinjiang, Qinghai, Ningxia, and Henan Provinces (14). During 1977–1988, a national program for brucellosis control was conducted with the introduction of diagnostic criteria, treatment protocols, and control measures, and
vaccination of domestic animals was used as the main control measure. National sentinel surveillance was established in 1990 to monitor the seroprevalence of brucellosis in humans and animals (16).

During the past decade, outbreaks of human brucellosis have been reported in increasing numbers and with an apparent geographic expansion from the historically affected north of China (17,18) to southern provinces where non-occupational exposure might be more common because of the increasing movement of humans, animals, and animal food products from brucellosis-endemic regions (19–21). The epidemiology of human brucellosis clearly presented major challenges in China during the past 60 years, but studies reporting the spatial–temporal patterns of human brucellosis with high-quality, nationwide incidence data are lacking (2,4,14,22,23). We describe the magnitude and distribution of human brucellosis in mainland China using the notifiable reporting data for 1955–2014 and emphasize its recent reemergence. Improving our understanding of the changing epidemiology of brucellosis and identifying high-risk areas can help in formulating plans for national strategies to prevent and control brucellosis.

Methods

Data Source and Ethical Considerations

On July 5, 1955, human brucellosis was made statutorily notifiable in China: all probable or laboratory-confirmed new brucellosis cases were required to be reported (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/3/16-1710-Techapp1.pdf). In this study, we used 2 datasets because the requirements for reporting changed during the study period (24). One comprises the number of brucellosis cases aggregated by case-patient sex, age group, and occupation; incidence rate; death rate; and case-fatality ratio (online Technical Appendix Table 2), reported monthly through paper-based post or electronic files during 1955–2003. The other consists of individual brucellosis cases reported by doctors within 24 hours after diagnosis to the online National Notifiable Infectious Disease Reporting Information System at the Chinese Center for Disease Control and Prevention during 2004–2014. (Variables in individual datasets are available in online Technical Appendix Table 3.) All data used in this study were anonymized so that individual patients could not be identified.

The National Health and Family Planning Commission of China determined that the collection of data from human cases of brucellosis was part of continuing public health surveillance of a notifiable infectious disease and was exempt from institutional review board assessment. All data were supplied and analyzed in an anonymous format, without access to personal identifying information.

Case Definition

Brucellosis cases have been classified as probable (clinically diagnosed) or confirmed (laboratory confirmed) in accordance with the guidelines for human brucellosis diagnosis issued by the Chinese national health authorities in 1977, 1988, 1996, and 2007, which were successively used during 1977–2014 (online Technical Appendix Table 4). Probable cases are diagnosed by local experienced physicians according to patient anamnesis, epidemiologic exposure, clinical manifestations, and/or positive results of presumptive laboratory tests, including the plate agglutination test and the intradermal allergic reaction test. Confirmed cases are probable cases with 1 positive result of the following tests: standard tube agglutination test, complement fixation test, Coombs test, cysteine test for serologic diagnosis, or positive Brucella spp. isolation (1).

Data Analysis

Our analysis comprised all probable and confirmed cases in persons with illness onset from January 1, 1955, through December 31, 2014. According to the National Mid-term and Long-term Animal Disease Control Plan of China (25), all the provinces in northern China were identified as the key regions for brucellosis control. Therefore, we aggregated the surveillance data of each province to northern and southern China (online Technical Appendix Table 5), as previously reported (26), to examine spatial–temporal patterns by region. To eliminate the potential effect of the introduction of Internet-based reporting on the increasing number of cases since 2004, we made a time-series prediction of the number of cases in 2004 on the basis of data for 1993–2003, using the Holt exponential smoothing method with a 95% CI (27). Then we compared the upper value of the 95% CI with the actual number of cases in 2004 to calculate the excess proportion of cases that might have contributed to improved data reporting. Adjusted incidence rates for 2004–2014 were estimated by using this excess proportion and plotted as an epidemic curve. We also predicted the monthly numbers of cases during the next 5 years (2015–2019) by Holt-Winters exponential smoothing on the basis of data reported during 2004–2014 to explore the trend of incidence with seasonality (27).

We created a heat map of the yearly incidence rate to visualize the long-term change over the 60-year period by province. We also created a heat map of the monthly number of cases reported during 2005–2014 by province, standardized by the yearly number in each province, and plotted a heat map of average weekly proportions of case numbers by province to explore the seasonal pattern during 2005–2014. To test the differences between northern and southern China, we used Mann-Whitney U tests with a significance level of α = 0.05 to test for differences in the time from illness onset to diagnosis and χ² with a significance
level of $\alpha = 0.05$ to test the differences in the proportion of imported cases. The R statistical software (version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria) with the package "forecast" (version 6.1), was used to produce the graphs and heat maps and to perform statistical analyses and prediction, and ArcGIS 10.2.2 (ESRI, Redlands, CA, USA) was used to plot the geographic patterns.

**Results**

**Demographic Features**

During 1955–2014, a total of 513,034 human brucellosis cases (median 3,504/year [interquartile range (IQR) 1,145–7,886]), including 170 deaths, were reported to the national human brucellosis surveillance system in mainland China (Figure 1, panel A). Among them, 346,682 (67.6%) cases were reported in the individual database during 2004–2014; the proportion of laboratory-confirmed cases ranged from 76.9% in 2004 to 93.2% in 2014 (Table; Figure 2). Most cases during 2004–2014 occurred in males; the male:female ratio was 2.9:1 for both northern (2.9:1) and southern (2.6:1) China (Table; online Technical Appendix Table 6). Median age of case-patients was 44 years (IQR 34–54 years), and case distribution was similar by sex and type of diagnosis and between northern and southern China (Figure 2). Most (88.8%) case-patients were farmers or veterinarians or worked in livestock husbandry, transport, and trade or food production during 2004–2014.

**Overall Incidence and Seasonality**

The annual incidence rate fluctuated during the 60 years studied (Figure 1, panel A). Before 1979, human brucellosis incidence was relatively steady (IQR 0.4–1.0 cases/100,000 residents) and peaked during 1957–1963 (range 0.9–1.8/100,000) and again during 1969–1971 (range 1.0–1.2/100,000). Incidence decreased dramatically beginning in 1979 and remained low until 1994 (IQR 0.05–0.10/100,000). However, the incidence increased from 1995 through 2014 (median 0.2/100,000 [IQR 0.1–0.2] during 1995–2003 and 2.5/100,000 [IQR 1.5–2.9] during 2004–2014); incidence was highest (4.2 cases/100,000 residents) in 2014. After removal of the excess proportion

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**Figure 1.** Reported human brucellosis cases (N = 513,034), mainland China, 1955–2014. A) Aggregated number of cases (blue bars) and annual incidence rate (orange line) per 100,000 residents reported by year. The adjusted incidence rate (green dashed line) was estimated by an excess proportion (22.06%) that might be attributed to the effect of Internet-based reporting since 2004 (see Methods). B) Forecast of the monthly number of cases (blue line) during 2015–2019 by Holt-Winters exponential smoothing with 80% CIs (light gray) and 95% CIs (dark gray) based on monthly numbers for 2004–2014.
This finding contrasts with the distribution of brucellosis being reported in almost every province and year since 2010. Reemergence in all provinces of southern China. Cases have increased since 2000, and human brucellosis has emerged or reemerged in all provinces of southern China. The highest incidences during 1995–2014 were Tibet (14.07 cases/100,000 residents), Qinghai (4.43), Shanxi (0.87), Xinjiang (0.35), and Inner Mongolia (0.35); during 1995–2014, highest incidences shifted to Inner Mongolia (25.80), Shanxi (7.33), Heilongjiang (6.07), Jilin (1.79), and Hebei (1.40).

Similarly, the incidence in southern China has increased since 2000, when the disease was limited to a few provinces in southern China, such as Sichuan, Guangxi, and Guangdong (Figures 3, 4; online Technical Appendix Figure 2). Additionally, the proportion of imported cases was higher in southern than northern China (58.5% vs. 40.5%; p < 0.001), but cases in southern China had a longer lag from illness onset to diagnosis than did those in northern China (21 vs. 20 days; p = 0.003) (Table).

Correspondingly, the number of counties reporting human cases in mainland China increased from 87 in 1993 to 1,723 in 2014; each year since 2004, hundreds of counties were newly affected (Figures 5, 6). The proportion of counties affected in southern China increased from 1.1% in 2004 to 20.5% in 2014, highlighting the spatial spread over the past decade. From a land cover perspective, during 2004–2014, affected areas seem to have expanded from the provinces in northern pastureland areas to the adjacent grassland and agricultural areas that have a high density of sheep and goats, then to coastal areas and southeastern China (Figure 6).

**Discussion**

We used a longitudinal surveillance dataset spanning 60 years in China to investigate changes in the epidemiologic characteristics of human brucellosis, especially during the period of dramatic socioeconomic changes during the past 3 decades, during which the urban population increased from 19% in 1980 to 54% in 2014 (30). Human brucellosis has reemerged in mainland China since the mid-1990s;
incidence has increased and the disease has expanded geographically from northern to southern China. Our study, with long-term and high-quality incidence data, complements a previously published map of human brucellosis in East Asia (2–4).

The temporal trend in human brucellosis incidence in this study (i.e., high incidence during 1955–1978, low incidence during 1979–1994, and high (and increasing) incidence from 1995 onwards) is consistent with the trend in *Brucella* spp. seroprevalence from animal and human serosurveys conducted in China during 1950–2014. An overall seroprevalence of 41.27% in cattle, sheep, and pigs in brucellosis-endemic areas and 8.43% in humans was reported for 1952–1981, but seroprevalence was only 0.55% in animals and 0.75% in humans during 1982–1990 after implementation of a national control program in 1979 (14,31). During 1990–2001, seroprevalence in humans was 3.28% and has increased steadily since 1995, despite the
lack of obvious change in domestic animals (0.36%) (16). In the 21st century, seroprevalence maintained an increasing trend in occupationally exposed populations (11.36% during 2001–2004, 13.31% during 2005–2006, 21.97% in 2007, and 22.75% in 2011) and livestock (0.28% during 2001–2004, 0.72% during 2005–2006, and 1.49% in sheep in 2009) (32–35). Although incidence appeared to increase in every province, Tibet seems to follow an inverse pattern and reported few cases during the past 10 years, but serologic surveys indicate that seroprevalence of *Brucella* infection in animals and humans remained high in some areas since 2005 (36). Additionally, this study found a low case-fatality rate in passive surveillance, and the poor follow-up for outcomes of human brucellosis with chronic infection and illness might have contributed.

After brucellosis reemerged in China, the geographic distribution of affected areas gradually expanded (14). The areas of brucellosis endemicity gradually shifted from pasturing areas (i.e., Inner Mongolia, Xinjiang, Tibet, Qinghai, and Ningxia) to grassland and agricultural areas (i.e., Shanxi, Liaoning, Hebei, Shandong, and Jilin Provinces), and the southern provinces became increasingly affected (Figures 4, 6) (14,17). This reemergence and the geographic expansion might be attributed to a variety of contributing factors.

Because brucellosis is not transmitted among humans, humans can be a sentinel for livestock brucellosis. For every human brucellosis case, 15 *B. melitensis* cases are expected in small ruminants or 150 *B. abortus* cases in cattle (37), and the large population of reservoir animals infected

![Figure 3. Heat map of provinces with human brucellosis cases, by north and south and the latitude of the capital city of each province, China. A) Time series of incidence rate per 100,000 residents during 1955–2014, standardized by the eighth root. B) Time series of monthly cases, 2005–2014, standardized by the annual number of cases reported by each province. C) Seasonal distribution of cases by province, plotted as the mean value of the proportion of cases in each week of the year from 2005 through 2014.](image-url)
Figure 4. Geographic distribution of the annual incidence rate per 100,000 residents of human brucellosis by 5-year periods, mainland China, 1990–2014.
Human Brucellosis, China

with *Brucella* spp. provides a source and is likely to be one of the main causes of infection for humans. The number of livestock dramatically increased during the past 3 decades to meet the growing demand for meat in China (e.g., the yearly numbers of cattle for meat production increased from 3.3 million in 1980 to 46.7 million in 2011, and numbers of sheep and goats increased 6-fold) (38), which would have resulted in an increase of the total population of infected animals, even with low-level constant seroprevalence in livestock. The spatial distribution of human brucellosis apparently overlaps with livestock density, especially high densities of sheep and goats (Figure 6), and high incidences of human brucellosis tended to occur most commonly in grasslands at moderate elevation, where sheep and goats are the predominant livestock (17,39). Another possible reason for the reemergence is the lack of vaccination, quarantine, and elimination of infected animals among backyard livestock. Moreover, the intensive modes of production, which accounted for the rearing of only 42.9% of cattle and 51.1% of sheep and goats in 2011, along with poor infrastructure and lack of high-standard and standardized protocols for maintaining good hygiene within the production cycle, might also result in increasing infections (38). Hence persons engaged in livestock husbandry, production, and trade are at high risk for brucellosis infection because of occupational exposure. Additionally, animal products supplied from brucellosis-endemic areas that have not undergone quarantine or pasteurization might increase the risk for infection in nonoccupational populations and urban settings, taking a longer time from illness to diagnosis for imported cases in southern China, which might create extra challenges for disease prevention and case management (19,20,40). Therefore, susceptible livestock animals as the host and infection source for human infections are key to brucellosis prevention and control.

Vaccination is an effective method to reduce brucellosis incidence in livestock and correlates to a decrease in reported human cases, although no vaccines are available for humans (37,41). Compared with those from other countries, new sequence types of *Brucella* strains have been found in China, and the predominant biovars and sequence types of *Brucella* strains has changed during past half century in some regions (42). Thus, new livestock vaccines different from those recommended by the International Office of Epizootics are needed in China.

Targets have been set for brucellosis control in animals in 2015 and 2020 to reach the standard of control and decontamination by province (online Appendix Table 7) (25). However, to achieve the targets for brucellosis reduction, improvements are needed in socioeconomic parameters, diagnostic and notification systems in animals and humans, and the high prioritization for eliminating the disease in livestock. The continuing existence of human (and animal) brucellosis in China, with potential for further increases in incidence, indicates that the control of brucellosis will not be an easy task without taking a One Health approach,
Figure 6. Geographic expansion of human brucellosis across counties and distribution of land covers (28) and density of sheep and goats (29), mainland China, 2004–2014.
integrating health professionals from the human and animal sectors and administrations. This effort extends beyond medical and veterinary duties and encompasses economic and even political factors (2).

Our study has some limitations. First, the data used were collected from passive public health surveillance that might be influenced by changes in surveillance protocols, such as modifications in case definitions and laboratory tests, reporting methods, and availability of health facilities and laboratory diagnostics over the years (online Technical Appendix Table 4). Second, individual case data were not reported before 2004, so demographic characteristics, laboratory confirmation, and case distribution could be analyzed only for 2004–2014. Third, data on Brucella strains and biotypes and on the varied clinical presentations, including asymptomatic brucellosis infections among humans, were unavailable in this study to explore the distribution of pathogens and the severity of disease. However, the data we used were the most nationally comprehensive for human brucellosis in China.

In view of the reemergence of brucellosis in mainland China and the high incidence, further studies should be conducted to explore the drivers of this situation during the past 2 decades. Livestock–human seroprevalence surveys are needed to understand the correlation between livestock and human brucellosis, to identify the most important animal host species, and to attempt to regress human seroprevalence to livestock prevalence or simply livestock numbers (43). The application of spatial–temporal transmission modeling, linking environmental and socioeconomic variables and density (e.g., http://www.worldpop.org) and mobility of livestock and humans (44) with the seroprevalence data, would improve understanding of the factors driving reemergence of brucellosis and enable us to better predict the risk in space and time. This information could further inform on potential causes of reemergence (45,46) and the economics of control in relation to ongoing control activities in China.

In summary, on the basis of notifiable surveillance data in mainland China during 1955–2014, we found that human brucellosis has reemerged since the mid-1990s, and the affected areas have expanded from northern to southern China, especially since 2004. Control strategies in animals and humans should be adjusted to account for these changes by adopting a One Health approach at different levels. Further research is warranted to explore the drivers behind the reemergence.

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References


SYNOPSIS


Multidrug-Resistant Candida haemulonii and C. auris, Tel Aviv, Israel

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Candida auris and C. haemulonii are closely related, multidrug-resistant emerging fungal pathogens that are not readily distinguishable with phenotypic assays. We studied C. auris and C. haemulonii clinical isolates from 2 hospitals in central Israel. C. auris was isolated in 5 patients with nosocomial bloodstream infection, and C. haemulonii was found as a colonizer of leg wounds at a peripheral vascular disease clinic. Liberal use of topical miconazole and close...
contact among patients were implicated in *C. haemulonii* transmission. *C. auris* exhibited higher thermotolerance, virulence in a mouse infection model, and ATP-dependent drug efflux activity than *C. haemulonii*. Comparison of ribosomal DNA sequences found that *C. auris* strains from Israel were phylogenetically distinct from isolates from East Asia, South Africa and Kuwait, whereas *C. haemulonii* strains from different countries were closely interrelated. Our findings highlight the pathogenicity of *C. auris* and underscore the need to limit its spread.

*Candida* species are leading causes of bloodstream infection (BSI) in hospitalized patients, particularly those in intensive care units who are exposed to broad-spectrum antimicrobial drugs, indwelling vascular catheters, parenteral nutrition, abdominal surgery, and immunosuppressive agents (1,2). High rates of attributable death have been associated with delayed initiation of appropriate antifungal treatment (3,4). This problem is compounded by the emergence of drug-resistant *Candida* species, notably *C. glabrata*, in many hospitals (5).

*C. auris* is an emerging opportunistic pathogen, first reported in 2009 as an isolate from the external ear of an inpatient at a hospital in Japan (6). It has since been identified as a cause of nosocomial BSI in numerous countries in East Asia, the Middle East, Africa, and Europe (7–11). *C. auris* might be resistant to multiple classes of antifungal agents and apparently has a potential for person-to-person transmission, challenging clinicians and infection control teams (12). *C. auris* often is misidentified by traditional microbiological methods as *C. haemulonii*, a phylogenetically related drug-resistant *Candida* species that also is increasingly reported in healthcare facilities worldwide (13).

We report on the detection of multidrug-resistant *C. auris* and *C. haemulonii* in clinical specimens in Tel Aviv, Israel, and specifically on the emergence of *C. auris* as a cause of nosocomial BSI. We highlight distinct clinical and epidemiologic characteristics of these 2 species and present experimental evidence for differences in their virulence.

**Materials and Methods**

We undertook this study after *C. auris* BSI was detected in 4 patients during May–October 2014 at the Tel Aviv Sourasky Medical Center (TASMC), a tertiary-level hospital in Tel Aviv. An additional *C. auris* bloodstream isolate was recovered in April 2015 from a patient at the Wolfson Medical Center in Holon (southern Tel Aviv metropolitan area). No additional *C. haemulonii* or *C. auris* isolates were identified through inquiries at additional clinical microbiology laboratories in Israel.

The TASMC Institutional ethics committee approved this study. Need for informed consent was waived because of the observational and anonymous nature of the study.

**Clinical Candida isolates**

*Candida* isolates recovered from clinical specimens were identified at the TASMC Clinical Microbiology Laboratory by growth characteristics on CHROMagar *Candida* (CHROMagar, Paris, France) and the Vitek 2 YST ID system (bioMérieux, Marcy-l’Étoile, France). The Vitek 2 database does not include *C. auris*, and this species is routinely misidentified as *C. haemulonii* (13). We therefore reviewed all isolates identified as *C. haemulonii* during January 2009–August 2015. Isolates recovered during May 2014–August 2015 were stored at -20°C and subjected to further analyses. We assessed thermotolerance by plating serial dilutions of yeast culture on Sabouraud dextrose agar (SDA) plates and assessing growth after 24 h incubation at 35°C–42°C.

**Sequence-Based Species Identification**

*Candida* isolates were streaked on SDA plates to ensure purity. We extracted DNA by using PrepMan Ultra solution (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions and amplified and sequenced the internal transcribed spacer (ITS) and D1/D2 large subunit (LSU) ribosomal DNA segments by using primer pairs ITS1/ITS4 and LSU1/LSU2 (14), respectively. PCR was performed in 0.2-mL tubes with 0.4 μmol/L or 0.2 μmol/L of each primer for ITS and LSU, respectively; 10 μL Larova Red Load Taq Master Mix (5×) (Larova, Jena, Germany); and ≈25 ng of template. PCR conditions were 95°C for 4.5 min (denaturation), 40 cycles of 95°C for 30 s (denaturation), 55°C (ITS) or 48°C (LSU) for 30 s (annealing), 72°C for 1 min (extension), and a final extension stage of 72°C for 7 min. PCR products were resolved on 0.7% agarose gel and stained with SERVA DNA stain clear G (Tamar, Mevseret Zion, Israel). Products were cleaned with QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced at Hy-Labs (Rehovot, Israel). We then aligned ITS and LSU sequences with the matching type strain sequences for CBS5149T (*C. haemulonii*), CBS7798T (*C. duobushaemulonii*), CNM-CL7239T (*C. haemulonii* var. *vulnere*), CBS10099T (*C. pseudohaemulonii*), and CBS10913T (*C. auris*). A similarity score of >98% in both ITS and LSU sequences was required for species-level identification. All new sequences were deposited in GenBank (Table 1, https://www.ncbi.nlm.nih.gov/EID/article/23/2/16-1486-T1.htm).

**Phylogenetic Analyses**

We aligned ITS and LSU sequences of *C. haemulonii* and *C. auris* isolates by using MUSCLE (15) and generated phylogenetic trees with the neighbor-joining method (16), using the Kimura 2-parameter method to compute evolutionary distances (17). We tested phylogeny with the bootstrap method (500 replicates) and used *Schizosaccharomyces pombe* strains ATCC 38366 and CBS 356 as outgroups. Evolutionary analyses were performed in MEGA7 (18).
Patient Characteristics
We retrospectively reviewed the medical records of patients from whom Vitek-identified *C. haemulonii* was recovered from any site and recorded patient demographics, hospital unit, co-morbidities, medications, and clinical characteristics by using a structured form. We found 40 patient-specific *C. haemulonii* isolates, 20 (50%) of which originated from patients receiving care at an outpatient peripheral vascular disease clinic (clinic A). We therefore conducted an investigation at clinic A, which included review of patient treatment protocols, observed patient care, and surveillance mycologic cultures from environmental surfaces, wound irrigation solutions, dressings, and the hands of medical staff. To define risk factors for *C. haemulonii* colonization, we conducted an unmatched case–control study using 40 noncolonized patients followed at clinic A as controls.

Antifungal Susceptibility Testing
We determined MICs of fluconazole, itraconazole, voriconazole, posaconazole, amphotericin B, anidulafungin, micafungin, caspofungin, and flucytosine by broth microdilution using Clinical and Laboratory Standards Institute methods (19). Results were read after 48 h for azoles, amphotericin B, and flucytosine and after 24 h for echinocandins.

Rhodamine 6G Efflux
To assess ABC-type drug transporter activity, we determined glucose-induced efflux of rhodamine 6G, as described previously (20,21). We grew *Candida* isolates to log-phase in liquid yeast extract glucose at 35°C. We then collected yeast cells by centrifugation, transferred 10⁶ cells to 20 mL fresh yeast extract glucose, and incubated them at 27°C for an additional 2 h. Next, we collected yeast cells by centrifugation, washed them twice in phosphate-buffered saline (PBS), and added 10 mL PBS containing 15 mM rhodamine 6G without glucose to the pellets. Suspensions were vortexed and incubated at 27°C for 90 min to enable rhodamine 6G uptake under carbon source-depleted conditions. We then collected cells by centrifugation, washed them twice in PBS, and suspended them in 750 μL PBS in microfuge tubes. To start rhodamine 6G efflux, we added 250 μL PBS with 8 mmol/L glucose. We prepared control tubes with glucose-free PBS, removed them after 5, 15, and 25 min of incubation at 35°C, and measured fluorescence in 200-μL aliquots of supernatant by using a spectrophotometer at excitation 527 nm and emission 555 nm (Synergy HT, BioTek, Winooski, VT, USA).

Mouse Model of Disseminated Candidiasis
To assess the relative virulence of *C. haemulonii* and *C. auris*, we determined the lethality and tissue fungal loads of representative strains in a mouse model of hematogenous disseminated candidiasis. Experiments were approved by the TASMC Institutional Animal Care and Use Ethics Committee. We used cyclophosphamide (150 mg/kg intraperitoneally) to immunosuppress 6-week-old female BALB/c mice weighing 16–20 g (Harlan, Rehovot, Israel) 3 days before and on the day of infection. *Candida* cells were collected from log-phase culture on the day of infection and washed twice in sterile PBS. Mice were infected in groups of 10 with *C. haemulonii* strain TA001-14, *C. auris* strain TA005-14, and *C. albicans* strain CBS 8837. We injected 100 μL of PBS containing 7 × 10⁷ yeast cells intravenously into the lateral tail vein of each animal. A control group received intravenous injection of cell-free PBS. Death was assessed over 30 days. Kidney tissue fungal loads were determined in separate experiments where mice were similarly immunosuppressed and infected intravenously with 4 × 10⁷ yeast cells/100 μL PBS. Seven days after infection, mice were killed by CO₂ inhalation, and kidneys were excised aseptically, weighed, and homogenized in a TissueLyser (QIAGEN). Homogenates were serially diluted 10- to 1,000-fold in sterile saline and plated on SDA. We calculated fungal loads (CFU per gram of tissue) from colony counts after 48 h incubation at 35°C.

Statistical Analyses
We compared continuous variables between case and control patients using the Student t test for normally distributed variables and the Wilcoxon rank-sum test for non–normally distributed variables. We compared dichotomous variables using Fisher’s exact test. Rhodamine 6G efflux, expressed as relative fluorescence units, was computed for each *Candida* strain and compared by using 1-way analysis of variance. We used Dunnett’s multiple comparisons test to compare relative fluorescence unit values of specific *C. haemulonii* and *C. auris* strains with averaged control values of 5 *C. glabrata* strains. Survival curves of mice infected with different *Candida* strains were plotted by using the Kaplan-Meier method and compared with the log-rank test. We considered 2-tailed p values <0.05 statistically significant.

Results
Sequence-Based Identification
We identified 40 patient-specific *Candida* strains as *C. haemulonii* by the Vitek-2 YST ID system during January 2009–July 2015. Isolates were recovered from wounds (n = 24), urine (n = 9), blood (n = 5), and central venous catheter tips (n = 2). Of these, 9 isolates recovered during May 2014–May 2015 were available for analysis; we identified 6 (including the 5 blood isolates) as *C. auris* and 3 as *C. haemulonii* by ITS and LSU sequencing (Table 1). Sequences were 100% identical among strains of each species. *C. auris* strains from Israel shared 98.6% and 98.3% similarity of ITS and LSU sequences, respectively, with the
C. auris type strain CBS10913. C. haemulonii strains were 100% identical to C. haemulonii CBS5149 on the basis of ITS and LSU sequences.

Phylogenetic trees based on ITS and LSU sequences showed that the C. auris isolates from Tel Aviv are distinct from other isolates from East Asia, Africa, and the Middle East. Specifically, isolates from Israel showed 98.6% similarity of ITS and LSU sequences with the India clone, represented by CBS12768, 96.2% similarity with the South Korea clone, and 96.7% similarity with strain CH1 from Kuwait. In contrast, ITS and LSU sequences from Israel C. haemulonii strains were 100% homologous with C. haemulonii from South Korea, Brazil, and Kuwait, suggesting worldwide predominance of a single C. haemulonii clone (Figure 1).

Clinical Features
Eight of 9 patients with sequence-validated isolates were hospitalized at TASMC (Table 1). An additional patient with C. auris infection was hospitalized at the Wolfson...
Medical Center, but was receiving regular care for HIV infection at TASMC. All 3 C. haemulonii isolates were recovered from chronic leg ulcers of patients with peripheral vascular disease, 2 of whom were treated at vascular outpatient clinic A. Five of 6 C. auris isolates represented BSI: 3 patients had vascular catheter–related candidemia, and 2 had primary nosocomial candidemia of unclear origin. Two of 5 patients with C. auris BSI died during hospitalization.

We reviewed the medical records of 40 patients with Vittek–identified C. haemulonii cultures. Thirty-three (83%) were male. Median age was 74 years (range 37–91 years). Nineteen (48%) had peripheral vascular disease, 20 (50%) had diabetes mellitus, 22 (55%) had ischemic heart disease, and 11 (28%) had end-stage renal disease. Twenty patients (50%) were receiving regular care at clinic A, representing 8% (20/261) of all clinic patients. In all 20 patients, C. haemulonii had been recovered from chronic leg ulcers, and none had documented wound infection at the time of culture. Cultures of environmental surfaces, medical devices, dressings, irrigation solutions, and hands of medical staff were negative for yeast. Compared with 40 control patients who were not carriers of C. haemulonii, carriers were older, had a lower glomerular filtration rate, and were more likely to be male and to have ischemic heart disease (Table 2). Observations revealed a practice among medical staff of routinely applying topical miconazole cream to chronic ulcers without evidence of infection. Periodic wound cultures were obtained regularly, irrespective of signs of ulcer inflammation or purulence. Multiple social interactions were noted among patients in a single room where wound care was performed.

**Antifungal Susceptibility**

All 3 C. haemulonii and C. auris isolates had fluconazole MICs >8 mg/L (range 16–64 mg/L; MIC50 32 mg/L). MICs of other azoles were also elevated: itraconazole, 0.25 to >50 (range 16–64 mg/L; MIC50 2 mg/L); voriconazole, 0.25–1 mg/L (MIC50 0.5 mg/L); and posaconazole, 0.06 to >8 mg/L (MIC50 0.25 mg/L). Amphotericin B MIC ranged from 1 to 2 mg/L for C. auris isolates and from 2 to 8 mg/L for C. haemulonii isolates. All isolates appeared susceptible to anidulafungin (MIC 0.03 mg/L) and all isolates except 1 C. haemulonii were susceptible to micafungin (MIC 0.12–0.5 mg/L; MIC50 0.12 mg/L). Caspofungin MIC was 0.5 mg/L for all isolates. All isolates except 1 C. auris were susceptible to flucytosine (Table 3).

**Rhodamine 6G Efflux**

Rhodamine 6G is a substrate of ATP binding cassette (ABC) type efflux pumps responsible for multiazole resistance in C. glabrata. C. haemulonii and C. auris strains exhibited robust rhodamine 6G efflux activity when glucose (8 mM) was present in the medium, consistent with ABC-type transport. Rhodamine 6G efflux of C. auris strains was significantly greater than that of C. glabrata strains (14.4–, 10–, and 6.7-fold higher at 5, 15, and 25 min, respectively; p<0.0001) and C. haemulonii (3.8–, 3.8–, and 3.6-fold higher at 5, 15, and 25 min, respectively; p<0.0001). C. haemulonii showed greater rhodamine 6G efflux than C. glabrata (3.8–, 2.7–, and 1.9-fold higher at 5, 15, and 25 min, respectively (p<0.0001) (Figure 2).

**Thermotolerance**

Survival and growth at physiologic temperature are prerequisites for microbial invasion and pathogenicity. C. haemulonii isolates grew well at 35°C, but growth at 37°C was poor or absent, and no growth occurred at 40°C and 42°C. In contrast, growth of C. auris isolates at 37°C and 40°C was similar to that of C. albicans, and 4 of 6 isolates grew at 42°C (Figure 3).

**Virulence in a Mouse Model of Disseminated Candidiasis**

We compared the virulence of C. auris and C. haemulonii isolates in a mouse model of hematogenous disseminated candidiasis. C. haemulonii was completely nonvirulent in this model; 100% of mice survived 12 days after inoculation with no visible signs of illness. In contrast, inoculation with C. auris resulted in rapid death and only 20% survival 5 days after infection (p = 0.0002, log-rank test). Death of mice infected with C. auris was significantly less rapid than that of mice infected with C. albicans (median survival 4 d

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**Table 2. Comparison of colonized and noncolonized patients with Candida haemulonii, clinic A, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases, n = 20</th>
<th>Controls, n = 40</th>
<th>Odds ratio (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, y (range)</td>
<td>77.5 (44–91)</td>
<td>63.0 (43–94)</td>
<td>NA</td>
<td>0.015</td>
</tr>
<tr>
<td>Male sex</td>
<td>18 (90)</td>
<td>23 (57.5)</td>
<td>6.65 (1.26–65.0)</td>
<td>0.017</td>
</tr>
<tr>
<td>Median time in clinic A, mo (range)</td>
<td>40 (8–228)</td>
<td>48 (8–192)</td>
<td>NA</td>
<td>0.44</td>
</tr>
<tr>
<td>eGFR, mL/min/1.73m2, mean ± SEM</td>
<td>47.7 ± 5.56</td>
<td>62.9 ± 3.61</td>
<td>NA</td>
<td>0.022</td>
</tr>
<tr>
<td>Chronic kidney disease, stage 3–4</td>
<td>13 (65)</td>
<td>15 (37.5)</td>
<td>3.05 (0.88–11.2)</td>
<td>0.057</td>
</tr>
<tr>
<td>Dialysis</td>
<td>6 (30)</td>
<td>4 (10)</td>
<td>3.85 (0.76–21.0)</td>
<td>0.069</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>13 (65)</td>
<td>10 (25)</td>
<td>5.5 (1.51–21.1)</td>
<td>0.003</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>13 (65)</td>
<td>19 (47.5)</td>
<td>2.05 (0.59–7.37)</td>
<td>0.27</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>17 (85)</td>
<td>35 (87.5)</td>
<td>0.80 (0.13–5.84)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*Values are no. (%) patients except as indicated. eGFR, glomerular filtration rate, estimated using the Modification of Diet in Renal Disease (MDRD) equation (22); NA, odds ratio is not applicable for continuous variables.
and 1 d, respectively; p = 0.01; Figure 4, panel A). Kidney tissue fungal load correlated with survival rates. Specifically, we recovered no viable yeast cells from kidneys of mice inoculated with C. haemulonii, whereas infection with C. auris and C. albicans yielded median tissue loads of 5.9 × 10⁶ CFU/g and 7.1 × 10⁶ CFU/g, respectively (p < 0.0001; Figure 4, panel B). Histopathologic analysis showed yeast cell aggregates in kidneys of C. auris–inoculated mice, distinct from tissue invasive hyphae observed in C. albicans–infected kidneys (Figure 4, panel C).

Discussion
Concern about the international emergence and spread of C. auris as a cause of invasive infection in hospitals stems from 3 characteristics of this opportunistic pathogen (12,13): 1) resistance to multiple antifungal drugs and possibly to all major classes of systemic antifungal drugs; 2) horizontal transmission among hospitalized patients, leading to nosocomial outbreaks (8,10,11,13); and 3) high associated death rates (7,8,10). C. auris and C. haemulonii are phylogenetically related species in the Metschnikowia clade that share a propensity for multidrug resistance. We identified C. auris and C. haemulonii in 2 hospitals in Israel and highlighted clinical and experimental evidence for differences in the drug-susceptibility patterns, drug efflux activity, pathogenicity, and global phylogenetics of these 2 species.

In our study, C. auris and C. haemulonii had high MICs of azoles and amphotericin B. Echinocandin MICs were within the susceptible range. An amphotericin B epidemiologic cutoff value of 2 mg/L previously was established (23), but clinical correlation between amphotericin B MIC and treatment outcomes is lacking (24). Compared with C. auris, C. haemulonii isolates had higher amphotericin B MICs. The relevance of these resistance patterns to treatment strategies remains to be determined.

ABC-type efflux activity, as evidenced by Rhodamine 6G transport, was significantly greater among C. auris than C. glabrata isolates. This observation provides a mechanistic basis for the intrinsic resistance of C. auris to azoles and is consistent with the identification of multiple putative transporter-encoding genes belonging to the ABC and major facilitator gene families in the C. auris genome (25).

![Figure 2. Comparison of rhodamine 6G efflux over time among Candida isolates from Tel Aviv, Israel. Rhodamine 6G efflux is expressed as relative fluorescence units measured in culture supernatants after the addition of 8 mM glucose. Statistical significance was measured with 1-way analysis of variance and Dunnell’s post-test comparing each C. haemulonii and C. auris strain with the averaged value of C. glabrata strains at the corresponding time point. White bars, 5 min; gray bars, 15 min; black bars, 25 min. *p < 0.0001.](image-url)
Of Vitek-identified *C. haemulonii* isolates at TASMC, 50% were wound cultures from patients cared for at clinic A. That most of these isolates were not available for sequencing is a limitation of our study. However, we identified the 2 *Candida* isolates from clinic A patients that were available by ITS and LSU sequencing as *C. haemulonii*, and all 3 sequence-identified *C. haemulonii* isolates were recovered from leg ulcers of patients with peripheral vascular disease. Colonization of patients treated in close proximity in 1 room strongly suggests person-to-person transmission and supports interim guidelines for contact isolation (26). However, we were unable to identify an environmental reservoir of *C. haemulonii*. We suggest that topical application of miconazole to wounds most likely caused selective pressure and facilitated the overgrowth of *C. haemulonii*. After this investigation and termination of routine topical azole use, no additional cases of *C. haemulonii* were detected in clinic A during April 2015–July 2016.

We recovered 5 of 6 sequence-identified *C. auris* isolates from patients with nosocomial BSI. In contrast, all *C. haemulonii* isolates were cultured from superficial wounds. This observation reflects the global epidemiology of these species. *C. haemulonii* has been isolated from chronic leg ulcers of patients in India and Brazil (27,28). *C. auris* has caused outbreaks of BSI in the United Kingdom (13), India (8), Kenya (11), South Africa (9), and South Korea (29), whereas reports of *C. haemulonii* as an agent of BSI have been infrequent (27,29–32). Moreover, *C. auris* fungemia is associated with high death rates (8,10), contrasting with reports of patients surviving prolonged *C. haemulonii* fungemia (31). Fatal *C. haemulonii* fungemia, although rare, has been reported in neonates and in patients with cancer and neutropenia (27,32).

In our study, *C. auris*, but not *C. haemulonii*, grew at 37°C–42°C and exhibited lethality and tissue invasion in a mouse model of invasive candidiasis only slightly less than those of *C. albicans*, the prototypical pathogenic *Candida* species. Both *C. auris* and *C. haemulonii* are unable to form hyphae, which contribute to virulence in *C. albicans*. Formation of large aggregates resulting from failure of budding yeast to separate has been noted in some *C. auris* isolates (33). We observed distinct yeast cell aggregates in the kidneys of mice with lethal *C. auris* infection, which suggests that aggregation might be a mode of immune evasion and persistence in tissue. The *C. auris* genome
contains *C. albicans* gene orthologs, such as secreted proteinases and mannosyl transferases, which might have roles in pathogenesis (25). However, *C. auris* has a genome that is highly divergent from those of other *Candida* species, and most of its genes have not yet been characterized (25).

Ribosomal DNA sequences were identical among *C. haemulonii* strains from Israel, Kuwait, East Asia, South America, and the United States. In contrast, the global phylogenetics of *C. auris* demonstrate distinct clones for each country, indicating greater genomic diversity for this species. Further study is needed to establish whether the divergence of *C. auris* clones translates into country-specific patterns of invasiveness, virulence, and drug resistance. Our findings affirm the need for intensified vigilance and mobilization of infection control measures to limit the spread of *C. auris*.

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### References


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**Figure 4.** Differing virulence of *Candida auris* and *C. haemulonii* assessed in a mouse model of hematogenous disseminated candidiasis. Virulence was assessed in immunosuppressed BALB/c mice after intravenous injection of yeast cell suspension. A) Survival curves showing significantly shorter survival of mice infected with *C. albicans* than *C. auris* and no death among mice infected with *C. haemulonii*. B) Kidney fungal load (CFU per gram of tissue) shown to be significantly higher in mice infected with *C. albicans* than in those infected with *C. auris*, whereas no viable yeast was cultured from kidneys of mice infected with *C. haemulonii*. C) In mouse kidneys, *C. auris* cells formed aggregates and no hyphae (top) whereas *C. albicans* formed extensive tissue-invasive hyphae (bottom); *C. haemulonii* was not detected in tissue sections (middle). Grocott methenamine silver staining, original magnification ×100 for panels, ×400 for insets.
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Streptococcus suis sequence type 7 emerged and caused 2 of the largest human infection outbreaks in China in 1998 and 2005. To determine the major risk factors and source of the infections, we analyzed whole genomes of 95 outbreak-associated isolates, identified 160 single nucleotide polymorphisms, and classified them into 6 clades. Molecular clock analysis revealed that clade 1 (responsible for the 1998 outbreak) emerged in October 1997. Clades 2–6 (responsible for the 2005 outbreak) emerged separately during February 2002–August 2004. A total of 41 lineages of S. suis emerged by the end of 2004 and rapidly expanded to 68 genome types through single base mutations when the outbreak occurred in June 2005. We identified 32 identical isolates and classified them into 8 groups, which were distributed in a large geographic area with no transmission link. These findings suggest that persons were infected in parallel in respective geographic sites.

Human infections caused by Streptococcus suis have been recognized as a global public health and economic problem in the swine industry (1–3). These infections afflict persons in close contact with infected pigs or pork-derived products (4,5). Although sporadic cases of S. suis infections in humans had been reported worldwide previously, in the summer of 2005, China recorded the largest and most highly diffused outbreak of S. suis infection in humans, with 215 cases reported and 39 deaths (6,7). Although the overall case-fatality rate was 18%, it reached 63% among patients with streptococcal toxic shock–like syndrome (8,9). The causative pathogen was identified as sequence type (ST) 7, which had evolved from ST1 to become a highly virulent strain with epidemic potential. So far, S. suis ST7 has only been isolated in China (7,9). The outbreak cases were widely distributed among persons in 203 villages of 12 cities in Sichuan Province. Outbreak investigations by the Chinese Center for Diseases Control and Prevention identified and confirmed 1 case per village in 194 villages (6). The outbreak appeared to be caused by pig-to-human direct transmission (6). A policy of strictly prohibiting backyard slaughtering was implemented, which ended the outbreak (3,10). However, the reasons why the outbreak reached such a large scale remained a mystery.

We used whole-genome sequencing (WGS) to dissect this outbreak through sequencing of 85 isolates from patients and 7 isolates from diseased pigs associated with those patients (7). The outbreak was probably caused by infected piglets and was amplified by the industrial scale of piglet supply operations in China. These findings uncovered a unique public health threat in China brought about by economic development.

Materials and Methods

Isolates
We selected 92 isolates from the 2005 outbreak investigation for genome sequencing and analysis, including 85 isolates from patients and 7 from 6 diseased pigs (7), in addition to 2 isolates from a patient and a diseased pig from a 1998 outbreak in Jiangsu Province and 1 from a patient with a sporadic case in Jiangsu in 1999 (11). These 85 isolates were selected from 203 villages of 12 cities in Sichuan Province.
human isolates represented 39.5% of all cases reported and were distributed among 10 of the 12 affected cities, including the 4 cities accounting for 90.7% (195/215) of all cases and 80% (68/85) of all isolates from patients. We obtained these isolates over a 21-day period during the outbreak, which lasted 41 days.

We used the complete genome sequence of isolate SC84, which was sequenced previously (12), as reference. We typed all of the isolates were typed as minimum core genome type 1 (13), sequence type 7 (14), and showed an identical pulsed-field gel electrophoresis (PFGE) pattern with restriction enzyme Smal (7). We obtained information on each patient infected by a given isolate from the enhanced surveillance and investigation we conducted in 2005, including demographic and clinical characteristics and information on type of exposure and place of residence. We did so by searching the original records and database from the previous investigation (6,7).

WGS and Analysis of Single-Nucleotide Polymorphisms
We extracted genomic DNA by using Wizard Genomic DNA Purification Kit (Promega, WI, USA). To obtain the genome sequences, we constructed 500-bp libraries and performed WGS by using an Illumina Genome Analyzer IIx system (Illumina, San Diego, CA, USA) to produce 100-bp paired-end reads. We then mapped the high throughput reads to the reference genome of S. suis strain SC84 (GenBank accession no. NC_012924) by using SOAP2 and detected single-nucleotide polymorphisms (SNPs) by using SOAPsnp version 1.03 (12,15,16). We named the SNPs by using our automatic pipeline described previously (13). We constructed the outgroup by using the consensus base of 6 non-ST7 isolates, RC1, YS14, 14636, YS12, S15, and GZ1 (13). We conducted recombination analysis by using RDP3 (17) and constructed phylogenetic trees by using the Bayesian evolutionary method. We determined the time of divergence of a branch and substitution rates by using BEAST version 1.8.2 (18). We found the best-fit evolutionary model for the dataset to be the TN93 model, with a normal distribution of among-site rate heterogeneity and a proportion of invariant sites. We selected a relaxed (uncorrelated exponential) molecular clock and an extended Bayesian Skyline tree prior for the analysis. We performed 3 independent runs with sampling every 10,000 generations of 100,000,000 Markov chain Monte Carlo chains and analyzed the output by using the Tracer module (18). We then deposited the sequencing data in the GenBank database (accession no. SRP064815).

Geographic Information Analysis
We obtained geographic information for administrative divisions, including the 4 levels of village, town, county, and city, and national and provincial roads and highways in Sichuan. We determined the locations of piglet breeding companies (PBC) in Sichuan in operation before 2005 by using a database maintained by the Animal Husbandry Agency of Sichuan (http://www.scxmsp.gov.cn). We defined geographic distance as the shortest distance between any 2 geographic sites, such as villages, PBCs, or highways. We calculated the means of geographic distances to estimate the ranges which could be affected. We exhibited the geographic distributions of patients, highways, and PBCs by using a visualized digital earth system (VGEGloba3D) (19), which was developed by our geographic information team from the Institute of Remote Sensing and Digital Earth at the Academy of Sciences of China. We performed statistical analyses by using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). We tested pairwise comparison of mean distances by using the Student t test and multiple comparisons by using the Kruscal-Walls test. We also tested the association between the geographic distribution of clades from the 2005 outbreak and PBCs in the affected area by using a χ² test. We considered a p value ≤0.05 to be statistically significant.

Results
Genome Sequencing of S. suis ST7 Outbreak Isolates
We sequenced 85 human isolates and 7 pig isolates from the 2005 outbreak, 2 ST7 isolates from the 1998 outbreak, and 1 isolate from 1999 from a patient with sporadic infection in Jiangsu, where S. suis ST7 was first isolated in China. We obtained ≈596–1,081 Mb reads of high quality per isolate, which covered on average 284–516 (400.4 ±115.7) fold of the complete genome of SC84. All of the assembled genomes covered >98.0% of the reference genome, except for isolate SC218, which covered 94.2%. When mapping these genome sequences to that of SC84, we identified 160 SNPs, with 4–29 SNPs per genome (online Technical Appendix 1 Table 1, https://wwwnc.cdc.gov/EID/article/23/2/16-0297-Techapp1.xlsx). Overall, we identified 4.1 SNPs per genome, with 1.3 SNPs among 3 isolates from 1998 Jiangsu outbreak and 3.8 SNPs per genome among the 92 isolates from the 2005 Sichuan outbreak. Among the 160 SNPs, 126 were located in 115 genes, including 35 synonymous SNPs and 91 nonsynonymous SNPs, whereas the remaining 34 SNPs were located in intergenic regions. Most genes had 1 SNP only. However, we identified 3 SNPs each for genes SSUSC84_0178 and SSUSC84_1795, which encode a hypothetical protein and serine protease, respectively. The 91 nonsynonymous sites were distributed among 84 genes. The number of nonsynonymous sites exceeded synonymous sites for all Sichuan isolates (6–18 vs. 3–7), leading to high ratios nonsynonymous to synonymous sites.
synonymous substitutions (dN/dS) ranging from 1.14 to 3.60 and indicating that positive evolutionary pressure during the evolution of ST7 in Sichuan.

**Phylogenetic Relationship of Outbreak-Associated Isolates**

We determined the phylogenetic relationships of the 95 isolates by using Bayesian evolutionary analysis (online Technical Appendix 2 Figure 1, https://wwwnc.cdc.gov/EID/article/23/2/16-0297-Techapp2.pdf). We classified these isolates into 6 clades (having 3, 6, 24, 3, 38, and 21 isolates, respectively), which were supported with multiple SNPs. We defined 21 SNPs supporting the clades as clade definition (CD) SNPs (online Technical Appendix 1 Table 1; online Technical Appendix 2 Figure 1) because they were present in all isolates of a given clade. However, we noted 2 exceptions; CD SNPs A656244G (SSUSC84_0604, synonymous) and A961560C (intergenic) had reverted to the reference base in isolates SC130 and SC218, respectively. We confirmed these 2 nucleotide mutations by PCR and sequencing. Of the 139 non–CD SNPs, 100 SNPs were isolate-specific SNPs, and the remaining 39 SNPs were shared by ≥2 isolates.

**Emergence of the 5 Clades of *S. suis* ST7 Responsible for the 2005 Outbreak**

By using all 160 SNPs and the known isolation dates for the sequenced isolates, we constructed a Bayesian tree to visualize the overall relationships between root-to-tip branch length and the divergence time of the major nodes. We estimated the substitution rate to be 8.58 × 10⁻⁷ substitutions per site per year (95% highest posterior density 1.98 × 10⁻⁷ to 1.55 × 10⁻⁶), corresponding to the accumulation of ≈1.8 SNPs per genome per year. The estimated time to the most recent common ancestor for ST7 lineage in China was May 1996. The 6 ST7 clades appear to have merged successively: clade 1 emerged in October 1997, clade 2 in February 2002, clade 3 in September 2002, clade 4 in November 2002, clade 5 in July 2003, and clade 6 in August 2004. All Sichuan ST7 isolates were of a single origin, and by the end of 2004, ST7 had diversified into 41 lineages and saw a rapid expansion to 68 genome types through single base mutations by June 2005, when the outbreak occurred (online Technical Appendix 2 Figure 1). Therefore, substantial diversity had already developed within ST7 before the outbreak.

**Geographic Distribution of Outbreak-Associated Isolates**

We classified 32 of 92 outbreak-associated isolates (25 from 25 patients and 7 from 6 diseased pigs) into 8 genome types, which we termed as epidemiologically informative (EI) groups (Figure 1). The whole genome sequences of all isolates in a given EI group were identical (i.e., whole-genome identical isolates), which provide critical information for epidemiologic tracing. The EI groups contained 2–8 isolates. All except 2 human isolates from these EI groups had geographic information associated with them based on the patients’ (or diseased pigs’) place of residence. All EI groups except EI 1 and EI 7 were distributed across different counties or cities. Most EI 6 isolates were confined to 1 county but spread across 5 villages in 2 different towns. In total, these isolates were distributed in 26 villages, 24 towns, 13 counties, and 6 cities. No epidemiologic evidence indicated that these whole-genome identical isolates from different towns were a result of direct transmission. The EI groups were highly unlikely to have originated from a single infectious source for the outbreak. To account for such strain distribution patterns, the most likely scenario was that these EI groups had been distributed widely before the outbreak (Figure 1). To achieve such a wide distribution, the most likely explanation is that piglets were infected with these strains in the breeding companies before distribution to the backyard farmers.

We further analyzed the geographic distribution of isolates with a genome difference of 1 SNP (Figure 2). This spread scenario might also apply because the single SNP diversity might have developed during the raising of the infected piglets by backyard farmers. We identified 2, 11, 28, and 19 isolates from clades 2, 3, 5, and 6, respectively, that can be grouped into 4 clonal complexes with 1 SNP difference (in addition to the EI groups), including almost two thirds (60/92) of outbreak-associated isolates (Figure 2). We further examined geographic distribution of the members of the clonal complexes. Except for 7 isolates that had no information associated with them regarding village, town, county, or city level, the clonal complexes were distributed among 49 villages, 42 towns, 22 counties, and 8 cities. This finding of the same clonal complexes (i.e., high genetic relatedness) with a wide geographic distribution lends further support to our hypothesis that the piglets in these cases had been distributed widely before the outbreak occurred. This scenario is likely given that the piglets were probably colonized by the pathogen before sale or distribution.

Our geographic and phylogenetic analyses support this hypothesis. The molecular clock analysis estimated that all Sichuan ST7 isolates were diversified into 41 lineages by the end of 2004, nearly 6 months before the outbreak, and rapidly expanded into 68 genome types, which were distributed throughout vast geographic areas (Figure 3). Typically, the production period of raising piglets until they are grown pigs fit for slaughter is ≈6 months. Therefore, those ST7 lineages most likely colonized the piglets before they arrived at the backyard farmers (Figure 3).
Outbreak of S. suis Human Infection, China

Association of Disease Spread with Major Roads and Piglet Breeding Companies

When we plotted the locations of villages in which 72 of the patients and six of the deceased pigs had resided (village information for 13 patients was missing), we saw clearly that most of the villages were along the major roads or highways (Figure 4). Therefore, we hypothesized that the pathogen was carried by piglets that were traded from the PBCs to backyard farmers by using public ground transportation (20). To test this hypothesis, we analyzed the geographic distances between the patients’ resident villages and major PBCs and between the patients’ resident villages and highways that existed at that time. Seven major PBCs (A–G) were in operation around the outbreak periods; of these, PBCs A and B and PBCs F and G were close to each other (within a range of 10 km), so we treated each pair as a single entity (i.e., PBC A/B and PBC F/G) (online Technical Appendix 1 Table 2).

Because piglets typically were distributed locally, we used the shortest distance of the patients’ resident villages to the nearest PBCs and highways to determine the likely sources of the clades (online Technical Appendix 1 Table 2). Notably, clade 4 had only 3 isolates for computation of the association. When the mean distance of...
each clade to nearest highways were compared with that of all isolates to each other, the association of clades 2, 3, 5, and 6 with their nearest respective highway (G5 and G42, G76, G76, and G76) were statistically significant (online Technical Appendix 1 Table 3). Most cases were in patients who lived within 50 km of the nearest highway (Figure 4; online Technical Appendix 2 Figure 2). Therefore, transportation of the piglets through the highways probably played a role in the spreading of the disease. We used similar methods to test the association of clades with PBCs. Clades 2, 3, 5, and 6 were statistically significantly associated with PBC A/B, PBC E, PBC D, and PBC D, respectively (online Technical Appendix 1 Table 3; online Technical Appendix 2 Figure 2).

**Discussion**

In this study, genome epidemiology was used to obtain a high-resolution dissection of the largest and most highly diffused human infection outbreak of *S. suis*, which occurred in Sichuan, Province, China, in 2005. Phylogenetic analysis with whole genome sequences divided the outbreak isolates into 68 lineages and 5 clades, showing substantial diversity among the outbreak isolates. The outbreak was most likely caused by the distribution of infected piglets from industrialized PBCs to farmers’ backyards across wide geographic regions. The wide distribution of piglets in the region and the massive backyard slaughtering of diseased pigs in a short period led to numerous parallel transmissions from infected pigs to humans (21). The outbreak in swine peaked around July 20, 2005, and *S. suis* caused 98% of the deaths among these pigs (6).

Phylogenetic analysis of the 92 isolates from the Sichuan outbreak, including 85 human isolates, showed that these isolates can be divided into 5 clades. The clades diverged at various points in time during February 2002–August 2004. Thus, the diversity was developed years before the outbreak and not during or months before the outbreak. However, most (59) of the outbreak isolates belonged to clade 5 and were closely related to clade 6 isolates. In addition, 8 groups of isolates (consisting of a total of 32 isolates) were identical in genome sequences; these were divided into 8 EI groups because they allowed epidemiological tracing given that identical isolates from diverse geographic regions implied the same source of infection, which most likely was the PBCs.

The timing of the outbreak was consistent with a scenario in which piglets were infected at the source rather than in the backyard. Piglets take 6 months to grow to adult pigs for slaughtering. The incubation period for the outbreak was nearly 6 months. The observation of isolates with 1 SNP difference (Figure 2) also supports a common source of *S. suis* infection given that our estimated mutation rate is 1.8 SNPs per genome per year. *S. suis* can also cause disease in pigs. The human outbreak followed a large swine outbreak that killed ≈10,000 backyard pigs, further indicating that the outbreak was caused by infection in pigs (6). The identification of 41 lineages belonging to 5 clades suggests that the diversity of the *S. suis* strain was...
Outbreak of S. suis Human Infection, China

Ye et al. (7) found that the small S. suis outbreak in Jiangsu in 1998 reported by Zhu et al. (11) was caused by ST7; therefore, they have suggested that the ST7 strains involved in the Sichuan outbreak originated phylogenetically from Jiangsu and were spread through interprovincial import of breeder pigs to Sichuan (11). The inclusion of 2 isolates associated with Jiangsu outbreak confirmed the evolutionary link of the 2 outbreaks. The Jiangsu outbreak isolates were clustered together as clade 1 and diverged earliest. The origin of the Jiangsu and Sichuan strains dates back to 1996, and ST7 very likely spread across the provinces through carriage by breeder pigs.

Previous studies found that all of the outbreak isolates were highly homogenous and belonged to ST7 (7). Further analysis with PFGE, which is considered to be a gold standard, showed that the 2005 outbreak isolates belonged to the same PFGE pattern (7). During the outbreak investigation in 2005, the outbreak was found to be caused by a single homogenous clonal strain (7). Our genomic data provided much higher resolution to reveal a high level of

Figure 3. Geographic spread of the 5 outbreak clades of Streptococcus suis minimum core genome type 1 sequence type 7 before and during outbreak of S. suis human infections, Sichuan Province, China, 2005. Phylogenetic relationship of isolates within a clade is detailed in Technical Appendix 2 Figure 1 (https://wwwnc.cdc.gov/EID/article/23/2/16-0297-Techapp2.pdf). Lineages that developed before 2005 are marked with dashed lines. Clades are displayed separately around the map for better visualization of their geographic distribution. Locations are marked by bubbles in different colors (clades 2–6 are purple, red, blue, green, and orange respectively). The isolates in the same epidemiologically interesting group are collapsed at the tip of the tree and identified by their group number. Bubbles in the red ellipse on the map represent isolates from the most concentrated outbreak region of Ziyang city; red plots on the tree represent these isolates; stars represent the piglet breeding companies.

developed in the PBCs, thus explaining the heterogeneity of strains from the same source before the outbreak.

Our mapping of the transportation route with the locations of the 5 S. suis clades and PBCs further support the distribution of infected piglets. In particular, PBC D was associated with clades 5 and clade 6. However, no isolates were available from the company to confirm the link. Overall, our integration of the genomic data and the geographic data explains the highly diffused pattern of the Sichuan outbreak.
heterogeneity within the outbreak involving 5 clades that had developed well before the outbreak occurred. Our findings also exposed the inadequacy of PFGE in tracing S. suis ST7 transmission.

The outbreak was an unforeseen consequence of economic development. To increase pork production, imported pig breeds replaced local breeds in China, and piglets were produced through large companies and distributed to backyard farmers. This combination of practices has been commonplace in Sichuan Province and many other parts of China. The farmers receive piglets from large industrial-scale companies to raise in their small backyards under poor hygienic conditions. In Sichuan, a sizable swine population was found in small backyard farms, and nearly every family was keeping a few swine at the time of the outbreak. The combination of a highly industrialized piglet supply system and the farmer’s backyard animal raising practices might have created a high risk for infectious disease outbreaks of unprecedented scale in terms of the number of persons infected and the geographic spread, posing an even greater public health threat (22, 23). A pathogen-free supply of piglets and improved hygiene for backyard farmers could help prevent such outbreaks (23–26). Alternatively, disease monitoring at the PBC level would be a very effective outbreak-prevention strategy. Clades 5 and 6 were associated with PBC D and accounted for the majority of the outbreak isolates. Therefore, PBC D was likely to be the primary contributor to the outbreak. Clades 5 and 6 shared the most recent common ancestor (Figure 1) with the time of divergence of the clades dating back to 2003, so piglets at PBC D probably were infected by S. suis ST7 for ≥2 years before the outbreak. Because ST7 also causes disease in pigs, monitoring and intervention at the PBC level could have averted the outbreak, underscoring the importance of disease monitoring at its source for zoonotic human infections.

In conclusion, the Sichuan outbreak of S. suis in humans was caused by the parallel transmission of infection from pigs to humans through distributed pig farming. The combination of centralized industrial-scale supply of infected piglets by PBCs and the backyard animal raising practices of farmers has created a unique environment for the incubation of a large outbreak. A pathogen that formerly only caused sporadic disease has now evolved to become a major threat to human health. Our findings provide important insights into S. suis epidemiology and demonstrate that novel intervention strategies are required for the prevention of such outbreaks.

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Figure 4. Geographic locations and affected region of major highways and piglet breeding companies associated with outbreaks of human infection with Streptococcus suis minimum core genome type 1 sequence type 7, Sichuan Province, China, 2005. Colored bubbles represent Sichuan clades 2–6 (purple, red, blue, green, and orange, respectively). Stars represent the piglet breeding companies, dashed lines represent the associated highways, and colors are consistent with the clades they are related to. Purple shadow, area in red line, area in green line, and orange shadow represent the affected regions related to clades 2, 3, 5, and 6, respectively.
Author Contributions: P.D., H.Z., J.Z., and R.L. contributed equally to this work. J.X. conceived the project. J.X. and H.Z. designed the study. H.Z., C.Y., H.J., D.J., X.B., and Z.C. collected the samples. P.D., H.Z., J.Z., L.L., J.L., L.X., W.Z., C.C., and J.X. analyzed the data. P.D., H.Z., R.L., and J.X. wrote the paper. All authors have discussed the results and commented on the manuscript.

Mr. Du is a research assistant at the National Institute for Communicable Disease Control and Prevention at the Chinese Center for Disease Control and Prevention. His primary research interests relate to the evolution of pathogenic bacteria and the mechanism of horizontal gene transfer.

References


Over the past decade, West Nile virus (WNV) has spread across the United States. We aggregated blood donor data from 2010–2012 and then calculated the incidence of WNV RNA–positive donations and compared the incidence with neuroinvasive disease (NID) case data from the ArboNET surveillance system. Of 10,107,853 donations, 640 were confirmed positive. The seasonal WNV incidence rate per 100,000 persons was 33.4 (95% CI 22–45) in 2010, 25.7 (95% CI 15–34) in 2011, and 119.9 (95% CI 98–141) in 2012. NID to blood donor ratios were 1 in 164 (95% CI 152–178) in 2010, 1 in 158 (95% CI 145–174) in 2011, and 1 in 131 (95% CI 127–136) in 2012. We updated estimates of the ratio of NID to WNV infection rates, demonstrating stable disease penetrance over the study period. Blood donor WNV RNA screening is a valuable public health tool for WNV surveillance.

West Nile virus (WNV), a mosquito-borne flavivirus, was first isolated in 1937 from a patient in Uganda (1). The virus was introduced into the United States in 1999, resulting in a focal epidemic that year in New York, New York. WNV then made a westward migration across the United States, becoming an endemic public health problem that is monitored through the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) ArboNET surveillance system (2). Although most WNV infections are asymptomatic, they can cause a syndrome of fever and myalgia in a minority of cases and can also cause neuroinvasive disease (NID) manifesting as meningitis, encephalitis, or acute flaccid paralysis (2–6). An estimated 20%–25% of infected persons become symptomatic (5). During 1999–2010, an estimated 2–4 million WNV infections, 0.4–1 million cases of febrile illness, and 13,000 reported cases of NID occurred in the United States (7). US public health authorities received >39,000 reports of WNV infections during 1999–2013 (8). Early data from 1999 indicated that NID develops in <1 in 150 WNV-infected persons (5). However, based on 2003 data, this estimate was revised to 1 in 256 based on a correlation of NID case-report data with total WNV infection projections derived from blood donor WNV testing data (9).

The risk of transmitting WNV through blood transfusion was anticipated by CDC investigators and estimated to be as high as 2.7 transmissions/10,000 transfused blood units during the peak of the 1999 outbreak (10); however, confirmed transfusion-transmitted infections were not reported until 2002 (11,12). After these reports, the US Food and Drug Administration, CDC, US blood centers, and test manufacturers quickly collaborated to develop and implement, beginning in summer 2003, routine nucleic acid testing (NAT) to screen blood donors for WNV RNA (13,14).

Predictions of the scale of annual outbreaks are not reliable (7). Environmental temperature and precipitation data may serve to estimate when WNV transmission rates in mosquitoes and birds will exceed specific thresholds, such that the probability of transmission to humans would be predicted to occur (15,16). Blood center data are a useful adjunct because testing laboratories monitor the number of WNV NAT–positive donations in near real time to determine if they should convert from NAT of minipools (MP-NAT) to more sensitive NAT of individual donations (ID-NAT) (17). In addition, WNV NAT–positive blood donations and NID cases are reported to CDC by state and territorial health laboratories via the ArboNET surveillance system. These reports showed that, after relatively low numbers of WNV outbreaks...
during 2004–2011, the WNV incidence rate in 2012 was one of the highest reported. The NID-associated death rate in 2012 was 9.9%, and the number of deaths (286) is the highest annual number reported to CDC; of the 286 deaths, 55.5% (159) were reported from 5 states: 89 (31%) from Texas, 20 (7%) from California, 16 (5.6%) in Louisiana, and 17 (6%) each from Michigan and Oklahoma (18).

Blood donors represent a readily accessible sample of the US population that is systematically screened for incident WNV infections. This screening provides an approximation of the magnitude of the WNV epidemic each year, and this estimate complements data reported to ArboNET on cases of symptomatic WNV-associated disease. Busch et al. (9) previously estimated national and state-specific WNV infection rates in 2003 from the number and frequency of WNV NAT–positive blood donations and used those rates to provide statewide projections, which were then correlated with NID case rates. In this study, we used a larger dataset of WNV NAT–positive blood donations to model the US population incidence of WNV during 2010–2012. We also provide updated ratios of the estimated number of WNV infections to NID cases.

**Methods**

**Study Population**

Blood donor data were extracted from operational blood center databases and provided without personal identifiers. The total number of donations and the number of NAT-confirmed WNV-positive donations were categorized by donor age, sex, and state of residence. Data were collected over the 5-month epidemic period from June 1 to October 31 during 2010–2012. During 2003–2012, only 5 WNV NAT-positive blood donations were reported outside the months (June–October) that we included in this analysis: 4 occurred in November 2012 and 1 in April 2010 (19). Data for this study were obtained from the American Red Cross (Washington, DC, USA), which collects blood in 44 US states and Washington, DC; Blood Systems, Inc. (Scottsdale, AZ, USA), whose centers collect mostly in the Southwest, the Central Plains, and parts of California; the New York Blood Center (New York, NY, USA), which collects in New York and New Jersey; Carter Blood Care (Bedford, TX, USA), which collects in northern Texas; and OneBlood (Tampa, FL, USA), which collects blood throughout Florida and southern Georgia. Altogether, the current dataset is estimated to capture ≤60% of US blood donations. Blood centers provided count data stratified by age, date of donation, sex, and geographic location, and Blood Systems, Inc. provided person-specific data on all donors to enable risk factor analysis. This analysis did not constitute human subjects research because only existing data without personal identifiers were available to the investigators.

**Blood Donor Screening**

During the time of the study, blood donations from Blood Systems, Inc., the New York Blood Center, and Carter Blood Care were tested at Creative Testing Solutions laboratories (Tempe, AZ, USA) for WNV RNA by minipool NAT (pools of 16) and ID-NAT for resolution of donations within reactive pools, both using transcription-mediated amplification (TMA) (Hologic, San Diego, CA, USA; Grifols Diagnostics, Emeryville, CA, USA). The American Red Cross performed WNV NAT at its National Testing Laboratories (Stockbridge, GA, USA) using the same TMA assay. OneBlood used MP-NAT (pools of 6) based upon a PCR technique (Roche Molecular Systems, Branchburg, NJ, USA) or MP-NAT (pools of 16; Hologic/Grifols); resolution of donations within reactive pools was done using ID-NAT. Results from MP-NAT testing are monitored to determine if epidemic activity requires the triggering of more sensitive ID-NAT testing in geographic areas experiencing outbreaks (17,20–25). The trigger for converting from MP-NAT to ID-NAT in response to ongoing WNV activity was 1–2 reactive blood donations; for the TMA system, this was restricted to those reactive donations having a high ID-NAT signal (17,25,26).

NAT-reactive blood donations can represent a WNV-infected donor or a falsely reactive test result; thus, results must be confirmed by repeating NAT on an independent sample, by demonstrating donor seroconversion, or both (17,25). In our study, we included all ID-NAT–confirmed positive donations whether they were initially screened by MP-NAT or ID-NAT.

**Statistical Analysis**

Using only Blood Systems, Inc., data for confirmed WNV-positive and negative donations, we performed a univariate analysis of WNV NAT reactivity by donation year and donor sex and age group. We compared categorical variables by using the χ² test; age as a continuous variable was compared using the Student t-test. We estimated odd ratios in a logistic regression model that included adjustment for age group, sex, region of residence, season, and month.

Most WNV RNA–positive persons who donate blood are asymptomatic or in the presymptomatic stage of infection; thus, we calculated the seasonal incidence of WNV on the overall dataset, assuming independence between blood donation and WNV infection (25,27,28). For this analysis, we used WNV RNA detection periods (i.e., number of days between first testing positive and testing negative) of ≈10.7 days by MP-NAT and ≈19.6 days by ID-NAT (29). These estimates were adapted from the method of Busch et al. (9), using data from Kleinman et al. (30). We did not have access to data on whether donations were screened by MP-NAT or ID-NAT. Given that roughly equal proportions of yield donations were derived from MP-NAT and ID-NAT.
screening, we used an average window of 15.1 days, assuming a 50% mixture of ID-NAT and MP-NAT screening donations during the epidemic period. We then multiplied the total donations screened for WNV RNA by 15.1 days to derive person-time for the denominator in incidence calculations; the number of corresponding NAT yield donations was used as the numerator (9).

We derived the monthly WNV incidence in each state from June through October by multiplying the number of NAT-positive donations for each month by the number of days in each month and dividing by the average period of time during which RNA is detectable (9). We calculated state-specific seasonal WNV incidence by adding the 5 monthly WNV incidence estimates for each year. An estimation of the number of WNV infections in each state was calculated by multiplying each state-specific seasonal WNV incidence by the corresponding population estimate obtained from the US Census Bureau (31). We then obtained a national seasonal estimate by summing over the estimates for participant states. An overall seasonal incidence for the 3 years was calculated weighted on the general population for each year. CIs were obtained assuming a Poisson distribution for NAT-positive donations.

We obtained the ratio of WNV infections to reported NID cases by state by dividing the estimated number of infections in the general population by state by the number of NID cases reported to ArboNET. This estimation was repeated for each year. We obtained CIs by applying Taylor series expansion (32). Analysis of correlation between WNV incidence and reported NID through ArboNet surveillance was done using a linear regression. Correlations and summarizations were expressed using $R^2$. All statistical analyses were performed using Stata 12.1 (StataCorp LP, College Station, Texas, USA). We prepared graphical (maps) displays of results using ArcGIS version 9.3.1 (ESRI, Redlands, CA, USA). We did not conduct a county-level analysis.

Results

Study Population and Demographic Predictors
A total of 10,107,853 blood donations collected during June–October in 2010–2012 were included in this study: total donations for 2010 were 3,470,405, total donations for 2011 were 3,360,443, and total donations for 2012 were 3,277,005. All donors included in the analysis were US residents; 20% lived in Western states, 27% in the Midwestern states, 32% in Southern states, and 21% in Northeastern states. Data from Blood Systems, Inc., indicated that blood donors ranged in age from 16 to 98 years (median age 45 years), and men accounted for 53% of donations.

Overall, 640 donations were WNV NAT positive (Figure 1). WNV RNA–positive blood donations clustered according to WNV epidemic activity and the catchment areas of the participating blood collection networks. Apparent clustering was observed in Southwest, Central, and Northeast states in 2010 and in Southwest and Northeast states in 2011; the pattern was much more dispersed in 2012, involving the North Central, Southwest, and Northeast states.

The frequency of WNV RNA was 63% higher among male than female donors and 122% higher among white than nonwhite donors. Donors from the Midwest had higher rates of WNV infection. Higher rates of NAT-positive donations were observed in 2012 versus 2010, but rates in 2011 were significantly lower than those in 2010 (Table).

Figure 1. Geographic distribution of blood donations confirmed positive for West Nile virus (WNV) RNA, United States, June–October 2010–2012. The 640 confirmed WNV DNA–positive donations are represented by dots. Shading indicates cumulative number of donations for 2010–2012, by state, for catchment areas of ≥1,000 donations.
WNV Seasonal Incidence Analysis

Seasonal rates were 3.7 cases/100,000 donations (≈1 in 26,700) in 2010, 2.6 cases/100,000 donations in 2011 (≈1 in 38,200), and 12.9 cases/100,000 donations (≈1 in 7,800) in 2012. Over the 3 years, WNV activity was highest in August and September, as evidenced by NAT-positive rates; in 2010, rates peaked in September (7.7 cases/100,000 donations), and in 2011, rates peaked in August (7.0 cases/100,000 donations) (Figure 2). In 2012, NAT-positive rates peaked in August (26.9 cases/100,000 donations), but activity was high from July (16.2 cases/100,000 donations) through September (16.2 cases/100,000 donations).

Diverse geographic incidence patterns were observed over the 3 years (Figure 3). In 2010 and 2011, the states with the highest activity were Arizona, New Mexico, Nebraska, and Kansas; the incidence in 2011 was lower than that in 2010. In 2012, the epidemic grew in scale and expanded to Texas and North Central states, including South Dakota, North Dakota, Minnesota, and Wyoming. High infection incidence was also observed in Alabama and Mississippi.

Overall seasonal WNV incidence estimates were 33.4 cases/100,000 persons in 2010 (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/2/16-1058- Techapp1.pdf) and 24.7 cases/100,000 persons in 2011 (online Technical Appendix Table 2). The estimated incidence for 2012 was 119.9 cases/100,000 persons. Among states, incidence ranged from 12.9 cases/100,000 persons in Virginia to 766.9 and 1,465.4 cases/100,000 persons in North and South Dakota, respectively (online Technical Appendix Table 3).

National and state-specific variability in projected WNV infections generally paralleled NID rates reported to ArboNET (Figure 3). In 2010, 2011, and 2012, 629, 486, and 2,872 NID cases, respectively, were reported. Cumulative national estimates of WNV cases were 103,450 cases in 2010 and 76,975 cases in 2011, and the ratio of NID cases to WNV infections was 1 to 164 (95% CI 152–178) in 2010. In 2011 and 2012, the epidemic grew in scale and expanded to Texas and North Central states, including South Dakota, North Dakota, Minnesota, and Wyoming. High infection incidence was also observed in Alabama and Mississippi.

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In 2012, an estimated 376,612 WNV infections occurred, and the ratio of NID cases to WNV infections was 1 to 131 (95% CI 126–136) (Figure 4; online Technical Appendix Table 3). Over the 3 years of the study, the weighted ratio of NID cases to WNV infections in the general population was 1 to 141 (95% CI 118–164). In addition, during 2010–2012, projected incidence correlated with NID case frequencies (R^2 value of 0.83 in 2010, 0.83 in 2011, and 0.79 in 2012) (Figure 4).

Discussion
Our findings, which extended previous findings (9), highlight the value of using WNV NAT–positive blood donation data to model population incidence in the United States. Our period of data collection covered 3 years, including the large WNV epidemic in 2012, enabling us to demonstrate seasonal and geographic variation in incidence. Using a large geographic catchment area and multiple years of data, we were able to provide updated estimates of the ratio of NID cases to WNV infections, demonstrating stable disease penetrance over the study period and that our estimate (1:141) is closer to that reported for the year 1999 (1:140) (5) than that reported for the year 2003 (1:256) (9).

Our analysis of demographic factors shows seasonal and geographic variations of WNV infection rates in blood donors. This study’s incidence estimate of 12.9 infections/100,000 blood donations in 2012 is the same as that reported by Francis et al. (33) for the 2010 outbreak in New York but lower than the 20 and 27 cases/100,000 persons reported for national data in 2003 (9,2). Kleinman et al. (20) reported a higher rate of 35 cases/100,000 blood donations for the 2003 epidemic for a specific group of blood centers. Using American Red Cross data and a well-defined confirmatory algorithm (similar to the method in our study), Stramer et al. (22) reported infection rates of 14.9 and 4.4 cases/100,000 blood donations in 2003 and 2004, respectively; these rates are higher than those we estimated for 2010 and 2011 (3.7 and 2.6 cases/100,000 donations, respectively), but rates in 2003 and 2012 appeared comparable. Projected WNV incidence in the general population and NID case frequency decreased slightly from 2010 to 2011 and then spiked upward in 2012 during an outbreak that spread to the Midwest with high incidence rates (online Technical Appendix Table 3); this pattern was similar to that observed during the 2003 epidemic (http://www.cdc.gov/westnile/resources/pdfs/data/2003stateincidencemap.pdf). These results are in agreement with our data showing
that the highest projected incidence generally correlated with NID case frequencies (Figure 4).

Patterns of WNV activity vary from year to year, exhibiting temporal and geographic variations of incidence, as shown by our data in the blood donor pool and corresponding projections in general population incidence. Fourteen years after their first appearance in the United States, WNV epidemics are still unpredictable and difficult to control (34), as confirmed by the surge of cases in 2012, resulting in 286 reported deaths, after years of relatively mild epidemic years (http://www.cdc.gov/westnile/resources/pdfs/data/2012wnvhumaninfectionsbystate.pdf).

Previous studies have noted older age and male sex as predictors of severe outcomes (e.g., NID) (5, 35, 36), but not for detection of WNV RNA, as observed in our study. The strong association that we found with white race/ethnicity is novel and is likely reflective of the fact that more white than nonwhite persons donate blood.

Using data for WNV NAT-positive donors, we estimated that 555,037 WNV infections occurred in the United States during 2010–2012. During that period, ArboNet reported a total of 7,407 NID and non-NID cases (http://www.cdc.gov/westnile/statsmaps/finalmapsdata/index.html). Busch et al. (9) estimated that 735,000 WNV infections occurred during 2003 in the United States, and ArboNet reported 9,862 NID and non-NID cases in 2003 (http://www.cdc.gov/westnile/resources/pdfs/data/2003wnvhumaninfectionsbystate.pdf). Cervantes et al. (29) reported an estimated 85,156 WNV infections in northern Texas during the 2012 epidemic, compared with our estimate of 93,004 infections in all of Texas that year.

We report a weighted ratio of 1 NID case per 141 WNV infections during 2010–2012, similar to the ratio of 1 NID case per 140 WNV infections reported during the 1999 New York outbreak (5). The difference between our estimate and the estimate reported by Busch et al. (9) for the year 2003 (1 NID case/256 WNV infections) may reflect yearly variations of the disease, data quality, or, most probably, the differences in the WNV NAT positivity window used in the study by Busch et al. (6.9 days) and in our study (15.1 days), which could have resulted in an overestimate of WNV incidence and NID ratio in 2003. Since the 1999 outbreak in New York, genetic evolution of WNV has been described or hypothesized in the United States and elsewhere with a hypothetical increase in virus fitness and pathogenicity (37–40). The virulent lineage 2 WNV has been implicated in increasing epidemics in Europe and Russia and with devastating cases of NID (41).

Our findings do not support a change in virus penetrance in the United States that might have resulted in the higher number of deaths reported during the 2012 season.

The ratio of infection cases to NID cases is a good surveillance strategy for WNV pathogenic evolution. Although issues with case recognition and passive reporting may result in underreporting of NID cases in the general population, data on NID incidence may be more reflective of the total population that is covered by public health surveillance (2, 28). On the other hand, decreased public health communication during low-incidence epidemic years could result in underreporting of WNV infections, and increased communication during more severe epidemics could result in more complete reporting.

Strengths of the current study include a very large study population spanning a large geographic region of the United States and a uniform blood donor sampling frame and test methods for WNV RNA. Limitations include geographic gaps in participating blood centers, leading to a potentially biased estimate of incidence in certain states. Blood donor incidence detected by NAT may underestimate infection rates in the general population by as much as 25% because of self-exclusion from donation due to WNV signs and symptoms (42), resulting in self-selected healthy
donors. Also, blood collection centers do not draw from entire states, so some areas are proportionally not represented (29). Because we used operational data, we had only a limited number of demographic variables and no information on potential exposures to WNV. Last, because we did not have data on whether MP-NAT or ID-NAT screening was used for each donation, we used an average RNA detection duration period of 15.1 days and, thus, may have slightly underestimated or overestimated WNV incidence (29).

In conclusion, we used a large nationwide dataset obtained from a consortium of blood collection organizations to strengthen the idea that monitoring US blood donations for WNV RNA is a useful surveillance tool for studying the evolution of epidemics and potentially associated pathogenicity. WNV RNA blood donation data are useful for tracking epidemics prospectively (because they are collected in real time) and retrospectively as a complement to existing case-based WNV surveillance networks in the United States.

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Highly Pathogenic Influenza A(H5Nx) Viruses with Altered H5 Receptor-Binding Specificity

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Emergence and intercontinental spread of highly pathogenic avian influenza A(H5Nx) virus clade 2.3.4.4 is unprecedented. H5N8 and H5N2 viruses have caused major economic losses in the poultry industry in Europe and North America, and lethal human infections with H5N6 virus have occurred in Asia. Knowledge of the evolution of receptor-binding specificity of these viruses, which might affect host range, is urgently needed. We report that emergence of these viruses is accompanied by a change in receptor-binding specificity. In contrast to ancestral clade 2.3.4 H5 proteins, novel clade 2.3.4.4 H5 proteins bind to fucosylated sialosides because of substitutions K222Q and S227R, which are unique for highly pathogenic influenza virus H5 proteins. North American clade 2.3.4.4 virus isolates have retained only the K222Q substitution but still bind fucosylated sialosides. Altered receptor-binding specificity of virus clade 2.3.4.4 H5 proteins might have contributed to emergence and spread of H5Nx viruses.

Highly pathogenic avian influenza A(H5N1) viruses have caused major economic losses in the poultry industry and might cause zoonotic infections. Recently, a novel H5 virus (clade 2.3.4.4) has emerged (1–4) and shown unprecedented intercontinental spread. Whereas highly pathogenic avian influenza A(H5N1) viruses previously detected in poultry in the Americas are believed to have descended from low pathogenicity viruses in this region (5), clade 2.3.4.4 viruses were the first highly pathogenic avian influenza virus of the A/goose/Guangdong/1/1996 lineage to appear in North America (2–4) during the winter of 2014–2015. Emergence of these novel viruses resulted in culling of >7 million turkeys and 42 million chickens in the United States (6). In Asia, 13 (mostly lethal) cases of human infection with clade 2.3.4.4 H5N6 viruses have been reported (7). Emergence of new viruses with clade 2.3.4.4 hemagglutinin (HA) that infect poultry and humans emphasizes the need for detailed characterization of molecular properties of these viruses.

Influenza A viruses are subtyped according to their envelope glycoproteins HA and neuraminidase (NA): 16 HA and 9 NA subtypes are found in aquatic birds and constitute the animal influenza A virus reservoir. Viruses with H5 or H7 subtypes occasionally acquire a multibasic cleavage site in their HA (8), which results in a highly pathogenic phenotype. In general, highly pathogenic H5 viruses have the N1 subtype (H5N1). In contrast, the novel highly pathogenic H5 clade 2.3.4.4 viruses have reassorted with different NA subtypes, including N1, N2, N3, N5, N6, and N8 (1,9–17).

HA proteins bind to sialoside receptors on the host cell surface. Avian and human influenza A viruses prefer binding to sialic acids linked to a penultimate galactose by an α2-3 or α2-6 linkage, respectively (18). Type and number of internal monosaccharides and their linkages determine fine specificity of virus receptors (19,20). NA removes sialic acids from glycans, which enables virus particles to be released from the cell surface after assembly and from decoy receptors (e.g., in mucus). The balance between activities of HA and NA proteins has a critical role in optimal viral fitness, tropism, and transmission (21).

Changes in HA receptor-binding properties might affect virus host range and within-host virus properties. These changes might have contributed to the remarkable spread of clade 2.3.4.4 viruses. Although a recent study (22) reported enhanced avidity of H5N6 viruses for human-type receptors, recombinant clade 2.3.4.4 highly pathogenic influenza A virus H5 proteins from virus isolates in North America show a strict avian receptor-binding preference (23). We compared receptor-binding properties for clade 2.3.4.4 H5 proteins from an H5N8 virus from Europe with those for an early ancestral clade 2.3.4 H5 protein from an H5N1 virus to identify differences in these properties.

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Materials and Methods

Genes, Expression Vectors, and Protein Expression and Purification
We cloned codon-optimized H5-encoding cDNAs (GenScript, Piscataway, NJ, USA) of H5N1 A/wild duck/Hunan/211/2005 (GenBank accession no. EU329186.1) and H5N8 A/chicken/Netherlands/14015526/2014 (GISAID, https://platform.gisaid.org, accession no. EPI-ISL_167905) in pCD5 expression vectors flanked by signal peptide-, GCN4- isoleucine-zipper trimerization motif-, and Strep-tag II-encoding sequences. Clones were mutagenized when indicated, expressed, and purified as described (24).

HA Receptor-Binding Assays
We assessed binding of HA to fetuin and transferrin (Sigma, St. Louis, MO, USA) and glycan arrays as described (24). Biolayer interferometry was performed by using Octet QK (ForteBio, Menlo Park, CA, USA) and in-house–synthesized saccharides NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (3′SLNLN), NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (6′SLNLN), NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc (3′SLex), and NeuAcα2-3Galβ1-4GlcNAc (3′SLN) coupled to LC-LC-biotin. Streptavidin sensors were loaded with 0.1 µmol/L of glycan for 15 min. We assessed association of HA proteins (0.2 µg/µL) with StrepMAB-Classic (IBA GmbH, Göttingen, Germany) at a molar ratio of 2:1 and performed binding of HA to tissues as described (25), except that H5 proteins were precomplexed with StrepMAB-Classic conjugated to horseradish peroxidase (IBA GmbH) and goat antimouse IgG (heavy plus light chain) conjugated to horseradish peroxidase (Invitrogen, Carlsbad, CA, USA). We performed immunohistochemical analysis with 3′SLex-specific antibody KM93 (26) (EMD Millipore, Darmstadt, Germany) at a 1:500 dilution by using standard procedures, including antigen retrieval (27) on an avian intestinal tissue microarray (28).

Modeling
The crystal structure of H5 protein from A/Vietnam/1194/2004 (H5N1) virus was complexed with 3′SLex (Protein Data Bank accession no. 3ZNM 28) (29) and used as a template for modeling the structures of A/wild duck/Hunan/211/2005 and A/chicken/Netherlands/14015526/2014 viruses with SWISS-MODEL (30). Subsequent energy minimizations were not necessary because inspection of modeled structures by using GROMOS (http://www.gromacs.org/Documentation/Terminology/Force_Field/GROMOS) showed no unfavorable energy interactions. Superpositioning of Cα backbone atoms of residues lining the receptor-binding site of A/wild duck/Hunan/211/2005 (H5N1) or A/chicken/Netherlands/14015526/2014 (H5N8) viruses with A/Vietnam/1194/2004 (H5N1) virus showed that the root mean square deviation of superpositioned atoms was <0.2 Å for both viruses. Molecular interactions were further examined by using Swiss-Pdb Viewer (31).

Receptor-Binding Properties of Different H5 Proteins
We compared receptor-binding properties of an H5 protein derived from an early clade 2.3.4 H5N1 virus isolate (A/wild duck/Hunan/211/2005) with a clade 2.3.4.4 isolate (H5N8 A/chicken/Netherlands/14015526/2014) detected in Europe (referred to as H5N12.3.4 and H5N8 HA proteins, respectively) by using recombinant soluble HA proteins (24). H5 proteins were analyzed for their binding to fetuin and transferrin (Figure 1). Fetuin contains α2-3–linked and α2-6–linked sialosides at a ratio of 2:1 (32,33), but transferrin contains only α2-6–linked sialosides (34). We used a clade 1 H5 protein (H5N1 A/Viet Nam/1203/2004; referred to as H5N11) and an H1 protein of a human seasonal H1N1 virus (A/
Kentucky/UR06–0258/2007; referred to as H1) with known receptor-binding properties as controls (24,27).

Results

Receptor-Binding Properties of Different H5 Proteins
All H5 proteins efficiently bound fetuin. However, only H5N8 virus HA showed limited binding to transferrin, which indicated that H5N12.3.4 and H5N8 HA proteins prefer binding to α2-3–linked sialic acids similarly as H5N1 HA (27). The H1 protein bound fetuin to a lower extent than the H5 proteins and bound transferrin, which was consistent with this protein preferentially binding α2-6–linked sialosides (24).

Receptor fine specificity of H5N12.3.4 and H5N8 HAs was determined by using glycan array analysis. The H5 proteins bound to a range of mono- and bi-antennary α2-3–linked glycan structures corresponding to N- and O-linked sialosides (Figure 2). Both proteins did not bind to α2-6–linked sialosides. The specificity of the H5N12.3.4 HA was similar to that of its H5N1 ancestor (27) (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/23/2/16-1072-Techapp1.pdf). However, glycan array analysis showed a fucosylation-specific change in receptor fine specificity for H5N8 HA.

We compared binding to 2 sets of α2-3–linked sialosides that differed only in the absence (glycans 1–9) or presence (glycans 11–19) of fucose at the GlcNAc of lactosamine repeats (Figures 2, 3). H5N12.3.4 HA bound only to 1 fucosylated glycan (glycan no. 13; 6-sulfo 3′Sialyl Lewis X [6S-3′SLeX]). In contrast, H5N8 HA bound to all α1-3–fucosylated glycan, as well as the single α1-4–fucosylated glycan (glycan no. 15). We also analyzed binding of these proteins to different glycans by using biolayer interferometry (Figure 4). Although H5N12.3.4 and the H5N8 HAs showed similar binding properties to 3′SLNLN, 6′SLNLN, and 3′SLN, they clearly differed in their binding to 3′SLeX, which is consistent with results of glycan array analysis (Figure 2).

Phylogenetic Analysis of Clade 2.3.4.4 H5 Proteins
To identify residues responsible for these differences in receptor binding, we mapped the amino acid substitutions along the trunk of an HA phylogenetic tree (Figure 5). Clade 2.3.4.4 viruses first emerged in 2008 (35). Eight amino acid substitutions, still maintained in the recently emerging clade 2.3.4.4 viruses, characterize the transition from the most closely related clade 2.3.4 ancestor (A/wild duck/Hunan/211/2005) (H5N1). A number of these amino acid substitutions in 2,562 HAs from highly pathogenic influenza A(H5N1) viruses were isolated during 1996–2015 (excluding clade 2.3.4.4 viruses) (Table 1). Substitutions K222Q and S227R (H3 numbering used here, corresponding to K218Q and S223R in H5 numbering), which have rarely occurred (0.08% and 0.9%, respectively), were found in the receptor-binding site. In the course of further evolution, a glycosylation site at the head domain at position 160 is lost, and there are 2 other unique substitutions (K193N, T193D) in the vicinity of the receptor-binding site. The Ser residue at position 227 present in H5N12.3.4 HA was again found in more recent clade 2.3.4.4 H5-containing viruses that have been detected in North America.

Receptor-Binding Properties of Mutant H5 Proteins
We introduced the amino acid substitutions in H5N8 HA into H5N12.3.4 HA and analyzed for their effects on receptor

Figure 2. Glycan array analysis of recombinant H5 proteins of influenza A viruses. A) Wild-type H5N12.3.4 (KS) and B) H5N8 (QR) H5 proteins were applied to the glycan array precomplexed with StrepMAB-classic (IBA GmbH, Göttingen, Germany) and fluorescent secondary antibodies. Letters in parentheses indicate amino acids at positions 222 and 227. Binding of hemagglutinins is indicated in relative fluorescence units (RFU). Binding is shown to sialylated glycans present in the array for nonfucosylated (glycans 1–9; red bars) and fucosylated (glycans 11–19; blue bars) forms. Glycan numbers indicated on the x-axes correspond to glycan structures shown in Figure 3. H5N12.3.4, novel H5N1 virus clade 2.3.4.
Influenza Viruses with Altered Binding Specificity

binding. Substitutions T160A or S227R did not affect fetuin binding (Figure 6, panel A), but K222Q reduced binding ≈2-fold. Reduction of binding was not observed when substitution K222Q was combined with S227R. Introduction of additional substitutions at positions 160, 193, and 199 in the order in which they occurred during evolution of clade 2.3.4.4 HAs did not affect fetuin binding.

We studied the receptor fine specificity of these proteins by using glycan array analysis. Substitution T160A did not change receptor fine specificity (online Technical Appendix Figure). In comparison with wild-type H5N1,2.3.4 HA (Figure 2, panel A), substitution K222Q (Figure 7, panel A) strongly decreased binding. However, substitution S227R (Figure 7, panel B) had a more specific negative effect. In contrast, combined substitutions K222Q and S227R (Figure 7, panel C) enhanced binding to the glycans bound by the wild-type H5N1,2.3.4 HA (Figure 2, panel A) and resulted in additional binding of fucosylated glycans that are also bound by H5N8 HA (Figure 2, panel B). Additional introduction of mutations found at positions 160, 193, and 199 in the background of Q222- and R227-containing H5N1,2.3.4 HA did not affect receptor fine specificity (online Technical Appendix Figure). We conclude that the combination of substitutions K222Q and S227R, already present in the earliest clade 2.3.4.4 H5Nx viruses (Figure 5), is largely responsible for the different receptor-binding properties of the H5N1,2.3.4 and the H5N8 HAs.

The branch of clade 2.3.4.4 H5 proteins that contains viruses from Taiwan and North America contains reverse substitution R227S (Figure 5). Therefore, we studied how residues at positions 222 and 227 affects receptor binding in the background of H5N8 HA. Substitutions R227S or Q222K hardly affected fetuin binding (Figure 6, panel B). In the glycan array, the R227S substitution did not affect binding of H5N8 HA to fucosylated sialosides (Figure 7, panel E), but the Q222K substitution, alone or in combination with the R227S substitution, inhibited binding of fucosylated receptors (Figure 7, panels D, F). We conclude that the identity of residue 222 plays a crucial role in binding of fucosylated sialosides, regardless of the background of highly pathogenic influenza A virus H5 protein. However, although R227 is required for binding
of fucosylated sialosides in clade 2.3.4 H5, this is not the case for clade 2.3.4.4 H5.

Modeling of Amino Acid Substitutions in H5 Protein Structure

We analyzed the interaction of the H5N1_{2,3,4} and the H5N8 HAs with a fucosylated and sialylated tetrasaccharide (3′SLeX; glycan no. 3) (Figure 3) by modeling on the structure of a clade 1 highly pathogenic influenza A(H5N1) virus H5 protein (H5N1_{A/Vietnam/1194/2004}) that was co-crystallized with 3′SLeX (28). The 3′SLeX ligand and major parts of the receptor-binding site (190-helix, 130-loop, and 220-loop) is shown in Figure 8. Poor binding of A/Vietnam/1194/2004 HA to 3′SLeX was explained (29) by steric hindrance between Lys at position 222 and fucose (Figure 8, panel A). This steric hindrance is maintained in H5N1_{2,3,4} HA but is lost after the K222Q substitution in H5N8 HA (Figure 8, panels B, C).

The effects of aa 227 on binding of fucosylated sialosides might result from the possibility of forming a hydrogen bond between the 220-loop and the amino-terminal end of the 190-helix through the side chains of R227 and N186, thereby influencing the flexibility of the receptor-binding site. At 2 positions that differ between H5N1_{2,3,4} and H5N8 HAs (S137A and S185P), we observed changes in the potential to form hydrogen bonds between major elements of the receptor-binding site (Figure 8). Such changes might affect the interaction of HA with sialic acid-containing glycans and might explain the background-dependent effect of the residue at position 227.

Binding of H5 Proteins to Avian Tissues

We studied binding of H5N1_{2,3,4} and H5N8 H5 proteins to avian tissues that differ in the presence of fucosylated sialosides. An antibody to 3′SLeX bound strongly to epithelial cells of chicken trachea, but not to duck intestinal tissue (Figure 9, panel A), which is consistent with previous findings (36). Removal of sialic acids by Vibrio cholerae neuraminidase inhibited binding. Tissue derived from another Anseriformes species (Greylag/Canada goose) also did not display 3′SLeX, and differential staining results were obtained for intestinal tissues of different Galliformes species (Table 2). The H5 proteins tested efficiently bound chicken trachea (Figure 9, panels B, C) and duck intestines that do not have 3′SLeX−containing glycans. Staining of duck tissues with H5N1_{2,3,4} HA was less intense.
Clade 2.3.4.4 HA in an H5N8 virus from Europe efficiently binds fucosylated sialosides, in contrast to an HA from the ancestral clade 2.3.4.4 (this study) and older highly pathogenic H5N1 virus HAs. We have shown that amino acid substitutions K222Q and S227R in the receptor-binding site of early clade 2.3.4.4 HAs are required for this change in receptor-binding specificity. HA residues K222 and S227 are extremely conserved among all clades of highly pathogenic H5N1 viruses; the double substitution K222Q/S227R was introduced only at the root of clade 2.3.4.4 (Fig 5). Structural analysis of a clade 1 HA indicates that the close proximity of the conserved K222 side chain and the fucose moiety of 3’SLeX most likely destabilizes their interaction (29). Modeling indicates that such destabilization is still present in H5N1 2.3.4 HAs but is absent in H5N8 HAs that contain Q222 and R227 residues (Figure 8).

Introduction of K222Q into H5N1 2.3.4 HA, which removes the competition with the fucose-moiety (29), in itself does not enable binding to fucosylated receptors (Figure 7). However, additional introduction of an arginine at position 227 (double substitution K222Q/S227R) in H5N1 2.3.4 HA was sufficient to cause a glycan array than for H5N8 HA. We conclude that 3’SLeX-containing sialosides might affect binding of H5N8 HA to avian tissues but is not essential.

Discussion

Clade 2.3.4.4 H5Nx viruses have shown unprecedented worldwide spread. Gene reassortments with other influenza A virus genotypes have generated a range of clade 2.3.4.4 viruses containing different NA subtypes, although they harbor an H5 protein that previously was almost exclusively associated with members of a monophyletic clade of N1 proteins descending from early highly pathogenic H5N1 virus isolates. We show that emergence of clade 2.3.4.4 H5Nx viruses is accompanied by a change in HA receptor-binding specificity. Altered receptor-binding properties might affect the balance between HA and NA, enable the virus to acquire different NA subtypes, and might result in altered host range and spreading.

Clade 2.3.4.4 HA in an H5N8 virus from Europe efficiently binds fucosylated sialosides, in contrast to an HA from the ancestral clade 2.3.4.4 (this study) and older highly pathogenic H5N1 virus HAs (28,37). We have shown that amino acid substitutions K222Q and S227R in the receptor-binding site of early clade 2.3.4.4 HAs are required for this change in receptor-binding specificity. HA residues K222 and S227 are extremely conserved among all clades of highly pathogenic H5N1 viruses; the double substitution K222Q/S227R was introduced only at the root of clade 2.3.4.4 (Figure 5). Structural analysis of a clade 1 HA indicates that the close proximity of the conserved K222 side chain and the fucose moiety of 3’SLeX most likely destabilizes their interaction (29). Modeling indicates that such destabilization is still present in H5N1 2.3.4 HAs but is absent in H5N8 HAs that contain Q222 and R227 residues (Figure 8).

Introduction of K222Q into H5N1 2.3.4 HA, which removes the competition with the fucose-moiety (29), in itself does not enable binding to fucosylated receptors (Figure 7). However, additional introduction of an arginine at position 227 (double substitution K222Q/S227R) in H5N1 2.3.4 HA was sufficient to cause a glycan array.

Figure 5. Phylogenetic analysis of influenza A virus clade 2.3.4.4 H5 proteins. A 362-aa full-length hemagglutinin (HA) sequence for H5 clade 2.3.4.4 was obtained from GenBank and the GISAID database (http://platform.gisaid.org). An HA protein tree was constructed by using the PHYLIP neighbor-joining algorithm (https://ugene.net/wiki/display/UUOM/PHYLIP+Neighbor-Joining) and the F84 distance matrix. This tree was used to construct a guide tree with 52 HA sequences representing all branches of the tree. These sequences were used to construct a summary tree of similar topology as the guide tree. Items above the branches indicate key residues that differ between different branches. Items in red above the branches indicate mutations introduced in this study. The HA protein tree is rooted by an early clade 2.3.4 isolate (A/goose/Guangdong/08). H5N1 2.3.4, and H5N8 HA proteins used in this study are indicated by red stars. H5N1 2.3.4, novel H5N1 virus clade 2.3.4.
binding profile nearly identical to that of H5N8 HA. Substitution S227R might result in the 220-loop interacting with the conserved loop at the N terminus of the 190-helix through 2 hydrogen bonds between R227 and N186 (Figure 8, panel C). This interaction, which potentially limits mobility of the 220-loop, might contribute to stabilizing the receptor-binding site in a conformation that enables binding of fucosylated receptors. R227 was required for binding of fucosylated sialosides in the background of H5N12.3.4 HA, but not H5N8 HA (Figure 7). Consistent with our findings, HAs of the Taiwanese/North American branch of clade 2.3.4.4 viruses, which obtained reverse substitution R227S (Figure 5), also efficiently bind fucosylated receptors (23).

Analysis of all HA sequences for all avian genotypes available in GenBank showed that the frequency of Q222 and R227, depending on the genotype, is 0 or extremely low. An exception is that Q222 is highly conserved in H7 and H10 genotypes, and R227 is the dominant amino acid in H6 and H13 genotypes. The combination Q222/R227 is present in a 1 clade of low pathogenicity H5N2 viruses represented by A/chicken/Ibaraki/1/05 (H5N2) (38), which was shown to be able to bind to fucosylated receptors, and for which residues 222 and 227 were shown to be essential (39). The HA of this virus appeared to show reduced binding to nonfucosylated glycans, in contrast to the clade 2.3.4.4 proteins analyzed in this study. However, the contribution of individual residues at these positions for receptor binding was not evaluated.

The isolates of this H5N2 clade in Japan originated from H5N2 viruses in Central America (38). Intercontinental transfer of avian influenza viruses is a rare event, drawing a remarkable parallel between 2 viral clades (highly pathogenic H5Nx and low pathogenicity H5N2), both of which have acquired the ability to bind fucosylated receptors and managed to spread intercontinentally. Similar to highly pathogenic H5Nx viruses, low pathogenicity H5N2 viruses might be spread by wild birds, although the possibility could not be ruled out that a virus

Table 1. Amino acids in hemagglutinins from 2,562 highly pathogenic influenza A(H5N1) viruses isolated during 1996–2015 (excluding clade 2.3.4.4 viruses)

<table>
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<tr>
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</table>

![Figure 6](image-url)
or vaccine was illegally introduced into Japan from Central America (40).

The ability of some low pathogenicity avian influenza A viruses to bind to fucosylated receptors of the 3′SLεX type has been considered a poultry-specific adaptation (20,36,39,41) although extensive studies on sufficient numbers of isolates across the complete range of HA genotypes are lacking. Except for low pathogenicity H7 viruses, which all bound efficiently to 6S-3′SLεX irrespective of their host species (42), duck viruses were suggested to bind poorly to 3′SLεX-type receptors (20,37,39,41,43). However, adaptation to binding of 3′SLε-type receptors has not been reported for high pathogenicity H5N1 viruses isolated from poultry.

Figure 7. Glycan array analysis of influenza A virus mutant H5 proteins. A) mutant H5N12.34 K222Q (QS); B) mutant H5N12.34 S227R (KR); C) mutant H5N12.34 K222Q/S227R (QR); D) H5N8 Q222K (KR); E) R227S (QS); F) Q227R/R227S (KS). Proteins were applied to the glycan array as detailed in the legend to Figure 2. Letters in parentheses indicate amino acids at positions 222 and 227. Binding of hemagglutinins is indicated in relative fluorescence units (RFU). Binding is shown to sialylated glycans present in the array in nonfucosylated (glycans 1–9; red bars) and fucosylated (glycans 11–19; blue bars) forms. Glycan numbers indicated on the x-axes correspond to glycan structures shown in Figure 3. H5N12.34 novel H5N1 virus clade 2.3.4.
A recent study showed poor binding of highly pathogenic H5 and H7 viruses to 3′SLe\(^\alpha\) (44). In another study, binding of a few avian influenza A viruses to fucosylated receptors correlated with their binding to α2-6-linked sialosides (41). However, we found only weak binding of H5N8 virus HA to α2-6–specific transferrin (Figure 1, panel B) and no measurable binding to α2-6–linked sialosides in the glycan array or by biolayer interferometry (Figure 4). HA of the Taiwanese/North American branch of clade 2.3.4.4 viruses did not bind α2-6–linked sialosides (23). Therefore, these results do not support the hypothesis that increased binding to

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**Figure 8.** Structural models of influenza A virus H5 proteins complexed with 3′SLe\(^\alpha\). A) Clade 1 H5 (H5N1, of A/Vietnam/1194/2004) complexed with 3′SLe\(^\alpha\) (PDB 3ZNM0 (29)). B) H5N1\(_{2,3,4}\) and C) H5N8 hemagglutinins were modeled into the structure shown in panel A as detailed in Materials and Methods. Key amino acids are indicated and shown in a stick representation. C (gray), O (red), and N (blue) in the side chains are colored. SIA, Gal, GlcNAc, and Fuc moieties of 3′SLe\(^\alpha\) are shown in purple, yellow, blue, and red, respectively. Hydrogen bonds are indicated by dotted lines. H5N1\(_{2,3,4}\), novel H5N1 virus clade 2.3.4; H5N1\(_1\), H5N1 virus clade 1.

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**Figure 9.** Histochemical analysis of binding of influenza A virus H5 proteins to avian tissues. A) Duck intestine and chicken trachea tissues stained with an antibody specific for 3′SLe\(^\alpha\) (anti-3′SLe\(^\alpha\)). Tissue sections treated with *Vibrio cholerae* neuraminidase (VCNA) before immunostaining were used as controls. Scale bars indicate 200 \(\mu\)m in left panel and 50 \(\mu\)m in center and right panels. B, C) Duck intestine and chicken trachea tissues incubated with H5 proteins H5N1\(_{2,3,4}\) and H5N8 after precomplexing with horseradish peroxidase (HRP)–conjugated antibodies. Scale bars indicate 200 \(\mu\)m in left panel and 50 \(\mu\)m in right panel. D) Chicken trachea tissues incubated with HRP–conjugated antibodies against H5N1\(_{2,3,4}\) (no hemagglutinin [HA]) were used as a negative control. H5N1\(_{2,3,4}\), novel H5N1 virus clade 2.3.4. Scale bar indicates 50 \(\mu\)m.
fucosylated receptors enhances the propensity of avian influenza A viruses to evolve into binding α2-6-linked sialoside receptors.

Clade 2.3.4.4 viruses are generally considered to have evolved in and being spread by wild birds and ducks before introduction into poultry. Phylogenetic analyses based on HA sequences (Figure 4) have shown the evolution into several branches/subclades often harboring multiple H5Nx virus genotypes. Substitutions K222Q and S227R were present in the earliest H5N5 virus isolates (A/duck/Guangdong/wy24/2008) and have been maintained in all branches except the Taiwanese/North American branch of clade 2.3.4.4 viruses, which obtained reverse substitution R227S. These findings strongly suggest that the capacity to bind to 3'SLe-x-type receptors has arisen in wild birds.

The potential role of altered receptor specificity in extended host range and the contribution to the rapid worldwide spread of influenza viruses is still unknown. The presence of 3'SLe-x-type receptors on intestinal epithelial cells varies between different avian species (Table 2) (36) and does not appear to be required for infection of these birds, but their presence in other tissues and species requires further investigation. Apart from determining host-range, altered receptor specificity might also influence other factors involved in spreading, such as virus titers, shedding, and pathogenesis in infected birds.

Outbreaks of clade 2.3.4.4 viruses in poultry might have contributed to enhanced spreading (45–47), However, adaptations in HA leading to evolution of poultry-specific clades have not yet been detected in HA-based phylogenetic analyses. Reassortments of the 6 internal gene segments are continuously associated with the further evolution of H5N5x viruses, but the potential contribution of the internal gene constellation to (poultry-specific) spreading remains to be determined.

Of particular interest are the recent outbreaks of influenza caused by H5N6 viruses in poultry and ducks in Southeast Asia, which might resulted in nonavian infections, including 13, mostly lethal, cases in humans (7). Although enhanced avidity of these H5N6 viruses for human-type receptors (carrying α2-6-linked sialosides) has been reported (22), the amino acid combination Q222/R227, which all H5N6 viruses have in their HA, is unlikely to be responsible, and other amino acid substitutions, which have been shown to contribute to binding of α2-6-linked sialosides by highly pathogenic H5N1 viruses, have not been detected in H5N6 viruses (48,49). Two clades of H5N6 viruses have been identified by phylogenetic analysis (50), one harboring an NA with a truncated stem and the other harboring a full-length stem. Truncation of the stem has been considered a poultry-specific NA adaptation. However, both H5N6 virus clades appear to have acquired their N6 segment in independent events from H6N6 viruses (50), one of which already contained the stem deletion. Also, both clades have caused infections in wild birds, poultry, and humans, but evidence for species-specific adaptations in NA is lacking.

A longstanding paradigm in influenza A virus biology is the requirement for an optimal balance between HA binding and NA cleavage. HA binding displays a clear receptor-NA balance, but substrate fine specificity of NAs has not been extensively investigated. A recent report showed that all NA genotypes (only N4 was not tested) displayed relatively poor digestion of fucosylated receptors (44). Possibly because of tight binding of such receptors by clade 2.3.4.4 viruses, N1 of highly pathogenic H5 viruses might have lost an unknown advantage over other NA genotypes in creating an optimal HA/NA balance, which lead to the remarkable success of novel H5Nx virus reassortants within this clade.

### Table 2. Detection of 3'SLe-x in intestine of avian hosts of influenza A(H5Nx) virus clade 2.3.4.4 subtypes*

<table>
<thead>
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<th>H5Nx virus infection†</th>
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<td>Galliformes</td>
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<td>+</td>
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<tr>
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<td>±</td>
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<td>Quail</td>
<td>−</td>
<td>H5N8</td>
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<td>H5N2</td>
</tr>
<tr>
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<td>Phasianidae</td>
<td>Partridge</td>
<td>−</td>
<td>ND</td>
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†Subtypes identified in different bird species on the basis of data from GenBank and GISAID (http://platform.gisaid.org).

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ND, not detected; NT, not tested; SLe, sialyl Lewis; −, no visible staining; ±, few cells weakly stained; +, intense staining of cells; ++, intense staining of many cells.
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References


Influenza Viruses with Altered Binding Specificity


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Livestock Susceptibility to Infection with Middle East Respiratory Syndrome Coronavirus

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Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV) first emerged in 2012 in Saudi Arabia and is currently a worldwide concern (1). As of September 21, 2016, the World Health Organization had confirmed ≈1,800 MERS cases and 643 associated deaths (2). Although during most reported outbreaks the virus is mainly transmitted by human-to-human contact, infection through contact with dromedary camels (Camelus dromedaries) plays a major role in the primary cases. In the Middle East and some countries from East Africa where MERS is endemic, high prevalence of MERS-CoV–specific antibodies in dromedaries has been reported (3–7). Moreover, a recent surveillance study in Saudi Arabia demonstrated that MERS-CoV strains isolated from humans were also detected in the upper respiratory tract of dromedaries of several geographic origins, indicating that the virus did not require mutations to jump between species (8). However, not all index cases can be explained by direct contact with dromedaries, and transmission from other domestic livestock or animal species to humans cannot yet be ruled out. Recently, evidence that alpacas (Vicugna pacos) were also susceptible to MERS-CoV infection was provided and confirmed by field studies performed in Qatar (9–11). In contrast, despite the ability of the virus to infect a plethora of cell lines and tissues from mammals of multiple species in vitro (12), serologic surveys of ruminants and horses did not conclusively determine circulation of MERS-CoV among these domestic animals (6,13,14). Sampling design could explain negative results, and experimental infections provide, in many instances, a more straightforward answer to virus host susceptibility. This knowledge is crucial for determining risk factors with regard to possible globalization of the disease.

Our aim with this study was to address these critical research gaps and to understand the potential role that other animals (besides dromedaries and alpacas) could play in MERS-CoV dissemination. We experimentally inoculated MERS-CoV into llamas (Lama glama), pigs (Sus scrofa), horses (Equus ferus caballus), and sheep (Ovis aries). We based our selection of species on epidemiologic interest and on sequence similarities in the MERS-CoV receptor binding domain of dipeptidyl peptidase-4 (DPP4).

Materials and Methods

Ethics

All experiments with MERS-CoV were performed at Biosafety Level 3 (BSL-3) facilities of the Biocontainment Unit of the Institut de Recerca i Tecnologia Agroalimentaries (IRTA) Centre de Recerca en Sanitat Animal (CReSA) in Barcelona, Spain. The study was approved by the Ethical and Animal Welfare Committee of IRTA and the Ethical Commission of Animal Experimentation of the Autonomous Government of Catalonia.
Cells and MERS-CoV
Vero cells were cultured in Dulbecco modified Eagle medium (DMEM; Lonza, Basel, Switzerland) supplemented with 1% fetal calf serum (EuroClone, Pero, Italy), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L glutamine. Passage 7 human isolate MERS-CoV stock (HCoV-EMC/2012) was propagated in Vero cells at 37°C in a CO₂ incubator for 3 days. The infectious virus titer was determined in Vero cells and calculated by determining the dilution that caused cytopathic effect in 50% of the inoculated cell cultures (50% tissue culture infectious dose endpoint [TCID₅₀]).

Animal Studies
All animals used in this study were obtained from France and Spain by private sale and housed at BSL-3 animal facilities (IRTA-CReSA, Barcelona, Spain). We obtained 8 llamas (6–8 months of age), 8 horses (6–8 months), 14 sheep (2–3 months), and 14 pigs (2 months). We intranasally inoculated a 10⁶ TCID₅₀ dose in 3 mL saline solution into each animal (1.5 mL in each nostril) by using a mucosal atomization device (LMA; North-America, Inc., San Diego, CA, USA) and monitored the animals daily for clinical signs (sneezing, coughing, nasal discharge, dyspnea). Rectal temperatures were recorded with a fast display digital thermometer (Digi-Temp; Kruuse Veterinary Products, Langeskov, Denmark) until postinoculation day 10. Nasal and rectal swabs were obtained on postinoculation days 1, 2, 3, 4, 7, 10, 14, and 24 in phosphate-buffered saline (PBS) (for PCR analysis) and DMEM (for virus isolation and titration) containing antimicrobial drugs (100 U/mL penicillin and 0.1 mg/mL streptomycin). All samples were stored at −80°C until tested. Serum samples were obtained before challenge and at postinoculation days 14 and 24 and were subsequently used to detect MERS-CoV–specific antibodies.

Virus Titration
Nasal swabs collected at different times after inoculation were evaluated for infectious virus by titration in Vero cells. We prepared 10-fold dilutions, starting with a dilution of 1:10, and transferred the dilutions to Vero cells. Plates were monitored daily under a light microscope, and wells were evaluated for cytopathic effect. The final determination was conducted on postinoculation day 5. The amount of infectious virus in swab samples was calculated by determining the TCID₅₀.

Pathology Studies
On postinoculation day 2, we euthanized 4 pigs and 4 sheep with an overdose of pentobarbital followed by exsanguination; using the same procedure, on postinoculation day 4, we euthanized 4 animals of each species (including llamas and horses) and on postinoculation day 24, the remaining animals. We performed complete necropsies and collected respiratory tissues (frontal, medial, and caudal turbinates; proximal, medial and distal trachea; large and small bronchi; and right cranial, mediadorsal, and caudal lung parenchyma) for virologic, histopathologic, immunohistochemical (IHC), and in situ hybridization (ISH) examination.

Tissues for pathology studies were fixed by immersion in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 3 µm for slides. Sequential slides were either stained with hematoxylin and eosin or used to detect the DPP4 receptor and MERS-CoV antigen by IHC and viral genome by ISH (15,16). In brief, we performed DPP4 IHC staining by using 5 µg/mL of polyclonal goat IgG anti-human DPP4 antibody (R&D Systems, Abingdon, UK) and peroxidase-labeled rabbit anti-goat IgG (1:200; DAKO; Agilent Technologies Company, Santa Clara, CA, USA) as a secondary antibody. To detect MERS-CoV antigen, we used a monoclonal antibody to the nucleocapsid protein (SinoBiological Inc., Beijing, China) as described (16). We performed ISH according to the manufacturer’s instructions by using probes targeting the nucleocapsid gene of MERS-CoV (Advanced Cell Diagnostics, Hayward, CA, USA). ISH staining was detected by using the Fast Red substrate as previously reported (17). For detection of mucous substances, we stained selected slides (from animals euthanized on postinoculation day 24) with periodic acid–Schiff (PAS) according to standard methods.

Viral RNA Detection by Reverse Transcription PCR
We collected tissues (0.2–0.5 g) for viral RNA quantification and placed them in cryotubes containing beads and 500 µL DMEM supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L glutamine. In brief, samples were homogenized at 30 Hz for 2 min by using a Tissuelyser II (QIAGEN, Hilden, Germany) and stored at −70°C until use. We extracted viral RNA from nasal swabs, rectal swabs, and tissue homogenates by using a NucleoSpin RNA virus kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. The RNA extracts were tested by UpE PCR (18). We conducted reverse transcription quantitative PCR (RT-qPCR) by using AgPath-ID One-Step RT-PCR reagents (Applied Biosystems, Foster City, CA, USA) and performed amplification by using a 7500 Fast Real-Time PCR System (Applied Biosystems) programmed as follows: 5 min at 50°C, 20 s at 95°C, and 45 cycles of 3 s at 95°C and 30 s at 60°C. We considered samples with a cycle threshold ≤40 positive for MERS-CoV RNA.

MERS-CoV S1 ELISA
We determined specific S1 antibodies in serum samples from postinoculation days 0, 14, and 24 by a MERS-CoV S1 ELISA as previously described (16), with some modifications. In brief, 96-well high-binding plates (Sigma-Aldrich,
St. Louis, MO, USA) were coated with 100 µL of S1 protein at 1 µg/mL in PBS overnight at 4°C. After blocking with 1% bovine serum albumin/PBS/0.5% Tween20 for 1 h at 37°C, individual serum samples were added at 1:100, followed by 1 h incubation at 37°C. Plates were washed 4 times with PBS, and the species-specific secondary antibody was added. We used the following conjugates: anti-llama IgG:horseradish peroxidase (HRP) (diluted 1:60,000; no. A160–100P, Bethyl Laboratories, Montgomery, TX, USA); anti-pig IgG:HRP (diluted 1:20,000; no. A5670, Sigma-Aldrich); anti-horse IgG(T):HRP (diluted 1:10,000; no. AA138P, Bio-Rad, Hercules, CA, USA); and anti-sheep IgG:HRP (diluted 1:10,000; no. 5184–2504, Bio-Rad). After 1 h of incubation at 37°C, wells were washed 4 times with PBS, and TMB (3,3′,5,5′-tetramethylbenzidine) substrate solution was added and allowed to develop for 8–10 min at room temperature, protected from light. We measured optical density at 450 nm.

**MERS-CoV Neutralization Assay**

We also tested serum samples collected on postinoculation days 0, 14, and 24 by a specific virus neutralization assay. First, the samples were inactivated at 56°C for 30 min. Following a previous protocol (16), we mixed 400 PFU of MERS-CoV (HCoV-EMC/2012) with serial 2-fold dilutions of heat-inactivated serum, incubated the mixture 1 h at 37°C, and inoculated it onto Huh7 cells. The presence of viral antigen was assessed 8 h after inoculation. Cells were fixed with formaldehyde and stained by using rabbit anti–MERS-CoV antibodies and fluorescein isothiocyanate–conjugated swine anti-rabbit immunoglobulins as secondary antibodies. We calculated 90% plaque-reduction neutralization test values for the MERS-CoV neutralization assay.

**Results**

**Clinical Signs**

After challenge, 3 of 8 llamas showed clinical signs (moderate mucus secretion in 1 nostril) at postinoculation days 4–18 (online Technical Appendix Figure 1, panel A, http://wwwnc.cdc.gov/EID/article/23/2/16-1239-Techapp1.pdf). Soon after inoculation, mild excretion of white mucus from the nose was noted for 3 pigs. Minimal mucus excretion in the nose was noted at variable times (postinoculation days 2–16) for 3 horses. We did not detect nasal discharge in any of the sheep during the experiment. No animal of any species showed a significant increase in body temperature after MERS-CoV inoculation (online Technical Appendix Figure 1, panel B).

**MERS-CoV RNA and Infectious Virus in Nasal Swab Samples and Viral RNA in Respiratory Tissues**

Pigs and llamas excreted virus in the nose, as evidenced by RT-qPCR of nasal swab samples from postinoculation days 1–10; in 1 llama, viral RNA was detected until postinoculation day 15 (Figure 1, panel A). At postinoculation day 7, the amount of MERS-CoV RNA was still high in all

![Figure 1](http://wwwnc.cdc.gov/EID/article/23/2/16-1239-Techapp1.pdf)
llamas, but a decrease in RNA level was noted at postinoculation day 10. In pigs, high levels of MERS-CoV RNA were demonstrated until postinoculation day 4 and started decreasing at postinoculation day 7; only 1 animal remained positive at postinoculation day 10 (Figure 1, panel A). We subsequently tested positive nasal swab samples for the presence of infectious virus. During the experiment, 7 of 8 llamas and 7 of 14 pigs excreted infectious virus during at least 1 day from postinoculation day 2 on. We detected infectious virus until postinoculation day 4 in pigs and postinoculation day 7 in llamas (Figure 1, panel B). Relatively low levels of viral RNA were detected only until postinoculation day 2 in 5 of 8 inoculated horses and only at postinoculation day 1 in 7 of 14 sheep, suggesting the presence of residual inoculum in these animals (data not shown). We did not detect virus in rectal swab samples from any animal.

To determine the presence of viral RNA in tissues, we euthanized representative numbers of animals on postinoculation days 2 (pigs), 4 (llamas and pigs), and 24 (llamas and pigs) and tested tissue homogenates by RT-qPCR. Early after infection, virus RNA transcripts were detected mainly in the nose, trachea, and small and large bronchi of llamas and pigs (Figure 2). At postinoculation day 24, viral RNA was detected only in some tissues (caudal nose and trachea) in 2 llamas (Figure 2).

Pathology, IHC, and ISH
We observed no substantial macroscopic changes attributable to MERS-CoV infection in any animals. All horses exhibited purulent inflammation of the guttural pouch (empyema), which was most likely of bacterial origin.

At postinoculation day 4, IHC and ISH demonstrated virus antigen and nucleic acid in a few nasal respiratory epithelial cells in 3 of 4 llamas and in 2 of 4 pigs (Figure 3). At postinoculation day 2, occasional bronchial cells were positive for antigen and nucleic acid in 1 of 4 pigs. Also at postinoculation day 2, occasional bronchial cells from 1 of 4 sheep were positive for MERS-CoV (online Technical Appendix Figure 2). MERS-CoV antigen was absent in the rest of the respiratory tissues of animals of all species collected on any of the days. Associated with the presence of virus antigen and nucleic acid, llamas and pigs demonstrated a mild to moderate rhinitis characterized by mild to moderate epithelial necrosis with infiltration of variable numbers of neutrophils in the epithelium (exocytosis) and in the lumen and mild to severe infiltration of the lamina propria with variable numbers of macrophages, lymphocytes, neutrophils, and plasma cells and multifocal mild edema. We also observed mild to moderate multifocal epithelial hypertrophy consistent with regeneration (Figure 2). We observed no other relevant microscopic lesions in horses (besides the evidence of purulent empyema) and sheep.

![Figure 2. MERS-CoV viral RNA in respiratory tissues of llamas (A) and pigs (B). Viral RNA was determined in tissue homogenates at postinoculation days 4 and 24. Error bars indicate SDs when results were positive in >1 animal. Dashed lines depict the detection limit of the assays (C, ≤40). C, cycle threshold; MERS-CoV, Middle East respiratory syndrome coronavirus; PI, postinoculation.](image-url)
DPP4 Receptor Distribution and Presence of Mucosubstances in Respiratory Tissues

DPP4 IHC staining of upper and lower respiratory tract tissues collected on postinoculation day 24 found DPP4 expression on the respiratory epithelium of the nose of llamas, pigs, and horses but only to a very limited extent on that of sheep (Figure 4, panel A). We also detected DPP4 expression in the lower respiratory tract (but mainly restricted to tracheal and bronchial epithelia) of horses, llamas, and pigs (Figure 4, panel B). PAS staining demonstrated the presence of mucosubstances in the nose of llamas, pigs, horses, and sheep, with a relatively higher number of mucous (goblet) cells in the lining epithelium of sheep and horses. In the horses only, there was also a layer of mucus covering lining epithelium with a multifocal distribution.

Specific Humoral Immune Response

ELISA results showed antibodies against the S1 protein in all llamas and pigs from postinoculation day 14 on, although the response in pigs was weaker than that in llamas (Figure 5, panel A). We confirmed the specificity of the response by virus neutralization assay (Figure 5, panel B). In all llamas, serum neutralizing MERS-CoV-specific antibody titers (1:80 to 1:320) were detected at postinoculation days 14 and 24. In addition, 14 days after challenge, in 5 of 6 pigs, MERS-CoV neutralizing antibodies were detected (1:80 to 1:160). However, 10 days later, these virus neutralizing antibodies decreased (1:20 to 1:40) (Figure 5, panel B). We detected no MERS-CoV–specific antibodies in serum of sheep and horses.

Discussion

Our study results indicate that pigs and llamas are susceptible to MERS-CoV infection. These animals shed infectious virus until postinoculation days 4 (pigs) and 7 (llamas), although titers were lower among pigs. In pigs and llamas, we detected virus predominantly in the nasal respiratory epithelium by IHC, ISH, or both, similar to what has been documented in dromedary camels (16) and alpacas (9). Accordingly, we mainly detected viral RNA, as assessed by RT-qPCR, in the nose, trachea, and bronchi of those animals. We also detected viral RNA in lung tissue from 2 of 4 pigs euthanized at postinoculation day 2. Virus shedding in dromedary camels and alpacas for longer periods, up to 14 days after experimental inoculation, has been reported (9,16,19). Of note, the level of MERS-CoV excreted in the
Figure 4. Presence of MERS-CoV receptor DPP4 (IHC) and of mucosubstances (PAS) in upper and lower respiratory tract tissues from sheep, pigs, llamas, and horses. A) In the nose, DPP4 (red cytoplasmic or membrane staining) was present on the lining epithelium of pigs, llamas, and horses but not sheep. PAS staining (magenta) demonstrated more mucous cells in the lining epithelium of sheep and horses and a layer of mucus on the lining epithelium of the horses. B) DPP4 (red cytoplasmic or membrane staining) was present on the lining epithelium of the trachea, bronchus/bronchioles, and alveoli in the pigs, llamas and horses but not in the sheep. Original magnification ×400 for all images. DPP4, dipeptidyl peptidase-4; IHC, immunohistochemistry; MERS-CoV, Middle East respiratory syndrome coronavirus; PAS, periodic acid–Schiff; term., terminal.
nose of dromedaries seems to be much higher (16,19) than that of other animal species described so far, suggesting a more prominent role of dromedaries in transmission of MERS-CoV to humans.

Differences in virus susceptibility and pathogenicity between animals of different species could be explained by a distinct tissue distribution of DPP4, the MERS-CoV receptor. In our study, llamas and pigs displayed a similar DPP4 distribution in the respiratory tract, comparable with that of dromedary camels (15). In contrast, DPP4 was barely detected in the respiratory tract of sheep, probably accounting for the lack of infection reported here. These results are in concordance with those reported by Adney et al., that MERS-CoV experimentally inoculated sheep showed no clinical disease and that only small amounts of virus were detected in nasal swab samples (20). Differences in susceptibility to MERS-CoV infection and level of virus excretion might also result from host factors associated with innate immunity. Surprisingly, horses were not susceptible to MERS-CoV despite high expression and wide distribution of the virus receptor along the respiratory tract. Moreover, the receptor binding domain, and in particular key amino acids on the docking site, are identical in horses and humans (21). Although human, camel, and horse DPP4 served as potent and nearly equally effective MERS virus receptors (22), horses were not productively infected by the strain of MERS-CoV used in this study. Detection of low levels of viral RNA in nasal swab samples until postinoculation day 2 can be attributed to residual inoculum. Similarly, a recent study with horses also showed low levels of MERS-CoV excretion and no virus neutralizing antibodies (20).

These results highlight that other mechanisms, such as epithelial cell permissibility or strong innate immune responses, may influence the establishment of infection. In that respect, PAS staining revealed differences in the number of goblet cells in the lining epithelium and mucus covering epithelial surfaces, which may have impeded the binding of the virus to the respiratory epithelium of horses. Also, virus tends to bind more to ciliated or nonciliated non–mucus-producing cells and, in proportion, these cells may be fewer in horses than in llamas and pigs. However, it is possible that the guttural pouch empyema, which most likely was of bacterial origin (probably Streptococcus spp.), may have influenced mucus production in the horses. Although these observations are in line with those from studies in the field indicating the absence of antibodies to MERS-CoV in equids (14), this aspect should be studied further.

Epidemiologic studies have provided evidence of endemic MERS-CoV infection among dromedaries in the Greater Horn of Africa as far back as 1983 (23,24) and in Saudi Arabia as far back as 1992–1993 (25). To implement optimal serologic surveillance in countries where MERS is and is not endemic, identifying which animal species might be potential reservoirs for MERS-CoV, besides dromedaries, is crucial. The finding that pigs can be infected with MERS-CoV suggests that other members of the family Suidae could be susceptible to the virus, such as common wart-hogs (Phacochoerus africanus), bushpigs (Potamochoerus...
larvatus), and wild boars (Sus scrofa scrofa). Indeed, these animals are commonly found in the Greater Horn of Africa or the Middle East, sharing territories and water sources with dromedaries. Thus, members of the family Suidae might merit inclusion in MERS surveillance programs. Further studies need to be done to investigate MERS-CoV transmission within and among species to provide a better understanding of the role of potential reservoirs during an outbreak. Moreover, studies comparing the innate immunity of horses with susceptibility of other animal species (i.e., dromedary camels, alpacas, llamas, or pigs) are needed.

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References

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Phylogenetic analysis of the influenza hemagglutinin gene (HA) has suggested that commercial pigs in Chile harbor unique human seasonal H1-like influenza viruses, but further information, including characterization of these viruses, was unavailable. We isolated influenza virus (H1N2) from a swine in a backyard production farm in Central Chile and demonstrated that the HA gene was identical to that in a previous report. Its HA and neuraminidase genes were most similar to human H1 and N2 viruses from the early 1990s and internal segments were similar to influenza A(H1N1) pdm09 virus. The virus replicated efficiently in vitro and in vivo and transmitted in ferrets by respiratory droplet. Antigenically, it was distinct from other swine viruses. Hemagglutination inhibition analysis suggested that antibody titers to the swine Chilean H1N2 virus were decreased in persons born after 1990. Further studies are needed to characterize the potential risk to humans, as well as the ecology of influenza in swine in South America.

In the past decade, variant swine-origin influenza A viruses (swIAVs) of subtypes H1N2 and H3N2 have been associated with human infections, particularly in persons with close contact to swine (1–7), although evidence of human-to-human transmission is minimal. The emergence and subsequent pandemic caused by the unique influenza A(H1N1)pdm09 virus (pH1N1) from swine in 2009 highlights the public health threat posed by swIAVs, especially in populations having little preexisting immunity (8). Therefore, it is imperative to understand the circulation of swIAVs on a global level.

While our knowledge of the global diversity and evolution of swIAVs has increased, less information has been available regarding swIAVs circulating in South America. Recently, subtypes H1N1, H1N2, and H3N2 were identified in commercial pigs in Chile (9). Phylogenetic analysis from 18 swIAVs sequenced from a 2012 cross-sectional sampling of 22 production companies in Chile (95% of their commercial swine) demonstrated extensive genetic diversity in the hemagglutinin (HA) segment (9). The human seasonal H1-like viruses were unique to swine in Chile and not closely related to other H1 swIAVs. These viruses represented 2 independent introductions from humans, with Chile H1 human I clade most closely related to human viruses from the late 1980s and Chile H1 human II clade most closely related to human viruses from the early 1990s (9). No further phylogenetic or virus characterization information was available.

In addition to commercial pig farming, Chile has an extensive backyard production system, a common form of animal production throughout the world. Backyard production farms (BPFs) are a particular public health concern given poor biosecurity and close interaction between humans and multiple animal species (10). During 2014, we conducted active backyard production system surveillance in Chile’s main swine production area (11) and isolated a subtype H1N2 swIAV virus (A/swine/Chile/YA026/2014; sw/Chile). We performed phylogenetic analyses and full genomic sequencing on this virus to determine its relationship with other known influenza isolates. We propagated it in primary human bronchial and swine respiratory epithelial cells, as well as C57Bl/6 mice, to determine its infectiousness. We also characterized its transmission using ferrets. Hemagglutination inhibition (HI) assays with human serum samples were done to assess the risk this virus poses for human infection, and serological analyses were conducted with pig serum samples from Chile to determine the prevalence of the virus and its risk for emergence.

Materials and Methods

Study Area and Nasal Swab Sampling

Active surveillance was conducted as described (11) in 40 BPFs in Central Chile, representing 90% of the backyard

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production system. During this time, only 3 farms had swine (n = 3) available for sampling. We collected nasal samples using disposable sterile swabs, which were placed in 1 mL universal transport media. None of the animals were symptomatic.

H1N2 Virus Isolation

We screened nasal swab samples by real-time reverse transcription PCR for matrix gene as described (12). PCR-positive samples were centrifuged at 8,000 rpm for 15 min and the clarified supernatant (200 µL) inoculated on MDCK cells in 24-well plates in duplicate and incubated at 37°C in 5% CO₂ for 1 h. Following incubation, cells were washed then incubated with Eagle’s minimum essential medium (MEM; MediaTech, Manassas, VA, USA) supplemented with 2 mM glutamine, bovine serum albumin, and 1 µg/mL TPC (L-1-tosylamide-2-phenylethyl chloromethyl ketone)–trypsin until cytopathic effect was observed. Then, cell supernatant was collected, centrifuged, and stored at −80°C as described (12). Only 1 virus was isolated: A/swine/Chile/YA026/2014 (H1N2) (sw/Chile).

Cells

MDCK cells were cultured in MEM supplemented with 2 mM glutamine and 10% fetal bovine serum (Gemini BioProducts, West Sacramento, CA, USA) and grown at 37°C in 5% CO₂. A549 cells were cultured in Dulbecco’s minimum essential medium (DMEM; Lonza, Walkersville, MD, USA) supplemented with 4.5 g/L glutamine and 10% fetal bovine serum and grown at 37°C in 5% CO₂. Well-differentiated (transepithelial resistance >300 Ω × cm²), primary normal human bronchial epithelial (NHBE) cells were grown as previously described (13). In brief, cells were plated on collagen-coated membranes in bronchial epithelial basal media (Lonza) supplemented with SingleQuots additives (Lonza) and media changed every 48 h. When cells became 100% confluent, basal media was replaced with DMEM:F12 (Lonza) supplemented with SingleQuots, and apical media was removed. Every 48–72 h,

Figure 1. Phylogenetic trees showing comparison of swine influenza virus (H1N2) from Chile (red) and reference viruses. We performed phylogenetic analyses on complete hemagglutinin (A) and neuraminidase (B) genome sequences using RAxML with 200-bootstrap replicates (21). Blue indicates control H1 viruses. Scale bars indicate number of substitutions per site.
the apical surface was washed with 0.9% sodium chloride solution (Sigma, St. Louis, MO, USA). Cells were incubated at the air-liquid interface at 37°C in 5% CO₂. Primary swine nasal epithelial cells (sNECs) were prepared as described (14). In brief, nasal swab specimens were collected, placed in 50 mL conical tubes, and incubated in phosphate-buffered saline (PBS) at 4°C for 10–15 min before centrifuging for 10 min at 300 × g at 4°C. The pellet was resuspended in supplemented DMEM:F12 and incubated on collagen-coated plates at 37°C in 5% CO₂. Cells were plated onto transwells and, when 100% confluent, moved to an air-liquid interface to differentiate for 1–3 wk. sNECs were moved to 33°C 1 d before infection.

Virus Sequencing
Sanger sequencing of viral stocks was performed by the St. Jude Hartwell Center (12), and deep amplicon sequencing and assembly were conducted as described previously (15). Sequences can be obtained in GenBank (accession nos. CY207245–CY207252).

Viral Propagation
A/swine/Chile/YA026/2014 (H1N2, sw/Chile), A/Memphis/3/1987 (H1N1, Mem/87), A/swine/Iowa/13-1015/2010 (H1N2, sw/IA), A/swine/Indiana/26-0818/2011 (H1N2, sw/IN), A/swine/North Carolina/18161/2002 (H1N1, sw/NC), A/swine/Italy/1310-2/1995 (H1N1, sw/IT), and

Figure 2. Phylogenetic trees comparing the internal genes of swine influenza virus (H1N2) from Chile (red) and reference viruses. We performed phylogenetic analysis for the matrix (A), nucleoprotein (B), nonstructural (C), polymerase acid (D), polymerase basic (PB) 1 (E), and PB2 (F) gene segments by using RAxML with 200- bootstraps replicates (21). Purple indicates location of control influenza A(H1N1)pdm09 CA/09 virus. Scale bars indicate number of substitutions per site. Detailed phylogenetic trees for these genes are provided in online Technical Appendix Figures 1–6 (https://wwwnc.cdc.gov/EID/article/23/2/16-1374-Techapp1.pdf).
A/California/04/2009 (pH1N1, CA/09) viruses were propagated in the allantoic cavity of 10-day-old, pathogen-free, embryonic chicken eggs at 37°C and viral titers determined by 50% tissue culture infectious dose (TCID\textsubscript{50}) analysis as previously described (16,17). These viruses were used as comparators for all studies except the ferret experiments.

**Phylogenetic Analysis**

We performed sequence assembly and editing by using BioEdit version 7.2.5 (18) and sequence alignment with MUSCLE version 3.8.3 (19). Reference sequences were obtained from the Influenza Virus Resource at NCBI (20). Phylogenetic relationships for each gene were inferred by maximum likelihood, incorporating a general time-reversible model of nucleotide substitution with a gamma-distributed rate variation among sites by using RAxML version 8.0 (21). A bootstrap resampling process of 200 replicates was implemented to provide statistical robustness to each node.

**Swine and Human Serum Samples**

During 2013–2015, swine serum samples (n = 266, 1–24 animals/BP) were collected from 80 BPFs in Central Chile, including the Valparaiso, Metropolitán, and Libertador General Bernardo O’Higgins regions. During 2009–2015, human serum samples were collected as part of an ongoing prospective observational study at the University of North Carolina Family Medicine Center (Chapel Hill, NC, USA). We chose a subset of these samples, collected 28–32 d after seasonal influenza vaccination; grouped them according to birth decade (1920–1929, 1930–1939, 1940–1949, 1950–1959, 1960–1969, 1970–1979, 1980–1989, and 1990–1999; n = 7–22/age group); and balanced for sex and race whenever possible (22).

**Hemagglutination Inhibition Assay**

Ferret antiserum was generated against sw/Chile, Mem/87, sw/IA, CA/09, sw/IN, sw/NC, and sw/IT viruses. HI assay was conducted as previously described (23). In brief, antisera was treated with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan), inactivated, and diluted 1:10. Serial dilutions of receptor-destroying enzyme–treated serum were incubated in duplicate with each virus for 15 min at room temperature, followed by 30 min incubation at 4°C with 0.5% turkey erythrocytes. HI titer was defined as the reciprocal dilution of the last well that reacted.

**In Vitro Replication**

MDCK cells were infected with a multiplicity of infection (MOI) of 0.01 for 1 h at 37°C. Cells were washed 3 times to remove unbound virus, and infected cells were cultured in MEM containing 0.075% bovine serum albumin and 1 µg/mL TPCK-treated trypsin. Aliquots of culture supernatants were collected at 6 h postinfection (hpi), 24 hpi, 48 hpi, and 72 hpi and immediately stored at −80°C. For infection of NHBE cells and sNECs, the apical surface was washed twice and incubated with serum-free DMEM containing virus for 2 h at 37°C, after which both apical and basal media were removed and fresh growth medium was added to the basal chamber as described (24). At 6 hpi, 24 hpi, 48 hpi, 72 hpi, and 96 hpi, DMEM was added to the apical surface, and cells were incubated for 30 min at 37°C (for NHBE) or 33°C (for sNEC). Apical media was collected and stored at −80°C. Viral titers were determined by TCID\textsubscript{50} on MDCK cells followed by Reed-Munch analysis (25).

**Animal Infections**

Six- to 8-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME, USA) (n = 11 mice/group) were lightly anesthetized with isofluorane and intranasally inoculated with PBS or 10\textsuperscript{3} TCID\textsubscript{50} units of virus in 25 µL PBS. Mice were monitored daily for signs of infection and weighed every 48 hpi (26). At 3 d postinfection (dpi) and 6 dpi, 3 control and 3 infected mice were humanely killed; lungs were harvested and homogenized in 1 mL PBS and nasal washes were collected as previously described (27). In brief, an incision was made in the trachea and 500 µL of PBS was flushed through the upper respiratory tract and collected. Viral titers were determined by TCID\textsubscript{50} (16,24). Data are representative of 2 separate experiments. For transmission studies, 9- to 15-week-old male ferrets (n = 2, Triple F Farms, Sayre, PA, USA) were intranasally inoculated with 10\textsuperscript{4} TCID\textsubscript{50} units in 1 mL PBS. Twenty-four hours later, naïve ferrets (n = 2 mice/group) were either

**Table.** Antigenic characteristics of swine influenza virus (H1N2) from Chile and control viruses *

<table>
<thead>
<tr>
<th>Virus</th>
<th>Subtype</th>
<th>Major clade</th>
<th>Subclade</th>
<th>Mem/87</th>
<th>sw/Chile</th>
<th>sw/IA</th>
<th>sw/NC</th>
<th>sw/IT</th>
<th>CA/09</th>
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<td>North America</td>
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<td>640</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
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<td>swH1N2</td>
<td>North America</td>
<td>δ</td>
<td>&lt;</td>
<td>640</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>sw/IA</td>
<td>swH1N2</td>
<td>North America</td>
<td>δ</td>
<td>&lt;</td>
<td>&lt;</td>
<td>320</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>sw/NC</td>
<td>swH1N1</td>
<td>North America</td>
<td>γ</td>
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<td>&lt;</td>
<td>80</td>
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<tr>
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<td>320</td>
<td>40</td>
<td>-</td>
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<td>pH1N1</td>
<td>North America</td>
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<td>&lt;</td>
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<td>40</td>
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<tr>
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<td>North America</td>
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<td>&lt;</td>
<td>320</td>
<td>160</td>
<td>40</td>
<td>-</td>
</tr>
</tbody>
</table>


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placed in direct contact with infected ferrets or housed in separate cages (respiratory contact) separated by ≈5 inches. Nasal washes were collected every 2 dpi for viral titration and serum samples collected at 21 dpi for HI analysis as described (28).

Ethics Statement
All sampling was approved by the ethics and biosecurity committee of Faculty of Veterinary Science, University of Chile, and collected by trained veterinarians according to Food and Agriculture Organization guidelines (http://www.fao.org/3/a-ak738e.pdf). Animal experiments were approved by the St. Jude Children’s Research Hospital institutional biosafety committee and animal care and use committee and were in compliance with the Guide for the Care and Use of Laboratory Animals. These guidelines were established by the Institute of Laboratory Animal Resources and approved by the

Figure 3. Testing for influenza in swine from backyard production farms (BPFs) in Central Chile. Swine serum samples collected from BPFs were analyzed for antibodies against influenza by nucleoprotein (NP)–specific ELISA, and hemagglutination inhibition analysis of serum samples from NP ELISA–positive farms was conducted to determine subtype. A) Results of influenza testing by BPF location. Red circles indicate location of positive farms, and gray circles indicate location of negative farms. B) Pie charts showing percentage of swine at each sampling site seropositive for each virus type: sw/Chile (red), CA/09 (yellow), sw/IA (green), and sw/NC (blue). Red star indicates location of sw/Chile H1N2 virus isolate. Insets indicate region of Central Chile covered by this study (red shading).
Governing Board of the US National Research Council. All human procedures were approved by the Biomedical Institutional Review Board at the University of North Carolina.

Statistical Analysis
We performed statistical analyses using JMP statistical software (SAS Institute, Cary, NC, USA) and GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). The Kruskal-Wallis test ($\alpha = 0.05$) was used to analyze nonparametric data, while normally distributed data were analyzed by 2-way analysis of variance with virus strain and time postinfection as main effects. The Student $t$-test was used for posthoc comparisons between the viruses, and Tukey’s honest significant difference was used for posthoc comparisons among the times postinfection. Differences were considered significant at $p<0.05$.

Results

Phylogenetic Similarity of Chilean H1N2 and Human Seasonal Viruses
Phylogenetic analysis demonstrated that sw/Chile H1N2 belongs to the $\delta$-like H1 subgroup (Figure 1, panel A) and is phylogenetically similar to the swIAVs from Chile described by Nelson et al. (9). The HAs of these swIAVs are closely related to the HAs of human seasonal H1 viruses from the late 1980s and early 1990s (closest relative A/Suiteda/1/89, 94% nucleotide identity), although they are separated by long branch lengths and represent over 15 years of evolution (9). Unfortunately, only the HA segments from the 2012 Chilean swIAVs were available in public databases, so we were unable to compare full genomes.

The sw/Chile neuraminidase (NA) segment arose from a reassortment between human seasonal H1N1 and H3N2 viruses (Figure 1, panel B). However, long branch length indicates extensive genetic divergence from human N2 sequences, with the closest sequence being an early 1990s North American H3N2 virus (A/New York/762/1993, 93% nucleotide identity). This difference can be indicative of possible temporal and species-specific adaptations, leading to genetic and antigenic differences with contemporary human N2 viruses. The sw/Chile virus internal genes are pH1N1 in origin, consistent with the high reassortment rate between pH1N1 and swine viruses (Figure 2; online Technical Appendix Figures 1–6, https://wwwnc.cdc.gov/EID/article/23/2/16-1374-Techapp1.pdf) (9,29). Overall, while sw/Chile does appear to originally be of human origin, detection of these viruses in multiple BPFs suggests these viruses might have become established in the swine population in Chile.

Antigenicity and Seroprevalence of Chilean H1N2 Virus
HI assays with a panel of ferret antiserum against classical swIAV (sw/IN and sw/NC from $\beta$ and $\gamma$ subgroups, respectively); pandemic CA/09 virus; sw/Iowa (from $\delta$ subgroup); and human seasonal H1 virus Mem/87 showed
that sw/Chile H1N2 virus was antigenically distinct from other H1 viruses (Table). To determine seroprevalence in swine within the surveillance area, serum samples (n = 266, 1–24 animals/BPF) were collected from swine on 80 BPFs in Central Chile in 2013–2015. Forty-eight (60%) BPFs were positive by nucleoprotein-specific ELISA, and the prevalence of positive pigs (86/266, 32.3%) on each BPF ranged from 14.2% to 100% animals (Figure 3, panel A). HI assays performed with a panel of influenza viruses on the ELISA-positive samples demonstrated that 14/48 (29.1%) BPFs and 22/86 pigs were positive for ≥1 of the test viruses. Antibodies against sw/Chile were identified in BPFs located in 3 regions of Central Chile (Figure 3, panel B). Of concern, 11/14 (78.6%) of the positive BPFs, representing 15/22 (68.2%) of the positive samples, were positive for multiple influenza strains, suggesting that different influenza viruses are co-circulating in backyard swine in Central Chile (Figure 3, panel B). Not all nucleoprotein ELISA–positive samples could be subtyped.

**Chile H1N2 Virus in Primary Swine and Human Cells**

Swine possess both mammalian (α-2,6) and avian (α-2,3) influenza receptors (30,31). Receptor binding assays (23) revealed that all viruses used in this study had greater binding affinity for mammalian-type (α-2,6–linked sialic acid) receptors (online Technical Appendix Figure 7). These viruses also replicated effectively in MDCK cells; differentiated, primary NHBE cells; and sNECs cultured at the air-liquid interface. Cells were infected at MOI 0.01 and viral titers determined at the indicated times by TCID<sub>50</sub> analysis on MDCK cells (23).

All viruses replicated to similar titers in MDCK cells (Figure 4, panel A) and sNECs (Figure 4, panel C), except for an increased replication of CA/09 virus in MDCK cells. However, significant differences in replication were observed in primary NHBE cells. The human seasonal Mem/87 and sw/Chile viruses replicated to lower titers than the other swine viruses, suggesting reduced fitness in human cells (Figure 4, panel B). Overall, these studies demonstrate that sw/Chile can productively replicate in human and swine cells.

**Chile H1N2 Virus in Mice**

Recently identified swine H1N2 viruses from China replicated efficiently in mice and pigs (32); however, no data were available on viruses from South America. Thus, we intranasally inoculated Balb/c mice with 10<sup>5</sup> TCID<sub>50</sub> units of each virus and monitored weight loss for 14 dpi. Viral titers were measured 3 dpi and 6 dpi (Figure 5). CA/09 virus caused considerable weight loss, as did sw/IA (H1N2, δ1 subclade), which was similar to CA/09, sw/IN (H1N2, β subclade), and sw/NC (H1N1, γ2 subclade) viruses. Increased weight loss was associated with efficient replication in lungs (Figure 5, panel B). In contrast, the Mem/87 and sw/Chile viruses caused minimal illness and had lower replication in lungs (Figure 5, panels A and B). The sw/Chile virus replicated the most effectively in the upper respiratory tract compared with the other swine viruses, which reached titers similar to that of CA/09 virus (Figure 5, panel C). These data show that while sw/Chile virus can replicate in mice, it causes minimal illness. The genetic basis for differences in pathogenicity and tropism between the swine strains warrants further investigation.

**Figure 5.** Replication of influenza viruses in vivo. To evaluate pathogenicity in mice, 6- to 8-week-old BALB/c mice (n = 11 mice/group/experiment) were infected with 10<sup>5</sup> 50% tissue culture infectious dose (TCID<sub>50</sub>) units of the indicated viruses and weight loss was monitored for 14 days postinfection (dpi) (A). At 3 dpi and 6 dpi, lungs (B) and nasal washes (C) were collected from 3 mice/group and viral titers were determined by TCID<sub>50</sub> analysis. Data are presented as mean ± SEM. *p<0.05 versus sw/Chile virus.
Chile H1N2 Virus in Respiratory Droplets

Eurasian swine H1N2 viruses transmit in ferrets by direct contact and, in some cases, by respiratory droplet (33,34). To test the transmissibility of sw/Chile virus, we intranasally inoculated ferrets with $10^6$ TCID$_{50}$ units of sw/Chile, Mem/87, or sw/IA viruses; at 1 dpi, naive ferrets were either placed in direct contact or housed in cages adjacent to the donor ferrets to monitor respiratory droplet transmission. Nasal washes were collected every 2 dpi to assess viral shed, and animals were monitored through 21 dpi. All of the donor animals shed virus, and all viruses transmitted through direct contact and respiratory droplet with some differences (Figure 6). Overall, the swine viruses replicated to a higher titer than the Mem/87 virus did in the donor and direct contact animals; swine viruses also transmitted faster by respiratory droplet. Respiratory contacts of sw/Chile virus–inoculated animals were shedding virus by 6 dpi, whereas Mem/87 virus–inoculated animals only showed detectable nasal washes after 8 dpi and 10 dpi. The sw/IA virus appeared to transmit faster and replicate to higher titer than did the sw/Chile and Mem/87 viruses (Figure 6). All animals seroconverted at 21 dpi. These studies highlight that both swine H1 δ viruses transmitted effectively in ferrets.

Decreased HI Antibody Titer in Persons Born after 1990

Because the HA and NA of sw/Chile were derived from human seasonal strains originating around 1990, we hypothesized that persons born after 1990 would have decreased HI antibody titers. To test this, we conducted HI analyses against CA/09, Mem/87, sw/IA, and sw/Chile viruses on serum samples from 137 persons in North America collected in 2010–2015; samples were grouped by decade of birth (n = 7–22 samples/group). Most persons born before 1990 had titers against all 4 viruses (Figure 7). However, few persons born after 1990 had titers against sw/Chile (9%; Figure 7, panel B); sw/IA (31.8%; Figure 7, panel C); or Mem/87 (27.2%; Figure 7, panel D) viruses. Persons with titers ranging from 1:10 to 1:160 were all born in 1990 or 1991. In contrast, most (91.7%) persons born after 1990 had titers against pH1N1 virus, ranging from 1:320 to 1:10,240 (Figure 7, panel A).

Discussion

Our data show that the swine H1N2 virus from Chile is antigenically distinct from other H1 viruses, replicates efficiently in mammalian cells, can transmit by respiratory droplet, and might pose a risk to immunologically naive persons born after 1990. Our study furthers the data recently published on prevalence and HA phylogeny of Chile swIAVs (9). We show that these viruses are circulating outside of commercial swine herds in BPFs, where animal–human interaction is high.

Since emergence of pH1N1 in 2009, reverse transmission of the virus from humans to pigs has been documented worldwide, resulting in several reassortments with classical swine and seasonal human viruses (1,2,4,5,9,12,35). As of July 2016, variant (v) influenza viruses have caused over 380 human infections in the United States alone. While
most of these infections have been H3N2v viruses, 8 have been H1N2v viruses. These infections are particularly concerning because most infections occurred in children (persons <18 years of age) who had direct or indirect exposure to swine (2). Census estimates indicate that 50% of BPFs in Chile have ≥1 person born after 1990, and 35% have ≥1 person born after 2000 (C. Hamilton-West, unpub. data). Our data indicate that younger age groups have reduced HI titers to sw/Chile virus; however, studies with serum samples obtained from throughout the population of Chile are warranted to better determine the risk these viruses pose to swine herds.

This study is subject to several limitations. First, these studies were limited to 1 virus isolate. A panel of swine viruses from Chile isolated from different years and regions warrant study, especially if genetic differences are found among viruses. Second, these results represent a limited number of the backyard production system within Chile. Enhanced swine surveillance throughout Chile and South America is needed. Third, studies at the animal–human interface are required to decipher the risk circulating swIAVs pose to humans. We were limited to serum samples from persons living in North America, and further work should be done on South America populations, especially with those exposed to swine. Antigenic cartography on serum samples from persons involved in backyard farming would provide invaluable new information on transmission of swine viruses to humans. Finally, further work is needed to improve understanding of swIAV genes involved in transmission in the ferret model.

Despite no evidence of sustained human-to-human transmission, increased risk for human infection with H1N2v viruses calls for further study and enhanced monitoring. Risk for possible emergence of zoonotic strains has been demonstrated worldwide. H1N2 viruses isolated from pigs in China replicated efficiently in mice and pigs (32,36,37), while viruses from South Korea and Europe transmitted to and even caused death in ferrets (33,34,38). Future work will focus on how these reassortants arose in Chile BPFs and the role of specific genetic differences in pathogenicity and transmissibility. In addition, further studies for seroprevalence in Chile BPF workers and antigenic cartography with the sw/Chile virus are necessary. Studies at the animal–human interface are needed to determine seroprevalence, identify broadly protective antibodies, and characterize T-cell responses, and active surveillance is needed to uncover infection rates to better determine the risk for zoonotic transmission of swine-origin H1N2 to human health. In summary, our findings highlight the need for continued, vigilant influenza virus surveillance in Chile and throughout South America.

Acknowledgments
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Figure 7. Evaluation of serologic responses of humans to H1 viruses by age. Hemagglutination inhibition (HI) studies were performed for CA/09 (A), sw/Chile (B), sw/IA (C), and Mem/87 (D) viruses by using human serum samples (n = 137) collected as part of an ongoing prospective observational study carried out at the University of North Carolina Family Medicine Center (Chapel Hill, NC, USA) in 2009–2015. A subset of the available samples were chosen from persons whose serum samples were collected 28–32 days after seasonal influenza vaccination. Samples were grouped by decade of birth (1920–1929, 1930–1939, 1940–1949, 1950–1959, 1960–1969, 1970–1979, 1980–1989, and 1990–1999). Groups consisted of samples from 7–22 persons and were balanced for sex and age whenever possible. Gray dots represent inverse HI titers of individual persons and bars represent mean HI titer ± SEM per decade born.
Sean Cherry, Cyndey Johnson, and Bridgett Sharp for expert technical assistance; and Andrés Lazo for sample collection.

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Dr. Bravo-Vasquez is a veterinarian and scientist in the Department of Preventive Veterinary Medicine at the University of Chile, Santiago, Chile. His primary research interests are virology and epidemiology of influenza viruses.

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http://wwwnc.cdc.gov/eid/articles/issue/22/7/table-of-contents
Ancylostoma ceylanicum
Hookworm in the Solomon Islands

Richard S. Bradbury, Sze Fui Hii, Humpressor Harrington, Richard Speare, Rebecca Traub

Although hookworm is highly prevalent in the Solomon Islands, the species involved are unknown. We initiated this study in response to finding Ancylostoma ceylanicum hookworm in a peacekeeper in Australia who had returned from the Solomon Islands. Kato-Katz fecal surveys performed in 2013 and 2014 in 2 village groups in East Malaita, Solomon Islands, identified hookworm-positive samples. These specimens were tested by cytchrome oxidase 1 (cox-1) gene multiplex PCR and sequenced. Of 66 positive specimens, 54 (81.8%) contained only Necator americanus, 11 (16.7%) contained only A. ceylanicum, and 1 (1.5%) contained both species. A. duodenale was not found. Haplotype analysis of cox-1 sequences placed all human isolates (99% bootstrap support) of A. ceylanicum within the zoonotic clade rather than the human-specific clade. This study confirms that A. ceylanicum is endemic in the East Malaita region of this Pacific Island nation. The strain of the A. ceylanicum in this region can be shared among humans, dogs, and cats.

Hookworm disease, caused by blood-feeding worms of the small intestine, affects nearly 1 billion persons worldwide, with more than half of infections in the Asia-Pacific region (1). This disease causes iron deficiency anemia and malnutrition, leading to illness in pregnant women and children. In pregnant women, hookworm disease is associated with increased risks for death and poor neonatal outcomes, including low birthweight and increased deaths in infants. In children, substantial impacts on physical and intellectual development occur (2,3). Currently, Necator americanus and Ancylostoma duodenale hookworms are believed to be the main causative agents of hookworm disease globally.

Recently, A. ceylanicum hookworm infection was described in a soldier from Australia who had returned from the Regional Assistance Mission to the Solomon Islands, a peacekeeping mission (4). Until the past decade, human infection with A. ceylanicum was considered to be a rare zoonotic disease. However, several recent studies have found this species to be far more prevalent than previously reported, and it is now recognized as the second most common hookworm infection of humans in parts of Asia (5). The prevalence of this species in the Pacific Islands, and specifically in Melanesia, remains unexplored.

Isolated case reports of A. ceylanicum infection from the Pacific Islands were made in the early to mid-20th century. In 1929, A. ceylanicum infection was diagnosed in a 5-year-old child from Europe returning from long-term residence in the Shortland Islands (an island group in the north of the Solomon Islands) (6). Infection with A. braziliense (at that time synonymous with A. ceylanicum) was also described in 2 soldiers from Australia returning from service in Papua New Guinea during World War II (7) and in 9 servicemen from the Netherlands who had returned from West New Guinea in the early 1960s (8). A. braziliense and A. ceylanicum have similar morphologic features, with minor distinguishing points, but are distinct taxa (9). To our knowledge, A. ceylanicum infection has not been reported in the other Melanesian islands of Vanuatu or New Caledonia. Until the report of Speare et al. (4), no further human cases of A. ceylanicum infection were reported from the Solomon Islands or from any other Pacific Islands. In Australia, 2 autochthonous human infections of A. ceylanicum infection have been described (10).

In response to the case report of Speare et al. (4), we determined the species of hookworms, and, specifically, the prevalence of human A. ceylanicum infections, in the Solomon Islands, a group of islands southeast of Papua New Guinea and northeast of Australia, located within the Melanesian archipelago. Five soil-transmitted helminth (STH) surveys in this nation have been published (11–15). This survey aimed to determine the species of hookworm infecting communities in the remote East Malaita region of the province of Malaita.

Methods

Study Site

East Malaita is on the east coast of the island of Malaita in the Solomon Islands. The region has a wet equatorial tropical environment, with high rainfall. The average temperature is 27°C, and the weather is humid and hot at all times of the year. Two STH surveys, one in December 2013 and one in

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*Deceased.
August 2014, were conducted in 2 villages of the province. Village 1 (Na’au) is on the mainland of Malaita, 0.95 km southeast of the major East Kwaio village of Atoifi (coordinates 8°52.729’S, 161°0.772’E). The village has a population of 195 persons and an average age of 30.1 years (median 28 years); 48.0% of the total population is female. Village group 2 (Kwai and Ngongosila) is found in adjacent coral atolls off the coast of East Malaita, 12.3 km north of Atoifi. Ngongosila is 0.83 km off the coast and Kwai is 0.55 km east of Ngongosila. At low tide, a land bridge is formed between the 2 islands. Kwai Island (8°46.20’S, 168°56.50’E) has a population of 458 persons, and Ngongosila (8°46.37’S, 168°56.28’E) has a population of 357 persons. The average age in village group 2 is 23.7 years (median 18 years), and 49.6% of the total population is female. All persons living in these villages are ethnic Malaitan Solomon Islanders. Although a small number of children and adults leave the villages to go to high school in larger population centers, most remain in their home village, to the extent that there is no underrepresentation of specific age groups in the village.

**Participant Recruitment**

Initial discussions with village leaders and key members of the community were begun up to 12 months before the studies were performed. In the months prior to each study, discussions were held with the community. Community-wide gatherings for leading to participant recruitment were held on December 14, 2013 for village 1 and on August 8, 2014 for village group 2. All residents were invited to participate. A census of residents was conducted before the survey to determine the number of persons and families on each island. Each person was assigned a code to preserve anonymity, and written informed consent was received from each participant or a parent or guardian. Each consenting person was provided with collection containers marked only with the participant code. Containers of fecal samples were left by the participants at the communal toilets in each village, and were collected twice a day by the researchers.

**Hookworm Specimen Collection and Preservation**

The presence of infecting STH was determined on the day of specimen collection by using a single Kato-Katz analysis (16). Kato-Katz slides were read within 1 hour and 30 minutes and at 4 hours after preparation. Samples positive for hookworms on Kato-Katz analysis were stored for later PCR analysis to determine the species of hookworm involved. Single fecal samples from egg-positive persons were preserved in 100% ethanol in a ratio of 1:1 and stored at room temperature until DNA extraction could be performed.

**Molecular Methods**

Ethanol-preserved samples were rehydrated by being centrifuged at 500 × g; the ethanol supernatant was removed and replaced with sterile distilled, deionized water and incubated at 4°C overnight. DNA was then extracted with the Powersoil Kit (Mo Bio, Carlsbad, CA, USA), according to the manufacturer’s instructions. Extracted DNA was stored at −80°C until PCR was performed.

A previously published multiplex conventional PCR targeting the internal transcribed spacer region (ITS) 1, 5.8S, and ITS2 region of *Necator americanus* and *Ancylostoma* spp. was performed (17). We submitted PCR products of 380 bp corresponding to *Ancylostoma* spp. to Macrogen, Inc. (Seoul, South Korea), for purification and bidirectional DNA sequencing. We subjected samples that were positive for *A. ceylanicum* to a second published conventional PCR, targeting a 377-bp region of the *cox-1* gene of *A. ceylanicum* for haplotype characterization (18).

We analyzed DNA sequences by using Finch TV version 1.4.0 (Geospiza, Inc., Billerica, MA, USA) and aligned them with BioEdit version 7.2.5 (http://www.mbio.ncsu.edu/bioedit/page2.html) with the *cox-1* gene from *A. ceylanicum* Malaysia isolates (GenBank accession nos. KC247728/30/31/34/36/39/40/42–45, Pos Iskandar [Human], and 5g Bumbun [Human]); Cambodia isolates (GenBank accession nos. KF896595/97 and KF896600–KF896605), and southern China isolates (GenBank accession nos. KP072071, KP072074, KP072080); *A. duodenale* (GenBank accession no. NC003415) and *A. caninum* (GenBank accession no. NC012309) isolates were also included in the analysis. We inferred the evolutionary history by the neighbor-joining method and computed evolutionary distances with the maximum composite likelihood method by base substitutions per site. The analysis involved 37 nt sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. The final data set had 296 positions. We conducted evolutionary analyses in MEGA6 (http://www.megasoftware.net/).

**Statistical Analysis**

We performed statistical analysis in Excel (Microsoft, Redmond, WA, USA) using the Student *t*-test (2-tailed) for comparison of the age of infected participants by the infecting species of hookworm. We used the χ² test for gender but the Fisher exact test was used for source village and correlation to co-infection with other STH because the numbers were insufficient for a valid χ² analysis. We considered any p value <0.05 to be significant.

**Ethical Approval**

Ethical approval for this study was granted by the Atoifi Adventist Hospital Ethics Committee (approval no. AAH008). Reciprocal ethical approval was granted by the Central Queensland University Human Research Ethics Committee (approval no. H15/05–099).
Results
A total of 65 fecal samples (33% participation rate) were received from village 1 and 576 fecal samples (71% participation rate) were received from village group 2. The prevalence of hookworms in village 1 and village group 2, according to the results of the Kato-Katz analysis, was 47.7% (31/65) and 24.1% (139/576), respectively.

The species of hookworm in 66 hookworm-positive samples was determined by multiplex ITS1, 5.8S, and ITS2 region PCR. Most samples, 81.8% (54/66), contained only *N. americanus* hookworm; 16.7% (11/66) contained only *A. ceylanicum* hookworm, and 1 sample (1.5%) contained a mixed infection with both *N. americanus* and *A. ceylanicum* hookworms. The species of hookworm infecting these persons had no major effect on the likelihood of infection with other STH; furthermore, we noted no association of age, sex, or village to a specific hookworm species (Table).

Of the 12 *A. ceylanicum* hookworm–positive samples, we successfully amplified the *cox-1* gene for 10. Haplotype analysis of *cox-1* sequences placed all human isolates (99% bootstrap support) within the clade comprising a mix of *A. ceylanicum* isolates sourced from humans, dogs, and cats in Malaysia, China, and Cambodia, as opposed to the clade comprising human-only isolates of *A. ceylanicum* (Figure).

Discussion
Human infection with *A. ceylanicum* hookworm is highly prevalent in the East Malaita region of the Solomon Islands, comprising 18.2% of tested samples found by the Kato-Katz method to contain hookworm eggs. Overall hookworm prevalence at Na’au was 47.7% and at Kwai-Ngongosila was 24.1%, and the prevalence of *A. ceylanicum* hookworm in tested samples at these villages was 11.9% and 3.9%, respectively. It appears that, as is the case in many Southeast Asia countries, *A. ceylanicum* is the second most common hookworm infecting humans in East Malaita. The presence of *A. ceylanicum* infection in the Eastern Malaita region of the Solomon Islands adds to the growing weight of evidence that *A. ceylanicum* is a widespread hookworm in humans and that this species may be present in other parts of Melanesia and the Pacific Islands. Indeed, the previously held assumption that human infection with this hookworm was a rare and unusual occurrence now appears false.

All *A. ceylanicum* isolates recovered in this study clustered within the zoonotic clade of this hookworm species, able to infect humans, dogs, and cats in China, Malaysia (19), and Cambodia (18). No isolates belonged to the second, more rare, clade also found in Southeast Asia, which, to date, consists only of human-infecting strains (18,20). Human settlement in the Pacific Islands is a more recent event than human colonization of Asia (21), and the human population density of the Pacific Islands is far lower than that in Southeast Asia. It is possible to propose a hypothesis that the human-adapted clade of *A. ceylanicum* might have evolved from the zoonotic clade in Asia, evolving to become specific to human hosts over an extended period of transmission only within that host species. Strains of *A. ceylanicum* brought to Melanesia with humans and their domesticated animals would be at a selective disadvantage to evolve from zoonotic to anthroponotic helminths because of the much lower human population density and relatively shorter time for evolution. Collection of multiple examples of each clade from throughout the Southeast Asian and Pacific regions, followed by analysis of evolutionary changes in mitochondrial DNA, might assist in clarifying this hypothesis.

| Table. Prevalence of *Ancylostoma ceylanicum* and *Necator americanus* among hookworm egg–positive fecal samples collected in East Kwao, Solomon Islands, 2013–2014. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| All patient samples | *A. ceylanicum*† | *N. americanus*† | Total | p value |
| Total no. samples | 12 | 55 | 68 | |
| Patient sex | | | | |
| M | 6 | 27 | 33 | 0.78 |
| F | 5 | 27 | 32 | |
| ND | 1 | 1 | 2 | |
| Patient age, y | | | | |
| Mean | 30‡ | 32§ | 32 | 0.78 |
| Median | 30 | 34 | 32 | |
| Range | 2–65 | 5–80 | 2–80 | |
| Village | | | | |
| Village 1 | 4 | 12 | 16 | 0.46 |
| Village group 2 | 8 | 42 | 50 | |
| Other | 0 | 1 | 1 | |
| STH co-infection | | | | |
| *Ascaris lumbricoides* | 5 | 20 | 25 | 0.75 |
| *Trichuris trichiura* | 1 | 5 | 6 | 1.00 |
| Both | 0 | 5 | 5 | 0.58 |

†Includes 1 mixed sample.
‡Two samples excluded from analysis because no data for age were recorded.
§Four samples excluded from analysis because no data for age were recorded.

ND, no data; STH, soil-transmitted helminth.
Dogs and cats are found in these rural villages. In a 2011 survey of Na’au, 17.5% of respondents reported owning or having ≥1 dogs in their household, and 40.8% reported similar close contact with cats (R. Speare, H. Harrington, unpub. data). In the East Malaita region of the Solomon Islands, this strain of *A. ceylanicum* may be both a zoonosis and an anthroponosis, transmitting in multiple directions among humans, dogs, and cats. Given the high prevalence in humans, it seems likely that if a wide selection of domestic cats and dogs were sampled, animal infections with this zoonotic clade of *A. ceylanicum* would also be discovered.

We did not recover *A. duodenale* hookworms from any of the 66 human infections sampled. This finding is consistent with the widely held assumption of early researchers that *A. duodenale* was a hookworm native to Europe, India, and China that had been introduced into the Pacific Islands by immigrants from those parts of the world. Early reports from American Samoa, Solomon Islands, Ellice Islands, Tonga, Cook Islands, and New Hebrides found exclusively *N. americanus* infection among the local populations, with *A. duodenale* hookworm seen only in immigrant populations from Europe, China, and India (22–24). Darling et al. (24) found infections with *A. ceylanicum*, but not *A. duodenale*, hookworm in native populations of Fiji in the 1920s but did not report the relative prevalence of these species. Walker and Bellmaine (7) reported a large number of *A. duodenale*
infections in servicemen from Australia returning from Papua New Guinea during World War II, but this hookworm was common in northern Australia at that time (25) and may have also been imported from the Northern Hemisphere with European colonization or acquired from other soldiers from Australia during the New Guinea campaign.

Several isolated cases of *A. ceylanicum* infection from the Pacific Islands and, more specifically, Melanesia have been reported previously, in which adult worms were recovered and identified (4,6,24). Only 1 study in the Pacific region (specifically, in Australia) has used PCR for species identification; this study found a relatively high percentage of *A. ceylanicum* infection (29%), although the sample size of autochthonous hookworms was low (n = 7) (10).

Given the established presence of human *A. ceylanicum* infections in Southeast Asia, Australia, and the Solomon Islands, and the rare historical reports of cases from Papua New Guinea and Fiji (8,24), it seems likely that this infection is common in other countries in the wider Pacific region, and particularly in Melanesia. Although numerous studies of hookworm prevalence in the regions have been performed previously, in very few were the species of hookworm identified. Those studies that did specific taxonomy almost exclusively used morphology of filariform larvae to differentiate *A. duodenale* from *N. americanus*. Although *N. americanus* hookworm lacks the prominent constriction of the intestine at the esophageal junction that is seen in filariform larva of *A. duodenale*, as well as several other subtle morphological differences, morphological differentiation of 2 species is established primarily by the far more prominent striations of the posterior sheath of *N. americanus*. These striations are not readily visible on the posterior sheath of *A. duodenale* larvae (26). The filariform larva of *A. ceylanicum* share the posterior sheath striations seen in *N. americanus* (27) and thus may easily have been misidentified as *N. americanus* in studies in which only the morphology of these larvae in culture was used for identification. Larger surveys using PCR for species identification to further elucidate the prevalence and extent of *A. ceylanicum* infections throughout the Pacific are warranted.

This study did not record data on clinical data such as hemoglobin, eosinophilia, and nutritional status among participants for comparison of the relative impact of infecting hookworm species on these indices. Experimental infection of 2 human volunteers with *A. ceylanicum* hookworm by Carrol and Grove (28) found that eosinophilia occurred initially, declined after 4 weeks, and then completely resolved despite ongoing infection. These volunteers also showed no abnormality in hemoglobin or other blood count parameters. This pattern is similar to that seen with experimental infections with *N. americanus* hookworm (29). In contrast, Anten and Zuidema (8) noted marked eosinophilia and iron deficiency anemia among Dutch servicemen returning from New Guinea who were infected with *A. ceylanicum* hookworm. Given the high prevalence of *A. ceylanicum* infection in many parts of the world, studies of the clinical and nutritional effects of this soil-transmitted helminth are needed to determine the role and potential pathogenicity of infection.

This study was not specifically intended to act as a prevalence survey; rather, it was an attempt to define whether *A. ceylanicum* might be present in Melanesia. However, the percentage of hookworm-positive samples recovered is likely to be broadly representative of the total hookworm burden in those villages. Hookworm can cause mild diarrhea in symptomatic patients, which may introduce a slight bias in prevalence data. However, PCR is more sensitive than single-sample microscopy for the detection of hookworm (18). Had this method been performed on all fecal samples, it is almost certain that a higher number of positive samples would have been found. Furthermore, the relative frequency of each hookworm species in the random subset of microscopy-positive samples preserved and tested by PCR in Australia may not reflect the true prevalence in the source population. Although a degree of correlation of species prevalence results might be reasonably expected, it is not known whether *A. ceylanicum* hookworms produce relatively fewer or more eggs per day than *N. americanus* hookworms or if one species causes more severe diarrhea than the other. Each of these factors could have led to a degree of bias in the selection of microscopy-positive samples for later species analysis.

In summary, this study presents findings showing that *A. ceylanicum* may be the second most common human hookworm infection in the East Malaita region of the Solomon Islands, after *N. americanus*. By contrast, infection with *A. duodenale* hookworm appears to be absent from this region. We recommend that further work be done to investigate the prevalence of this species in humans and their domestic animals throughout the Pacific Island nations and territories and that its clinical significance as a human pathogen be elucidated as soon as feasible. This finding also highlights that hookworm control programs in Solomon Islands would benefit from considering a One Health approach, because, to be successful, these programs may have to control hookworms in humans, dogs, and cats simultaneously (5).

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This article is dedicated to the memory of our co-author, Emeritus Professor Richard Speare. Rick’s kindness, tireless work, and dedication to others leaves a profound legacy and testament to a great man in the fields of parasitology, zoonotic disease, and public health.

Dr. Bradbury is an Australian parasitologist with an interest in all fields of parasitology. He was recently appointed as the team lead of the Reference Diagnostics Laboratory in the Division of Parasitic Diseases and Malaria, Center for Global Health, Centers for Disease Control and Prevention, Atlanta, Georgia, USA. He wrote this article in both his personal capacity and in his capacity as an adjunct academic at Central Queensland University.

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In 2014, inactivated poliovirus vaccine (IPV) campaigns were implemented in Nigeria and Pakistan after clinical trials showed that IPV boosts intestinal immunity in children previously given oral poliovirus vaccine (OPV). We estimated the effect of these campaigns by using surveillance data collected during January 2014–April 2016. In Nigeria, campaigns with IPV and trivalent OPV (tOPV) substantially reduced the incidence of poliomyelitis caused by circulating serotype-2 vaccine–derived poliovirus (incidence rate ratio [IRR] 0.17 for 90 days after vs. 90 days before campaigns, 95% CI 0.04–0.78) and the prevalence of virus in environmental samples (prevalence ratio [PR] 0.16, 95% CI 0.02–1.33). Campaigns with tOPV alone resulted in similar reductions (IRR 0.59, 95% CI 0.18–1.97; PR 0.45, 95% CI 0.21–0.95). In Pakistan, the effect of IPV+tOPV campaigns on wild-type poliovirus was not significant. Results suggest that administration of IPV alongside OPV can decrease poliovirus transmission if high vaccine coverage is achieved.

The live attenuated oral poliovirus vaccine (OPV) is cheap and easy to administer; therefore, it has been the vaccine of choice for the Global Polio Eradication Initiative. However, the immunogenicity and efficacy of OPV is reduced in tropical developing countries, perhaps as a result of the high burden of other enteric pathogens (1,2). Furthermore, although OPV offers lifelong protection against paralytic poliomyelitis, intestinal immunity against infection and poliovirus shedding appears to wane quite rapidly (3), meaning that OPV-immunized persons may contribute to community transmission of poliovirus (4,5). OPV also causes vaccine-associated paralytic poliomyelitis in ≈1 child per million vaccinated, and, in rare instances, may revert to a neurovirulent and transmissible form, causing outbreaks of poliomyelitis associated with vaccine-derived polioviruses (VDPV) (6,7).

In developing countries, seroconversion induced by inactivated poliovirus vaccine (IPV) is better than that induced by trivalent OPV (tOPV); thus, in these countries, IPV provides better protection against paralytic poliomyelitis (8,9). However, immunization with IPV alone induces very limited intestinal mucosal immunity against viral shedding compared with that induced by OPV, and therefore, IPV may be permissive to poliovirus circulation even when vaccination coverage is high (10,11). Recent studies showed that in children previously exposed to OPV (and therefore mucosally primed), the administration of 1 dose of IPV substantially boosted intestinal immunity against fecal shedding of poliovirus measured after challenge with the live attenuated OPV (12,13). Of note, this boost was greater than that observed after an additional dose of OPV.

These encouraging findings motivated the introduction, beginning in 2014, of IPV to mass vaccination campaigns in Nigeria and Pakistan, 2 countries that had circulating VDPV and wild-type poliovirus at that time. However, it is not yet known whether the IPV boost of intestinal immunity observed against a challenge dose of vaccine (Sabin) poliovirus will translate to an effect of IPV campaigns on transmission of wild-type polioviruses and VDPVs at the community level.

To determine the effectiveness of campaigns using IPV+tOPV or tOPV alone in preventing the circulation of wild-type polioviruses or VDPVs, we analyzed poliovirus surveillance data for 2014–2016 from Nigeria and Pakistan. We report the results of that analysis and discuss their implications for the polio endgame strategy.

Materials and Methods

Data
We analyzed polio vaccination data reported from Nigeria and Pakistan during January 1, 2014–April 30, 2016, by combining information on the dates and locations (districts) of vaccination campaigns, the district-level incidence of poliomyelitis reported through surveillance for acute flaccid paralysis, and the presence or absence of poliovirus in environmental samples collected at regular
Statistical Analysis

We defined the incidence rate ratio (IRR) for a campaign as the rate of reported poliomyelitis cases in the period after the campaign divided by the rate reported before the campaign. We focused on a 90-day period before and after the campaign, but we explored the sensitivity of our results to the choice of the length of this time period. We created a line-list containing every campaign for each district in Pakistan and Nigeria and recorded the 1) campaign location (district and state/province); 2) vaccine(s) used (i.e., IPV+tOPV, tOPV alone, bivalent OPV [bOPV] alone, or serotype-1 monovalent OPV [mOPV] alone); 3) polio incidence before and after the campaign; and 4) length of the observation period. To avoid including information more than once in the statistical analysis, we censored data following entry into the line-listed database. To avoid bias, we entered campaigns with each vaccine(s) into the database in random order, according to a sequence of vaccine types (i.e., IPV+tOPV, tOPV, bOPV, mOPV); this system of entry maximized available information on campaigns that used IPV+tOPV and their effect compared with campaigns that used tOPV alone.

We used a Poisson regression model to estimate the IRR by vaccine type(s) administered in a campaign. The incidence of poliomyelitis in a district was modeled as a log-linear function of the following 3 independent variables: 1) an indicator variable (i) for whether observations were before or after a campaign; 2) the natural log of the number of child-years of observation (t) with a coefficient equal to 1, such that the incidence rate per child-year was modeled; and 3) an interaction term, such that the change in incidence after a campaign depended on a categorical variable (v) describing the type of vaccine(s) used in the campaign. We used a mixed-effects model with a random effect for each state or province and vaccine combination on the model intercept (baseline incidence). We fit this model using the lme4 package in the R statistical programming language [regression equation: \( \text{n\_cases} \sim \text{i} + \log\text{(t)} + (1|\text{state}:v) \), offset = \( \log\text{(t)} \), where : indicates an interaction term and (1|state:v) indicates the random effect on the intercept] (16,17). We compared this mixed-effects regression model with a model that included only the fixed effects using the Akaike Information Criterion (AIC). We used a similar approach with a mixed-effects binomial regression model to estimate the prevalence ratio (PR) for the proportion of environmental samples that were positive for poliovirus isolation before and after campaigns with different vaccines.

Results

Nigeria

In Nigeria, 5 campaigns with IPV+tOPV in different locations and at different scales took place during the analysis period, resulting in 55 district-campaign observations (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/2/16-1210-Techapp1.pdf). These campaigns used a total of 3.8 million doses of IPV and targeted children 14 weeks–59 months of age; although, 1 campaign included only children 6–35 months of age. The campaigns were followed by a reduction in the incidence of poliomyelitis associated with circulating VDPV serotype 2 (cVDPV2). The IRR, based on the mixed-effects regression, for cases in the 90 days after the campaigns compared with 90 days before the campaign was 0.17 (95% CI 0.04–0.78; \( p = 0.023 \)) (Figure 1; online Technical Appendix Table 1). After these campaigns, the prevalence of cVDPV2 was reduced among environmental samples collected in the same districts as the campaigns; however, this reduction was not statistically significant (PR 0.16 based on the mixed-effects regression, 95% CI 0.02–1.33; \( p = 0.09 \)). Campaigns with tOPV alone did not significantly reduce the incidence of poliomyelitis associated with cVDPV2 (IRR 0.59, 95% CI 0.18–1.97; \( p = 0.215 \) for comparison with IPV+tOPV campaigns), but they did significantly reduce the prevalence of cVDPV2 in the environment (PR 0.45, 95% CI 0.21–0.95; \( p = 0.02 \)). Campaigns with bOPV containing serotypes 1 and 3 did not show any effect on cVDPV2 prevalence or poliomyelitis cases. In areas of Nigeria where IPV campaigns were conducted, the number of polio cases associated with wild-type poliovirus (n = 1) was insufficient to enable a comparable analysis for this poliovirus type. Changing the length of time examined before and after each campaign did not substantially change the results. Reducing the period to <90 days led to a loss of statistical power; increasing the period to 150 days, resulted in a statistically significant effect of IPV+tOPV campaigns
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on the prevalence of cVDPV2 in environmental samples (online Technical Appendix Table 2).

The mixed-effects regression models gave a significantly better fit to the data compared with models that included only fixed effects, indicating heterogeneity among states in the incidence of poliomyelitis and prevalence of poliovirus isolation; the AIC for mixed versus fixed-effects models was 110.4 versus 194.9 for the IRR and 137.5 versus 171.6 for the PR. This heterogeneity is also apparent in plots of the location of poliomyelitis cases and environmental surveillance results before and after campaigns with IPV+tOPV (Figure 2).

Pakistan
During the analysis period in Pakistan, 15 IPV+tOPV campaigns were conducted, resulting in a total of 133 district-campaign observations (online Technical Appendix Table 3). These campaigns targeted children up to 23 months of age and used a total of 3.9 million doses of IPV. The effect of these campaigns on the number of cases of poliomyelitis associated with wild-type poliovirus serotype 1 (WPV1) and isolation of this virus in the environment was not apparent (IRR 1.01, 95% CI 0.50–2.02; PR 0.88, 95% CI 0.56–1.38) (Figure 1; online Technical Appendix Table 3). Campaigns using only tOPV had a modest but statistically significant effect on the incidence of poliomyelitis associated with WPV1 (IRR 0.79, 95% CI 0.63–0.99; p = 0.039). However, this effect was not apparent from environmental data (PR 0.93, 95% CI 0.71–1.22; p = 0.586). In areas of Pakistan where IPV campaigns were conducted, the number of poliomyelitis cases associated with cVDPV2 (n = 2) was insufficient to enable analysis. Changing the length of time examined before and after each campaign did not substantially change these results, although the effect of campaigns with IPV+tOPV on the incidence of poliomyelitis became significant when the period was increased to 150 days (online Technical Appendix Table 4).

Our results showed evidence for significant heterogeneity in the incidence of poliomyelitis and prevalence of WPV1 isolation among provinces in Pakistan. The AIC for mixed-effects versus fixed-effects models was 187.3 versus 1393.3 for the IRR and 133.7 versus 169.3 for the PR.

Discussion
We assessed the effect of vaccination campaigns that used IPV alongside OPV on the transmission of wild-type and VDPV. In Nigeria, we observed a substantial reduction in the incidence of poliomyelitis associated with cVDPV2 and in the detection of cVDPV2 in environmental samples after mass campaigns with IPV+tOPV. This reduction was greater than that observed after campaigns that used tOPV alone, although the difference was not statistically significant. This finding suggests that the substantial boost to intestinal immunity after vaccination with IPV, as observed in recent OPV challenge studies (12,13), translates into a measurable effect of IPV campaigns on poliovirus transmission in the community. These encouraging findings support the use of IPV in campaigns during the polio

Figure 1. Effect of mass vaccination campaigns with inactivated poliovirus vaccine plus trivalent oral poliovirus vaccine (IPV+OPV) or tOPV alone on poliovirus detection in persons or the environment, Nigeria and Pakistan, 2014–2016. The incidence rate ratio for poliomyelitis and the prevalence ratio for poliovirus detection in environmental samples (sewage) during 90 days after compared with 90 days before mass vaccination campaigns are shown for Nigeria (A) and Pakistan (B) and can be compared with the complete data and estimates (online Technical Appendix Tables 1, 3 (https://wwwnc.cdc.gov/EID/article/23/3/16-1210-Techapp1.pdf). The estimates (diamonds) are shown with 95% CIs (error bars); the dashed error bars indicate when the upper CI exceeded the plot limit of 1.4.
Effect of Inactivated Poliovirus Vaccine Campaigns

In Pakistan, an effect of mass campaigns with IPV+tOPV on persistent WPV1 circulation was not apparent. This finding may partly reflect a lack of statistical power. In the sensitivity analysis, using a longer time window of 150 days resulted in a significant reduction in the incidence of poliomyelitis: IRR 0.56 (95% CI 0.33–0.95) for IPV+tOPV campaigns and IRR 0.67 (0.53–0.86) for tOPV-only campaigns. However, this reduction was not apparent for WPV1 isolated in the environment (PR 0.85 [95% CI 0.59–1.23] for IPV+tOPV campaigns, and PR 1.00 [95% CI 0.76–1.30] for tOPV-only campaigns). In Pakistan, the lack of evidence for an effect from IPV campaigns may also reflect low coverage during the campaigns and restrictions on the age groups that were targeted for vaccination. Campaign coverage was suboptimal in Pakistan during much of 2014–2015; in September 2015, in areas at high risk for polio, just 39% of union councils were estimated to have achieved >80% campaign coverage, although this improved to 62% in November 2015 (18). Estimates of campaign coverage during January 2014–June 2015, which were based on the vaccination histories of children reported with nonpolio acute flaccid paralysis, also suggest that coverage among undervaccinated communities in Pakistan was poorer than that in Nigeria (19). Suboptimal vaccination coverage means that fewer children will benefit from a boost in intestinal immunity by IPV, not only as a result of smaller numbers receiving the vaccine, but also because fewer children will have been mucosally primed by OPV given in earlier campaigns or though routine immunization.
systems. According to WHO/UNICEF, routine immunization coverage with OPV in 2015 was suboptimal (≈75% on average) in both Pakistan and Nigeria.

During vaccination campaigns, it is more challenging to deliver IPV than OPV because IPV must be administered by trained healthcare workers and is thus offered at specific locations only (e.g., health centers) rather than being delivered directly to households, as with OPV. Nonetheless, in Nigeria and Pakistan, IPV campaign coverage has been reported to be comparable to that for OPV-only campaigns (20,21). However, in Pakistan, IPV was administered only to children <2 years of age, and in Nigeria (with the exception of 1 campaign), children <5 years of age were included. The wider age group in Nigeria may have contributed to the greater effect of IPV on poliovirus transmission because children 2–4 years of age may shed poliovirus and contribute to transmission despite being protected against poliomyelitis.

The difference in the effect of campaigns that used IPV in Pakistan and Nigeria may also reflect differences in the circulating poliovirus types (cVDPV2 in Nigeria and WPV1 in Pakistan). However, this seems unlikely because the transmissibility and pathogenicity of cVDPV2 in Nigeria appears equivalent to that for wild-type poliovirus (22).

Our analysis has several limitations, including its observational nature and lack of randomization of vaccines used during campaigns, which could have resulted in systematic differences in the areas that used IPV+tOPV compared with tOPV alone. We attempted to account for these differences in our statistical analysis by allowing for random variation in the incidence of poliomyelitis and prevalence of virus isolation by state or province and by vaccine(s) used in the campaign. The statistical power of our analysis was also limited by the low incidence of poliomyelitis in areas with IPV+tOPV campaigns. In Nigeria, the estimate of the effect of IPV+tOPV campaigns against poliomyelitis was driven by the results from Borno State, where an emerging cVDPV2 outbreak was apparently stopped by these campaigns. Environmental surveillance data from other states (Sokoto, Kaduna) support the effectiveness of IPV in campaigns, but the number of sampling sites informing the estimates was limited. As further experience with the use of IPV in campaigns is acquired, and with the planned expansion of environmental surveillance, it will be possible to refine our estimates. It may also become possible to increase the strength of our analyses by using additional statistical techniques, such as interrupted time-series methods, which are currently not appropriate, given the limited number of observations. Although a cluster-randomized trial would deal with any biases introduced through a lack of randomization in our study, such a trial would not be ethical, and to achieve sufficient statistical power would require such a large study as to be impractical.

Another limitation of our study is that we used poliovirus isolation in environmental (wastewater/sewage) samples as a proxy for poliovirus transmission in the community. Although environmental surveillance is known to be highly sensitive for poliovirus circulation in the catchment population, even in the absence of poliomyelitis cases, it may not capture more subtle effects of vaccination campaigns on the extent of poliovirus transmission within an area (23). Last, our before-and-after comparisons may be confounded with seasonal and longer term trends in poliovirus transmission. However, in Nigeria and Pakistan, the median months for IPV+tOPV campaigns were May and June, respectively, compared with June and July, respectively, for tOPV campaigns; the similarity of this timing suggests that the comparison of these campaigns was not confounded by seasonal trends. In addition, the absence of any effect of bOPV against cVDPV2 in Nigeria, as was expected, suggests that any confounding, if present, was minimal.

Our analysis offers support for the use of IPV in mass vaccination campaigns to stop poliovirus transmission, provided good coverage can be achieved. This use of IPV in campaigns should be pursued while maintaining sufficient supply of IPV for routine immunization in high-risk OPV-using countries (24). Any vaccine shortage could be compensated for by the use of fractional dose IPV, provided there is sufficient evidence of its efficacy in boosting immunity. The greater effect in Nigeria of IPV+tOPV campaigns compared with tOPV-only campaigns suggest that IPV-only campaigns may also offer substantial benefit. However, campaigns with IPV alone have not been implemented. Evaluation of the potential benefits of IPV-only campaigns in the context of waning intestinal immunity, a growing cohort of children who have not received OPV2, and global containment of OPV2 after withdrawal of the vaccine serotype should be a priority. Results of continuing programmatic experience will also inform efforts to improve the levels of coverage and the benefits of including children 3–4 years of age in IPV campaigns. Regardless, it is now clear that IPV will play a major role in securing the global eradication of poliomyelitis and in maintaining a polio-free world.

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References


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In February 2012, the novel respiratory syncytial virus (RSV) group A, genotype ON1, was detected in Kilifi County, coastal Kenya. ON1 is characterized by a 72-nt duplication within the highly variable G gene (encoding the immunogenic attachment surface protein). Cases were diagnosed through surveillance of pneumonia in children at the county hospital. Analysis of epidemiologic, clinical, and sequence data of RSV-A viruses detected over 5 RSV seasons (2010/2011 to 2014/2015) indicated the following: 1) replacement of previously circulating genotype GA2 ON1, 2) an abrupt expansion in the number of ON1 variants detected in the 2014/2015 epidemic, 3) recently accumulation of amino acid substitutions within the ON1 duplicated sequence, and 4) no clear evidence of altered pathogenicity relative to GA2. The study demonstrates the public health importance of molecular surveillance in defining the spread, clinical effects, and evolution of novel respiratory virus variants.

Respiratory syncytial virus (RSV) is a major cause of pneumonia and bronchiolitis among infants and children globally (1, 2). Although immune responses develop in those who have had RSV infection during childhood, these persons remain susceptible to RSV upper respiratory tract reinfection throughout life (3). No licensed RSV vaccine exists. Of the 11 proteins encoded by the RSV genome, the attachment glycoprotein (G) is the most variable and has been shown to accumulate amino acid changes over time (4). RSV is classified into 2 groups, RSV-A and RSV-B (5); each group is divided into genotypes (6), and these are further characterized into variants (7). Globally, RSV viruses belonging to different groups, genotypes, and variants often co-circulate in epidemics (7, 8). The phenomenon of reinfection and difficulty in developing a vaccine may in part be due to the antigenic diversity and variability in the virus (9).

Two novel RSV genotypes with large duplications of amino acids in the attachment G glycoprotein have been detected globally. In 1999, the BA genotype was detected in Buenos Aires, Argentina; the genotype had a 60-nt duplication within the C-terminal region of the G gene (10). The BA variant subsequently spread rapidly throughout the world, becoming the predominant group B genotype, and in some regions replacing all previous circulating RSV-B genotypes (11). More recently, in December 2010, genotype ON1, with a 72-nt duplication (also within the C-terminal region of the G gene), was detected in Ontario, Canada (12). Viruses belonging to this genotype have rapidly spread and diversified globally (13–20). Such emergent genotypes appear to have a fitness advantage over preceding genotypes of the same RSV group (21). Of public health interest is whether increased fitness is associated with increased severity and immune evasion (with potential vaccine modality implications).

The temporal progression of RSV genotypes can be followed directly because of the unique tags (the duplications), which provides a rare opportunity to learn more about the introduction, spread, severity, and related selection processes (including immune evasion) for RSV and to obtain insights into the nature of emergence of novel virus variants. In this regard, we undertook an in-depth analysis of RSV-A genotype ON1 epidemiology in Kilifi, a county in coastal Kenya. In Kilifi, RSV epidemics typically begin during September–November of 1 year and continue until July–August of the following year, with a peak in cases during January–March. We have analyzed sequence data collected over 5 RSV epidemic seasons in Kilifi (2010/2011 to 2014/2015), which includes the period after the initial detection of this novel genotype within Kilifi.

Spread and Evolution of Respiratory Syncytial Virus A Genotype ON1, Coastal Kenya, 2010–2015


DOI: http://dx.doi.org/10.3201/eid2302.161149
Materials and Methods

Study Location and Population
The study was undertaken in Kilifi County and is part of surveillance aimed at understanding the epidemiology and disease effects of RSV-associated pneumonia cases in this region (22). Respiratory swab samples (combined nasopharyngeal and oropharyngeal) were collected from September 2010 through August 2015 from children ages 1 day to <5 years admitted to Kilifi County Hospital (KCH) with syndromically defined severe or very severe pneumonia (referred to here as lower respiratory tract infections, LR-TIs), as defined in Table 1 and previously (22)

Study Samples and Laboratory Procedures
All specimens were screened for RSV by 2 methods (22–24). Raw samples were tested for RSV antigen by immunofluorescence antibody test (Chemicon International Inc., Temecula, CA, USA). Viral RNA was extracted from respiratory samples using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and tested for RSV (differentiating groups A and B) by multiplex real-time reverse transcription PCR. All RSV-positive samples by either test were taken forward for processing. In addition, a small number of RSV-negative samples were sequenced.

The viral RNA was reverse transcribed into cDNA by using the Omniscript RT Kit (QIAGEN). The cDNA was then amplified with primers targeting the G ectodomain region (3,25), and the amplicons were sequenced by using BigDye version 3.1 chemistry on an ABI 3130xl (Applied Biosystems, Waltham, MA, USA). Sequence reads were assembled into contigs by using Sequencher version 5.0.1 (Gene Codes Corp., Ann Arbor, MI, USA). The sequences analyzed here have been deposited in GenBank (accession nos. KX453303–KX453534); previously reported sequences from Kilifi added to this analysis had also been deposited in GenBank (accession nos. KF587911–KF588014) (13).

Global Comparison Dataset
To determine the relatedness of the Kilifi viruses to those circulating around the world and thereby clarify their global context, we downloaded all RSV-A G-gene sequences collected during 2010–2015; deposited in GenBank as of January 19, 2016; and 241 nt to 687 nt in length. A total of 995 sequences from 24 countries were used in this analysis. For the whole dataset and for some countries, we further grouped sequences by calendar year for temporal analysis. We subsampled unique sequences (sequences that differed by ≥1 nt from any other sequence over the sequenced region) by epidemic season (Kilifi only) or per calendar year.

Sequence Alignments and Diversity Analysis
All sequences, from Kilifi and the global dataset, were collated and aligned using MAFFT (multiple alignment using fast Fourier transform) alignment software version 7.272 (26). We calculated the variability of nucleotides and amino acids using MEGA 6.06 (27).

Phylogenetic Analyses
We used MEGA 6.06 to infer maximum-likelihood phylogenetic trees under the general time reversible model with the site heterogeneity gamma model (27). This model was the best substitution model as determined by IQ-TREE version 1.4.2 (28). Bootstrapping with 1,000 iterations was implemented to evaluate branch support of the phylogenetic clusters. We assigned RSV-A genotypes as previously determined by Peret et al. (6) and Eshaghi et al. (12). To position the genotype ON1 viruses in the global context, we examined ON1 lineages as recently assigned by Duvvuri et al. (20).
**RSV-A Variants Analysis**

We determined the number of genotype GA2 and ON1 variants circulating in Kilifi and globally using a recently developed pragmatic criterion \((7,8)\). In brief, a variant is a virus or a group of viruses within a genotype that possesses \(\geq 4\) nt differences in the G ectodomain region when compared with other viruses. This analysis was done using usearch v8.1.1861 \((29)\).

**Protein Substitution and Selection Analysis**

The \(N\)-glycosylation sites were predicted by using the NetNGlyc 1.0 server \((30)\). We only considered the default Asn-X-Ser/Thr sequon (when X was not proline) for prediction. We also analyzed for patterns of change in amino acids using python scripts. Finally, we looked for potential positively selected and co-evolving sites using the Datamonkey server (http://www.datamonkey.org/). For positive selection analysis, we used 3 methods: SLAC (single likelihood ancestor counting), FEL (fixed effects likelihood), and MEME (mixed effects model for evolution).

**Statistical Analyses**

We explored associations between demographic, clinical, or outcome variables and RSV genotypes for all cases of RSV-positive severe and very severe pneumonia. We used logistic regression computing odds ratios (ORs) in Stata version 13 (StataCorp LP, College Station, TX, USA).

**Results**

Over the 5 RSV epidemics examined (2010/2011 to 2014/2015), a total of 4,010 samples were collected from eligible children; 3,561 (88.8%) were tested for RSV and 881 (24.7%) RSV-positive samples were identified (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/2/16-1149-Techapp1.pdf). Of these samples, 600 (68.1%) were RSV-A. The G gene was successfully sequenced in 442 (73.7%) samples. An additional 41 sequences were available from samples that were negative by both immunofluorescent antibody test and PCR or from patients with mild pneumonia (data for these cases were not included in the clinical severity analysis). Thus, we carried 483 sequences for phylogenetic analysis. The sequences ranged from 618 nt to 690 nt in length, corresponding to nucleotides 295–912 of the reference strain A2 (M74568).

We found that 2 RSV-A genotypes were circulating in Kilifi: ON1 \((n = 283, 58.6\%)\) and GA2 \((n = 200, 41.4\%)\). The temporal prevalence of the total RSV, RSV-A, and genotypes ON1 and GA2 is shown in Figure 1 and online Technical Appendix Table 1. We observed rapid replacement of the previously circulating dominant GA2 genotype by ON1 in Kilifi, from a prevalence of 0% in the 2010/2011 epidemic to a prevalence of 67.4% in 2011/2012 when ON1 was first detected in Kilifi, and to 96.1% in the recent 2014/2015 epidemic. In addition, RSV-A predominated in 3 consecutive RSV epidemics from 2012/2013 to 2014/2015.

To investigate the demographic and clinical effects of RSV-A genotype ON1 in Kilifi, we compared the proportions of GA2- and ON1-infected case-patients by sex, age, clinical features of cough, difficulty in breathing, chest wall indrawing, inability to drink, hypoxia, prostration/consciousness, pneumonia status (severe or very severe pneumonia), length of hospital stay, and death at the hospital (Table 1). The proportions of both genotypes were very similar for the demographic and clinical characteristics analyzed. However, the proportion of patients with ON1 infection who were unable to eat was more than double that for GA2-infected case-patients \((18.9\% \text{ vs. } 8.8\%)\), and this difference was found significant by logistic regression \((OR 2.40, 95\% CI 1.31–4.36; Table 2)\). Nonetheless, the proportion with very severe pneumonia was no higher in ON1 infections than in GA2 infections \((OR 0.89, 95\% CI 0.57–1.39)\).

The maximum-likelihood tree shows the clustering of unique genotype ON1 sequences in Kilifi (Figure 2). Two
genotype ON1 lineages recently defined by Duvvuri et al. (20) are shown to be circulating in Kilifi: lineages ON1 [1.1] and ON1 [1.3]. Of these 2 lineages, ON1 [1.3] was the most prevalent in 2011/2012 and 2012/2013. However, a potential new lineage, denoted here as ON1 [1.4], clustered away from ON1 [1.3] and seemed to have recently arisen comprising sequences from the strains circulating in the 2013/2014 and 2014/2015 epidemics. The genetic divergence (p distance) between ON1 [1.4] and the other ON1 lineages identified in Kilifi ranged from 0.013 to 0.045, similar to the genetic distances between the previously defined ON1 lineages.

We detected a total of 66 RSV-A variants during the entire surveillance period in Kilifi (online Table 3, http://wwwnc.cdc.gov/EID/article/23/2/16-1149-T3.htm). The variants comprised 1–82 sequences; 39 (59.1%) of the 66 variants were singletons. Most variants did not persist between epidemics (46/66 (69.7%)). However, 14 variants persisted for 2 consecutive seasons, 1 for 4 consecutive seasons, and 5 for 2 nonconsecutive seasons. Therefore, the number of variants (accumulated by epidemic) increased to 86 by 2014/2015. The number of GA2 variants declined consistently, from 17 variants in 2010/2011 (before ON1 arrived) to only 4 variants in 2014/2015. On the other hand, the number of ON1 variants assigned remained at 5 variants between 2011/2012 and 2012/2013 before increasing to 8 variants in 2013/2014, then rising markedly to 25 variants in 2014/2015.

Seven codon sites were predicted to be N-glycosylated within the G protein for the Kilifi sequences: 4 sites for genotype ON1 viruses (codons 103, 135, 237, 318) and 6 sites for genotype GA2 viruses (codons 103, 135, 237, 251, 273, 294). However, none of the potential N-glycosylation sites occurred within the 72-nt duplication of the ON1 viruses. Notably for GA2 viruses, sites 237 and 273 were mutually exclusive: a virus belonging to this genotype had either these sites potentially N-glycosylated but not both (online Technical Appendix Figure 1).

The nucleotide variability and amino acid variability over the 4 seasons are shown in online Technical Appendix Table 2. Amino acid substitutions over the sequenced portion of the G protein are shown in Figure 3, panel A. Two codon positions possessed amino acid substitutions that distinguished between ON1 (232G, 253K) and GA2 (232E, 253T) viruses. In addition, the new ON1 [1.4] lineages viruses seem to have fixed a threonine (I/T136T) and acquired a unique substitution (P206Q) that distinguishes them from the other ON1 lineages.

Figure 3, panels B and C, illustrates amino acid substitutions within the duplication region of the Kilifi ON1 viruses. We designated the first set of 72 nt as duplication sequence I and the second set as duplication sequence II. Within this region, we observed that over the 3 seasonal epidemics from 2011/2012 to 2013/2014 and early (September–November) in the 2014/2015 epidemic, amino acid substitutions only occurred within the duplicated sequence I except for 3 substitutions in 2 viruses within the duplicated sequence II. Beginning in December 2014, however, we found numerous substitutions in duplicated sequence II with 2 adjacent and corresponding positions between the duplicated sequences I and II acquiring similar amino acid substitutions (i.e., Y273H and Y297H, P274L/S and P298L/R). Furthermore, sites 273 and 297 were detected to be co-evolving from the Spidermonkey analysis (http://www.datamonkey.org/). However, only 1 ON1 codon site (251) was identified to be positively selected with p<0.05 by >1 method (SLAC, FEL, or MEME).

Using global datasets for genotypes ON1 and BA, we compared the temporal detection of RSV variants within each of these genotypes during the first 5 and 10 years, respectively, from initial detection (online Technical Appendix Table 3). We observed an explosion of new ON1 variants globally, from 8 variants in 2011, to 78 variants in 2012, to 153 variants in 2013. However, the number of ON1 variants decreased in 2014 and 2015, which corresponded with a substantial decrease in both the number of ON1 sequences available in GenBank and the countries that have deposited sequences from these years. On the other hand, the number of BA variants seemed to follow a stepwise or punctuated pattern, whereby the number of variants was stable at 1–6 during 1996–2001, dramatically increased and stabilized at 20–30 variants during 2002–2004, and again sharply rose to 82 variants in 2005 (online Technical Appendix Table 3). At the country level (online Technical Appendix Table 4), the rapid rise in the number of ON1 variants detected in Kilifi was also observed in the Philippines and Germany, although the number of BA variants detected in some of the countries sampled remained relatively stable over time.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Unadjusted odds ratio (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt;1 y</td>
<td>0.86 (0.49–1.49)</td>
<td>0.579</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.76 (0.52–1.12)</td>
<td>0.172</td>
</tr>
</tbody>
</table>

Table 2. Clinical severity comparison between cases of pneumonia caused by respiratory syncytial virus A genotypes ON1 and GA2 in children admitted to Kilifi County Hospital, Kenya, September 2010–August 2015*
Discussion
We provide a detailed analysis on the spread and the associated demographic, clinical, and evolutionary characteristics of the novel RSV-A genotype ON1 in Kilifi. ON1 was first detected in Kilifi in February 2012, and within that RSV epidemic (2011/2012), it displaced the previous dominant genotype GA2 by attaining a prevalence of 67%. Its dominance has continued to rise to 96% within a span of 4 epidemics. This rapid rate of replacement is unlike previous replacement rates in the same location; for example,
when GA5 was displaced by GA2, it took GA2 ≈7 years to reach a prevalence of 95% (8). ON1 seems to possess a fitness advantage over GA2. If such fitness is the result of immune evasion, this characteristic has potential implications for vaccines to deliver population level immunity by herd protection (31).

We found evidence that infections caused by RSV ON1 are more severe than those caused by GA2, showing a higher prevalence of patients’ inability to eat. However, overall numbers of cases of very severe pneumonia were equal for both genotypes. The data, therefore, do not provide a strong indication of more severe disease arising from the ON1 variant. Duvvuri et al. (20) reported significant association of RSV ON1 infection with female patients, which was not evident in our study. Yoshihara et al. (32) reported that cases of upper respiratory infection caused by RSV ON1 in Vietnam caused were significantly associated with clinically severe manifestations of wheezing, tachypnea, and difficulty in breathing compared to infections caused by RSV NA1, whereas Panayiotou et al. (33), on the contrary, reported that children infected with RSV ON1 in Cyprus experienced significantly milder illness than those infected with RSV GA2. Some studies have reported no differences at all between infections with these genotypes. The discordant results may arise from methodologic differences in analyses, clinical disease definitions, and study designs, chance effects resultant from inadequate sample sizes, differences between viruses in different locations, or even host/environmental differences. Prospective studies specifically designed to evaluate virulence or clinical differences between genotypes may offer more reliable insight.

We noted a change in the alternation of RSV subgroup dominance pattern in Kilifi since ON1 was introduced into this community. While ON2 was replacing GA2, it also appeared to also exclude group B strains. RSV-A predominated over RSV-B in 3 consecutive epidemics from 2012/2013 to 2014/2015. Previously, according to data collected during 2002–2012 in Kilifi, RSV-A predominated in up to 2 consecutive epidemics (8). However, it is unclear whether this is a direct effect of ON1, a general change in RSV epidemiologic patterns, or a chance occurrence.

Globally, the prevalence of ON1 seems to vary by location. In Ontario, Canada, where ON1 was first detected in December 2010, the prevalence of ON1 has remained stable at 11%–13% (20). Other countries that have similarly reported ON1 prevalence rates <20% include South Africa (34) and China (35). Reports from Italy (14), South Korea (36), United States (17), Malaysia (37), Japan (19), Thailand (16), Latvia (38), and Cyprus (33) indicate varied RSV ON1 prevalence of 20%–70%. A recent article reported that ON1 was the sole (100%) RSV-A genotype in Buenos Aires, Argentina, in 2014 (39). The varying prevalence suggests that even though ON1 is rapidly spreading globally, host or ecological differences may determine RSV spread. However, the conclusions of host and ecological differences potentially driving varying prevalence rates in different countries may have been confounded by inadequate/short surveillance periods in these countries.

As shown here and in previous research from Kilifi (7,8), RSV epidemics are composed of multiple variants, each differing sufficiently to suggest separate introductions into the community (as opposed to arising from diversification during the epidemic). In addition, these variants often do not persist between epidemics, which suggests that each year 1) variants generate local herd immunity, leading to their demise, thus requiring reintroductions; or 2) that many invading variants compete in seeding new seasonal epidemics, and that the preceding year variants lose out (perhaps on a chance basis or as stated above because they are less fit due to variant specific immunity).

RSV accumulates amino acid changes over time (4), and we have similarly observed accumulation of amino acid changes in the Kilifi ON1 viruses. It is of interest that in the first 3 RSV epidemic seasons in which we detected ON1 in Kilifi, amino acid substitutions were almost always restricted to the duplicated sequence I of the 72-nt duplication. However, in the 2014/2015 epidemic a virtual explosion in amino acid substitutions was observed within the duplicated sequence II that coincided with a surge in the number of detected ON1 variants. In addition, similar amino acid substitutions occurred in 2 adjacent and corresponding sites in the duplicated sequences I and II, with 1 set of these sites co-evolving. The longer attachment protein of the 72-nt duplication in ON1 viruses appears to offer more opportunities for variable changes and thus greater diversity and increased fitness over previous group A genotypes.

Two codon sites, 232 and 253, within the G protein region analyzed were found to distinguish between genotype ON1 and GA2 viruses. The amino acid change Glu-232-Val has been reported for RSV-A escape mutants that result in loss of reactivity to a specific monoclonal antibody (4). Furthermore, a functional analysis of the 60-nt duplication in BA strains has shown that the duplicated region in the G protein of these viruses augment their fitness (21). While a similar analysis has not been reported for ON1, it is plausible that the increased fitness observed in ON1 is largely due to the 72-nt duplication. However, G-protein N-glycosylation seems to play no role in the increased fitness of ON1 as similar potential N-glycosylation codon sites were detected in both ON1 and GA2 and no additional N-glycosylation sites were detected within the ON1 duplication region.

The rapid diversification of ON1 observed in Kilifi seems to reflect rapid expansion at the global level. While
sampling variability may play a role, and similar to varying prevalence, there was variability in the diversification of ON1 viruses in different countries. The number of ON1 variants seemed stable in some countries (e.g., Japan) while expanding in others (e.g., Germany and Philippines). The temporal distribution of BA variants in different countries, however, was mostly stable. Comparisons in the temporal patterns of genotypes BA and ON1 variants may highlight differences between the RSV group B and A viruses. Increased sampling and surveillance will help illuminate on whether such inter-genotypic and RSV group differences are due to ecologic differences or variable sampling.

In conclusion, it is evident that genotype ON1 is not only rapidly spreading globally but also fast evolving. The result is the near exclusion of the previous dominant group A GA2 genotype. The implications of this apparent increased fitness of RSV-ON1 have yet to be resolved. There is some evidence for increased severity of the virus but this is by no means clear or consistent across studies. Continued surveillance for cases together with collection of detailed standardized clinical data are warranted. The possibility exists that ON1 and other similar new RSV variants (e.g., the BA genotype) gain dominance by evading host immunity. It is reasonable to assume this could lead to evasion of future vaccine protection, lessening the herd immunity potential of vaccination, similar to influenza A vaccines.

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References
51. Kinyanjui TM, House TA, Kiti MC, Cané PA, Nokes DJ, Medley GF. Vaccine induced herd immunity for control of respiratory syncytial virus.
During 2006–2015, we analyzed 70 dengue virus (DENV) strains isolated from febrile travelers returning to Germany. High genetic diversity, including multiple co-circulating DENV lineages and emerging new lineages of DENV-3 and DENV-4, was demonstrated. Our passive surveillance system based on returning travelers yielded substantial information on DENV diversity.

Although dengue virus (DENV) infects ≈390 million persons annually and one third of the world’s population is at risk for infection, there is no effective vaccine or specific antiviral therapy for infection with DENV (1). Dengue is a rapidly spreading mosquito-borne viral disease and the most frequent cause of febrile illness among international travelers returning from DENV-endemic tropical areas, such as Southeast Asia, the western Pacific region, and the Americas (2,3). Viremic travelers have the potential to introduce DENV into DENV-free or nonendemic areas where competent mosquito vectors are present (4). Reintroduction of DENV in regions that had been considered free of the disease for many years has also been observed (5–7).

Phylogenetic analysis has elucidated the origins, epidemiology, and forces that shape DENV molecular evolution in nature (8). For example, according to official German air travel statistics reports, 4,855,763 air trips were taken in 2011 from Germany to countries listed as DENV-endemic areas by the World Health Organization; 10%–20% each of these trips were made to India, Thailand, and Brazil; and 5%–10% each flew to Singapore, Mexico, and the Dominican Republic (9). We determined the genetic relatedness and molecular epidemiology of DENV isolates from travelers returning to Germany during 2006–2015.

The Study
During 2006–2015, we analyzed 15,876 acute-phase serum samples from patients with suspected DENV infection; the samples had been submitted to the World Health Organization Collaborating Centre for Arbovirus and Hemorrhagic Fever Reference and Research for diagnostic testing. We tested all samples by using DENV type-specific real-time reverse transcription PCR (rRT-PCR) (10) or an antigen-capture ELISA (Platelia Dengue NS1 Ag; Bio-Rad, Hercules, CA, USA) and in-house DENV IgG and IgM indirect immunofluorescence assays. rRT-PCR—and nonstructural protein 1–positive serum samples that tested negative for DENV IgG and IgM were spread onto Vero E6 cells and incubated for 7 days at 37°C; successful DENV isolation was identified by rRT-PCR. We extracted viral RNA from cell culture supernatants by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany).

We successfully isolated 70 DENV strains originating from 20 countries (online Technical Appendix, http://www.cdc.gov/EID/article/23/2/16-0751-Techapp1.pdf). We amplified the complete envelope glycoprotein (E) gene using DENV type–specific degenerate primers (online Technical Appendix). Sequence assembly, analysis, and multiple alignments were performed with Geneious version 7.1.8 (Biomatters, Auckland, New Zealand). All available complete envelope gene sequences of DENV serotypes 1–4 (DENV-1–4), except laboratory strains and potential recombinants, were retrieved from GenBank and compared with those sequenced in this study. The phylogenetic relationships and origin of the imported DENV isolates were analyzed by the maximum likelihood method in the RAxML program (11) with general time-reversible plus gamma distribution substitution model and a rapid bootstrap (100 replicates) procedure, and visualized in FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Most DENV infections were acquired in Thailand (35.7%), followed by Indonesia (12.8%), the Philippines (10%), and India (7.1%). The proportion of cases imported from other countries in Asia, Africa, and the Americas ranged between 1.4% and 4.3% (Figures 1, 2). Phylogenetic analysis revealed a high genetic diversity of DENV-1–4 in travelers from Germany, including co-circulation of multiple genetically diverse viral lineages that were closely related to those previously circulating in the Americas and Southeast Asia (mostly Thailand, Indonesia, and...
the Philippines) (online Technical Appendix). DENV-1 was the predominant and most genetically diverse type, representing 33 sequences and clustering into 23 phylogenetically distinct lineages. Most of the isolates belonged to genotypes I and II, which are circulating in Southeast Asia (12). These genotypes represent dominant regional variants; only a few strains are closely related to viruses circulating in the Americas (genotype V, lineages 3–6) or to a genotype III virus in India. All but 3 DENV-2 isolates were the Cosmopolitan genotype, and the source population was limited mostly to countries in Southeast Asia (online Technical Appendix).

DENV-2 phylogeny provides evidence for 7 phylogenetically distinct introductions of the virus into travelers from Germany; the origins of these isolates were predominantly in Bali and the Philippines. DENV-3 sequences belonged to genotypes I–III; these isolates were closely related to lineages representing regional or local variants, except lineages 8 and 9 (online Technical Appendix).

A key phylogenetic pattern was the presence of a new lineage, L6 (online Technical Appendix), within DENV-3 phylogeny comprising closely related isolates sampled from German, Taiwanese, and Chinese travelers returning from Thailand and Laos during 2012–2014. A similar pattern of clustering to that for DENV-3 was observed for DENV-4 isolates. Thus, most DENV-4 from travelers were infected with genotype I and III strains closely related to lineages commonly sampled within Southeast Asia and to a genotype II isolate from Bolivia. A key phylogenetic pattern was the presence of a new lineage, L3, within DENV-4 phylogeny (online Technical Appendix) with sequences found in samples from patients tested during 2013–2015 with travel histories to Thailand, China, and the Philippines (lineage 3). Lineage 3 was the most recently circulating lineage detected in this study. This clustering is compatible with extensive viral traffic between Thailand, China, and the Philippines: Thailand, where most of the DENV infections were acquired, is thus a possible source of a virus population responsible for local or regional outbreaks.

Conclusions
Countries in Southeast Asia that are considered DENV hyperendemic are increasingly popular tourist destinations for residents of Germany; thus, German travelers to these countries are potentially exposed to multiple types and genotypes of DENV. A high prevalence of DENV infection has been reported in travelers returning from DENV-endemic areas, emphasizing the importance of international travelers as potential sources of imported disease or sentinels for local outbreaks in DENV-free or non–DENV-endemic areas (2,3). The relative risk of infection by country is difficult to calculate without attention to seasonal fluctuations in dengue fever incidence and travel patterns. Broadly speaking, though, among the top contributing countries mentioned, the comparative risk of infection with travel-associated DENV appears much higher in the Philippines and Indonesia (10% and 12.8% of the cases, compared with 1.7% and 2.8%, respectively, of travelers from Germany to DENV-endemic countries) than in Thailand (35.7% of the cases versus 15.0% of the

Figure 1. Geographic origin of dengue viruses isolated from travelers returning to Germany, 2006–2015.
travelers) and the lowest in India (7.1% of the cases versus 18.5% of the travelers).

We investigated DENV diversity and origin of infection in travelers returning to Germany from DENV-endemic areas and identified a high genetic diversity of DENV genotypes and lineages. Notably, 2 of these lineages (DENV-3, genotype III, lineage 6 and DENV-4, genotype III, lineage 6) appear to have emerged very recently and are still responsible for local outbreaks in countries in Southeast Asia, thus reiterating the need to monitor the appearance and spread of novel lineages. Most investigated isolates were closely related to lineages known to have circulated in Thailand, the Philippines, and Indonesia, indicating that these countries serve as a major source of multiple DENV lineages (12). The observed high numbers of co-circulating lineages in the Thai, Indonesian, and Philippines source populations support the hypothesis of multiple geographic origins or extensive virus interchange among these countries (online Technical Appendix).

Surveillance of symptomatic returned travelers can provide information on circulating DENV genotypes and lineages in heavily visited tourist areas and DENV-endemic regions. In Europe, the emergence of arboviruses should be particularly monitored because of the introduction and expansion of the DENV vector Aedes albopictus mosquito. In Germany, where recently introduced Aedes albopictus mosquitoes have spread in the southwestern part of the country, international travelers and the presence of competent vectors could potentially facilitate seasonal local transmission of DENV (7,13,14). Our findings indicate a diverse array of imported DENV infections in travelers from Germany and emphasize the need for a continued surveillance of DENV infections in non–DENV-endemic regions as well as prompt and rapid serologic and molecular testing for DENV infection in febrile patients returning from DENV-endemic countries.

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References

Dengue Virus in Returning Travelers, Germany


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http://wwwnc.cdc.gov/eid/articles/etymologia

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We compared the epidemiology of hepatitis A and hepatitis E cases in China from 1990–2014 to better inform policy and prevention efforts. The incidence of hepatitis A cases declined dramatically, while hepatitis E incidence increased. During 2004–2014, hepatitis E mortality rates surpassed those of hepatitis A.

Hepatitis A virus (HAV) and hepatitis E virus (HEV) cause acute hepatitis in humans and are transmitted mainly through the fecal–oral route. Hepatitis A and hepatitis E became notifiable in China in 1990 and 1996, respectively. Since the introduction of the hepatitis A vaccine and the start of mass vaccination in several countries in the 1980s, hepatitis A incidence declined substantially, not only among vaccinated children but in the population as a whole (1,2). China first licensed its live attenuated hepatitis A vaccine in 1992 and later the inactivated hepatitis A vaccine in 2002 (3). The hepatitis A vaccine was initially introduced into the private market, although some provinces provided subsidies through the World Health Organization Expanded Programme on Immunization (http://www.wpro.who.int/china/areas/immunization/en/). Starting in May 2008, hepatitis A vaccinations were incorporated into the routine national childhood immunization program for children >18 months of age (3).

The Study

We obtained data on cases of hepatitis A reported during 1990–2014 and hepatitis E for 1997–2014 from China’s National Notifiable Disease Report System and collated demographic information from the China National Bureau of Statistics. We defined confirmed cases on the basis of dates of disease onset and updated diagnostic criteria issued by the Chinese Ministry of Health in 2008; these criteria are based on epidemiologic history, clinical signs, and laboratory test results (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/23/2/16-1095-Techapp1.pdf).

We used R version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria) and SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) to estimate annual incidence and mortality rates for hepatitis A and hepatitis E according to patient age and sex. Notified cases were geocoded into provinces and mapped by ArcGIS 10 (Esri Inc., Redlands, CA, USA). To examine seasonality, we created heat maps by using monthly incidence normalized to the maximum incidence each year. We used a similar approach to examine seasonality across latitudes. We assessed potential associations between incidence and demographic and economic factors by using Poisson regression.

Hepatitis A incidence dropped from 55.7 cases/100,000 person-years in 1991 to 1.9 cases/100,000 person-years in 2014, a decrease of 96.6% (Figure 1). In contrast, hepatitis E incidence increased significantly over this period, from 0.21 cases/100,000 person-years in 1997 to 1.99 cases/100,000 person-years in 2014, an 8-fold increase (p<0.0001 by Poisson regression) (Figure 1). The mortality and incidence rates for hepatitis E overtook those for hepatitis A in 2004 and 2011, respectively (Figure 1). Hepatitis E cases across the country were most frequently reported in March (online Technical Appendix Figures 4, 5). This change may result from increased temperature and rainfall in the spring, which could increase the likelihood of acquiring HEV.

HEV is a substantial cause of illness and death worldwide, particularly among pregnant women (4). Until the introduction of the first hepatitis E vaccine to private markets in China in 2011, there were no specific pharmaceutical interventions for HEV (5). Given the similarity in diseases caused by HAV and HEV and the recent decline in hepatitis A incidence, we compared the epidemiology of human cases infected with the 2 pathogens in China.

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DISPATCHES

1These authors contributed equally to this article.
2These authors are joint senior authors.
HEV infection from exposure to contaminated water, such as water sourced from a stream near a free-range pig farm (6–8). In contrast, the seasonal pattern of HAV infections varied by latitude; cases were reported most frequently in the spring in southern provinces and in the autumn in most northern provinces (online Technical Appendix Figure 5). The decline in the incidence of hepatitis A cases in winter and summer varies by province, and reasons for the differential latitudinal pattern are unclear (7–9).

Hepatitis A incidence was highest among children and young adults (Figure 2). In contrast, hepatitis E incidence was highest among older adults and low among children and young adults (Figure 2). For both diseases, incidence and mortality rates were higher among male patients, and mortality rates tended to increase with age (Figure 2). The percentage of infections resulting in death were generally similar among men and women within each age group for most years (online Technical Appendix Figure 2).

We found considerable changes in the epidemiology of hepatitis A and E over time in mainland China. The major decline in hepatitis A incidence during 1992–2014 cannot be explained solely by the introduction of the vaccine because implementation of vaccinations in the general population had been relatively low until the inactivated hepatitis A vaccine was included in the national Expanded Program on Immunization in May 2008 (3). Other key reasons could be heightened public awareness, improved social hygiene, and upgrades in sewage treatment and water quality (9–11). Decreasing incidence was also accompanied by a change in the age distribution of reported hepatitis A case-patients; the average age increased over time (Figure 2), possibly a consequence of inclusion of the vaccine in the national Expanded Program on Immunization, which targets children >18 months of age. The incidence of hepatitis A is now highest in northwestern China, which is a comparatively less developed region of the country (online Technical Appendix Figure 1).

Our findings in China are similar to those documented in other countries, where HEV infection is now more common than HAV infection (12,13). The increase in hepatitis E incidence could result from either a true increase in the number of cases or from improved case diagnosis. Hepatitis E case-patients are mostly adults, particularly older adults (Figure 2). Although we did not have data on HEV genotypes for this study, it is possible that the change in age distribution of hepatitis E patients may result from the shift of the prevalent HEV genotype in China from genotype 1 to genotype 4 (and, to a lesser extent, genotype 3). Genotype 3 and genotype 4 are known to infect older men (14,15). The increase in the number of HEV infections in eastern China (online Technical Appendix Figure 1) could also be caused by this genotype shift and improved surveillance in these more developed provinces (15), rather than by true geographic heterogeneity in risk factors.

There are some limitations to our study (online Technical Appendix). Our findings are inferred from case notification data, and the data quality could vary because of changes in case definitions. Other limitations include
variable availability of laboratory diagnostics and lack of hepatitis E genotype data.

Conclusions
Reports of hepatitis A in China have declined substantially, while reports of hepatitis E cases have continued to rise. The mortality rate for hepatitis E surpassed that for hepatitis A in 2004. Decreasing trends of hepatitis A incidence after implementation of a vaccination program targeting children >18 months of age indicate a similar strategy for hepatitis E could be considered as a means to curtail incidence. In addition, variations in demographic, geographic and seasonal distributions of hepatitis A and E may inform future prevention strategies in China.

Acknowledgments
We thank the staff members at the hospitals, local health departments, and county-, district-, prefecture-, and provincial-level centers for disease control and prevention for their valuable assistance in coordinating data collection.
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Fatal Infection with Murray Valley Encephalitis Virus Imported from Australia to Canada, 2011

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Murray Valley encephalitis virus (MVEV), a flavivirus belonging to the Japanese encephalitis serogroup, can cause severe clinical manifestations in humans. We report a fatal case of MVEV infection in a young woman who returned from Australia to Canada. The differential diagnosis for travel-associated encephalitis should include MVEV, particularly during outbreak years.

In 2011, an outbreak of 17 confirmed cases of Murray Valley encephalitis (MVE) occurred in Australia, mostly in Western Australia and Northern Territory, where the virus is considered enzootic in water fowl (1). We report a travel-associated case of MVE detected in Canada related to that outbreak.

The Case-Patient
A previously healthy 19-year-old woman from Alberta, Canada, returned from Australia with increasing fatigue, fever, lethargy, and confusion. She had spent 6 months in New Zealand as part of an agricultural exchange, in which her activities included hay bundling, heavy machinery operation, and manure management but no direct zoonotic contacts.

Before returning to Canada, she took a solo 10-day vacation in Australia. Her itinerary and activities were reconstructed from tickets, receipts, and email correspondence. She flew to Darwin, in the tropical region (Top End) of Northern Territory, and then left on a 3-day tour of Kakadu, Mary River, and Litchfield National Parks. She then took a bus to Alice Springs, where she spent another 3 days touring local sites, after which she flew from Alice Springs to Calgary, Alberta, Canada, through Sydney, Australia; Auckland, New Zealand; and Los Angeles, California, USA. She was not previously vaccinated for Japanese encephalitis virus.

Symptoms of excessive fatigue developed the day of her return to Canada (day 1) and were initially attributed to jet lag. The next day, she was drowsy, confused, and febrile. The patient was admitted to a rural community hospital where empiric high-dose intravenous ceftriaxone, vancomycin, and acyclovir were administered.

Because of progressive neurologic deterioration, the patient was airlifted to Foothills Medical Centre in Calgary. On arrival (day 3), she was febrile (temperature 39.7°C) and lethargic and showed worsening confusion, incomprehensible speech, inappropriate verbal responses, and a fluctuating level of consciousness. At examination, she had mild tachypnea. She did not have nuchal rigidity, focal neurologic signs, or a rash. Initial clinical tests results are shown in Table 1. Test results for malaria were negative. Chest radiography showed fine, diffuse, interstitial markings.

Results of computed tomography (CT) of the brain were within reference ranges (Figure 1, panel A). Results of cerebrospinal fluid (CSF) testing were abnormal (Table 1). Gadolinium-enhanced magnetic resonance imaging showed areas of restricted diffusion in the splenium of the corpus callosum and T2 flipped attenuation inversion recovery sequence hyperintensity in the posterior aspects of both thalami (Figure 1, panel B). A provisional diagnosis of flavivirus encephalitis was made. High-dose intravenous meropenem was given because of possible melioidosis encephalomyelitis, which has been reported in the Top End of Northern Territory (2).

The patient became increasingly agitated and had worsening hypoxemia, which required transfer to the intensive care unit for tracheal intubation and mechanical ventilation. Results of repeat chest radiography were consistent with development of the acute respiratory distress syndrome. On day 4, she began to show decerebrate posturing with increased deep tendon reflexes, diffuse rigidity, unresponsiveness, and a downward gaze preference. Repeat CT of the brain showed evolving hypodensity of both thalami with extension into the brainstem.

On day 5, a presumptive diagnosis of MVE was made on the basis of results obtained for CSF by reverse transcription PCR. Primers specific for flavivirus nonstructural protein 5 coding region (3) yielded a 770-nt sequence obtained from a 863-bp amplicon, which showed 98% identity with that of Murray Valley encephalitis virus (MVEV) strain 1–51 (GenBank accession no. AF161266).

Serum samples collected on day 4 and tested by using an ELISA were positive for MVEV IgM but negative for IgG and neutralizing antibodies (Table 2). CSF abnormalities...
were most pronounced on day 5 (Table 1), after which time CSF cell counts decreased rapidly. All cultures of blood, urine, CSF, and respiratory secretions were negative for MVEV. A broad investigation into possible etiologies was conducted for blood, saliva, CSF, brain tissue, and respiratory samples (Table 2).

During the ensuing 2 days, decerebrate posturing worsened, rigidity increased, and the patient became deeply comatose. Continuous electroencephalography monitoring showed onset of progressively worsening seizure activity refractory to phenytoin and levetiracetam. Infusions of intravenous diprivan and midazolam were required to produce burst-suppression (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/23/2/16-1161-Techapp1.pdf).

On day 8, a dilated, nonreactive right pupil developed. A CT scan of the brain showed marked thalamic hypodensity with sulcal effacement, acute obstructive hydrocephalus, and cerebellar tonsillar herniation (Figure 1, panel C). An emergent external ventricular drain was placed, and standard measures were taken to treat intracranial hypertension. Despite intervention, refractory intracranial hypertension developed (intracerebral pressure >70 mm H₂O). A decompressive craniectomy was considered but the patient died on day 10 of worsening obstructive hydrocephalus (Figure 1, panel D) and cerebellar tonsillar herniation.

Autopsy showed severe active encephalomyelitis (Figure 2). Postmortem brain biopsy specimens from the corpus callosum, upper spinal cord, and thalamus were positive for MVEV by reverse transcription PCR (online Technical Appendix Figure 2) with amplicon sequences identical to those obtained from CSF. MVEV was readily isolated on Vero cells from fresh homogenates prepared from each of

<table>
<thead>
<tr>
<th>Variable</th>
<th>Reference range or value</th>
<th>Day 3</th>
<th>Day 5†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong>&lt;br&gt;Leukocyte count, × 10⁶ cells/L</td>
<td>4.0–11.0</td>
<td>11</td>
<td>142</td>
</tr>
<tr>
<td>Gram stain</td>
<td>NA</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

*MVEV, Murray Valley encephalitis virus; NA, not applicable.
†All blood test results for day 5 were within reference ranges.

Figure 1. Neuroimaging during course of illness for a patient with a fatal infection of Murray Valley encephalitis virus imported from Australia to Canada, 2011. Each image corresponds to an axial cross-section through the thalamus and basal ganglia. A) Computed tomography (CT) at day 3. B) Magnetic resonance imaging (T2 flipped attenuation inversion recovery sequence) at day 3 showing abnormalities in the posterior thalami and splenium of the corpus callosum. C) CT when a dilated, right pupil developed in the patient with marked thalamic hypodensity and obstructive hydrocephalus. D) CT before death (day 10) showing necrosis of both thalami and a dilated left lateral ventricle.
the 3 biopsy specimens. The genomic sequence has been deposited in GenBank under accession no. KX229766. Additional autopsy findings included lymphocytic myocarditis, pulmonary edema, and acute tubular necrosis of the kidney. The liver and spleen were congested. The pancreas and ovaries were histologically normal.

Conclusions
Historically, epidemics of MVE were recognized on the eastern coast of Australia; 6 known outbreaks were documented in the early twentieth century. Since the late 1970s, MVEV has largely been maintained in enzootic cycles involving mosquitoes and water fowl in the northern regions of Central and Western Australia; there have been multiple reported epidemics (4). Before the outbreak in 2011, heavy rains across Australia created ideal conditions for *Culex annulirostris* mosquitoes, the vector of MVEV, thus intensifying transmission to humans throughout the country (5). A shift in the demographic pattern of MVE cases toward non-Aboriginal, adult workers and tourists engaged in high-risk activities for mosquito exposure was observed (1). Australian States and Territories routinely use MVEV surveillance methods (mosquito monitoring, virus isolation from mosquitoes, sentinel chicken flocks, and climate surveillance) (6). Each state and territory has its own public health response and communications strategy (1), which target tourists to various degrees.

Human infection with MVEV is generally asymptomatic or mild with nonspecific symptoms, including headache, myalgia and, less commonly, rash. MVE is estimated to occur in <0.1% of infected persons but has a mortality rate of 15%–30% and produces long-term neurologic sequelae in ≤50% of survivors (7–9). Several distinct clinical patterns of MVE have been observed: encephalitis with complete recovery; cranial nerve/brainstem involvement with tremor; spinal cord involvement (poliomyelitis-like); and relentless progression to death, as seen in the patient we report (8). The presence of widespread magnetic resonance imaging abnormalities of the thalamus, midbrain, spinal cord, and cerebellum during acute illness predicted a devastating neurologic outcome (10). A novel feature of this case was the postmortem finding of viral myocarditis, which could account for early and unexpected respiratory decompensation of the patient.

Despite increased awareness of MVE, imported cases in Europe, Asia, and the Americas are rare (11). This case serves as a cautionary reminder of other viral etiologies of encephalitis that should be considered for returning travelers, although many of these etiologies might be outside the diagnostic capability of many clinical laboratories. Appropriate samples should be referred to centers in which specialized testing is available.

Acknowledgments
We thank Jim Burrow, Bart Currie, Stephen Doggett, and Peter Markey for generously sharing knowledge and experiences; and the Centers for Disease Control and Prevention Fort Collins, CO, USA, for providing supplemental diagnostics for the case.

Dr. Niven is a specialist in critical care medicine in the Calgary Zone of Alberta Health Services, Calgary, Alberta, Canada. His major research interest is clinical and health services research for critically ill patients.

References

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**Table 2. Laboratory test results for a patient with a fatal infection of MVEV imported from Australia to Canada, 2011***

<table>
<thead>
<tr>
<th>Sample (collection day) and test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebrospinal fluid (3)</td>
<td></td>
</tr>
<tr>
<td>Enterovirus RT-PCR</td>
<td>Negative</td>
</tr>
<tr>
<td>West Nile virus RT-PCR</td>
<td>Negative</td>
</tr>
<tr>
<td>Herpes simplex virus type 1 and 2 PCR</td>
<td>Negative</td>
</tr>
<tr>
<td>Varicella zoster virus PCR</td>
<td>Negative</td>
</tr>
<tr>
<td>MVEV RT-PCR†</td>
<td>Positive</td>
</tr>
<tr>
<td>Intrathecal antibody production</td>
<td>ND</td>
</tr>
<tr>
<td>Saliva (4)</td>
<td></td>
</tr>
<tr>
<td>Rabies virus RT-PCR</td>
<td>Negative</td>
</tr>
<tr>
<td>Blood (4)</td>
<td></td>
</tr>
<tr>
<td>HIV 1 and HIV-2 antibody</td>
<td>Negative</td>
</tr>
<tr>
<td>Antibodies against arbovirus antigens‡</td>
<td>Negative</td>
</tr>
<tr>
<td>MVEV§</td>
<td>Positive</td>
</tr>
<tr>
<td>Rickettsial antibody</td>
<td>Negative</td>
</tr>
<tr>
<td>Syphilis antibody</td>
<td>Negative</td>
</tr>
<tr>
<td>Cryptococcal antigen</td>
<td>Negative</td>
</tr>
<tr>
<td>Hepatitis B virus surface antigen</td>
<td>Negative</td>
</tr>
<tr>
<td>Hepatitis C virus antibody</td>
<td>Negative</td>
</tr>
<tr>
<td>Hantavirus antibody</td>
<td>Negative</td>
</tr>
<tr>
<td>Respiratory (5)</td>
<td></td>
</tr>
<tr>
<td>Nasopharyngeal swab specimen for respiratory virus panel¶</td>
<td>Negative</td>
</tr>
<tr>
<td>BAL of RML for respiratory virus panel¶</td>
<td>Negative</td>
</tr>
<tr>
<td>BAL of LUL for respiratory virus panel¶</td>
<td>Negative</td>
</tr>
<tr>
<td>Brain tissue (10)</td>
<td></td>
</tr>
<tr>
<td>MVEV RT-PCR and tissue culture#</td>
<td></td>
</tr>
<tr>
<td>Upper spinal cord</td>
<td>Positive</td>
</tr>
<tr>
<td>Thalamus</td>
<td>Positive</td>
</tr>
</tbody>
</table>

*‡Arbovirus antigen standard panel: St. Louis encephalitis, Powassan, dengue, and West Nile viruses. §MVEV IgM ELISA positive, MVEV IgG ELISA negative, and MVEV plaque-reduction neutralization test negative (Centers for Disease Control and Prevention, Fort Collins, CO, USA). †TAG respiratory virus panel (Luminex Molecular Diagnostics, Inc., Toronto, Ontario, Canada), which tests for respiratory syncytial virus; human coronaviruses HKU1, OC43, NL63, and 229E; parainfluenza viruses 1, 2, 3, and 4; enteroviruses/rhinoviruses; human metapneumovirus; adenovirus; and influenza viruses A and B. #Pan-flavivirus RT-PCR and sequencing of amplicon (3). MVEV was isolated on Vero cells from fresh homogenates of biopsy specimens prepared at the time of autopsy on day of clinical illness.

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Infection with Murray Valley Encephalitis Virus


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To determine oral transmissibility of the L-type bovine spongiform encephalopathy (BSE) prion, we orally inoculated 16 calves with brain homogenates of the agent. Only 1 animal, given a high dose, showed signs and died at 88 months. These results suggest low risk for oral transmission of the L-BSE agent among cattle.

The epidemic of bovine spongiform encephalopathy (BSE) in cattle is thought to be caused by oral infection through consumption of feed containing the BSE agent (prion). Since 2003, different neuropathologic and molecular phenotypes of BSE have been identified as causing ≈110 cases of atypical BSE worldwide, mainly in aged cattle. Although the etiology and pathogenesis of atypical BSE are not yet fully understood, atypical BSE prions possibly cause sporadic cases of BSE (1).

The L-type BSE (L-BSE) prion has been experimentally transmitted to cattle by intracerebral challenge, and the incubation period was is shorter than that for classical BSE (C-BSE) prions (2–6). The origin of transmissible mink encephalopathy in ranch-raised mink is thought to be caused by ingestion of L-BSE–infected material (7). Although L-BSE has been orally transmitted to mouse lemurs (8), it remains to be established whether L-BSE can be transmitted to cattle by oral infection. We therefore investigated the transmissibility of L-BSE by the oral route and tissue distribution of disease-associated prion protein (PrPSc) in cattle. All experiments involving animals were performed with the approval of the Animal Ethical Committee and the Animal Care and Use Committee of the National Institute of Animal Health (approval nos. 07–88 and 08–010).

The Study
We divided a group of 16 Holstein female calves, 3–5 months of age, into 4 groups of 2–6 animals each. Each group of calves was orally administered 1 g (n = 4), 5 g (n = 6), 10 g (n = 4), or 50 g (n = 2) of pooled whole-brain homogenate prepared from cattle experimentally infected with L-BSE (3,6) (Table). The endpoint titer of the pooled brain homogenate assayed in bovinized transgenic (TgBoPrP) mice was 10^6.9 of 50% lethal dose/g tissue (data not shown). As noninfected controls, 3 female calves were obtained at 3–4 months of age and euthanized at 60, 92, and 103 months of age, and samples were analyzed as for the experimental animals.

At 88 months after inoculation, 1 of the animals (91 months of age) that had received 50 g of L-BSE–infected brain homogenate was unable to get up. The animal extended her forelimbs and hind limbs rigidly forward but did not show persistent knuckling of her fetlock; she did not have difficulty eating and drinking. Seven days after appearance of clinical signs, the animal was found dead, having shown no characteristic signs of L-BSE, such as dullness, lowering of the head, and overreactivity to external stimuli, which had previously been observed after intracerebral inoculation of animals under experimental conditions (4).

Histopathologic examination of tissues from this animal revealed minimal or mild spongiform changes of the gray matter neuropil in the thalamic and brainstem nuclei; however, these changes were not visible in the cerebral and cerebellar cortices, the olfactory bulb, or the dorsal motor nucleus of the vagus nerve at the obex. Higher amounts of proteinase K–resistant PrPSc, analyzed by Western blotting with monoclonal antibody T2 (9), were detected in the thalamus, brainstem, cerebellum, spinal cord, and retina (Figure 1, lanes 8–16; Figures 2, panels A, B), whereas PrPSc accumulation was lower in the cerebral cortices and the olfactory bulb (Figure 1, lanes 1–6). The molecular characteristics of proteinase K–resistant PrPSc, such as the molecular weight and the glycoform profile in the brain of the animal, were identical to those observed in the inoculum. The most conspicuous PrPSc finding, obtained by using immunohistochemistry with monoclonal antibody F99/97.6.1 (VMRD, Pullman, WA, USA), was fine and coarse granular deposits in the neuropil of the thalamus, brainstem, and gray matter of the spinal cord, and in the retina. Perineuronal PrPSc staining was conspicuous in the large neurons of the thalamic and brainstem nuclei (Figure 2, panel C) but less common in other brain areas. Fewer PrPSc deposits were dispersed in the dorsal motor nucleus of the vagus nerve at the obex (Figure 2, panel A). No amyloid plaques were detectable in any brain section. In the extracerebral tissues, PrPSc was lower in most of the samples from the nerve ganglia.
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Conclusions

Our results suggest that the risk for oral transmission of L-BSE among cattle may be very low; after 88 months, the only case of transmission occurred in a cow that had been inoculated with a high dose of L-BSE–infected brain homogenate. The incubation period was much longer for cattle dosed orally with L-BSE–infected brain homogenate than for cattle dosed orally with C-BSE–infected tissue (34–74 mo for C-BSE) (10). This finding may suggest that the L-BSE prion requires much longer to propagate from the gut to the central nervous system. In addition, the lack of clinical signs, except for difficulty in rising, may present a genuine clinical picture of L-BSE under natural conditions (11). In most cases of naturally occurring atypical BSE identified so far, the animals were >8 years of age, except for 3 cases: 1 H-BSE and 1 L-BSE in Spain (1) and 1 H-BSE in Germany (12). Therefore, we cannot exclude the possibility that L-BSE developed sporadically/spontaneously. However, this case may not have naturally occurred, in view of the low prevalence of L-BSE in Japan during October 2001–August 2016, which was 0.065 cases/1 million tested adult animals. In our study, the remaining live animal, challenged with 50 g of L-BSE brain homogenate, will provide the further information about the oral transmissibility to cattle. Bioassays of brain samples in TgBo-PrP mice are ongoing.

Table. Experimental oral inoculation of 16 calves with brain homogenate of L-type bovine spongiform encephalopathy prions

<table>
<thead>
<tr>
<th>Inoculum dose, g</th>
<th>No. inoculated</th>
<th>No. died (postinoculation mo)</th>
<th>No. euthanized (postinoculation mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>0</td>
<td>2 (51), 2 (52)</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>0</td>
<td>1 (54), 2 (70), 1 (73), 1 (75), 1 (82)</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>0</td>
<td>1 (74), 1 (81), 1 (85), 1 (86)</td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>1 (88)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Necropsies were performed on all animals except the 1 still alive as of December 2016.

( trigeminal, dorsal root, stellate, cervical cranial, nodose, and celiac and mesenteric), cauda equina, vagal nerve, optic nerve, neurohypophysis, ocular muscle, and adrenal medulla (Figure 1, lanes 17–33; Figures 2, panels D–H). However, no PrP<sup>Sc</sup> signal was detected in most of the somatic nerve fibers (Figure 1, lanes 25, 26, 29, 30), the enteric nervous system (Figure 1, lanes 32, 33), and any lymphoid organs including the remaining Peyer’s patches (data not shown).

The only other animal inoculated with 50 g of L-BSE brain material was alive and clinically healthy as of postinoculation month 94 (December 2016). Calves that received 1 g, 5 g, or 10 g of L-BSE brain tissues showed no clinical signs of BSE and were euthanized and underwent necropsy 51–86 months after inoculation (Table). For all of these animals and the uninfected controls, PrP<sup>Sc</sup> results were negative by Western blot and immunohistochemical analysis.

Figure 1. Western blot analysis of proteinase K–resistant disease-associated prion protein (PrP<sup>Sc</sup>) in tissue samples obtained from a cow at 88 months after oral inoculation with brain homogenate of L-type bovine spongiform encephalopathy (BSE) agent. The tissues tested are shown by lane: 1, olfactory bulb; 2, frontal cortex; 3, piriform cortex; 4, parietal cortex; 5, occipital cortex; 6, hippocampus; 7, putamen; 8, thalamus; 9, hypothalamus; 10, midbrain (superior colliculus); 11, obex; 12, cervical enlargement (C7) of spinal cord; 13, lumbar enlargement (L5) of spinal cord; 14, cerebellar cortex; 15, cerebellar white matter; 16, retina; 17, neurohypophysis; 18, trigeminal ganglion; 19, dorsal root ganglion (L5); 20, cervical cranial ganglion; 21, stellate ganglion; 22, celiac and mesenteric ganglion complex; 23, optic nerve; 24, cauda equina; 25, facial nerve; 26, hypoglossal nerve; 27, cervical vagus nerve; 28, sympathetic chain; 29, brachial nerve plexus; 30, sciatic nerve; 31, adrenal gland (medulla); 32, ileum; 33, colon. Lanes 1–16 and lanes 17–33 were loaded with 0.5 mg and 100 mg tissue equivalent, respectively. As controls, lanes L and C were also loaded with 0.5 mg of L-BSE and 0.17 mg of C-BSE cattle brain equivalent, respectively. The relative percentages of PrP<sup>Sc</sup> (below each lane, upper panel) are normalized against midbrain. The PrP<sup>Sc</sup> signals in the extracerebral tissues (below each lane, lower panel) are indicated as positive (+) or negative (–).
The neuroanatomical PrP\textsubscript{Sc} distribution pattern of orally challenged cattle differed somewhat from that described in cattle naturally and intracerebrally challenged with L-BSE (2–6,11,13,14). The conspicuous differences between the case we report and cases of natural and experimental infection are 1) higher amounts of PrP\textsubscript{Sc} in the caudal medulla oblongata and the spinal cord coupled with that in the thalamus and the more rostral brainstem and 2) relatively low amounts of PrP\textsubscript{Sc} in the cerebral cortices and the olfactory bulb. Furthermore, fewer PrP\textsubscript{Sc} deposits in the dorsal motor nucleus of the vagus nerve may indicate that the parasympathetic retrogressive neuroinvasion pathway does not contribute to transport of the L-BSE prion from the gut to the brain, which is in contrast to the vagus-associated transport of the agent in C-BSE (15). PrP\textsubscript{Sc} accumulation in the extracerebral tissues may be a result of centrifugal trafficking of the L-BSE prion from the central nervous system along somatic or autonomic nerve fibers rather than centripetal propagation of the agent (4,6,9). Consumption of L-BSE–contaminated feed may pose a risk for oral transmission of the disease agent to cattle.

**Acknowledgments**

We thank Naoko Tabeta, Naomi Furuya, Junko Yamada, Ritsuko Miwa, Noriko Shinozaki, and the animal caretakers for their expert technical assistance.

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**References**


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**Figure 2.** Immunohistochemical detection of disease-associated prion protein (PrP\textsubscript{Sc}) in a cow at 88 months after oral inoculation with brain homogenate of L-type bovine spongiform encephalopathy agent. A) Low amount of PrP\textsubscript{Sc} deposition in the dorsal motor nucleus of the vagus nerve (DMNV) compared with the more pronounced depositions in the solitary tract nucleus (NST), the spinal tract of trigeminal nerve (STN), and the reticular formation (RFM) in the medulla oblongata at the obex level. Scale bar indicates 200 µm. B) Conspicuous PrP\textsubscript{Sc} deposition in the gray matter of spinal cord (L6). Scale bar indicates 2 mm. C) Granular staining in the neuropil and perineuronal staining (arrows) in the oculomotor nucleus of the midbrain. Scale bar indicates 50 µm. D) Granular PrP\textsubscript{Sc} deposition in the muscle spindle of the ocular muscle. Scale bar indicates 20 µm. E–H) PrP\textsubscript{Sc} in the trigeminal (E), dorsal root (F), cervical cranial (G), and nodose ganglion (H). Scale bars indicate 50 µm.


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Valerie Cortez, Pamela Freiden, Zhengming Gu, Elisabeth Adderson, Randall Hayden, Stacey Schultz-Cherry

Human astroviruses are a major cause of pediatric gastroenteritis, especially in immunocompromised children. We conducted a retrospective study to demonstrate that diverse astrovirus genotypes can co-circulate in pediatric oncology patients. A subset of cases is associated with long-term virus shedding (range 17–183 days).

Astroviruses are a leading cause of diarrhea, and children <2 years of age and immunocompromised persons are at higher risk for systemic and severe disease (1). Few studies have investigated the diversity of astroviruses that infect these populations despite there being 3 distinct phylogenetic clades of human astroviruses (HAstVs) (canonical genotypes HAstV1–8 and noncanonical genotypes MLB1–3 and VA1–5), which makes surveillance challenging (2). To explore the genetic diversity of astroviruses in persons with high-risk for infection, we performed a retrospective study in immunocompromised pediatric oncology patients, analyzing remnant fecal samples collected in 2008 and 2010–2011.

The Study
A total of 909 remnant fecal samples were collected from 419 patients; 473 samples from 220 patients were collected during January–December 2008, and 436 samples from 199 patients were collected during January 2010–June 2011 (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/23/2/16-1436-Techapp1.pdf). All samples were de-identified, so that only a patient identification code, sample identification code, and date were known. No other clinical data were available. The St. Jude Children’s Research Hospital Institutional Review Board approved this study with a waiver of consent.

We extracted viral RNA from homogenized fecal samples by using the MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems Life Technologies, Carlsbad, CA, USA). We screened samples from 2008 by using a singleplex real-time reverse transcription PCR (rRT-PCR) (3), an in-house multiplex PCR to identify canonical (HAstV1–8) and noncanonical (MLB1 and VA2) genotypes (4), and endpoint PCRs by using primers targeting open reading frame (ORF) 1(5) and ORF2 (6,7). The singeplex method detected HAstV in 23/72 samples, compared with 61/67 (5 samples were not tested) by the multiplex method. We screened samples from 2010–2011 solely by the multiplex method; 14 were positive for HAstV. Overall, we detected HAstV in 86 (9.5%) of 909 samples from 60 (14.3%) of 419 patients (Table; online Technical Appendix Table), giving a detection rate comparable to those reported for other immunocompromised populations (8,9).

To place these findings in the context of those for other enteric virus infections, we also used rRT-PCR methods to determine the prevalence of norovirus and sapovirus in the fecal samples from 2008 (10,11). Due to sample quantity limitations, we were unable to test 31 samples for sapovirus. We detected HAstV in the highest proportion of patients and samples (22% [49/220] and 15% [72/473], respectively), followed by norovirus (11% [39/220] and 12% [58/473]) and sapovirus (12% [25/211] and 6.6% [29/442]) (Figure 1). Together, these data demonstrate that HAstVs are a major contributor to the enteric virus infections in this patient population. Furthermore, co-infection occurred in only 16% of HAstV-positive patients (Table; online Technical Appendix Table), much lower than the 33%–65% of patients reported in other studies (2).

A median of 40 (range 18–54) samples were collected each month in 2008; cases peaked in spring and decreased in summer and early fall (online Technical Appendix Figure 2). We further investigated temporal trends in HAstV infections by using longitudinal samples from 34 patients (2–12 samples/patient) collected over a median of 81 (range 3–328) days. Of the 34 patients, 12 were previously HAstV negative, indicating that more than one third of patients had newly acquired infections (online Technical Appendix Table). Of the 12 patients with >1 positive specimen, 6 experienced prolonged HAstV shedding (defined by positive samples collected >2 weeks apart [range 17–183 days]) (online Technical Appendix Table). Subsequent fecal samples for 2 patients (SJ35 and SJ209) were negative for HAstV, suggesting that the virus had cleared, whereas the other 4 patients (SJ22, SJ175, SJ245, and SJ275) had detectable HAstV in their final fecal samples.
Despite being co-infected with HAstV and norovirus, patient SJ275 persistently shed only HAstV, highlighting the need to further explore enteric virus co-infections and the potential for virus interference.

We genotyped 50 of the 86 HAstV-positive samples by using the aforementioned endpoint PCR methods, with patient-specific primers and the 3’ RACE System (Invitrogen Life Technologies, Carlsbad, CA, USA) for rapid amplification of cDNA ends to obtain partial open reading frame (ORF) 1b (RNA-dependent RNA polymerase) and ORF2 (capsid protein) sequences. We were unable to genotype the remaining positive samples because of inadequate sample quality or quantity. We used 50 sequences, collectively representing 38 unique infections, in the phylogenetic analysis (online Technical Appendix Figure 3) and BLAST (https://blast.ncbi.nlm.nih.gov) searches for samples with only shorter sequence reads available. Tree topology of ORF1b and ORF2 sequences indicated that none of the samples contained recombinant viruses or was co-infected with different genotypes. Overall, HAstV1 was the most prevalent genotype identified (n = 19, 50%), followed by the noncanonical genotypes VA2 (n = 8, 21%) and MLB1 (n = 5, 13%) (Figure 2). Fecal samples from 4 (11%) patients were positive for HAstV2; 1 each was positive for HAstV5 and HAstV8. Of note, 4 patients were sequentially infected with >1 genotype, and 3 patients from 2008 had serial infections with canonical and noncanonical genotypes (Table; online Technical Appendix Table). The average interval between these infections was ~7 months.

Conclusions
In a previous study of immunocompromised children hospitalized with diarrhea, the prevalence of HAstV infection was 5% (9). HAstV detection in HIV-infected persons of all ages with and without gastroenteritis has been reported to be as high as 12% (8). Thus, the 14% detection rate we observed in pediatric oncology patients is consistent with the rate in previous reports. Some patients showed prolonged virus shedding, which has been reported in immunocompetent and immunocompromised children, in some cases for as long as 3 months (12,13). However, 4 of the patients in our study shed virus beyond 3 months, including 1 who shed for ~6 months, highlighting the ability of HAstV infections to persist within this population. In addition, 3 of the 4 patients with prolonged shedding were infected with the noncanonical genotypes MLB1 or VA2. Although genotypic differences in virus load and long-term shedding have been reported for canonical viruses (HAstV1–8) (12), we are not aware of such studies with noncanonical viruses. A larger, prospectively followed, longitudinal cohort would be required to investigate these differences.

Our identification of 4 patients with sequential HAstV infections is notable, especially because all cases occurred during 1 year of observation. One previous report also described a child who was first infected with HAstV3 and then, 9 months later, with HAstV1 (14). Although our study showed more than one third of the HAstV infections detected in samples from 2008 were newly acquired, we do not know the precise circumstances of these infections—whether they were associated with healthcare settings, or if they occurred at particular times during the patients’ treatment. The trend of HAstV infections in our study appears to mirror that in reports from Egypt, Brazil, and the eastern United States, which also observed a peak of infections in late spring and early summer (13).

In summary, we used multiple molecular methods to identify HAstV infections in immunocompromised pediatric oncology patients, enabling us to examine a broad
representation of the infections experienced by this population and to identify 6 co-circulating viruses. We showed that the singleplex rRT-PCR method (3) was unable to capture infections caused by noncanonical viruses and is, therefore, limited in its utility for accurate surveillance and diagnosis. Although our multiplex method was able to identify all canonical and 2 noncanonical viruses (4), it is possible that we missed infections with other strains. Given the limited knowledge about HAstV genotypes and clinical disease, particularly in immunocompromised persons, additional studies will be crucial to help develop better diagnostics to capture all known HAstV genotypes.

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mcr-1–Harboring Salmonella enterica Serovar Typhimurium Sequence Type 34 in Pigs, China

Linxian Yi,1 Jing Wang,1 Yanling Gao, Yiyun Liu, Yohei Doi, Renjie Wu, Zhenling Zeng, Zisen Liang, Jian-Hua Liu

We detected the mcr-1 gene in 21 (14.8%) Salmonella isolates from pigs at slaughter; 19 were serovar Typhimurium sequence type 34. The gene was located on IncHI2-like plasmids that also harbored InCF replicons and lacked a conjugative transfer region. These findings highlight the need to prevent further spread of colistin resistance in animals and humans.

Since our report of emergence of a plasmid-mediated colistin resistance mechanism involving the mcr-1 gene in Escherichia coli (1), mcr-1 has been found in >30 countries in 5 continents in <1 year (2). E. coli is the main host of mcr-1, although several other Enterobacteriaceae, including Salmonella, have also been implicated as carriers of this gene (3,4). We screened pigs at slaughter for Salmonella isolates to elucidate the epidemiology of mcr-1 in this major foodborne pathogen, which is a serious public health problem.

The Study
During July 2013–May 2014, a total of 1,780 cecum samples were obtained from pigs at 5 slaughter houses in southern and central China. Samples were incubated in buffered peptone water for 20 h and then inoculated into chromogenic medium selective for Salmonella spp. (CHROMagar Microbiology, Paris, France) for 24 h. Suspected Salmonella colonies were selected (1 isolate was selected from each sample) and identified by using PCR for detection of the invA gene.

We obtained 142 Salmonella isolates and screened them for mcr-1 by using PCR and sequencing with primers described (1); 21 (14.8%) were positive for mcr-1. These isolates were serotyped as described (5). We determined the clonal relationship of mcr-1–positive isolates by using pulsed-field gel electrophoresis (PFGE) with XbaI restriction enzyme and identified sequence types (STs) of these isolates by using multilocus sequence typing (http://mlst.warwick.ac.uk/mlst/dbs/Senterica).

Most of the isolates had indistinguishable PFGE patterns and were clonally related to serovar Typhimurium ST34 (n = 19), which was the dominant type (Table). ST34 is common in isolates from humans in China and other countries and has been linked to food-producing animals (6,7).

We determined MICs for 12 antimicrobial drugs for all mcr-1–positive isolates by using agar dilution methods or a broth microdilution method. Colistin showed MICs of 1–2 mg/L for these isolates (Table). More than 80% of the isolates were also resistant to ampicillin, streptomycin, florfenicol, tetracycline, sulfamethoxazole/trimethoprim, and gentamicin; 76.2% showed reduced susceptibility (MIC >0.06 mg/L) to ciprofloxacin.

We identified 2 lipopolysaccharide regulatory genes (pmrA and pmrB) by using and PCR and sequencing (8). Plasmid-mediated resistance genes floR (florfenicol resistance) and oqxAB (olaquindox and ciprofloxacin resistance) were detected by using PCR. None of the 21 isolates had mutations in the pmrA and pmrB genes, which are associated with colistin resistance in Salmonella isolates. A total of 20 and 19 isolates had the floR and oqxAB genes, respectively. Salmonella Typhimurium ST34 has also been associated with the spread of the oqxAB gene in human clinical isolates in China (9), and Salmonella Typhimurium ST34 coproducing oqxAB or floR genes has also been isolated from humans in Europe and Canada (4,10).

To determine the genetic context of mcr-1, we used PCR mapping with primers ISAPF-5’-CGAAGCAC-CAAGACATCA-3’ and MCR-R (5’-CCACAAGAA-CAAA CGGACT-3’). Insertion sequence ISApf1 was found upstream of mcr-1 for all isolates.

We determined transferability and location of mcr-1 by using conjugation, transformation, and hybridization (with S1-PFGE nuclease). These procedures showed that mcr-1 genes were located on an 180-kb plasmid, except for those in strains SH33 (±110-kb plasmid) and SH239 (±180-kb plasmid and ±138-kb plasmid) (Table). However, the mcr-1 gene could not be transferred to E. coli C600 by conjugation.

We then randomly selected 6 mcr-1–positive Salmonella isolates (SH138, SH149, SH175, SH36, SH39, and Z4P319S) for chemical transformation, which was successful for SH175, SH36, and Z4P319S. S1-PFGE con-
firmed that transformants harbored single plasmids and had 8–16-fold higher MICs for colistin than the recipient E. coli DH5α. The floR and oqxAB genes were also transferred with the mcr-1 gene.

We used Hiseq Technology (Illumina, San Diego, CA, USA) to sequence plasmid DNA purified from transformants of Salmonella Heidelberg SH36 and Salmonella London Z4P319S and genomic DNA extracted from the original isolate (Salmonella Typhimurium SH138, which was selected as a representative ST34 strain). We assembled sequence reads were assembled into contigs by using SOAPdenovo version 2.04 (http://soap.genomics.org.cn/soapdenovo.html) and the separated plasmid contigs of the 3 mcr-1–carrying plasmids from chromosomal contigs and compared them with pHNSHP45–2 by using blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BRIG (11). We then analyzed replicon sequence types of these plasmids by using the Plasmid MLST Database (http://pubmlst.org/plasmid/) and performed analysis and annotation of the partial sequence of mcr-1–carrying plasmids by using the RAST Server (12), ISfinder (https://www.is.biotoul.fr), BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and Gene Construction Kit 4.0 (Textco BioSoftware, Inc., West Lebanon, NH, USA).

Table. Characteristics of mcr-1–positive Salmonella isolates from pigs at slaughter, China, 2013–2014*. 

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serovar</th>
<th>PFGE type</th>
<th>Sequence type</th>
<th>S1-PFGE, plasmid, kb</th>
<th>Colistin MIC, mg/L</th>
<th>Polymyxin B MIC, mg/L</th>
<th>Other antimicrobial drug resistance†</th>
<th>Resistance gene‡</th>
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<tr>
<td>SH149</td>
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*According to recommendations of the joint Clinical and Laboratory Standards Institute (CLSI)–European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Polymyxin Breakpoints Working Group (http://www.eucast.org/), the International Organization for Standardization standard broth microdilution method (20776–1) was used to determine colistin MICs for these strains. Antimicrobial susceptibility was determined and evaluated according to CLSI document no. M100-S25 (http://shop.clsi.org/site/Sample_pdf/M100S25_sample.pdf). Resistance to florfenicol was interpreted according to EUCAST clinical breakpoints and epidemiological cutoff values (>16 mg/L) (http://mic.eucast.org/Eucast2/). MICs of olaquindox for all isolates, except SH36, were >64 mg/L. AMP, ampicillin; CIP, ciprofloxacin; FFC, florfenicol; GEN, gentamicin; ND, not determined; STR, streptomycin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline.

†Boldface indicates resistance phenotypes transferred to a recipient by transformation.
‡Boldface indicates resistance genes transferred to a recipient by transformation.
§Original strains of transformants.
Figure 1. Sequence comparison of scaffolds (portions of genome sequences reconstructed from end-sequenced whole-genome clones) identified in mcr-1–positive plasmids pHNSH36, pHNZ319S, and pHNSH138 with 2 mcr-1–bearing plasmids pHNSHP45-2 (GenBank accession no. KU341381) and pMR0516mcr (GenBank accession no. KX276657), and contigs identified in mcr-1–positive genomes of Escherichia coli strain NT1 in BRIG (11) (GenBank accession LSBW01000090.1) obtained during analysis of mcr-1–positive Salmonella isolates from pigs at slaughter, China, 2013–2014. Arrows indicate positions and direction of transcription of genes. Reference plasmids pHNSHP45–2 (A) and pMR0516mcr (B) are indicated in black in the outer circles.
For the 3 isolates, we found \textit{mcr-1} on derivatives of IncHI2-like plasmids, which also harbored IncF (F4) and IncFIB (B53) replicons. The 3 plasmids were named pHNSH36 (from strain SH36), pHNZ319S (from strain Z4P319S), and pHNSH138 (from strain SH138). IncHI2 plasmids, such as pHNSHP45–2, have been frequently associated with global spread of \textit{mcr-1} genes (3,13,14).

Compared with pHNSHP45–2 (in \textit{E. coli} isolated from a pig in China [14]), the 3 plasmids all had a typical IncHI2-type backbone coding replication, transfer, maintenance, and stability functions, and a multiresistance region. However, we did not identify conjugative transfer region 1 of IncHI2 plasmids, including \textit{tra}G\textit{I}H and \textit{trh}\text{R}XY\text{F}\text{H}G, in the 3 plasmids (Figure 1, panel A), which might be the reason why these plasmids were not transferred by conjugation.

The multiresistance region contained numerous resistance genes, such as \textit{oqxAB}, \textit{floR}, \textit{sul1}, \textit{cmlA}, \textit{aadA2}, and complete or truncated insertion sequences and transposons (IS26, Δ\textit{Tn}2, \textit{Tn}21, IS\text{I}006, and IS\text{C}R2), which was similar to that of pHNSHP45–2, except that pHNSH36, pHNZ319S, and pHNSH138 were missing \textit{fos}A3 and \textit{bla}\text{CTX-M-14} (Figure 1, panel A). The 2,635-bp module (\textit{mcr-1–ΔIS}Apl1) was similar to that of pHNSHP45–2, but different in some aspects, such as insertion sites and orientation, which were identical to those of the novel IncHI2-IncF plasmid pMR0516mcr (GenBank accession no. KX276657) found in a clinical \textit{E. coli} isolate from the United States (15), and human \textit{E. coli} strain TN1 from Vietnam (GenBank accession no. LSBW0100090.1) (Figure 2).

Similar to pMR0516mcr (IncHI2-F18:A-:B1) (Figure 1, panel B), the 3 plasmids also contained an IncF-type backbone, including regions coding replication and partial regions coding transfer, maintenance, and stability functions. Similar to pHNSHP45–2, the plasmid carrying \textit{mcr-1} in strain NT1 belonged to the ST3-IncHI2 plasmid group. However, pMR0516mcr and the 3 plasmids described in this study were not typeable because there were no \textit{smr}0199 loci in the IncHI2 plasmid (Figure 1, panel B). These data suggest that the \textit{mcr-1–ΔIS}Apl1 module might have first been inserted into IncHI2 plasmids and that recombination between IncF- and IncHI2-type plasmids might have occurred subsequently, resulting in acquisition of the IncF-backbone fragment and deletion of the IncHI2 transfer region 1 or combination with the replication region in some instances.

\textbf{Conclusions}

We found that spread of \textit{mcr-1} in pigs at slaughter in China was associated with clonal dissemination of \textit{Salmonella} Typhimurium ST34. This organism has also been detected in Portugal and England (3,4). The presence of indistinguishable IncHI2-F4:A-:B5 plasmids in different \textit{Salmonella} serovars indicates that horizontal transfer of \textit{mcr-1}–harboring plasmids might have also contributed to spread of \textit{mcr-1} in \textit{Salmonella} spp.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Genetic organization of scaffolds (portions of genome sequences reconstructed from end-sequenced whole-genome clones) containing \textit{mcr-1} harbored by plasmids pHNSH36, pHNZ319S, and pHNSH138 obtained during analysis of \textit{mcr-1}–positive \textit{Salmonella} isolates from pigs at slaughter, China, 2013–2014, and structural comparison with plasmids pHNSHP45–2, pMR0516mcr, and \textit{Escherichia coli} TN1 contig 18. Arrows indicate positions and direction of transcription of genes. Regions with >99% homology are indicated in gray or blue. Triangles indicate truncated genes. Information in parentheses after isolates indicate source, location, and GenBank accession number.}
\end{figure}
Other drug-resistance genes, such as floR and oqxAB, were always transferred with mcr-1 by IncHI2-F4:A:-B5 plasmids, which led to successful flow of mcr-1–harboring Salmonella isolates under various selective pressures because florfenicol and olagquinod are widely used in farm animals in China. In addition, spread of similar IncHI2-like plasmids and Salmonella Typhimurium ST34 clones carrying mcr-1 in different countries highlights the need for internationally coordinated intervention strategies to limit its further dissemination.

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References


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Low Circulation of Zika Virus, Cambodia, 2007–2016

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We describe a retrospective study on circulation of Zika virus in Cambodia during 2007–2016 among patients with dengue-like symptoms and Aedes aegypti mosquitoes. Our findings suggest that Zika virus in Cambodia belongs to the Asia genotype, is endemic, has low prevalence, and has had low-level impact on public health.

Zika virus (family Flaviviridae, genus Flavivirus) is an arthropod-borne virus mainly transmitted by the Aedes mosquito. Zika virus was first isolated in 1947 in Africa from rhesus macaques, and human illness was first recognized in Uganda in 1964 (1). This virus is known to cause various and nonspecific symptoms, such as fever, rash, arthralgia, headache, and conjunctivitis. In the second half of the 20th century, although the virus was detected in Africa and in Malaysia, only serologic evidence of Zika virus was reported in India, Thailand, Pakistan, Indonesia, Vietnam, and the Philippines (2). The first well-documented outbreak of Zika virus occurred in 2007 in Yap State, part of the Federated States of Micronesia (3), was followed by further outbreaks in the region (e.g., French Polynesia, New Caledonia, and Easter Island) in 2014 (2). Zika virus was detected in mainland South America in March 2015 in Brazil (4), subsequently spreading in South and Central America and most of the Caribbean over a short period (2). Before recent introduction and outbreaks of Zika virus in Singapore and Thailand (5), only sporadic autochthonous cases have been reported in Southeast Asia, whereas travel-associated cases from Zika-endemic countries have been detected in the United States, Europe, Southeast Asia, and Australia (2).

Zika virus infections have been associated recently with evidence of microcephaly related to transmission from mother to fetus during the first trimester of pregnancy (6). Moreover, other neurologic syndromes and nonvector modes of Zika virus transmission, including congenital, perinatal, and sexual, have been also described (7).

Cambodia’s National Dengue Surveillance System is a pediatric hospital–based syndromic surveillance system of suspected dengue cases managed by the Ministry of Health’s National Dengue Control Program (NDCP). Acute- and convalescent-phase samples from ≈5 patients randomly selected from each of the 5 sentinel sites in the NDCP system are collected and tested each week at Institut Pasteur du Cambodge (IPC) to ensure virologic diagnosis and surveillance (8). The first case of Zika virus infection in Cambodia was diagnosed in a 3-year-old child through community surveillance of acute fevers conducted during 2006–2010 in parallel to the NDCP sentinel system (9).

The Study

We conducted a retrospective study by using samples in our biobank: 1) acute-phase serum and supernatant from NDCP collected during January 2007–July 2016; 2) acute- or convalescent-phase serum and supernatant samples collected by private clinics for dengue diagnosis during the same period; and 3) urine from dengue-negative patients and mosquito samples included in the DENFREE study conducted in Cambodia during 2012–2013 (online Technical Appendix 1, http://wwwnc.cdc.gov/EID/article/23/2/16-1432-Techapp1.pdf). The use of samples was approved by the National Ethics Committee for Health Research of Cambodia. We also tested 3,159 Ae. aegypti female mosquitoes negative for dengue infection by quantitative real-time reverse transcription PCR (qRT-PCR). In total, 2,400 serum samples and 173 supernatants from C6/36 cell cultures of serum from the dengue surveillance system, 270 urine samples from the DENFREE study, and 3,159 mosquitoes were tested for Zika virus by qRT-PCR (10). Positive results were confirmed by using conventional RT-PCR targeting the nonstructural protein 5 gene (11) and the Triplex qRT-PCR kit (12). DNA products of conventional RT-PCR were sent for sequencing to a commercial sequencing facility (Macrogen, Inc., Seoul, South Korea). Serum samples were also tested for Zika virus IgM, dengue virus (DENV), and Japanese encephalitis virus (JEV) by using an in-house IgM capture ELISA (MAC-ELISA) (13). The interval between the date of symptom onset and the date of sampling ranged from 0 to 26 days (n = 1,922; mean 4.13 days, 95% CI 1.66–6.6 days).

Five human serum samples were positive for Zika virus by qRT-PCR; these samples were collected in 2007 (n = 1), 2008 (n = 1), 2009 (n = 2), and 2015 (n = 1).
No other samples, including urine and mosquito samples, were positive. Among the 5 positive samples, 3 were also positive by conventional RT-PCR. Phylogenetic analysis of partial sequences of the nonstructural protein 5 gene from human samples (1 from 2008 and 2 from 2009) showed that all 3 strains belonged to the Asia genotype. Zika virus strain S1118214 (GenBank accession no. KX455424) isolated in 2008 clustered closest with strains from the outbreak in Micronesia (2007); 1 of the strains isolated in 2009, T0706225 (accession no. KX455425), clustered closest with outbreak or endemic strains from New Caledonia (2014), Thailand (2014), China (2016), Chile (2014), and French Polynesia (2013); and the other strain isolated from 2009, T1002464 (accession no. KX455426), clustered closest with the Cambodia strain previously isolated in 2010 (9) (Figure 1). These findings suggest that Zika virus strains in Cambodia are closely related.
to strains causing outbreaks in the Pacific and South America and to endemic strains circulating in Asia; however, longer sequences are needed to confirm this finding.

The serologic study of 1,992 acute-phase serum samples showed that 16 samples from 2007 (n = 2), 2008 (n = 1), 2010 (n = 2), 2012 (n = 4), and 2015 (n = 7) were positive for Zika virus IgM, with no cross-reactivity with DENV and JEV, suggesting a presumptive diagnosis of recent infection. The interpretation of the 1,976 remaining samples is distributed as follows: 146 recent DENV infections; 4 recent JEV infections; 743 recent flavivirus infections (defined by IgM detection for ≥2 of 3 antigens tested [DENV, JEV, and Zika virus]); and 1,087 samples negative for antibodies for any of the 3 viruses tested.

The PCR- and IgM-positive Zika virus infections we detected came from 9 provinces in north, central, and south Cambodia (Figure 2). All 5 acute-phase samples that tested positive by RT-PCR were negative by MAC-ELISA. The convalescent-phase serum samples exhibited a pattern of recent flavivirus infection in 2 samples and recent Zika virus infection in 1 sample (online Technical Appendix 2). The confirmed case in 2015 was considered to be an autochthonous case because no history of recent travel to Zika-endemic areas was reported by the patient. During January–May 2016, all suspected dengue samples that tested negative for DENV were also tested for Zika virus; no cases were detected, either by serology or RT-PCR.

We show that Zika virus circulated across Cambodia during the past 10 years with low intensity and limited effect on public health. No clear explanation exists for the low detection of Zika virus by RT-PCR or by MAC-ELISA, but it might be attributable to 1) the low incidence of Zika virus, which usually causes mild disease compared with DENV, in Cambodia; 2) possible bias in the selection of viremic cases, which were mainly reported in hospitalized suspected dengue patients and therefore were more severe; 3) less than ideal timing of specimen collection for the detection of Zika virus, which has been shown to be transient and frequently low-titer and too early for detection of Zika virus IgM on acute-phase serum samples; or 4) the difficulty in interpreting MAC-ELISA results owing to the cross-reactivity of IgM between DENV and JEV, which are widely present in Cambodia.

Conclusions
The epidemiology of Zika virus is changing, and the risk for reintroduction in Cambodia is a real threat that should not be underestimated. Thailand, Vietnam, and Laos have reported cases since December 2015, and, local transmission has been observed recently in Singapore (5,15). *Ae. aegypti* and *Ae. albopictus* mosquitoes are widespread throughout Cambodia. The risk for reemergence or reintroduction of Zika virus should be monitored by extending the existing surveillance program to ambulatory suspected...


Thirty-five human influenza A(H5N1) cases were reported in Cambodia during 2013–2014 after emergence of a clade 1.1.2 reassortant virus. We tested 881 villagers and found 2 cases of pauci- or asymptomatic infection. Seroprevalence after emergence of the reassortant strain (0.2%) was lower than the aggregate seroprevalence of 1.3% reported in earlier studies.

Thirty-five human influenza A(H5N1) cases were reported in Cambodia during 2005–2012. In January 2013, researchers at the Institut Pasteur du Cambodge (IPC) in Phnom Penh, Cambodia, identified a new influenza A(H5N1) clade 1.1.2 reassortant virus in humans exposed to poultry (1). A sudden surge of 26 new human cases was observed in 2013, of which 15 (57.9%) resulted in death. In the first quarter of 2014, a total of 9 confirmed cases were reported: 8 (including 4 deaths) were caused by the clade 1.1.2 reassortant virus and 1 by clade 2.3.2.1 virus. Of the 8 clade 1.1.2 H5N1 cases, 3 occurred in villages in Kratie and Kompong Cham Provinces. We conducted studies in the 2 affected villages in these provinces in 2014 and compared our findings with those from 7 community seroprevalence studies our team conducted during 2005–2010.

The Study
In the first week of February 2014, a suspected case and 2 laboratory-confirmed cases of the new influenza A(H5N1) clade 1.1.2 reassortant virus occurred in a village in Kratie Province (village 1; population 695); The cases were in 2 separate households. At the same time, a third confirmed case was identified in a village in Kompong Cham Province (village 2; population 921). The 3 patients with confirmed H5N1 virus infection were young children who had close contact with sick or dying poultry. Cases of H5N1 virus infection had also been reported in village 2 in April 2007 and December 2009.

We selected these 2 villages to conduct seroprevalence studies within a month of case occurrence to determine point-seroprevalence in the general population (Figure). Epidemiology teams from IPC and the Cambodia Ministry of Health interviewed and obtained serum samples from all village residents who provided informed consent. Sampling was repeated ≥2 weeks later to test for a possible increase in H5N1 virus antibody levels. We obtained National Ethics Committee for Human Research approval for all serostudies conducted as part of pandemic risk assessments.

We used hemagglutination inhibition (HI) and microneutralization assays as described previously (2) to test paired serum samples for H5N1 virus antibodies. Patients whose first serum sample was antibody-negative and whose second blood sample showed seroconversion (HI titer ≥80 and a microneutralization titer ≥40) were considered H5N1 virus positive (3). Patients who had detectable antibodies in the first serum sample and a ≥4-fold rise in antibody titer (minimum HI titer of 40 and microneutralization titer of 20) for the second sample were also considered H5N1 positive (3).

We used EpiData (EpiData Association, Odense, Denmark) to double-enter questionnaires. We excluded index cases from the analysis to assess risk of infection due to co-exposure or secondary transmission and calculated prevalence by using Poisson confidence intervals. We used the Fisher exact test to compare our data to those from community seroprevalence studies conducted before emergence of this H5N1 reassortant virus.

Paired samples were obtained from 238 (34.2%) of the 695 children and adults in village 1 and from 643 (69.8%) of 921 persons in village 2 (Figure). All persons in direct contact with the index case-patient in village 1 were screened, but 2 healthy family contacts of the second case-patient in village 1 refused participation. By laboratory testing, we found 1 additional case of pauci- or asymptomatic human
Seroprevalence studies to document the incidence and clinical spectrum of H5N1 virus infections help evaluate the effects of emergence of a reassortant virus on virus transmission in affected communities (4). We therefore compared these 2014 results to the 10-year findings of 7 community seroprevalence studies (Table). These comparisons were undertaken by the same teams of IPC epidemiologists and virologists around confirmed cases in H5N1 epidemic foci in Cambodia with the cooperation and support of the Cambodian Ministry of Health.

Studies we conducted prior to the emergence of the reassortant strain, including 2 unpublished serosurveys, tested 2,713 persons, of whom 35 (1.3%) were positive for H5N1 antibodies (Table). Our surveys of the population surrounding cases of infection by the reassortant H5N1 virus in 2014 shows a seroprevalence of 0.2%, lower than the aggregate seroprevalence of 1.3% for 2005–2010. This difference is statistically significant (p = 0.004).

Even if the 2 healthy but untested contacts in village 1 had been found positive, the seroprevalence of H5N1 virus in humans would still be significantly lower following the emergence of the clade 1.1.2 reassortant virus when compared with historical IPC data (2,5,6). Despite the 2013–2014 surge in cases, there is no evidence of increased H5N1 virus transmission to humans or altered clinical and epidemiologic characteristics associated with the emergence of this reassortant virus. A review of literature showed that with the exception of 3 studies in China (7–9), all community seroprevalence studies conducted around H5N1 cases outside Cambodia found 0% prevalence (references not listed).

Conclusions
Routine biocontrol measures in Cambodia are poor, and poultry surveillance is lacking. Farmers either delay reporting and detection, or sell sick poultry, due in part, to lack of compensation from the government. This enables poultry outbreaks to spread. These factors could explain why prevalence in populations around confirmed cases was 10-fold higher in Cambodia than elsewhere until 2012.

The drop in the number of positive cases in the 2014 community serosurveys raises the possibility that villagers’ knowledge of avian influenza and prevention may have improved after 2012, probably as a result of Cambodian authorities’ commitment to and international funding for community education campaigns. Recent studies, however, have shown a >2.5-fold increase in the circulation of avian H5N1 virus in live bird markets in Cambodia during...
specific public health and research questions that can be reassortant virus must be carefully investigated to address However, each new epidemic phase or emergence of a new community-wide seroprevalence studies may not be required. virus. Direct-contact tracing is essential, but regular com Cambodian after emergence of the clade 1.1.2 reassortant H5N1 infections.

levels associated with pauci- and asymptomatic influenza methods and the lack of consensus on neutralizing antibody roprevalence results between studies due to differences in clusters and foci. There was also some minor loss to fol ral seroprevalence studies were conducted around H5N1 suggest increased transmissibility of the virus to humans. may also reflect improved surveillance in the public health circulation of H5N1 reassortant virus among poultry, but it 2013-2014 may therefore be linked to this increased cir human infections with H5N1 reassortant virus during 2013 compared with 2011 (10,11). The peak in confirmed human infections with H5N1 reassortant virus during 2013-2014 may therefore be linked to this increased circulation of H5N1 reassortant virus among poultry, but it may also reflect improved surveillance in the public health sector because our 2014 seroprevalence study does not suggest increased transmissibility of the virus to humans.

Our study has limitations. Although there were 9 hu human cases of infection with H5N1 virus reported in 2014, all followed by direct-contact tracing, only 2 spatiotemporal seroprevalence studies were conducted around H5N1 clusters and foci. There was also some minor loss to fol Transmission risk estimates in villages with clusters might be influenced by the role of host genetics in susceptibilit to infection (12,13). Our findings may therefore apply to genetically vulnerable persons and may have been overestimated, but they remain comparable to or lower than historical data in Cambodia. It is difficult to compare se vore prevalence results between studies due to differences in methods and the lack of consensus on neutralizing antibody levels associated with pauci- and asymptomatic influenza H5N1 infections.

In conclusion, our findings provide no evidence for increased human-to-human H5N1 virus transmission in Cambodia after emergence of the clade 1.1.2 reassortant virus. Direct-contact tracing is essential, but regular community-wide seroprevalence studies may not be required. However, each new epidemic phase or emergence of a new reassortant virus must be carefully investigated to address specific public health and research questions that can be answered only in an outbreak setting.

Acknowledgments

We thank hospital clinical teams and local public health officers for the notification and testing of suspected cases of infection with influenza A(H5N1) virus, and the persons enrolled in the study.

This work was funded by the Office of the Assistant Secretary for Preparedness and Response, US Department of Health and Human Services (grant no. IDSEP 110011-01-00). P.B. is currently an employee of GSK Vaccines R&D. Dr. Ly is a medical epidemiologist in the Epidemiology and Public Health Unit of the Institut Pasteur du Cambodge, Phnom Penh, Cambodia. His research focuses on epidemiologic investigations involving the influenza A(H5N1) virus and other emerging diseases.

References


Table. Seroprevalence of influenza A(H5N1) virus in affected villages (excluding index cases), Cambodia, 2005–2014*

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country, population type</th>
<th>Year</th>
<th>Clade</th>
<th>Testing method</th>
<th>Village population</th>
<th>No. positive/no. tested</th>
<th>% Positive (95% CI)‡</th>
</tr>
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<tbody>
<tr>
<td>(5)</td>
<td>Cambodia, villagers</td>
<td>2005</td>
<td>1</td>
<td>MN, WB</td>
<td>1,146</td>
<td>0/351</td>
<td>0 (0–0.01)</td>
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<tr>
<td>(6)</td>
<td>Cambodia, villagers</td>
<td>2006</td>
<td>1</td>
<td>MN</td>
<td>1,192</td>
<td>7/674</td>
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<td>(2)</td>
<td>Cambodia, villagers</td>
<td>2007</td>
<td>1.1.1</td>
<td>MN, HI</td>
<td>847</td>
<td>18/700</td>
<td>2.6 (0.2–4.1)</td>
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<tr>
<td>Unpub. data</td>
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<td>2009</td>
<td>1.1.1</td>
<td>MN, HI</td>
<td>927</td>
<td>10/622</td>
<td>1.6 (0.9–3.0)</td>
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<tr>
<td>Unpub. data</td>
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<td>2010</td>
<td>1.1.2</td>
<td>MN, HI</td>
<td>452</td>
<td>0/366</td>
<td>0 (0–0.01)</td>
</tr>
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<td>This study</td>
<td>Cambodia, villagers</td>
<td>2014</td>
<td>1.1.2R‡</td>
<td>MN, HI</td>
<td>695</td>
<td>1/238</td>
<td>0.4 (0.1–3.0)</td>
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<td>This study</td>
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<td>2014</td>
<td>1.1.2R‡</td>
<td>MN, HI</td>
<td>921</td>
<td>1/643</td>
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<td></td>
<td>4,564</td>
<td>35/2,713</td>
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<td>Cambodia, 2014</td>
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<td></td>
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<td>1,616</td>
<td>2/881</td>
<td>0.2 (0.1–0.9)</td>
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<td>6180</td>
<td>37/3,594</td>
<td>1.0 (0.7–1.4)</td>
</tr>
</tbody>
</table>

*Positive results were determined by using World Health Organization criteria. HI, hemagglutination inhibition assay; MN, microneutralization assay; WB, Western blot.
‡Poisson interval. An additional study conducted in 2008 in Cambodia focused on 394 soldiers (majority), support personnel, and their families in a confirmed H5N1 virus hotspot. No infections were found (prevalence 0%; 95% CI 0.0%–0.01%). The collective exposure was different from previous studies (soldiers had little or no exposure to poultry), so these data were not included in the table.
‡Clade 1.1.2 reassortant strain with internal and matrix genes originating from clade 2.3.2.1.


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Nosocomial Infections with IMP-19–Producing Pseudomonas aeruginosa Linked to Contaminated Sinks, France

Lucie Amoureux, Karena Riedweg, Angélique Chapuis, Julien Bador, Eliane Siebor, André Péchinot, Marie-Lorraine Chrétien, Claire de Curraize, Catherine Neuwirth

We isolated IMP-19–producing Pseudomonas aeruginosa from 7 patients with nosocomial infections linked to contaminated sinks in France. We showed that bla\textsubscript{IMP-19} was located on various class 1 integrons among 8 species of gram-negative bacilli detected in sinks: P. aeruginosa, Achromobacter xylosoxidans, A. egyptiacens, P. putida, Stenotrophomonas maltophilia, P. mendocina, Comamonas testosteroni, and Sphingomonas sp.

Acquired metallo-β-lactamases (MBLs) belong to the families IMP, VIM, NDM, SPM, GIM, SIM, DIM, KHM, TMB, FIM, and AIM (1). IMP and VIM are the most common families. MBLs have been reported worldwide among Pseudomonas aeruginosa isolates (2). Therapeutic options for infected patients are severely limited because isolates are resistant to many classes of antimicrobial drugs. Genes for MBLs are found mostly in class 1 integrons, which carry additional drug resistance genes. To date, 33 of the 51 known IMP variants have been detected in P. aeruginosa; there has been only 1 report of an IMP-19 producer (3). This MBL is widespread among Acinetobacter spp. in Japan and has also been reported in Achromobacter xylosoxidans (4,5).

During 2009–2016, infections with IMP-19–producing P. aeruginosa isolates were detected in 7 patients in the Hematology Department of University Hospital François Mitterrand, a 1,600-bed hospital in Dijon, France. We describe these infections and report results of environmental investigations.

The Study

The hematology department of the hospital contains a 15-bed conventional unit and a 9-bed protective isolation unit. At the entrances of rooms in the conventional unit, there is a hand hygiene sink (for staff and visitors) and a bathroom in a separate area (shower stall, sink, and toilets). In the protective isolation unit, air is filtered through a laminar flow system, and a sink and toilets are located next to each bed (distance 1.5 m). The ceramic sinks have no counter space, and drains are made of stainless steel. All faucets in the department are hand-operated and provided with antibacterial filters (0.22 µm). Surfaces are cleaned daily (once in the conventional unit and twice in the protective isolation unit) with a solution containing quaternary ammonium compounds (0.25% didecylmethylammonium chloride).

During 2009–2016, a total of 7 patients (P1–P7) in the department were infected or colonized by IMP-19–producing P. aeruginosa, which were isolated from blood samples (2 patients), urine samples (2 patients), throat swab samples (2 patients), and a central venous catheter (1 patient). All patients underwent throat, urine, and feces sampling at admission and were free of P. aeruginosa, thus indicating nosocomial acquisition. All isolates were resistant to ceftazidime, imipenem, meropenem, doripenem, ciprofloxacin, and most aminoglycosides; 4 isolates were susceptible to piperacillin and 3 to amikacin.

We conducted an environmental investigation in the hospital (Figure 1, https://wwwnc.cdc.gov/EID/article/23/2/16-0649-F1.htm). More than 100 environmental samples were obtained when no patients were colonized (except for samples collected in room 32 a few hours after patient P7 had been transferred to an intensive care unit). Water samples were collected from different faucets (nursing room, medication preparation rooms, and rooms of some patients). First-catch lukewarm water (500 mL) was collected in sterile bottles containing 20 mg/L sodium thiosulfate and concentrated by filtration (0.45-µm membrane filters). All sinks and shower drains were sampled by rotating a cotton swab inserted through the drain. Toilets were sampled with swabs inserted under the rim of the toilet bowl.

We plated samples from filters (for water) and swab specimens (from sinks) on Drigalski medium containing ceftazidime (32 mg/L). When a positive culture was observed after 48 hours of incubation, a PCR specific for bla\textsubscript{IMP-19} was performed for the culture mixture. For samples with a positive PCR result, we then plated a dilution of the culture mixture on Drigalski medium containing ceftazidime (32 mg/L) and imipenem (16 mg/L) to obtain isolated colonies, which we further subjected to identification and confirmation of IMP-19 production.
Many resistant organisms were detected in these environmental samples. We used mass spectrometry and \textit{nrndA} gene sequencing to identify \textit{Achromobacter} isolates (6). \textit{bla}_{\text{IMP-19}} and integrons were detected as described (7,8). We used pulsed-field gel electrophoresis after \textit{XbaI} digestion to genotype \textit{P. aeruginosa} isolates (9). Pulsotypes were compared by calculating the Dice correlation coefficient with DendroUPGMA software (http://genomes.urv.cat/UPGMA/).

The 7 clinical isolates belonged to 3 distinct genotypes A, B, and C (Table; Figure 2). We detected environmental IMP-19–producing isolates belonging to 8 species of gram-negative nonfermenting bacilli: \textit{P. aeruginosa}, \textit{Achromobacter xylosoxidans}, \textit{A. aegrifaciens}, \textit{P. putida}, \textit{Stenotrophomonas maltophilia}, \textit{P. mendocina}, \textit{Comamonas testosteroni}, and \textit{Sphingomonas} sp. Of the 7 environmental isolates of \textit{P. aeruginosa} we identified, 6 belonged to the same genotype as clinical isolates (genotype A).

The \textit{bla}_{\text{IMP-19}} gene was located in various integrons, mainly on a \textit{sul}-type class 1 integron and \textit{Tn402}-like class 1 integron. In these integrons, \textit{bla}_{\text{IMP-19}} was associated with different gene cassettes, including \textit{aac(6\prime)}-Ib, \textit{aadA13}, \textit{aadB}, or fused \textit{qacG-aac(6\prime)}-Ib (Figure 3). Few isolates had several copies of \textit{bla}_{\text{IMP-19}} located on integrons of different structures (Table).

\textbf{Conclusions}

The incidence of MBL producers among imipenem-resistant \textit{P. aeruginosa} in France is low compared with incidences for other countries (2,10). Reports of outbreaks are scarce and usually involve VIM producers (11,12). Therefore, detection of IMP-19 producers in our hospital was unusual. The long intervals without cases, the absence of any overlap between cases, and genotypic diversity of clinical isolates did not suggest a single common source of infection. These findings prompted us to conduct environmental investigations.

IMP-19 producers were detected in 9 of 15 rooms in the conventional unit and 2 of 9 rooms in the protective isolation unit. These producers were \textit{P. aeruginosa} and a wide variety of gram-negative nonfermenting bacilli. Most of these producers have little clinical relevance, but they are silent reservoirs for dissemination of \textit{bla}_{\text{IMP-19}} to major pathogens. The role of these environmental bacterial species in the spread of MBL suggested in previous studies (13,14) is confirmed by our findings.

The diversity of species found and genetic structures involved with \textit{bla}_{\text{IMP-19}} indicated that the environmental contamination occurred a long time ago. One isolate of IMP-19–producing \textit{Aeromonas caviae} was found in a patient in the same building in 2006 (7). This phenomenon is probably endemic to our hospital, in which sink drains are not accessible for removal of biofilms without complete dismantling (inappropriate sink design).

Transfer of pathogens from sinks to patients might occur in several ways. Water from faucets is directed...
straight into the drain, resulting in splashing that can lead to contamination of an area ≤1 m from the sink (13). Therefore, patients can be contaminated when they brush their teeth, wash their hands, or take a shower. Health-care personnel are also at risk for hand contamination, which might lead to transfer of pathogens to patients during care.

All patients had ≥1 stay in rooms that were positive for IMP-19-producing organisms. After patient P7 died of sepsis, all drains in the ward were changed. However, this

Table. Characteristics of bacterial isolates from patients with nosocomial infections linked to contaminated sinks, France*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin (date)</th>
<th>Site</th>
<th>PFGE profile</th>
<th>Integron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa PA1</td>
<td>Patient P1 (2009 Feb)</td>
<td>Urine</td>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>P. aeruginosa PA2</td>
<td>Patient P2 (2009 Apr)</td>
<td>Blood</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>P. aeruginosa PA3</td>
<td>Patient P3 (2009 Jun)</td>
<td>Throat</td>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>P. aeruginosa PA4</td>
<td>Patient P4 (2013 Oct)</td>
<td>Urine</td>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>P. aeruginosa PA5</td>
<td>Patient P5 (2015 Oct)</td>
<td>Central catheter</td>
<td>C</td>
<td>F</td>
</tr>
<tr>
<td>P. aeruginosa PA6</td>
<td>Patient P6 (2015 Dec)</td>
<td>Throat</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>P. aeruginosa PA7</td>
<td>Patient P7 (2016 Jul)</td>
<td>Blood</td>
<td>A</td>
<td>ND</td>
</tr>
<tr>
<td>P. aeruginosa PA8</td>
<td>Room 10 (protective unit)</td>
<td>Sink</td>
<td>Related</td>
<td>G</td>
</tr>
<tr>
<td>P. aeruginosa PA9</td>
<td>Room 40</td>
<td>Shower drain</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>P. aeruginosa PA10</td>
<td>Room 40</td>
<td>Shower drain</td>
<td>A</td>
<td>E, F</td>
</tr>
<tr>
<td>P. aeruginosa PA11</td>
<td>Room 40</td>
<td>Sink</td>
<td>A</td>
<td>B, C</td>
</tr>
<tr>
<td>P. aeruginosa PA12</td>
<td>Room 46</td>
<td>Shower drain</td>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>P. aeruginosa PA13</td>
<td>Room 32</td>
<td>Sink</td>
<td>A</td>
<td>ND</td>
</tr>
<tr>
<td>P. aeruginosa PA14</td>
<td>Room 32</td>
<td>Toilet bowl</td>
<td>A</td>
<td>ND</td>
</tr>
<tr>
<td>Achromobacter xylosoxidans</td>
<td>Room 48</td>
<td>Sink</td>
<td>ND</td>
<td>F</td>
</tr>
<tr>
<td>A. aegrifaciens</td>
<td>Room 32</td>
<td>Toilet bowl</td>
<td>ND</td>
<td>E, F</td>
</tr>
<tr>
<td>A. aegrifaciens</td>
<td>Room 37</td>
<td>Shower drain</td>
<td>ND</td>
<td>E, F</td>
</tr>
<tr>
<td>P. putida</td>
<td>Room 32</td>
<td>Toilet bowl</td>
<td>ND</td>
<td>E, F</td>
</tr>
<tr>
<td>P. putida</td>
<td>Room 4</td>
<td>Shower drain</td>
<td>ND</td>
<td>E, F</td>
</tr>
<tr>
<td>P. putida</td>
<td>Room 64</td>
<td>Shower drain</td>
<td>ND</td>
<td>G</td>
</tr>
<tr>
<td>P. putida</td>
<td>Room 65</td>
<td>Toilet bowl</td>
<td>ND</td>
<td>C</td>
</tr>
<tr>
<td>Sphingomonas sp.</td>
<td>Room 12 (protective unit)</td>
<td>Sink</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Comamonas testosteroni</td>
<td>Room 46</td>
<td>Sink</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>P. mendocina</td>
<td>Room 40</td>
<td>Shower drain</td>
<td>ND</td>
<td>E, F</td>
</tr>
<tr>
<td>Sphingomonas maltophilia</td>
<td>Room 44</td>
<td>Sink</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND, not determined; PFGE, pulsed-field gel electrophoresis.
measure did not eradicate biofilms found in the plumbing system. Because the building tested was 16 years old, it has been decided to completely rebuild the ward in early 2017, paying special attention to water distribution and discharge systems to minimize hospital-acquired infections. In conclusion, our findings might help other hospitals to identify potential reservoirs of carbapenemase-producing bacteria and lead to implementation of rapid control measures to contain outbreaks.

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References

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Risk Factors for Disseminated Coccidioidomycosis, United States

Of 150,000 new coccidioidomycosis infections that occur annually in the United States, ≈1% disseminate; one third of those cases are fatal. Immunocompromised hosts have higher rates of dissemination. We identified 8 patients with disseminated coccidioidomycosis who had defects in the interleukin-12/interferon-γ and STAT3 axes, indicating that these are critical host defense pathways.

Coccidioidomycosis is acquired by inhaling spores of Coccidioides immitis. The Centers for Disease Control and Prevention reported 22,401 cases (42.6 cases/100,000 population) in 2011, an increase from 2,265 cases (5.3/100,000) reported in 1998 (1). Although Coccidioides infection usually produces little illness and results in lifelong immunity, 25%–30% of infections result in protracted but self-limited illness; <1% are complicated by dissemination, which is serious and sometimes fatal (2–4). Diagnosis and treatment remain challenging, especially in persons with disseminated, severe, or chronic disease, where host immunity plays an important role.

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During January–March 2014, we reviewed risk factors for dissemination and summarized all coccidioidomycosis cases in patients with primary immunodeficiency (PID). These cases highlight the importance of the interleukin (IL)–12/interferon (IFN)–γ and signal transducer and activator of transcription 3 (STAT3) pathways in host defense. Dissemination of this typically self-limited pathogen should prompt consideration of underlying host genetic factors.

Literature Review
Our systematic literature search resulted in 370 case reports of disseminated coccidioidomycosis (DC) published during 1975–2014 (online Technical Appendix, https://www.cdc.gov/EID/article/23/2/16-0505-Techapp1.pdf). DC was defined as a positive culture or histologic finding from a nonpulmonary site. For comparative purposes, patients were further classified by exogenous immunosuppression, pregnancy, or 1 versus >2 extrapulmonary affected sites.

How the host responds to and contains coccidioidomycosis is unclear, but dissemination occurs in 30%–50% of immunosuppressed hosts. Dissemination can be single-site or multisite, is associated with more severe outcomes than disease limited to the respiratory tract, and requires prolonged treatment (4). Literature review confirms critical interactions of *Coccidioides* spp. with race/ethnicity, sex, pregnancy, and immune status (Table 1).

The rate of DC is higher for pregnant women than for the general population (5,6). We found dissemination to the nervous system reported in 37% of pregnant women, approximately one third of whom died (https://www.cdc.gov/EID/article/23/2/16-0505-Techapp1.pdf). Of total deaths, 75% occurred among women during their third trimester; fetal or infant death occurred in 40% of reported cases. Although one third of pregnant women affected were black, survival did not differ by race.

Despite overall improved survival, immunocompromised persons remain at high risk for fatal DC; the crude mortality rate (CMR) was ≈50% for persons immunocompromised by HIV, cancer, organ transplantation, antirejection medications, antiinflammatory biologicals, or chemotherapy (https://www.niaid.nih.gov/sites/default/files/HollandTechnicalAppendix.docx). CMRs were lower, but still substantial, for patients receiving steroids (https://www.niaid.nih.gov/sites/default/files/HollandTechnicalAppendix.docx). In HIV-infected exogenously immunocompromised patients, coccidioidomycosis was similar to that in persons without HIV/AIDS. CMRs were lower for persons who were able to stop exogenous immunosuppression. Patients with exogenous immunosuppression were 37% white, 20% Hispanic, and 11% black (https://www.niaid.nih.gov/sites/default/files/HollandTechnicalAppendix.docx), similar to the racial/ethnic distribution in DC-endemic areas (California, Arizona: 48% white, 34% Hispanic, 6% black) (7,8). However, these racial/ethnic differences should be interpreted cautiously because race/ethnicity data were unavailable for 24% of patients with exogenous immunosuppression. Regardless of age, immunosuppressed patients were substantially more likely to have extrapulmonary dissemination, require hospitalization, have progressive infection, or die of coccidioidomycosis.

Most (84%) patients with multisite infection were male, and the number of blacks was double that of any other race (https://www.niaid.nih.gov/sites/default/files/HollandTechnicalAppendix.docx). Additionally, osteomyelitis was more common among blacks (82%) than whites (29%); central nervous system (CNS) infection was more common among whites (59%) than blacks (13%). Hispanics and Asians also had higher rates of osteomyelitis (69% and 60%, respectively) and lower rates of CNS dissemination (38% and 13%, respectively) than whites (https://www.niaid.nih.gov/sites/default/files/HollandTechnicalAppendix.docx). In contrast, among patients with exogenous immunosuppression, differences in rates of osteomyelitis and CNS infections by race were much smaller (44% of blacks with osteomyelitis vs. 24% of whites and 33% of blacks with CNS infection vs. 21% of whites). These data suggest that different immunologic factors that track with race might variably control susceptibility to DC, osteomyelitis, and CNS disease. However, exogenous immunosuppression apparently overrides these racial/ethnic variations.

Consistent with the demographic characteristics of patients with multisite disease, 83% of those with single-site infection were male (https://www.niaid.nih.gov/sites/default/files/HollandTechnicalAppendix.docx).

| Table 1. Summary of disseminated coccidioidomycosis cases reported in the literature* |
|--------------------------|----------|-----------------|-------------------|-----------------|
| Predisposition/no. sites affected | Sex, no. | Race/ethnicity, % | Age, y, median (range) | Site of disease, % | Survival, % |
| Pregnancy, N = 52 | M, 59; F, 19 | Black, 19; white, 14; Hispanic, 11; Asian 3 | 27 (17–38) | CNS, 18; bone, 5 | 42 |
| Immunosuppression,† N = 79 | M, 84; F, 16 | Black, 39; white, 17; Hispanic, 13; Asian, 15 | 36 (1–84) | CNS, 29; bone, 62 | 72 |
| Multisite dissemination, N = 100 | M, 115; F, 24 | Black, 32; white, 21; Hispanic, 9; Asian, 17 | 33 (1–73) | CNS, 9; bone, 50 | 99 |

*†CNS, central nervous system.
†Oncologic, n = 8; HIV, n = 12; transplant, n = 24; steroids/immune-modulation, n = 35.
Overall, blacks had more single-site osteomyelitis than whites (64% vs. 41%), and whites had more CNS infection than blacks (17% vs. 2%)([https://www.niaid.nih.gov/sites/default/files/HollandTechnicalAppendix.docx](https://www.niaid.nih.gov/sites/default/files/HollandTechnicalAppendix.docx)). Thus, despite the lower CMR in single-site disease, racial/ethnic differences in infection site were largely consistent between those with single-site and multisite infection.

Single-site and multisite disease accounted for 86% of extrapulmonary Coccidioides infections in blacks and 91% in Asians but for only 56% in whites and 52% in Hispanics. Furthermore, blacks accounted for approximately one third of single-site and multisite infections despite constituting only 6% of the population in coccidioidomycosis-endemic areas. In contrast, only 10% of patients with single-site and multisite disease were Hispanic, even though Hispanics accounted for 35% of the general population in those areas (7). The more population-consistent number of blacks with DC among exogenously immunosuppressed persons and the blunting of racial/ethnic differences with exogenous immunosuppression suggest that exogenous immunosuppression overwhelms intrinsic racial/ethnic variations in host defense. Interpretation of these differences is limited by self-identified race/ethnicity, an imprecise surrogate for ancestral genetic origins. Future studies using established ancestral markers will help solidify associations between coccidioidomycosis infection and race/ethnicity.

We identified 8 cases of proven PID with DC (Table 2). Mutations in the IL-12/IFN-γ or STAT3 pathways were diagnosed in PID patients (online Technical Appendix Figure); these patients were younger and more racially/ethnically diverse than immunosuppressed single-site and multisite infected groups. All patients with discrete immune defects had prolonged, refractory infection; some were controlled with exogenous IFN-γ. Of the 8 patients, 3 had no relevant prior medical histories, suggesting that discrete mutations in these pathways might go unrecognized until DC develops.

In Coccidioides-susceptible mice, exogenous IL-12 is protective, whereas disease in resistant strains is exacerbated by its neutralization (13). In vitro, human macrophage killing of phagocytosed Coccidioides depends on IL-12/IFN-γ signaling (14). Furthermore, peripheral blood mononuclear cells from nonimmune (delayed-type hypersensitivity-negative) donors produce significantly less IFN-γ in response to Coccidioides antigens than do such cells from immune (delayed-type hypersensitivity-positive) donors. In vivo, 3 patients with DC improved substantially after therapy with IFN-γ. Immune function studies in 2 of those patients showed blunted IFN-γ-mediated responses (15).

The involvement of STAT3 in resistance to Coccidioides infection is complex. STAT3 is a critical mediator of IL-23 signaling, a cytokine involved in producing IFN-γ, IL-12, and IL-17, all of which are required for immunity to

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**Table 2. Patients with disseminated coccidioidomycosis and discrete primary immune deficiencies**

<table>
<thead>
<tr>
<th>Case no. (ref)</th>
<th>Age, y/sex</th>
<th>Race/ethnicity</th>
<th>Medical history</th>
<th>Extrapulmonary disease</th>
<th>Relapse</th>
<th>Method of diagnosis</th>
<th>Genetic findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/F</td>
<td>White</td>
<td>HIES, recurrent pneumonia, otitis, skin infections, eczema, thrush</td>
<td>Meningitis</td>
<td>No</td>
<td>BAL/CSF cultures</td>
<td>STAT3: heterozygous (c.2137G&gt;A)</td>
</tr>
<tr>
<td>2 (9)</td>
<td>17/F</td>
<td>Not reported</td>
<td>HIES, Staphylococcus aureus skin and soft tissue infections, recurrent sinus infections, pneumonia</td>
<td>Meningitis, cerebral abscess</td>
<td>No</td>
<td>Coccidioides Ab, CSF culture</td>
<td>STAT3: heterozygous (p.T412S)</td>
</tr>
<tr>
<td>3 (10)</td>
<td>11/M</td>
<td>White</td>
<td>11 mo: Mycobacterium chelonel pneumonia 22 y; disseminated M. kansasii</td>
<td>Osteomyelitis, lymphadenitis</td>
<td>Yes</td>
<td>Ab level, lymph node biopsy</td>
<td>IFN-γR1: deficiency (c.818del4fs)</td>
</tr>
<tr>
<td>4 (11)†</td>
<td>22/F</td>
<td>Palestinian</td>
<td>11 y: Salmonella serogroup D lymphadenitis</td>
<td>Diffuse lymphadenitis</td>
<td>Yes</td>
<td>Coccidioides IgM and IgG, lymph node biopsy</td>
<td>IL-12Rβ1: homozygous (p.C186Y)</td>
</tr>
<tr>
<td>5 (11)</td>
<td>6/M</td>
<td>Palestinian</td>
<td>No other significant history</td>
<td>Osteomyelitis, lymphadenitis, nasal lesion</td>
<td>Yes</td>
<td>Lymph node, nasal lesion, bone biopsies</td>
<td>IL-12Rβ1: homozygous (p.C186Y)</td>
</tr>
<tr>
<td>6</td>
<td>15/M</td>
<td>Black</td>
<td>No other significant history</td>
<td>Osteomyelitis, soft tissue</td>
<td>Yes</td>
<td>BAL cultures, bone and soft biopsies</td>
<td>IL-12Rβ2: heterozygous (p.C101Y)</td>
</tr>
<tr>
<td>7 (12)</td>
<td>17/F</td>
<td>Hispanic</td>
<td>14 y: extensive, persistent tinea capitis and kerion caused by Trichophyton tonsurans</td>
<td>Osteomyelitis, soft tissue, cutaneous lesions</td>
<td>Yes</td>
<td>Coccidioides Ab, skin biopsy</td>
<td>STAT1: gain of function mutation (p.E353K)</td>
</tr>
<tr>
<td>8 (12)</td>
<td>9.5/F</td>
<td>White</td>
<td>No other significant history</td>
<td>Osteomyelitis, cerebral lesions, intrathoracic lymphadenitis</td>
<td>Yes</td>
<td>Coccidioides Ab</td>
<td>STAT1: gain of function mutation (p.A267V)</td>
</tr>
</tbody>
</table>

*Ab, antibody; BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; HIES, hyperimmunoglobulin E (Job’s) syndrome; ref, reference.†Patients 4 and 5 are siblings, and their parents are first cousins.
**Coccidioidal** in vivo. It also might be involved downstream of dectin-1, which is required for resistance to *Coccidioides* in mice and induces the phosphorylation of STAT3.

**Conclusions**

Risk factors for DC include exogenous immunosuppression (steroids and biologicals), pregnancy, race/ethnicity, and discrete genetic defects. Although racial/ethnic associations with DC were evident in patients without known underlying risks, they were submerged by exogenous immunosuppression.

Functional and genetic studies indicate that the IL-12/IFN-γ axis and STAT3-mediated immunity are central to protection against *Coccidioides*. We identified mutations affecting these pathways in 8 patients with especially severe or refractory DC, some of whom responded to IFN-γ therapy. Younger patients with severe DC or patients whose illness relapses should be considered for genetic screening for discrete primary immune defects. The discrete defects demonstrated here clearly do not account for all occurrences of coccidioidomycosis in the general population but highlight the importance and nature of genetic control.

Coccidioidomycosis is distinguished by its geography and relative virulence in many persons who otherwise appear immunologically competent. Because most persons in whom DC develops are previously healthy, *Coccidioides* most likely exploits a very narrow vulnerability. The demonstration that DC has an underlying genetic predisposition indicates that the advent of newer genetic techniques, such as whole exome/genome sequencing, will inevitably identify coccidioidomycosis-specific genetic factors. These, in turn, should enable us to better understand, preempt and treat coccidioidomycosis.

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Dr. Odio completed this work while she was a medical student at the Cleveland Clinic Lerner College of Medicine, Cleveland, OH, USA. She now an internal medicine resident at Yale–New Haven Hospital. Her research interests include infectious diseases, immunology, and host-pathogen interactions.

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Incidence of Norovirus-Associated Diarrhea, Shanghai, China, 2012–2013

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We conducted sentinel-based surveillance for norovirus in the Pudong area of Shanghai, China, during 2012–2013, by analyzing 5,324 community surveys, 408,024 medical records, and 771 laboratory-confirmed norovirus infections among 3,877 diarrhea cases. Our analysis indicated an outpatient incidence of 1.5/100 person-years and a community incidence of 8.9/100 person-years for norovirus-associated diarrhea.

Norovirus is the most common cause of gastroenteritis (i.e., diarrhea or vomiting) (1). Diarrhea represents the second greatest burden of infectious disease in the world, and globally, >20% of diarrhea cases are associated with norovirus infection (2). China is one of the 15 highest-burden countries for diarrhea in the world (3). To guide the planning, implementation, and evaluation of disease control programs, nationwide sentinel-based surveillance for diarrhea across all age groups has been conducted in China since 2009, in which the prevalence of norovirus is monitored regularly (4). However, incidence rates of norovirus are not readily available from previous studies because of the lack of population denominators (4,5). To assess the population-based burden of norovirus disease based on this surveillance platform, we conducted community surveys and reviewed medical records for diarrhea in the Pudong New Area (a district of the city of Shanghai, China) during 2012–2013. We then estimated age-stratified rates for norovirus-associated diarrhea.

The Study

We conducted surveillance for diarrhea (defined as ≥3 passages of watery, loose, bloody, or mucoid stools within a 24-hour period) at outpatient clinics (mainly enteric, pediatric, and internal medicine clinics) of 10 sentinel hospitals in Pudong during February 1, 2012–December 31, 2013. We determined the catchment population, market share of sentinel hospitals, and weighted proportion of persons with diarrhea in the community who sought medical care by conducting the age-stratified Hospital Utilization and Attitudes Survey among respondents residing in Pudong (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/2/16-1153-Techapp1.pdf). In total, an estimated population of 1,722,580 was under surveillance during the study period.

We obtained the number of outpatient visits for diarrhea each year at 10 sentinel hospitals by reviewing and analyzing medical records. We extracted medical records of acute gastroenteritis (AGE), encoded as A00–A09 or K52.9 by the International Classification of Diseases, 10th Revision (ICD-10), from 10 sentinel hospitals’ hospital information systems (HIS) in 2012 and 2013. In total, 408,024 episodes of AGE were identified (189,645 in 2012 and 218,379 in 2013). To validate these AGE records with diarrhea cases likely meeting the study case definition, we ascertained numbers of diarrhea cases at 7 sentinel hospitals’ enteric clinics during February 2012–December 2013 (28,030 eligible diarrhea case-patients during the same period). We used the ratio of 0.712 (28,030/39,365) as a contractive factor to narrow down the ICD-10–coded AGE episodes to diarrhea cases likely meeting the study case definition (Figure 1). After validation, we estimated that 290,513 (408,024 × 0.712) diarrhea case-patients visited the 10 sentinel hospitals in 2012 and 2013.

To measure the proportion of diarrhea episodes associated with norovirus, physicians working at outpatient clinics of the 10 sentinel hospitals screened visiting patients for...
eligibility of enrollment; the first 1–3 eligible diarrhea case-patients for each week in each sentinel hospitals were recruited by using a convenience sampling method. Fecal specimens were collected for enrolled case-patients and tested for norovirus genogroups GI and GII in the local public health laboratory by using reverse transcription PCR (RT-PCR) assays as described previously (4). In total, we enrolled 3,877 diarrhea case-patients (2,235 in 2012 and 1,642 in 2013). We detected norovirus in 771 cases (19.9%). We observed no significant difference in norovirus detection between surveillance years (19% in 2012 vs. 21% in 2013). However, detection of norovirus was significantly different between age groups (22% in persons >5 years of age vs. 12% in children <5 years of age; p<0.001) (Table 1). The highest norovirus detection rates were in October (35%–38%) and the lowest in June (5%–6%), but a secondary peak in detection occurred in March (19%–34%) (Figure 2).

We calculated outpatient incidence rates for norovirus-associated diarrhea in Pudong as the number of outpatient visits associated with norovirus divided by the total population at risk, which was based on total episodes of AGE in sentinel hospitals, the contractive factor used to narrow down the total episodes of ICD-10–coded AGE to diarrhea cases likely meeting the study case definition, the percentage of diarrhea-case patients with norovirus, and the total study catchment population. We estimated community incidence rates by dividing the number of outpatient visits associated with norovirus by the product of population at risk and the proportion of persons with diarrhea in the community who sought medical care, based on results of the Hospital Utilization and Attitudes Survey. We calculated 95% CIs by using bootstrap methods with 1,000 samples for each rate.

We estimated the annual outpatient incidence rates of norovirus-associated diarrhea in Pudong to be 1.3/100 person-years (95% CI 1.2–1.4) in 2012 and 1.6/100 person-years (95% CI 1.4–1.8) in 2013, with an average rate of 1.5/100 person-years (95% CI 1.4–1.6) for the 2 years combined (Table 2). Children <2 years of age (7.4/100 person-years, 95% CI 5.4–9.3) and adults >65 years of age (2.6/100 person-years, 95% CI 2.0–3.2) exhibited increased rates of outpatient visits, compared with adults 25–44 years of age (1.0/100 person-years, 95% CI 0.9–1.1) (Table 2). The annual community incidence rates for norovirus-associated diarrhea were 8.1/100 person-years (95% CI 7.2–9.0) in 2012 and 9.8/100 person-years (95% CI 8.7–11.0) in 2013, with an average rate of 8.9/100 person-years (95% CI 8.2–9.7) for the 2 years combined.

### Conclusions

This 2-year study provides age-stratified incidence rates for norovirus infections among medically attended diarrhea

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**Table 1.** Norovirus test results among diarrhea case-patients, by sex, surveillance year, and age group, Pudong New Area, Shanghai, China, 2012–2013

<table>
<thead>
<tr>
<th>Variable</th>
<th>Positive, no. (%)</th>
<th>Negative, no. (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>426 (21)</td>
<td>1,646 (79)</td>
<td>0.278</td>
</tr>
<tr>
<td>F</td>
<td>345 (19)</td>
<td>1,460 (81)</td>
<td></td>
</tr>
<tr>
<td><strong>Surveillance year</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>422 (19)</td>
<td>1,813 (81)</td>
<td>0.074</td>
</tr>
<tr>
<td>2013</td>
<td>349 (21)</td>
<td>1,293 (79)</td>
<td></td>
</tr>
<tr>
<td><strong>Age group</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>0–11 mo</td>
<td>30 (10)</td>
<td>277 (90)</td>
<td></td>
</tr>
<tr>
<td>12–23 mo</td>
<td>23 (16)</td>
<td>124 (84)</td>
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</tr>
<tr>
<td>24–59 mo</td>
<td>28 (13)</td>
<td>184 (87)</td>
<td></td>
</tr>
<tr>
<td>5–24 y</td>
<td>69 (18)</td>
<td>306 (82)</td>
<td></td>
</tr>
<tr>
<td>25–44 y</td>
<td>268 (21)</td>
<td>1,028 (79)</td>
<td></td>
</tr>
<tr>
<td>45–64 y</td>
<td>263 (24)</td>
<td>828 (76)</td>
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<tr>
<td>&gt;65 y</td>
<td>90 (20)</td>
<td>359 (80)</td>
<td></td>
</tr>
</tbody>
</table>

---

**Figure 1.** Registration, enrollment, and testing of diarrhea case-patients in Pudong New Area, Shanghai, China, 2012–2013. (A pilot study was conducted during the first month of the year 2012. No case enrollment was conducted during that period.) AGE, acute gastroenteritis; ICD-10, International Classification of Diseases, 10th Revision.

**Figure 2.** Proportion of norovirus detected among diarrhea case-patients in outpatient settings, by month of symptom onset, Pudong New Area, Shanghai, China, 2012–2013.
patients in Pudong, Shanghai. We used a sentinel-based surveillance approach and combined multiple data sources to generate incidence, including 5,324 community surveys, 408,024 retrospectively collected electronic medical records, and 771 laboratory-confirmed norovirus infections identified among 3,877 diarrhea cases. Each year in Pudong, ≈1 in 11 persons became ill with norovirus-associated diarrhea in the community (corresponding to 526,000 visits) and ≈1 in 67 persons visited a healthcare provider for norovirus-associated diarrheal illness (corresponding to 88,000 ambulatory visits). These findings suggest that norovirus was a substantial burden on the community and healthcare system of Pudong.

In our study, outpatient rates of norovirus-associated diarrhea were consistent with the 2 consecutive surveillance years of 2012 and 2013. The average annual incidence for norovirus-associated outpatient visits was estimated to be 1.5/100 person-years, which was broadly consistent although slightly higher than that reported in studies conducted in other countries, such as the United States (0.4–1.2/100 person-years) (6), the United Kingdom (0.48–0.6/100 person-years) (7), Germany (0.29–1.07/100 person-years) (8), and the Netherlands (0.5–1.5/100 person-years) (9). The average prevalence of norovirus across all age groups in our study was 19.9%, similar to the global estimate of 20% from outpatient studies by Ahmed et al. (2). This prevalence was higher when compared with some other studies that were conducted in other countries (4.4%–16%) (6–8). We believe that high exposure to norovirus in our study population might have been a source of the elevated rates. The new norovirus GII.4 variant Sydney_2012 emerged during our study period and has caused several outbreaks in eastern China since 2012 (10,11). Although sequencing information was lacking in our study and we have only 2 years of surveillance data, given the elevated rates and substantial burden of norovirus in our study population, we should remain vigilant and continue to monitor norovirus, ideally with genotype information, in future studies.

Acknowledgments

We wish to thank all the patients, nurses, clinicians, and laboratory, research, and administrative staff who took part in our active surveillance study at 10 sentinel hospitals.

This study was funded by the Key Discipline Construction of Health System in Pudong New Area (grant no. PWZx2014-14). Dr. Yu is an epidemiologist at Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College in China. Dr. Yu’s work focuses on the epidemiology of enteric and respiratory viral infections, including norovirus and rotavirus.

Table 2. Estimated outpatient and community incidence of norovirus-associated diarrhea, by surveillance year, age group of patients, and season, Pudong New Area, Shanghai, China, 2012–2013

<table>
<thead>
<tr>
<th>Variable</th>
<th>Outpatient incidence, no. cases/100 person-years (95% CI)</th>
<th>Community incidence, no. cases/100 person-years (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surveillance year</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>1.3 (1.2–1.4)</td>
<td>8.1 (7.2–9.0)</td>
</tr>
<tr>
<td>2013</td>
<td>1.6 (1.4–1.8)</td>
<td>9.8 (8.7–11.0)</td>
</tr>
<tr>
<td>2 y combined</td>
<td>1.5 (1.4–1.6)</td>
<td>8.9 (8.2–9.7)</td>
</tr>
<tr>
<td>Age group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–11 mo</td>
<td>8.4 (5.8–11.6)</td>
<td>20.3 (13.8–27.9)</td>
</tr>
<tr>
<td>12–23 mo</td>
<td>6.8 (4.2–9.6)</td>
<td>14.7 (9.2–20.8)</td>
</tr>
<tr>
<td>24–59 mo</td>
<td>1.4 (1.0–2.0)</td>
<td>2.5 (1.7–3.5)</td>
</tr>
<tr>
<td>5–24 y</td>
<td>1.1 (0.8–1.3)</td>
<td>8.8 (7.0–10.8)</td>
</tr>
<tr>
<td>25–44 y</td>
<td>1.0 (0.9–1.1)</td>
<td>13.0 (11.6–14.7)</td>
</tr>
<tr>
<td>45–64 y</td>
<td>1.7 (1.5–1.9)</td>
<td>3.2 (2.8–3.6)</td>
</tr>
<tr>
<td>≥65 y</td>
<td>2.6 (2.0–3.2)</td>
<td>7.8 (6.1–9.5)</td>
</tr>
<tr>
<td>Season</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring, Mar–May</td>
<td>1.2 (1.0–1.4)</td>
<td>7.9 (6.7–9.0)</td>
</tr>
<tr>
<td>Summer, Jun–Aug</td>
<td>0.8 (0.7–1.0)</td>
<td>4.9 (3.9–6.0)</td>
</tr>
<tr>
<td>Autumn, Sep–Nov</td>
<td>2.3 (2.1–2.5)</td>
<td>13.4 (11.7–15.1)</td>
</tr>
<tr>
<td>Winter, Dec–Feb</td>
<td>1.7 (1.4–1.9)</td>
<td>10.5 (8.8–12.3)</td>
</tr>
</tbody>
</table>

References


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August 2015: Surveillance

- Drivers of Emerging Infectious Disease Events as a Framework for Digital Detection
- Real-time Microbiology Laboratory Surveillance System to Detect Abnormal Events and Emerging Infections, Marseille, France
- Underrecognition of Dengue during 2013 Epidemic in Luanda, Angola
- Health Care–Associated Infection Outbreak Investigations in Outpatient Settings, Los Angeles County, California, USA, 2000–2012
- Influenza A Viruses of Human Origin in Swine, Brazil
- Response Strategies against Meningitis Epidemics after Elimination of Serogroup A Meningococci, Niger
- Phylogeneography of Influenza A(H3N2) Virus in Peru, 2010–2012
- Differentiation of Acute Q Fever from Other Infections in Patients Presenting to Hospitals, the Netherlands
- Susceptibility of Carrion Crows to Experimental Infection with Lineage 1 and 2 West Nile Viruses
- Hospital Resource Utilization and Patient Outcomes Associated with Respiratory Viral Testing in Hospitalized Patients
- Development of Framework for Assessing Influenza Virus Pandemic Risk
- Community-Based Outbreak of Neisseria meningitidis Serogroup C Infection in Men who Have Sex with Men, New York City, New York, USA, 2010–2013
- Risk for Mycobacterial Disease among Patients with Rheumatoid Arthritis, Taiwan, 2001–2011
- Prevalence of Hepatitis E Virus Infection in Pigs at the Time of Slaughter, United Kingdom
- Bartonella spp. and Coxiella burnetii Associated with Community-Acquired, Culture-Negative Endocarditis, Brazil
- Genomic Assays for Identification of Chikungunya Virus in Blood Donors, Puerto Rico, 2014
- Seasonal Patterns of Buruli Ulcer Incidence, Central Africa, 2002–2012
- Occupational Exposure to Dromedaries and Risk for MERS-CoV Infection, Qatar, 2013–2014
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- Bovine Viral Diarrhea Virus Infection in Cattle, United States, 2011
- Influenza A Virus Subtype H5 Infections in Pigs, China, 2011
- Phylogeography of Influenza A(H3N2) Virus in The Netherlands, 2009–2014
- Influenza A Virus Infections in Pigs, The Netherlands, 2014
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- Development of Framework for Assessing Influenza Virus Pandemic Risk

http://wwwnc.cdc.gov/eid/articles/issue/21/08/table-of-contents
Noroviruses are a leading viral cause of epidemic and sporadic acute gastroenteritis in humans of all ages, causing substantial illness and death. Each year, noroviruses cause ≈21 million infections in the United States and ≈200,000 deaths worldwide. Among the 6 known norovirus genogroups (GI–VI), all GI, most GII, and a few GIV noroviruses infect humans (human noroviruses). Each genogroup includes up to 22 genotypes based on the sequences of major capsid protein 1 (VP1). Although GI.4 noroviruses were predominant globally for 2 decades, the previously rare GII.17 genotype emerged during the 2014–15 epidemic season in China and other Southeast Asian countries/regions, causing major epidemics of acute gastroenteritis (1,2). Infection of domestic pigs, cattle, dogs, and rhesus macaques with human norovirus has been reported (3–7). We report the detection and characterization of norovirus GII.17 that extensively and naturally infected farm-raised rhesus monkeys in southwestern China.

The Study
In January 2015, a total of 50 fecal samples were randomly collected from the general monkey population at a farm with ≈2,000 monkeys in Kunming City, Yunnan Province, China, in accordance with the guidelines for humane treatment of animals and approved by the Institutional Animal Care and Use Committee of the Institute of Medical Biology at the Chinese Academy of Medical Science. Viral RNA was extracted from 10% fecal suspensions in physiologic saline by use of a QIAGEN Mini RNA kit (Hilden, Germany). We randomly selected 28 RNA samples for calicivirus detection with a 1-step reverse transcription PCR that used the primer pair P289 and P290 (δ), designed to amplify a genome fragment encoding the calicivirus RNA-dependent RNA polymerase. One of the samples showed the expected 310-bp calicivirus RNA-dependent RNA polymerase gene fragment, which was confirmed by DNA sequencing. Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis indicated that this gene fragment was from a GII.17 norovirus, which we named Mk/KM1509/Yunnan/CHN/2015 (monkey GII.17 norovirus).

Next, we amplified and sequenced the full ≈7.5-kb genome of this norovirus. Sequence analysis showed that the monkey GII.17 norovirus genome sequence shared 99% nt identity with the human GII.17 norovirus recently detected in China (2). Phylogenetic analysis among representative full-length VP1-encoding genes revealed 3 clusters of GII.17 noroviruses (A, B, C) (Figure 1) (9). The monkey GII.17 norovirus grouped with cluster C of the recently detected GII.17 human noroviruses in China. To estimate the infection rate of monkey GII.17 norovirus in the monkey population, we designed a new pair of specific primers (199 and 200) based on our newly isolated genome sequence to reanalyze the 50 extracted RNA samples. PCR amplification and DNA sequencing of the PCR products indicated identical GII.17 noroviruses in 16 (32%) samples.

We then performed a challenge experiment to assess infection and replication of this GII.17 norovirus in monkeys. We randomly selected 2 monkeys for which fecal samples were negative for norovirus and intragastrically administered (by nasogastric tube) a GII.17-positive fecal sample (consisting of 1 mL filtered 20% fecal suspension containing $8.3 \times 10^4$ norovirus genome copies). Despite the absence of typical signs (watery diarrhea and fever), both challenged animals shed norovirus RNA in their feces for at least 16 days; by postinoculation day 3, shedding peaks were $2.573 \times 10^5$ genome copies/gram feces for 1 monkey and $1.33 \times 10^5$ for the other (Figure 2, panels A, B). These great increases of the shed genome copies indicated successful infection and replication of the GII.17 norovirus in monkeys.

We also measured possible seroconversion in the challenged animals by using recombinant VP1 protein of the...
monkey GII.17 norovirus developed after the challenge was performed. ELISA with monkey norovirus VP1 as the capture antigen showed that both monkeys had high norovirus IgG titers (1:320) before the challenge. As a result, norovirus-specific antibody titer increases for both challenged animals were only 2-fold (Figure 2, panels C, D). The observed high preexisting norovirus antibody titers in both monkeys selected for challenge may have resulted from previous infection with the GII.17 norovirus, although their fecal samples were norovirus negative by the time of selection for challenge. The observed low antibody responses and the lack of typical clinical signs after norovirus infection via virus challenge may result from relatively high preexisting GII.17 antibody titers. Further study to define the role of preexisting norovirus antibodies in norovirus infection of rhesus monkeys is needed.

Histo-blood group antigens (HBGAs) are norovirus host factors in which hosts with matched HBGAs exhibit increased susceptibility to norovirus infection (10). To improve understanding of the HBGAs binding profile of this monkey GII.17 norovirus, the recombinant VP1 proteins of the new monkey strain and a recent human GII.17 norovirus were expressed in Escherichia coli (online Technical Appendix Figure, panel A), as previously described (11). HBGA binding assays, performed by using defined human saliva samples with known HBGA types, revealed that the VP1 protein of the monkey GII.17 norovirus bound to human saliva samples with significantly lower binding signals (optical densities) than the human GII.17 norovirus (online Technical Appendix Figure, panels B and C). Accordingly, sequence comparisons of the P domain (the HBGA binding domain) between the human and monkey noroviruses and structural analysis based on the known GII.17 P dimer crystal structure (12) revealed 2 residue mutations, D377G and N342S, near the HBGA binding site of the monkey GII.17 norovirus (online Technical Appendix Figure, panel D). The D377G mutation of the monkey GII.17 norovirus replaces the negatively charged aspartic acid with a small, neutral glycine; the N342S mutation replaces the larger, strongly polar asparagine with a tiny, weakly polar serine. These 2 mutations may be the reason why binding of the monkey GII.17 norovirus to HBGAs is weaker than that of the GII.17 human norovirus. We also noted that the monkey GII.17 VP1 protein bound saliva samples with significantly higher binding signals to saliva samples of type B, which also happens to be the major blood type of rhesus monkeys (13).

**Conclusions**

Although limited success during monkey challenge studies using human noroviruses has been reported (14,15), our study showed that GII.17 noroviruses were able to infect a monkey population, indicating extensive human norovirus infection of farm-raised rhesus monkeys under natural conditions. Our findings suggest that it may be possible to...
establish a useful animal model of norovirus infection to evaluate human norovirus vaccines and antiviral drugs and to study human norovirus pathogenesis, although further testing needs to be done to confirm such possibility. Our findings also raise new concerns about possible viral reservoirs and cross-species transmission of noroviruses.

Considering the fact that a new GII.17 variant emerged as the predominant norovirus and caused major epidemics in China during the same period (1,2), the detected monkey GII.17 norovirus probably originated from a human GII.17 norovirus. However, the mutations near the HBGA binding site might imply an initial adaptation of the monkey GII.17 norovirus to the new host. To provide a better understanding of its infection, pathogenesis, host specificity, epidemiology, and cross-species transmission, further characterization of this monkey GII.17 norovirus is warranted. This information may also be valuable for the future establishment of a monkey model of norovirus infection for vaccine and antiviral evaluation and for addressing the concerns of unknown viral reservoirs and potential zoonotic infection of noroviruses.

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Mr. He is dean of the Kunming National Primate Research Center, Institute of Medical Biology, Chinese Academy of Medical Science, Kunming. His research interests focus on the development and application of nonhuman primate animal models of infectious diseases.

References

Figure 2. Challenge testing of 2 norovirus-negative macaques with GII.17 norovirus. A, B) Challenged macaques shed norovirus-specific RNA (genome copies) for at least 16 days; shedding peaked on postinoculation day 3. C, D) Serum norovirus antibody titers before (postinoculation day 1) and after (postinoculation day 7–35) challenge.


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Determination of *Elizabethkingia* Diversity by MALDI-TOF Mass Spectrometry and Whole-Genome Sequencing

Helle Brander Eriksen, Heidi Gumpert, Cecilie Haase Faurholt, Henrik Westh

In a hospital-acquired infection with multidrug-resistant *Elizabethkingia*, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and 16S rRNA gene analysis identified the pathogen as *Elizabethkingia miricola*. Whole-genome sequencing, genus-level core genome analysis, and in silico DNA-DNA hybridization of 35 *Elizabethkingia* strains indicated that the species taxonomy should be further explored.

*Elizabethkingia* are gram-negative, nonmotile, nonspore-forming rods that are rarely associated with human disease. *E. meningoseptica* is the most recognized clinically, mainly causing hospital-acquired infections, including sepsis in immunocompromised patients and meningitis in neonates (1). These bacteria are intrinsically resistant to β-lactam antimicrobial drugs, including second- and third-generation cephalosporins and carbapenems, because they naturally carry an Ambler class A serine β-lactamase gene *bla*<sub>CME</sub>, which encodes a penicillin- and cephalosporin-hydrolyzing enzyme (2), and 2 Ambler class B metallo-β-lactamase genes, *bla*<sub>B-4</sub> and *bla*<sub>GOB</sub>, which encode carbapenem-hydrolyzing enzymes (3).

The species *E. anopheles* and *E. miricola* are increasingly being isolated and cause similar infections to *E. meningoseptica* (4,5). *E. miricola* was discovered in condensed water collected onboard the Russian space station Mir in 2003 (6), and *E. anopheles* was isolated from the midgut of the *Anopheles gambiae* mosquito in 2011 (7). We report infection with a carbapenem-resistant *Elizabethkingia* species in a patient in Copenhagen, Denmark in September 2015; we identified the bacterium as *E. miricola* by using MALDI-TOF Mass Spectrometry (MALDI Biotyper 3.1, Bruker Daltonics Microflex LT, database MBT DB-5627) identified the colonies as *E. miricola* (score 2.105). Antimicrobial susceptibility testing showed that the isolate was susceptible to ciprofloxacin (MIC 0.25 mg/L) and co-trimoxazole (MIC 0.125 mg/L); intermediate susceptible to piperacillin/tazobactam (MIC 16 mg/L); amoxicillin/clavulanic acid (MIC 8 mg/L), and tigecycline (MIC 0.5 mg/L); and resistant to all other drugs tested (Table). The imipenem/imipenem-EDTA Etest (bioMérieux, Marcy l’Etoile, France) for detection of metallo-β-lactamase was positive.

To confirm the species identity and identify the antimicrobial resistance genes, we performed WGS of the isolate with the Illumina MiSeq platform producing 2 × 150-bp paired-end reads by using the Nextera XT library preparation kit (Illumina Denmark ApS, Copenhagen, Denmark). Reads were assembled by using SPAdes, which produced a 4.13-Mbp genome termed HvH-WGS333 (BioProject PRJNA335686, BioSample SAMN05450241). We performed annotation of the genome using Prokka (8) and used ARG-ANNOT (9) to identify 2 metallo-β-lactamase genes, namely *bla*<sub>B-4</sub> and *bla*<sub>GOB</sub>, and an extended-spectrum β-lactamase gene sharing 90.6% nucleotide identity with *bla*<sub>CME</sub>. The isolate was identified by EzTaxon (10) as *E. miricola* on the basis of its 16S rRNA gene with 99.69% similarity. However, single-nucleotide variant (SNV)
Determination of Elizabethkingia Diversity

Table. Antimicrobial susceptibility of Elizabethkingia isolate HvH-WGS333 from patient with hospital-acquired septic arthritis, Copenhagen, Denmark 2015

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC, mg/L</th>
<th>MIC breakpoint, S≤/R&gt; mg/L</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>0.25</td>
<td>0.5/1</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>0.125</td>
<td>4/4</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>0.5</td>
<td>0.25/0.5</td>
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</tr>
<tr>
<td>Piperacillin/tazobactam†</td>
<td>16</td>
<td>4/16</td>
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<td>Amoxicillin/clavulanic acid‡</td>
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<td>2/8</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&gt;256</td>
<td>2/8</td>
<td>Resistant</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>&gt;256</td>
<td>4/8</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;256</td>
<td>4/8</td>
<td>Resistant</td>
</tr>
<tr>
<td>Meropenem</td>
<td>&gt;32</td>
<td>2/8</td>
<td>Resistant</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>12</td>
<td>4/4</td>
<td>Resistant</td>
</tr>
<tr>
<td>Colistin</td>
<td>&gt;256</td>
<td>4/4</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

*Submitted isolate to GenBank (accession no. NZ_MCFJ00000000.1). The isolate contains antimicrobial resistance genes bla₄₅, bla₄₆, and an extended-spectrum β-lactamase, sharing 90.6% nucleotide identity to bla₄₅. Antimicrobial susceptibility testing was performed by using Etest (bioMérieux, Marcy l’Etoile, France) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org). For interpretation, non–species-related (pharmacokinetics/pharmacodynamics) breakpoints were used except for co-trimoxazole, in which Stenotrophomonas maltophilia breakpoints were used, and for gentamicin and colistin, in which Pseudomonas sp. breakpoints were used. R, resistance; S, susceptibility.
†For susceptibility testing purposes, the concentration of tazobactam was fixed at 4 mg/L.
‡For susceptibility testing purposes, the concentration of clavulanic acid was fixed at 2 mg/L.

Because the isolate from this patient was not closely related to the E. miricola type strain, we performed a genus-level core genome analysis of 35 publicly deposited Elizabethkingia strains from GenBank to identify a more closely related isolate. Each genome was annotated (8) and the core genome was approximated by ROARY (11), yielding a 419,813-bp core genome of 426 genes. We constructed a phylogenetic tree of the core genome using RAxML (12) with 100 bootstrapping replicates (Figure). The ATCC 13253 E. meningoseptica strain did not cluster with the other E. meningoseptica strains and was the most distantly related Elizabethkingia sp. annotated. Before WGS was commonly used, high levels of divergence were reported among E. meningoseptica (formerly Flavobacterium meningosepticum) isolates, with 2 groups detected, Ursing and Bruun (UB) I and UBII (13), with ATCC 13253 belonging to UBII. Our HvH-WGS333 isolate was most closely related to EM_CHUV, an endotracheal isolate submitted as E. miricola; these 2 isolates are next most closely related to G4071, which belongs to the UBII:2 (13), and then to GTC 862 E. miricola. E. miricola ATCC 33958 is most closely related to G4075, a clinical blood culture E. meningoseptica in the UBII:3 group. Strains belonging to groups UBII and UBII are phenotypically very similar, explaining why DNA-DNA hybridization (DDH) was previously used to distinguish these groups (14).

Additionally, we performed in silico DDH (15) to other sequenced Elizabethkingia isolates to determine whether our isolate could be defined as the same species (i.e., having a DDH value >70%). The in silico DDH value is calculated by summing up the identities in matches between the genome sequences and dividing by the total match hit lengths. HvH-WGS333 only had an in silico DDH >70% with EM_CHUV and G4071, which clustered most closely together in the tree but was also closely related (67.1%) to strain GTC 862 (Figure). A previous DDH study investigating the Elizabethkingia genus found DDH values at 70°C of 52% between UBII:2 and UBII:3, 45% between UBII:2 and UBII:4, and 36% between UBII:3 and UBII:4 (14), in agreement with our in silico DDH results (Figure). Our analyses support the identification of the 3 UBII subgroups 2, 3, and 4 as being 3 different Elizabethkingia species, with UBII:2 being the closest related to the E. miricola type strain.

Conclusions

The wide adoption of MALDI-TOF mass spectrometry for bacterial identification is likely to increase the detection of infections caused by Elizabethkingia, and thus the number of infection reports. However, the applied MALDI-TOF database contained only 3 E. meningoseptica and 1 E. miricola isolates at the time of our study. Identification on the basis of the 16S rRNA gene corroborated our MALDI-TOF mass spectrometry identification of E. miricola. When exploring this further using WGS, we discovered our isolate shared 80.5% of the GTC 862 E. miricola genome and the in silico DDH value was 67.1%, just below the 70% species threshold. It thus appears that our isolate, along with EM_CHUV and G4071, is closely related to the E. miricola species and that the taxonomic position of the UBII:3 and UBII:4 strains should be reconsidered. In light of our WGS analysis demonstrating considerable Elizabethkingia genetic diversity, we suggest that the MALDI-TOF database needs to be updated and the nomenclature needs to be re-examined. WGS is proving to be a valuable tool for the
correct identification of new species and detailed characterization of multidrug-resistant bacteria.

Dr. Eriksen is training for specialization in Clinical Microbiology and is affiliated with the Department of Clinical Microbiology, Copenhagen University Hospital, Hvidovre, Denmark. Her primary research interests are epidemiology, bacteriology, and whole genome sequencing.

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October 2016: Disease Patterns

- Outbreaks of Human *Salmonella* Infections Associated with Live Poultry, USA, 1990–2014
- Vaccine-Derived Polioviruses and Children with Primary Immunodeficiency, Iran, 1995–2014
- Infection-Related Deaths from Refractory Juvenile Idiopathic Arthritis
- Accuracy of Diagnosis of Human Granulocytic Anaplasmosis in China
- Population-Level Effects of Human Papillomavirus Vaccination Programs on Infection with Nonvaccine Genotypes
- Cat-Scratch Disease in the United States, 2005–2013
- Community- and Healthcare-Associated *Clostridium difficile* Infections, Finland, 2008–2013
- Carbapenem Resistance in Clonally Distinct Clinical Strains of *Vibrio fluvialis* Isolated from Diarrheal Samples
- Whole-Genome Characterization of Epidemic *Neisseria meningitidis* Serogroup C and Resurgence of Serogroup W in Niger, 2015
- Ebola Virus Disease in Children, Sierra Leone, 2014–2015
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- Estimation of Severe MERS-CoV Cases in the Middle East, 2012–2016
- Distinct Zika Virus Lineage in Salvador, Bahia, Brazil
- *Streptococcus suis* Serotype 2 Capsule In Vivo
- Chikungunya Virus in Febrile Humans and *Aedes aegypti* Mosquitoes, Yucatan, Mexico
- Viral RNA in Blood as Indicator of Severe Outcome in Middle East Respiratory Syndrome Coronavirus Infection

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http://wwwnc.cdc.gov/eid/articles/issue/22/10/table-of-contents
Zika virus has recently been introduced to the Americas and is spreading rapidly. We evaluated the characteristics of US travelers to Zika virus–affected countries who were seen at Global TravEpiNet sites during March 2015–October 2016. Nearly three quarters of travelers were men or women of reproductive age.

In 2014, a total of 30.8 million US residents traveled internationally, and 39% of trips were to the Caribbean, Central America, and South America (the Americas) (1). Travelers to this region are at risk for mosquito-borne illnesses, including Zika virus infection (2). As of November 1, 2016, a total of 49 countries and territories in the Americas have reported Zika virus transmission (3).

Zika virus spreads primarily through mosquito bites and sexual contact (2,4,5) and is of particular concern to persons of reproductive age because Zika virus infection in pregnancy can cause microcephaly and brain defects (6,7). We describe the demographics of US travelers to Zika virus–affected countries in the Americas, with a focus on persons of reproductive age.

The Study

Global TravEpiNet (GTEN), supported by the Centers for Disease Control and Prevention (CDC), is a consortium of US clinical practices providing pretravel healthcare to international travelers. GTEN sites include academic practices, healthcare consortia, health maintenance organizations, pharmacy-based clinics, private practices, and public health clinics (8). We collected data on persons seen for pretravel consultation during March–October 2016 at 20 participating clinics (8).

We evaluated the destinations, purpose of travel, accommodations, departure month, time to departure, length of travel, and age for all travelers to Zika virus–affected countries in the Americas. We defined a man of reproductive age as being ≥15 years of age and a woman of reproductive age as being 15–44 years of age (9). Among women of reproductive age, we evaluated the frequency of pregnancy, breastfeeding, possible pregnancy in the next 3 months, and use of prescription birth control.

We considered all countries and territories with autochthonous Zika virus transmission as reported by CDC as of November 1, 2016: Anguilla, Antigua and Barbuda, Argentina, Aruba, the Bahamas, Barbados, Belize, Bolivia, Brazil, British Virgin Islands, Cayman Islands, Colombia, Costa Rica, Cuba, Dominican Republic, Ecuador, El Salvador, French Guiana, Grenada, Guadeloupe, Guatemala, Guyana, Haiti, Honduras,amaica, Martinique, Mexico, Netherlands Antilles, Nicaragua, Panama, Paraguay, Peru, Puerto Rico, Saint Kitts and Nevis, Saint Lucia, Saint Vincent and the Grenadines, Suriname, Trinidad and Tobago, Turks and Caicos, US Virgin Islands, and Venezuela (3).

A total of 22,736 travelers were seen for a pretravel consultation during March 2015–October 2016. Of these, 6,440 (28%) planned trips to ≥1 Zika virus–affected country in the Americas. Peru was the most common Zika virus–affected destination (accounting for 25% of all travelers to Zika virus–affected countries), followed by Brazil (12%). Of the 6,440 travelers to Zika virus–affected countries, 4,819 (75%) were persons of reproductive age.

More than half (59%) of travelers to Zika virus–affected countries were women; nearly two thirds (63%) of these women were of reproductive age (Table 1). Overall, the most common reason for travel was leisure (59%). More than one quarter (26%) of women of reproductive age were traveling for missionary work or nonmedical service work, and 15% were traveling for research or education. Only 1% of women of reproductive age were visiting friends and relatives (hereafter referred to as VFR travelers).

Less than 1% of women of reproductive age traveling to Zika virus–affected countries reported being pregnant...
US Travelers to Zika Virus–Affected Countries

(n = 7) or breastfeeding (n = 9) at the pretravel consultation (Table 2). Overall, 42 women (2%) reported they were planning pregnancy in the next 3 months; nearly 5% of women ages 30–39 years were planning pregnancy. Approximately one third of women (34%) reported using prescription birth control; the highest rate of prescription birth control use (44%) was among women 20–29 years of age.

Conclusions

Zika virus transmission has increased in the Americas. Providing pretravel counseling on mosquito bite prevention and risk for sexual transmission of Zika virus and recommending that pregnant women not travel to areas with Zika virus transmission are public health priorities. We describe the characteristics of US travelers seeking health advice before travel to Zika virus–affected countries in the Americas. Our findings suggest areas for intervention.

First, we found that three quarters of travelers seen at GTEN sites before visiting countries with Zika virus transmission in the Americas were of reproductive age. Nearly two thirds of women traveling to Zika virus–affected countries were of reproductive age, and only approximately one-third reported using prescription birth control. Five percent of women ages 30–39 years reported planning pregnancy; not all might have disclosed their plans (nearly half of all pregnancies in the United States are unplanned) (10). Our findings underscore that women of reproductive age, some with immediate plans for pregnancy, are traveling to Zika virus–affected countries in the Americas. CDC has issued Zika virus–related recommendations regarding pregnancy planning for travelers (http://www.cdc.gov/zika/pregnancy/thinking-about-pregnancy.html). Healthcare providers should stay abreast of these recommendations to counsel travelers to the Americas appropriately.

Second, we found that one quarter of women of reproductive age traveling to Zika virus–affected countries in the Americas were traveling for mission or nonmedical service trips; another 15% were traveling for research or education. Previous studies show that volunteer travelers are likely to pursue health information but might not adhere to mosquito

Table 1. Demographic characteristics of travelers to Zika virus–affected countries in the Americas, February 2015–October 2016*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All travelers, N = 6,440</th>
<th>Women of reproductive age, n = 2,373</th>
<th>Men of reproductive age, n = 2,446</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>3,783 (59)</td>
<td>2,373 (100)</td>
<td>0</td>
</tr>
<tr>
<td>Male</td>
<td>2,657 (41)</td>
<td>0</td>
<td>2,446 (100)</td>
</tr>
<tr>
<td>Median age (IQR), y</td>
<td>32 (22–51)</td>
<td>26 (20–32)</td>
<td>36 (25–54)</td>
</tr>
<tr>
<td>Traveler type†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leisure traveler</td>
<td>3,816 (59)</td>
<td>1,209 (51)</td>
<td>1,483 (61)</td>
</tr>
<tr>
<td>Business traveler</td>
<td>696 (11)</td>
<td>211 (9)</td>
<td>357 (15)</td>
</tr>
<tr>
<td>Visiting friends and relatives</td>
<td>110 (2)</td>
<td>29 (1)</td>
<td>30 (1)</td>
</tr>
<tr>
<td>Medical care, providing or receiving</td>
<td>430 (7)</td>
<td>242 (10)</td>
<td>135 (6)</td>
</tr>
<tr>
<td>Research or education</td>
<td>714 (11)</td>
<td>366 (15)</td>
<td>237 (10)</td>
</tr>
<tr>
<td>Mission or nonmedical service</td>
<td>1,288 (20)</td>
<td>622 (26)</td>
<td>439 (18)</td>
</tr>
<tr>
<td>Adventure</td>
<td>600 (9)</td>
<td>254 (11)</td>
<td>231 (9)</td>
</tr>
<tr>
<td>Destination type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban only</td>
<td>1,280 (20)</td>
<td>456 (19)</td>
<td>497 (20)</td>
</tr>
<tr>
<td>Rural only</td>
<td>937 (15)</td>
<td>332 (14)</td>
<td>359 (15)</td>
</tr>
<tr>
<td>Both urban and rural</td>
<td>4,223 (66)</td>
<td>1,585 (67)</td>
<td>1,590 (65)</td>
</tr>
<tr>
<td>Accommodations†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camping</td>
<td>645 (10)</td>
<td>295 (12)</td>
<td>262 (11)</td>
</tr>
<tr>
<td>Hostel or dormitory</td>
<td>1,382 (21)</td>
<td>662 (28)</td>
<td>519 (21)</td>
</tr>
<tr>
<td>Home stay, relatives</td>
<td>362 (6)</td>
<td>107 (5)</td>
<td>110 (5)</td>
</tr>
<tr>
<td>Home stay, nonrelatives</td>
<td>1,021 (16)</td>
<td>475 (20)</td>
<td>366 (15)</td>
</tr>
<tr>
<td>Hotel</td>
<td>4,378 (68)</td>
<td>1,516 (64)</td>
<td>1,683 (69)</td>
</tr>
<tr>
<td>Cruise</td>
<td>427 (7)</td>
<td>68 (3)</td>
<td>177 (7)</td>
</tr>
<tr>
<td>Other</td>
<td>953 (15)</td>
<td>336 (14)</td>
<td>364 (15)</td>
</tr>
<tr>
<td>Country of birth, United States</td>
<td>5,704 (89)</td>
<td>2,106 (89)</td>
<td>2,147 (88)</td>
</tr>
<tr>
<td>Departure months</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mar–Jun 2015</td>
<td>1,050 (16)</td>
<td>416 (18)</td>
<td>380 (16)</td>
</tr>
<tr>
<td>Jul–Oct 2015</td>
<td>1,110 (17)</td>
<td>389 (16)</td>
<td>441 (18)</td>
</tr>
<tr>
<td>Nov 2015–Feb 2016</td>
<td>1,278 (20)</td>
<td>428 (18)</td>
<td>492 (20)</td>
</tr>
<tr>
<td>Mar–Jun 2016</td>
<td>1,587 (25)</td>
<td>644 (27)</td>
<td>552 (23)</td>
</tr>
<tr>
<td>Jul–Oct 2016</td>
<td>1,177 (18)</td>
<td>423 (18)</td>
<td>489 (20)</td>
</tr>
<tr>
<td>After Nov 1, 2016</td>
<td>238 (4)</td>
<td>73 (3)</td>
<td>94 (4)</td>
</tr>
<tr>
<td>Median time to departure (IQR), d</td>
<td>22 (10–40)</td>
<td>21 (10–39)</td>
<td>21 (10–39)</td>
</tr>
<tr>
<td>Median length of travel (IQR), d‡</td>
<td>10 (7–15)</td>
<td>10 (7–15)</td>
<td>10 (7–16)</td>
</tr>
<tr>
<td>Median no. of destination countries (IQR)</td>
<td>1 (1–1)</td>
<td>1 (1–1)</td>
<td>1 (1–1)</td>
</tr>
</tbody>
</table>

*Values are no. (%) travelers except as indicated. Women of reproductive age were defined as those 15–44 y, men of reproductive age as those ≥15 y.
†Travelers could choose >1 response.
‡Includes travel up to 2,000 d.
avoidance measures (11,12). A survey of volunteers traveling to the Dominican Republic in 2014 demonstrated that only 30% reapplied mosquito repellant, and <5% stayed in accommodations with screens (12). Service organizations might consider educating on mosquito avoidance and distributing mosquito repellant and permethrin-treated clothes for appropriate destinations (13) when their members travel to Zika virus–affected areas. Providing bed nets should be considered for preventing other mosquito-borne diseases.

We previously reported that ≈11% of all travelers seen at GTEN sites were VFR travelers (8,14), and the US Office of Travel and Tourism Industries estimates that 27% of travelers are VFR travelers (1). Only 1% of the population in our study were VFR travelers. This finding suggests that VFR travelers to Zika virus–affected countries in the Americas might seek pretravel advice at a lower rate than VFR travelers to other locations (8) and is noteworthy because VFR travelers are at elevated risk for mosquito-borne illnesses (2).

Our analysis has limitations. Travelers at GTEN sites might not represent all US international travelers, and clinical practice at GTEN sites might differ from other settings where pretravel health care is provided. Also, we did not collect data on contraceptive practices in male travelers; this information would be of interest given the current recommendation for men to use condoms for sexual intercourse. Last, we did not correlate dates of travel with the time that Zika virus transmission was identified in each country.

In conclusion, our findings show that many persons of reproductive age are traveling to Zika virus–affected countries in the Americas. We observed that VFR travelers represent an unexpectedly small proportion of those seeking health advice before travel to these Zika virus–affected countries; outreach efforts to increase the frequency of pretravel health encounters for these travelers are warranted. Clinicians should provide education on mosquito bite prevention for all travelers to Zika virus–affected countries and should discuss use of condoms or abstinence to reduce the risk for sexual transmission during and after travel. In addition, clinicians should assess reproductive plans, review use of effective birth control, and discuss waiting for conception when returning from areas with Zika virus.

Acknowledgments
We thank all the members of the Global TravEpiNet Consortium (http://www.globaltravepinet.org).

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Ms. Lammert is a research assistant at the Travelers’ Advice and Immunization Center at the Massachusetts General Hospital, Boston, and a doctoral student in epidemiology at the University of Minnesota, Minneapolis. Her research interests include travel medicine and infectious diseases.

References

**Table 2. Reproductive status of women of reproductive age traveling to Zika virus–affected countries in the Americas, March 2015–October 2016**

<table>
<thead>
<tr>
<th>Status</th>
<th>No. (%) travelers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All women, N = 2,373</td>
</tr>
<tr>
<td>Pregnant</td>
<td>7 (0.3)</td>
</tr>
<tr>
<td>Breastfeeding</td>
<td>9 (0.4)</td>
</tr>
<tr>
<td>Possible pregnancy in next 3 mo</td>
<td>42 (1.8)</td>
</tr>
<tr>
<td>Using prescription birth control</td>
<td>803 (33.8)</td>
</tr>
</tbody>
</table>

*Women of reproductive age were defined as those 15–44 y.
US Travelers to Zika Virus–Affected Countries


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May 2016: Vectorborne Diseases

- An Operational Framework for Insecticide Resistance Management Planning
- Outbreak of Middle East Respiratory Syndrome at Tertiary Care Hospital, Jeddah, Saudi Arabia, 2014
- Differences in Genotype, Clinical Features, and Inflammatory Potential of Borrelia burgdorferi sensu stricto Strains from Europe and the United States

- Expansion of Shiga Toxin–Producing Escherichia coli by Use of Bovine Antibiotic Growth Promoters
- Projecting Month of Birth for At-Risk Infants after Zika Virus Disease Outbreaks
- Genetic Characterization of Archived Bunyaviruses and Their Potential for Emergence in Australia
- Plasmodium falciparum In Vitro Resistance to Monodesethylamodiaquine, Dakar, Senegal, 2014

- Astrovirus MLB2, a New Gastroenteric Virus Associated with Meningitis and Disseminated Infection
- Spectrum of Viral Pathogens in Blood of Malaria-Free Ill Travelers Returning to Canada
- Expanded Geographic Distribution and Clinical Characteristics of Ehrlichia ewingii Infections, United States
- Rickettsia parkeri Rickettsiosis, Arizona, USA
- Acute Human Inkoo and Chatanga Virus Infections, Finland

http://wwwnc.cdc.gov/eid/articles/issue/22/05/table-of-contents
Biofilm-Forming Capability of Highly Virulent, Multidrug-Resistant Candida auris

Leighann Sherry, Gordon Ramage, Ryan Kean, Andrew Borman, Elizabeth M. Johnson, Malcolm D. Richardson, Riina Rautemaa-Richardson

The emerging multidrug-resistant yeast pathogen Candida auris has attracted considerable attention as a source of healthcare–associated infections. We report that this highly virulent yeast has the capacity to form antifungal resistant biofilms sensitive to the disinfectant chlorhexidine in vitro.

The yeast pathogen Candida auris was first detected in 2009 from an ear canal infection in Japan (1). This species initially attracted attention because of its reduced susceptibility to afoles and amphotericin B, combined with the lack of reliable culture-based methods for its identification (2). More recently, C. auris has been associated globally with life-threatening invasive diseases, such as bloodstream and wound infections. C. auris has also caused hospital outbreaks across Asia and South America, as highlighted in a 2016 clinical alert (3). In addition, in a UK intensive care unit, candidemia developed in 20% of patients colonized with C. auris (4). Although the mode of transmission within hospitals is unknown, C. auris may substantially contaminate rooms of colonized or infected patients (5). Phospholipase and proteinase activity have been identified as virulence factors (6); however, because previously used assessment techniques were rudimentary, this pathogen’s ability to form biofilm remains under question (7). The draft genome identifying various proteins involved in biofilm formation (8), coupled with recent descriptions of aggregative and nonaggregative phenotypes, the latter of which are more virulent in vivo (9), indicate the possibility of heterogeneous C. auris biofilm formation, as described for C. albicans (10). We sought to examine these aggregative and nonaggregative C. auris phenotypes in the context of biofilm-forming capacity, investigate their susceptibility to a panel of antifungal agents and the skin disinfectant chlorhexidine, and investigate their virulence in vivo.

The Study

Throughout this study, we used C. albicans SC5314 and Candida glabrata WT2001 as comparators for C. auris nonaggregative strains NCPF 8971 (strain 10) and NCPF 8973 (strain 12) and aggregative strains NCPF 8977 (strain 2) and NCPF 8978 (strain 6), as previously described (9). Strains were propagated in YPD broth (Sigma-Aldrich, Dorset, UK), incubated overnight at 30°C, and adjusted to 10⁶ cells/mL in RPMI 1640 medium (11). On 3 separate occasions, 8 biofilms of each Candida species were grown in flat-bottomed, 96-well polystyrene microtiter plates and incubated for 24 h at 37°C, after which biomass was assessed by crystal violet assay (12). C. albicans displayed the greatest biofilm mass (Figure 1, panel A), consistent with previous findings (10). Compared with C. albicans, all C. auris strains formed significantly reduced biofilms (p<0.0001): biomass for nonaggregative C. auris strains 10 and 12 was 2.4 and 1.5 times less, respectively, than those for C. albicans, and biomass for aggregative C. auris strains 2 and 6 was 3.0 and 3.1 times less, respectively. However, these strains formed significantly greater biofilms (p<0.0001) than those formed by C. glabrata (3.8, 6.0, 3.0, and 2.9 times more for strains 10, 12, 2, and 6, respectively). We confirmed these findings for each species by scanning electron microscopy after growing the strains on Thermanox Coverslips (Thermo Fisher Scientific, Paisley, UK) for 24 h, as previously described (12). C. albicans biofilms were typically densely packed with hyphae (Figure 1, panel B), whereas C. glabrata formed a sparse biofilm consisting of yeast cells only, without extracellular matrix (Figure 1, panel C). C. auris strain 10 biofilm formation was intermediate to the C. albicans and C. glabrata phenotypes, showing predominately budding yeast and occasional pseudohyphae (Figure 1, panel D). In agreement with previous findings (9), all tested C. auris strains displayed the same phenotype.

To determine MICs for planktonic and sessile cells of the C. auris strains, we performed antifungal susceptibility testing using standardized Clinical Laboratory Standards Institute M27-A3 broth microdilution (visual inspection) and standardized candidal biofilm testing (metabolic viability) with fluconazole, voriconazole, caspofungin, micafungin, liposomal amphotericin B, amphotericin B, and chlorhexidine (13,14). Antifungal agents were tested in serial 2-fold dilutions (0.06–32.0 mg/L) for planktonic and sessile cells. Fluconazole was ineffective (MICs of >32 mg/L) against planktonic and sessile communities, whereas...
voriconazole displayed minimal activity against planktonic cells (Table 1, 2). Although liposomal amphotericin B was active against planktonic *C. auris* at 0.25–1.0 mg/L, up to 16 mg/L was required to reduce biofilm metabolic viability by 90%. Amphotericin B was more effective, requiring 4 mg/L to kill biofilms. Micafungin was the most active echinocandin, requiring <0.5 mg/L to inhibit planktonic cells, compared with 2–32 mg/L for caspofungin. However, these 2 antifungal agents were ineffective against biofilms, requiring >32 mg/L to inhibit sessile cells. Of note, chlorhexidine exhibited the greatest activity, requiring <0.02% to effectively inhibit planktonic and sessile cells across all strains tested. All strains showed similar sensitivity profiles, with the exception of strain 10, for which voriconazole was required in higher concentrations and caspofungin in lower concentrations to effectively inhibit planktonic growth.

Killing assays in *Galleria mellonella* were performed, as previously described (12), to assess the pathogenicity of each *Candida* species. Ten *G. mellonella* larvae (Livefoods Direct Ltd, Sheffield, UK) with bodyweights of ≈300 mg were used for each test group. Standardized inoculums of 10⁶ and 10⁵ and to 5 × 10⁵ and 5 × 10⁴ cells/larvae (Figure 2) in PBS, were injected into the hemocoel, as previously described (9). We assessed pathogenicity using a Kaplan-Meier plot, monitoring the percent survival over 5 days. Survival data for 5 × 10⁵ cells/larvae showed a significant difference in the killing of larvae by *C. glabrata* and the other *Candida* species (*p*<0.0001) (Figure 2, panel B). Although *C. albicans* and *C. auris* had similar kill kinetics in this model, infection with nonaggregative *C. auris* strain 10 achieved a 100% death rate within 48 h, compared with a rate of ≈87% with *C. albicans* (*p* = 0.3076). Moreover,

### Table 1. Planktonic susceptibility profiles of 7 antifungals against *Candida auris* yeast

<table>
<thead>
<tr>
<th>Drug</th>
<th>Strain 2</th>
<th>Strain 6</th>
<th>Strain 10</th>
<th>Strain 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>8</td>
<td>8</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>32</td>
<td>32</td>
<td>2</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Micafungin</td>
<td>0.5</td>
<td>≤0.0625</td>
<td>&lt;0.06</td>
<td>≤0.0625</td>
</tr>
<tr>
<td>Liposomal amphotericin B</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Chlorhexidine, %</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

*Values are mg/L except as indicated. All MIC tests were performed on 3 independent occasions, showing identical results each time.*
nonaggregative *C. auris* was significantly more pathogenic than *C. albicans* when a lower inoculum of 10^5 (p<0.05) and 5 × 10^4 cells/larvae (p<0.01) was administered. These data, along with those of Borman et al. (9), suggest that the nonaggregative *C. auris* phenotype has the capacity to form biofilms with enhanced virulence capacity.

**Conclusions**

Biofilm formation is a key driver of *C. albicans* pathogenicity and is associated with patient death (10,15). We show that *C. auris* can differentially adhere to polymeric surfaces, form biofilms, and resist antifungal agents that are active against its planktonic counterparts. Of particular interest, caspofungin was predominately inactive against *C. auris* biofilms; this finding was unexpected because caspofungin is normally highly effective against *Candida* biofilms. These features contribute not only to *C. auris* virulence but also to its survival in hospital environments, increasing its ability to cause outbreaks (5). The results of the in vivo model used in this study are in line with our clinical experience and validated by findings in other in vivo studies (9), affirming that *C. auris* is highly virulent or more virulent than *C. albicans*.

Although unable to form biofilms equivalent to *C. albicans*, *C. auris* has a noteworthy virulence capacity that merits further exploration, particularly given the apparent heterogeneity associated with aggregative capacity. These factors, together with the innate resistance of *C. auris* to most antifungal agents, may explain why it is an emerging pathogen. Our findings suggest it is improbable that the spread and prevalence of *C. auris* can be controlled with antifungal stewardship approaches alone. We showed that chlorhexidine is effective against *C. auris* planktonic and sessile communities. Thus, use of this disinfectant can be advocated for topical control of *C. auris* at standard concentrations used for skin and wound cleansing and disinfection (0.05%–4.0%). Infection-prevention measures targeting *C. auris* biofilms in patients, on medical devices (e.g., equipment in contact with patients), and in the hospital environment will be required.

### Table 2. Sessile susceptibility profiles of 7 antifungals against *Candida auris* yeast

<table>
<thead>
<tr>
<th>Drug</th>
<th>Sessile MIC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Micafungin</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Liposomal amphotericin B</td>
<td>2</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>2</td>
</tr>
<tr>
<td>Chlorhexidine, %</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

*Values indicate mg/L except as indicated. Sessile MICs are defined as a 90% inhibition of the metabolic dye XTT, 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (Sigma-Aldrich, Dorset, UK) compared with the untreated control; MIC tests were performed on 3 independent occasions and showed identical results each time.*

---

**Figure 2.** Pathogenicity of *Candida* species yeast infections in vivo. *Galleria mellonella* larvae were infected with 10^6 (A), 5 × 10^6 (B), 10^7 (C), and 5 × 10^6 (D) cells/larvae of *C. albicans*, *C. glabrata*, and 4 *C. auris* strains, and larvae survival measured every 12 h over 5 d. Ten samples of each yeast were used, and experiments were performed on 3 independent occasions. Data represents the mean percentage survival, as determined using a Kaplan-Meier plot. PBS and controls, which were pierced only, were also included and had no effect on larvae survival. Results show that *C. auris* and *C. albicans* infection exhibit similar pathogenicity.
Dr. Sherry works as a medical mycologist at the University of Glasgow, Glasgow, UK. Her specific interest is fungal biofilms of clinical significance.

References
7. Borman AM, Szekely A, Johnson EM. Comparative pathogenicity of United Kingdom isolates of the emerging pathogen *Candida auris* and other key pathogenic Candida species. mSphere. 2016;1:pii:e00189-16

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Biofilm-Forming Capability of *Candida auris*

In 1974, Lewis Thomas (1913–1993), physician, professor, and dean, published *The Lives of A Cell*, the first of 2 books subtitled *Notes of a Biology Watcher*. The phrase “lives of a cell” refers to the independent yet interrelated parts of a human cell—including mitochondria, centrioles, and basal bodies—that once led independent lives. Without these previously independent lives working together, we would not have the capacity for thought, communication, and movement. Dr. Thomas wrote, “Our membranes hold against equilibrium, maintain imbalance, bank against entropy... We are shared, rent-ed and occupied.”

Our human lives do not depend just on the lives in our individual cells. Our lives depend fully on the lives in our individual cells. Our lives depend on the earth, including the atmosphere, and the many other human and nonhuman lives that occupy it. In explaining this complex interdependence, Dr. Thomas observed that the earth is “most like a cell.” This second interpretation of lives of a cell refers to the many interrelated earthly entities, such as plants, whales, humans, and even viruses, that “dart rather like bees from organism to organism, from plant to insect to mammal to me and back again,” all protected by the sky—a membrane that “works, and for what it is designed to accomplish it is as infallible as anything in nature.”

**EID Podcast: Lives of a Cell: 40 Years Later, A Third Interpretation**

In 1974, Lewis Thomas (1913–1993), physician, professor, and dean, published *The Lives of A Cell*, the first of 2 books subtitled *Notes of a Biology Watcher*. The phrase “lives of a cell” refers to the independent yet interrelated parts of a human cell—including mitochondria, centrioles, and basal bodies—that once led independent lives. Without these previously independent lives working together, we would not have the capacity for thought, communication, and movement. Dr. Thomas wrote, “Our membranes hold against equilibrium, maintain imbalance, bank against entropy... We are shared, rent-ed and occupied.”

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Increasing Antibiotic Resistance in *Shigella* spp. from Infected New York City Residents, New York, USA

Kenya Murray, Vasudha Reddy, John S. Kornblum, HaeNa Waechter, Ludwin F. Chicaiza, Inessa Rubinstein, Sharon Balter, Sharon K. Greene, Sarah L. Braunstein, Jennifer L. Rakeman, Catherine M. Dentinger

Approximately 20% of *Shigella* isolates tested in New York City, New York, USA, during 2013–2015 displayed decreased azithromycin susceptibility. Case-patients were older and more frequently male and HIV infected than those with azithromycin-susceptible *Shigella* infection; 90% identified as men who have sex with men. Clinical interpretation guidelines for azithromycin resistance and outcome studies are needed.

*Shigella* bacteria are transmitted through the fecal–oral route by direct contact with an infected person, by ingestion of contaminated food or water, or by fomites. Shigellosis is associated with travel to disease-endemic areas, men who have sex with men (MSM), crowding, poverty, and attendance at childcare centers (1); illness is generally self-limited. Antibiotics may shorten the duration and decrease the illness severity (2,3). Because *Shigella* spp. may be resistant to ampicillin and trimethoprim/sulfamethoxazole (TMP/SMX), azithromycin and ciprofloxacin are often prescribed to treat shigellosis. In 2016, the Clinical Laboratory Standards Institute (CLSI) published MICs of azithromycin that indicated *Shigella* resistance; values are based on in vitro data and are not clinical breakpoints (4).

In 2013, public health laboratories in New York City (NYC), New York, USA, began testing susceptibility of *Shigella* isolates to azithromycin. We identified factors associated with infection with isolates that exhibited decreased susceptibility to azithromycin (DSA) or resistance to ciprofloxacin.

The Study

After submission to NYC public health laboratories, representative colonies of *Shigella* isolates are identified with conventional biochemical tests and tested for susceptibility to ampicillin, cefixime, ciprofloxacin, azithromycin, and TMP/SMX using the Etest antibiotic gradient (bioMérieux, Durham, NC, USA). MICs are interpreted according to CLSI guidelines (5). After consultation with the Centers for Disease Control and Prevention (CDC), we defined DSA isolates as those with an MIC of azithromycin of $\geq 32$ µg/mL (J. Whichard, CDC, pers. comm., 2013).

Using a standard questionnaire, we interviewed persons infected with DSA or ciprofloxacin-resistant *Shigella* isolates, diagnosed during March 22, 2013–May 31, 2015; we abstracted antibiotic use data from medical charts. *Shigella*-infected case-patients were matched to the NYC HIV Surveillance Registry (6). We determined neighborhood poverty level as described (7) and compared proportions of those infected by age group, sex, and HIV status using $\chi^2$ tests. To identify factors associated with DSA or ciprofloxacin-resistant *Shigella* infection and with hospitalization, we used logistic regression analysis (SAS version 9.2; SAS Institute, Cary, NC, USA).

During 26 months, 978 *Shigella* isolates were submitted; 295 were associated with an outbreak (8) and analyzed separately, and 683 were defined as sporadic. Among patients with sporadic infections, 129 (19%) were infected with isolates displaying DSA, and 29 (4%) were infected with ciprofloxacin-resistant isolates; 5 isolates displayed both characteristics. The median age of case-patients was 27 years (range 0–93 years); 446 (65%) were male. Nearly all infections were caused by *S. sonnei* (65%) or *S. flexneri* (34%). Antibiotic resistance of isolates was as follows: 416 (61%) to ampicillin, 10 (1%) to cefixime, 29 (4%) to ciprofloxacin, and 481 (70%) to TMP/SMX (Table 1).

Persons infected with DSA or ciprofloxacin-resistant *Shigella* spp. were older and more likely to be male than those with DSA- or ciprofloxacin-susceptible isolates; no association with neighborhood poverty was found. Although most infections were caused by *S. sonnei*, most isolates displaying DSA were *S. flexneri*. Isolates displaying DSA or ciprofloxacin resistance were more likely to be ampicillin- and TMP/SMX-resistant than were azithromycin- and ciprofloxacin-susceptible isolates (Tables 1, 2).
Table 1. *Shigella* case-patient characteristics, by azithromycin or ciprofloxacin resistance, New York City, New York, USA, March 22, 2013–May 31, 2015*  

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>DSA, n = 129</th>
<th>Ciprofloxacin resistant, n = 29</th>
<th>Susceptible, n = 530</th>
<th>Total, n = 683†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–17</td>
<td>3 (2)</td>
<td>7 (24)</td>
<td>254 (48)</td>
<td>262 (38)</td>
</tr>
<tr>
<td>18–64</td>
<td>119 (92)</td>
<td>19 (66)</td>
<td>261 (49)</td>
<td>397 (58)</td>
</tr>
<tr>
<td>&gt;65</td>
<td>7 (5)</td>
<td>3 (10)</td>
<td>15 (3)</td>
<td>24 (4)</td>
</tr>
<tr>
<td><strong>Race</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>58 (45)</td>
<td>12 (41)</td>
<td>107 (20)</td>
<td>174 (25)</td>
</tr>
<tr>
<td>Black</td>
<td>39 (30)</td>
<td>1 (3)</td>
<td>75 (14)</td>
<td>115 (17)</td>
</tr>
<tr>
<td>Other</td>
<td>3 (2)</td>
<td>4 (14)</td>
<td>11 (2)</td>
<td>17 (2)</td>
</tr>
<tr>
<td>Unknown</td>
<td>29 (22)</td>
<td>12 (41)</td>
<td>337 (64)</td>
<td>377 (55)</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>15 (12)</td>
<td>3 (10)</td>
<td>74 (14)</td>
<td>91 (13)</td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td>78 (60)</td>
<td>15 (52)</td>
<td>131 (25)</td>
<td>222 (33)</td>
</tr>
<tr>
<td>Unknown</td>
<td>36 (28)</td>
<td>11 (38)</td>
<td>325 (61)</td>
<td>370 (54)</td>
</tr>
<tr>
<td><strong>Borough</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bronx</td>
<td>19 (15)</td>
<td>1 (3)</td>
<td>100 (19)</td>
<td>120 (18)</td>
</tr>
<tr>
<td>Brooklyn</td>
<td>32 (25)</td>
<td>7 (24)</td>
<td>192 (36)</td>
<td>230 (34)</td>
</tr>
<tr>
<td>Manhattan</td>
<td>61 (47)</td>
<td>12 (41)</td>
<td>119 (22)</td>
<td>191 (28)</td>
</tr>
<tr>
<td>Queens</td>
<td>16 (12)</td>
<td>7 (24)</td>
<td>111 (21)</td>
<td>131 (19)</td>
</tr>
<tr>
<td>Staten Island</td>
<td>1 (1)</td>
<td>2 (7)</td>
<td>8 (2)</td>
<td>11 (2)</td>
</tr>
<tr>
<td><strong>Neighborhood poverty, %‡</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>23 (18)</td>
<td>8 (29)</td>
<td>87 (17)</td>
<td>117 (18)</td>
</tr>
<tr>
<td>10–20</td>
<td>48 (38)</td>
<td>10 (36)</td>
<td>137 (27)</td>
<td>195 (29)</td>
</tr>
<tr>
<td>20–30</td>
<td>37 (29)</td>
<td>7 (25)</td>
<td>111 (22)</td>
<td>152 (23)</td>
</tr>
<tr>
<td>30–100</td>
<td>20 (16)</td>
<td>3 (11)</td>
<td>176 (34)</td>
<td>199 (30)</td>
</tr>
<tr>
<td><strong>HIV diagnosed</strong></td>
<td>76 (59)</td>
<td>7 (24)</td>
<td>101 (19)</td>
<td>183 (27)</td>
</tr>
<tr>
<td><strong>Antibiotic resistance by species§</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. sonnei</em></td>
<td>42 (33)</td>
<td>23 (79)</td>
<td>381 (72)</td>
<td>443 (65)</td>
</tr>
<tr>
<td>DSA</td>
<td>42 (100)</td>
<td>3 (13)</td>
<td>381(100)</td>
<td>42 (9)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>3 (7)</td>
<td>23 (100)</td>
<td>380 (100)§</td>
<td>23 (5)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>39 (93)</td>
<td>4 (17)</td>
<td>177 (46)</td>
<td>218 (49)</td>
</tr>
<tr>
<td>Cefixime</td>
<td>1 (1)</td>
<td>2 (9)</td>
<td>4 (1)</td>
<td>6 (1)</td>
</tr>
<tr>
<td>TMP/SMX</td>
<td>37 (88)</td>
<td>20 (87)</td>
<td>252 (66)</td>
<td>308 (70)</td>
</tr>
<tr>
<td><em>S. flexneri</em></td>
<td>86 (67)</td>
<td>5 (17)</td>
<td>140 (26)</td>
<td>230 (34)</td>
</tr>
<tr>
<td>DSA</td>
<td>86 (100)</td>
<td>1 (20)</td>
<td>140 (100)</td>
<td>86 (37)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1 (1)</td>
<td>5 (100)</td>
<td>139 (100)§</td>
<td>5 (2)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>79 (92)</td>
<td>5 (100)</td>
<td>109 (78)</td>
<td>192 (83)</td>
</tr>
<tr>
<td>Cefixime</td>
<td>2 (2)</td>
<td>1 (20)</td>
<td>1 (1)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>TMP/SMX</td>
<td>73 (85)</td>
<td>4 (40)</td>
<td>89 (64)</td>
<td>166 (72)</td>
</tr>
</tbody>
</table>

†N = 683 sporadic cases. DSA, decreased susceptibility to azithromycin; TMP/SMX, trimethoprim/sulfamethoxazole.  
‡5 isolates resistant to ciprofloxacin also displayed DSA.  
§Percentage of census tract residents below federal poverty level, per American Community Survey, 2009–2013; 19 missing.  
§S. boydii (n = 7) and S. dysenteriae (n = 3) omitted.

Of the 683 shigellosis case-patients, 183 (27%) had diagnosed HIV infection. Among these, 76 (42%) were infected with DSA isolates, and 7 (4%) were infected with ciprofloxacin-resistant isolates; 108 (59%) were infected with *S. flexneri*, 73 (40%) with *S. sonnei*, and 1 each (0.5%) with *S. boydii* and *S. dysenteriae*. Of 47 (62%) HIV-diagnosed persons with DSA *Shigella* infection, 45 (95%) identified as MSM.

Of the 153 persons with DSA- and/or ciprofloxacin-resistant *Shigella* infection, chart reviews were completed for 111 (73%). Interviews were completed for 97 (64%), and isolates of 80 (82%) of those had DSA to *Shigella*, 15 (15%) had ciprofloxacin-resistant isolates, and 2 (2%) had isolates resistant to azithromycin and ciprofloxacin. Most case-patients were male (140 [91.5%]); of 120 who completed interviews or were listed in the HIV Surveillance Registry, 102 (85%) identified as MSM. Eleven (12%) of 93 interviewed case-patients who answered the question reported international travel. All interviewees reported symptoms; most common were diarrhea (98%) and abdominal cramps (82%). Median illness duration was 7 days (range 2–45 days). Of 31 (32%) reported hospitalizations, 28 (90%) were infected with DSA and 3 (10%) with ciprofloxacin-resistant isolates; median duration of stay was 3 days (range 1–10 days). Twenty-five (81%) hospitalized case-patients were infected with *S. flexneri*. In a model that considered age, sex, species (*S. flexneri* and *S. sonnei*), HIV status, and neighborhood poverty level, only infection with *S. flexneri* was associated with hospitalization (odds ratio 4.04, 95% CI 1.46–11.18).

Antibiotics, most commonly ciprofloxacin, were prescribed for 114 (89%) of 128 case-patients (for whom
data were available; 16 (13%) received antibiotics to which their *Shigella* isolates was not susceptible. Fifteen (17%) of 90 patients had taken antibiotics in the 4 weeks before illness onset; all were men, 10 (67%) were HIV-positive, and 12 (92%) of 13 for whom data were available were MSM.

Median illness duration for the 15 (52%) interviewed case-patients infected with ciprofloxacin-resistant *Shigella* spp. was 7 days (range 2–17 days); 3 (20%) reported hospitalization, and 5 (33%) reported recent international travel. Five (63%) of 8 case-patients for whom data were available identified as MSM.

**Conclusions**

In NYC, 19% of nonoutbreak shigellosis cases were caused by organisms with an azithromycin MIC $\geq 32$ µg/mL, a much higher proportion than the national estimate of 3.8% (9). This finding is troubling because, when antibiotics are used empirically, they are often less effective in treating more resistant strains. Although *Shigella* infections are generally self-limited, resistant organisms could lead to complications among those who develop systemic infection if they cannot be adequately treated. Studies of clinical breakpoints for azithromycin and clinical outcomes are needed. In the meantime, providers should avoid treating resistant infections caused by antibiotic-resistant *Shigella* spp. due to transmission-facilitating behavior (10) or because of increased exposure to macrolides and fluoroquinolones used to treat sexually transmitted infections, which could increase selective pressure on *Shigella* organisms (12). HIV infection may increase the risk of acquiring and transmitting *Shigella* infection due to increased carriage and shedding time or altered immune response (2).

To limit the emergence of resistance, the NYC Department of Health and Mental Hygiene and CDC recommend that antibiotics be avoided in treating *Shigella* infections except in cases of severe illness or among those at risk for systemic infection (13,14). When antibiotics are prescribed, therapy should be modified on the basis of sensitivity testing; however, without CLSI-defined clinical breakpoints for azithromycin, this process will be challenging (4,14,15).

HIV-positive MSM may be at increased risk for acquiring infections caused by antibiotic-resistant *Shigella* spp. due to transmission-facilitating behavior (10) or because of increased exposure to macrolides and fluoroquinolones used to treat sexually transmitted infections, which could increase selective pressure on *Shigella* organisms (12). HIV infection may increase the risk of acquiring and transmitting *Shigella* infection due to increased carriage and shedding time or altered immune response (2).

Table 2. Characteristics associated with azithromycin or ciprofloxacin resistance among case-patients with *Shigella flexneri* and *S. sonnei* infections, New York City, New York, USA, March 22, 2013—May 31, 2015*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Resistant, n = 152†</th>
<th>Susceptible, n = 521‡</th>
<th>Crude OR (95% CI)§</th>
<th>Adjusted OR (95% CI)¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>Referent</td>
<td>Referent</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>Age, y</td>
<td>140 (92.1)</td>
<td>304 (58.4)</td>
<td>8.33 (4.50–15.40)</td>
<td>3.27 (1.63–6.55)</td>
</tr>
<tr>
<td>0–17</td>
<td>8 (5.3)</td>
<td>251 (48.2)</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>18–64</td>
<td>136 (89.5)</td>
<td>256 (49.1)</td>
<td>16.67 (8.00–34.73)</td>
<td>7.77 (3.52–17.14)</td>
</tr>
<tr>
<td>≥65</td>
<td>8 (5.3)</td>
<td>14 (2.7)</td>
<td>17.93 (5.86–54.84)</td>
<td>11.94 (3.63–39.33)</td>
</tr>
<tr>
<td>Neighborhood poverty, %¶¶</td>
<td>30 (19.7)</td>
<td>85 (16.9)</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>&lt;10</td>
<td>58 (38.2)</td>
<td>135 (26.9)</td>
<td>1.22 (0.73–2.04)</td>
<td>1.29 (0.73–2.29)</td>
</tr>
<tr>
<td>10–20</td>
<td>41 (27.0)</td>
<td>108 (21.5)</td>
<td>1.08 (0.62–1.86)</td>
<td>1.32 (0.71–2.47)</td>
</tr>
<tr>
<td>20–30</td>
<td>23 (15.1)</td>
<td>174 (34.7)</td>
<td>0.38 (0.21–0.68)</td>
<td>0.57 (0.29–1.10)</td>
</tr>
<tr>
<td>Species</td>
<td>Referent</td>
<td>Referent</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td><em>S. flexneri</em></td>
<td>90 (59.2)</td>
<td>140 (26.7)</td>
<td>3.95 (2.71–5.76)</td>
<td>1.91 (1.25–2.92)</td>
</tr>
<tr>
<td>HIV-diagnosed</td>
<td>Yes</td>
<td>Referent</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>Yes</td>
<td>82 (54.0)</td>
<td>99 (19.0)</td>
<td>4.99 (3.39–7.35)</td>
<td>1.44 (0.91–2.30)</td>
</tr>
</tbody>
</table>

* N = 683 sporadic cases. OR, odds ratio. †S. boydii (n = 1) omitted. ‡S. boydii (n = 6) and S. dysenteriae (n = 3) omitted. §Values reported in bold are significant, calculated by using bivariate or multivariable logistic regression. ¶Percentage of residents below federal poverty level, per American Community Survey, 2009–2013; 19 missing.
otherwise healthy shigellosis patients with antibiotics. When antibiotics are indicated, providers should use available susceptibility results and monitor patient outcomes.

Acknowledgments
We thank Madeline Sankaran, Jasmine Abdelnabi, Jasmine Parks, Alexander Davidson, and Eric Peterson for their assistance with interviewing case-patients and data abstraction.

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Neisseria meningitidis ST11 Complex Isolates Associated with Nongonococcal Urethritis, Indiana, USA, 2015–2016

Evelyn Toh, Dhanaraj Gangaiah, Byron E. Batteiger, James A. Williams, Janet N. Arno, Albert Tai, Teresa A. Batteiger, David E. Nelson

At a clinic in Indianapolis, Indiana, USA, we observed an increase in Neisseria gonorrhoeae–negative men with suspected gonococcal urethritis who had urethral cultures positive for N. meningitidis. We describe genomes of 2 of these N. meningitidis sequence type 11 complex urethritis isolates. Clinical evidence suggests these isolates may represent an emerging urethrotropic clade.

Neisseria meningitidis and N. gonorrhoeae are exclusive human pathogens. N. meningitidis is a leading cause of sepsis and meningitis, whereas N. gonorrhoeae (gonococcus) traditionally causes gonorrhea, a sexually transmitted infection involving the genitals, rectum, and throat. These species usually occupy distinct niches but may cause reciprocal diseases when N. meningitidis colonizes the anogenital tract or gonococcus colonizes oropharyngeal mucous membranes (1,2).

Sporadic cases of meningococcal urethritis have been reported since the 1930s (3). It was recognized by the 1970s that urethral N. meningitidis infections could be spread by oral sex and were more common in men who have sex with men (4). Most N. meningitidis urethritis cases identified before 1993 were caused by strains from serogroups A and B (5), but outbreaks involving other serogroups and nontypeable strains have been reported (6–10). These cases have typically presented with purulent urethritis or proctitis with gram-negative intracellular diplococci (GNID) identified by Gram stain of urethral exudate. The current prevalence of meningococcal urethritis is unknown in the United States but was thought to be low (4). However, a recent spike in heterosexual N. meningitidis urethritis cases beginning in 2013 in Ohio and Michigan, reported by Bazan et al. in association with the Gonococcal Isolate Surveillance Project, was linked to a nongroupable clonal N. meningitidis strain (sequence type 11 [ST11], clonal complex [CC] ET-37) (11). Whether N. meningitidis ST11 strains are common causes of nongonococcal urethritis or if these strains have adaptations that enhance their virulence for the urethra is unknown because genomes of urethral N. meningitidis isolates have not been previously reported.

Beginning in early 2015, we observed that 4 (6%) of 59 men we enrolled in gonococcal treatment trials in our clinic in Indianapolis, Indiana, USA, and who had urethral specimens that were positive on Gram stain tested negative for gonococcus by specific nucleic acid amplification tests (NAATs). Sugar fermentation reaction profiles of the isolates from all 4 of these men suggested that they were infected with N. meningitidis. We describe the recent epidemiology of these suspected N. meningitidis urethritis cases and the genomes of 2 of these N. meningitidis isolates.

The Study

We enrolled 59 men (age range 20–61 years, median age 31 years) in 3 gonococcal treatment trials from April 2014 through February 2016 at the Bell Flower Clinic, a public sexually transmitted infections clinic, in Indianapolis, Marion County, Indiana. All of these men had purulent urethral discharge with >10 leukocytes and GNID on Gram stains of urethral exudate. However, 4 men tested negative for gonococcus with specific NAATs (APTIMA Combo 2, Gen-Probe Inc., San Diego, CA, USA; or COBAS 4800, Roche Diagnostics, Indianapolis, IN, USA). All 4 men reported recent vaginal and oral sexual exposures. Urethral cultures yielded growth with colony morphology, oxidase, and Gram stain results consistent with gonococcus, but the isolates fermented glucose and maltose but not lactose, consistent with N. meningitidis. A pharyngeal swab from 1 of these men also grew N. meningitidis, whereas rectal swab specimens from all 4 were culture negative. All 4 cases responded to investigational gonococcal antibiotic regimens and were culture plus NAAT negative by test of cure assessed at day 7 post-treatment. Two of these isolates (NM1 and NM2), which were susceptible to ampicillin, ceftriaxone, chloramphenicol, levofloxacin and meropenem, were subcultured and confirmed to be N. meningitidis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Whole-genome sequencing of NM1 and NM2 (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/23/2/16-1434-TechnicalAppendix.pdf) revealed that these 2 isolates closely resembled strains of ST11 and CC11, with fine type PorA VR 1.5–1, PorA2 10–8; FetA3–6 ST11 (CC11). These authors contributed equally to this article.
Figure. Rooted phylogenetic tree of Neisseria meningitidis sequence type 11 urethral isolates from men in Indianapolis, Indiana, USA, 2015–2016, compared with representative serogroup strains of N. meningitidis. Tree was inferred by using the neighbor-joining method constructed with MEGA7 (13). The percentage of replicate trees in which the associated taxa clustered together in 500 bootstrap tests is indicated next to the branches. The tree is drawn to scale, and branch lengths correspond to evolutionary distances used to infer the phylogenetic tree. Arrow indicates urethritis strains NM1 and NM2. GC strain FA1090 was used as the outgroup. Scale bar indicates nucleotide substitutions per site.
Phylogenetic analysis indicated that NM1 and NM2 likely share a common ancestor with NM L93/4286, a serogroup C invasive strain from the United Kingdom (12). A rooted tree constructed by the neighbor-joining method indicated that NM1 and NM2 are closely related to *N. meningitidis* serogroup C strain, FAM18 (Figure) (13). Gene-to-gene comparison with FAM18 loci revealed that 29 out of 1,975 loci were missing in >1 of the urethral isolates, although 24 of these were near the end of a contig in either NM1 or NM2. Of the remaining loci, 1,075 (56%) were identical in all three strains, and 1,848 (96%) were identical in NM1 and NM2.

The capsule locus encompasses ≈24 kb in most NM isolates and contains genes that mediate capsule synthesis, transport, assembly and translocation, as well as LPS synthesis. NM1 and NM2 lack cssC, cssB, and cssA (former designation siaC, siaB, and siaC [14]), whereas these are intact in NM FAM18 and MC58 (online Technical Appendix). Our analysis indicated that these genes are highly conserved in diverse isolates from *N. meningitidis* serogroups B, C, W, and Y, but are also absent in gonococcal strain FA1090.

To estimate the prevalence of presumptive NM urethritis at BFC, we retrospectively examined 107 cases from men seen between 2013 and 2016 who had Gram stain results positive for WBCs and GNID but who had negative GC NAATs. Rates of negative GC NAATs among men with positive Gram stains were 12/436 (2.8%) in 2013, 8/552 (1.4%) in 2014, 37/533 (6.9%) in 2015, and 50/510 (9.8%) in the first 3 quarters of 2016, indicating a significant increase in these cases during the study interval by both a $\chi^2$ test ($p<0.0001$) comparing the 4 years and the Mantel-Haenszel test for a trend over time ($p = 0.005$). Analysis of cases before 2013 was not

### Table. Characteristics of 87 men with presumed urethral *Neisseria meningitidis* infection seen at the Bell Flower Clinic, Indianapolis, Indiana, USA, January 1, 2015–September 30, 2016*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>2015</th>
<th>2016</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Race</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>6 (16)</td>
<td>5 (10)</td>
</tr>
<tr>
<td>Black</td>
<td>29 (78)</td>
<td>45 (90)</td>
</tr>
<tr>
<td>Other</td>
<td>2 (5)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td>35 (95)</td>
<td>50 (100)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>2 (5)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Self-identified sexual orientation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterosexual</td>
<td>34 (92)</td>
<td>48 (96)</td>
</tr>
<tr>
<td>Homosexual</td>
<td>2 (5)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Bisexual</td>
<td>1 (3)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Modes of sexual contact†</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insertive vaginal intercourse</td>
<td>33 (89)</td>
<td>45 (90)</td>
</tr>
<tr>
<td>Insertive oral sex</td>
<td>35 (95)</td>
<td>44 (88)</td>
</tr>
<tr>
<td>Receptive oral sex</td>
<td>19 (51)</td>
<td>33 (66)</td>
</tr>
<tr>
<td>Insertive anal intercourse</td>
<td>1 (3)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Receptive anal intercourse</td>
<td>1 (3)</td>
<td>1 (2)</td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Discharge</td>
<td>35 (95)</td>
<td>49 (98)</td>
</tr>
<tr>
<td>Dysuria</td>
<td>2 (5)</td>
<td>0</td>
</tr>
<tr>
<td>Discharge/dysuria</td>
<td>37 (100)</td>
<td>49 (98)</td>
</tr>
<tr>
<td>No discharge/dysuria</td>
<td>0</td>
<td>1 (2)†</td>
</tr>
<tr>
<td><strong>Most recent HIV status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative, documented or self-reported</td>
<td>32 (86)</td>
<td>45 (90)</td>
</tr>
<tr>
<td>Not tested and unknown</td>
<td>5 (14)</td>
<td>5 (10)</td>
</tr>
<tr>
<td><strong>Exchange sex for drugs or money</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2 (5)</td>
<td>0</td>
</tr>
<tr>
<td>No</td>
<td>33 (89)</td>
<td>49 (98)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (6)</td>
<td>1 (2)</td>
</tr>
<tr>
<td><strong>Noninjection recreational drug use, excluding alcohol, preceding 60 d</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>17 (46)</td>
<td>31 (62)</td>
</tr>
<tr>
<td>No</td>
<td>18 (49)</td>
<td>19 (38)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (5)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Treatment provided</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceftriaxone plus azithromycin</td>
<td>34 (92)</td>
<td>49 (98)</td>
</tr>
<tr>
<td>Ceftriaxone plus doxycycline</td>
<td>1 (3)</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>2 (5)</td>
<td>1 (2)</td>
</tr>
<tr>
<td><strong>Urethral coinfection with <em>Chlamydia trachomatis</em> by NAAT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>6 (16)</td>
<td>8 (16)</td>
</tr>
<tr>
<td>Negative</td>
<td>31 (84)</td>
<td>42 (84)</td>
</tr>
</tbody>
</table>

*NAATs, nucleic acid amplification tests.
†One man had none.
‡Had irritated meatus.
possible because of the lack of discrimination in the NAAT used at the clinic.

Similar to the case-patients recently reported from Ohio and Michigan (11), our 37 case-patients in 2015 had an average age of 32 (range 20–61) years and were symptomatic with either discharge (n = 35) or dysuria (n = 2) and predominantly heterosexual (n = 34) (Table). Most received oral sex (n = 35) and were HIV negative (n = 32/32 tested).

Conclusions
Together, our results and those of Bazan (15) suggest that \textit{N. meningitidis} ST11 could be a notable emerging cause of nongonococcal urethritis; more extensive sequencing and comparisons of recent \textit{N. meningitidis} ST11 isolates from around the United States are underway. However, our results support the observation that both the rates and geographic distribution of \textit{N. meningitidis}–associated urethritis cases in the United States are increasing. Sequencing additional isolates should clarify whether these isolates correspond to an emerging urethrotropic clade of \textit{N. meningitidis}. Retrospective comparisons of NAAT-negative, GNID-positive urethritis case rates might also help discern whether these cases have been increasing elsewhere. As fewer clinics perform routine Gram staining, a serious concern is that infections with urethral discharge, coupled with a negative gonococcus-specific NAAT, could be misdiagnosed as a \textit{Chlamydia} or \textit{Trichomonas} infection. We speculate that an increased number of \textit{N. meningitidis} cases may occur if we limit our clinical diagnoses solely on the results of current diagnostic methods, thereby causing asymptomatic cases to go untreated. Therefore, continued investigation into diagnostic methods targeting urethral specific \textit{N. meningitidis} isolates is pressing, to control the transmission of sexually transmitted \textit{N. meningitidis}. Finally, we note that because the meningococcal \textit{ctrA} gene is highly conserved in NM1 and NM2, they should be able to be differentiated from gonococcus by using meningococcal \textit{ctrA} reverse transcription PCR assays (15).

Acknowledgments
We thank Rosemary Batteiger for assistance with mass spectrometry. We also thank the Bell Flower Clinic staff for the enrollment of patients into the gonorrhea study and for their meticulous documentation of clinical characteristics of patients. In addition, we are grateful to Stan Spinola for his advice and review of this manuscript.

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COMMENTARY

H5Nx Panzootic Bird Flu—Influenza’s Newest Worldwide Evolutionary Tour

Jeffery K. Taubenberger, David M. Morens

Influenza A viruses (IAVs) cause annual epidemics, periodic pandemics, and enzootic infections of numerous animals, including horses, dogs, pigs, seals, and whales (1). The natural reservoir for IAV is wild aquatic birds, including diverse species of Anseriformes (ducks and geese) and Charadriiformes (shorebirds and gulls), which continually transport an incredible array of genetically diverse IAVs over vast distances during migration.

In wild birds, IAVs usually cause inapparent, self-limited, lower gastrointestinal tract infections. Such low pathogenicity avian influenza (LPAI) viruses represent most of the avian influenza viruses. IAVs (mostly of low pathogenicity) also occasionally host switch to domestic poultry (mainly chickens and turkeys). Because gallinaceous poultry are not natural hosts of IAVs, sustained epizootic and enzootic transmission in poultry leads to viral genetic changes not found in IAVs adapted to other hosts, such as wild birds or mammals.

IAVs have 1 of 18 hemagglutinin (HA) subtypes (HA being the major surface glycoprotein that elicits immune responses). Two of these subtypes, H5 and H7, can spontaneously undergo mutations at the HA cleavage site to become highly pathogenic avian influenza (HPAI) viruses for gallinaceous poultry, typically resulting in fatal systemic infection in poultry and sometimes in wild birds that come into contact with poultry. We should emphasize that the designation of highly pathogenic and low pathogenicity refers only to bird pathogenicity) also occasionally host switch to domestic poultry (mainly chickens and turkeys). Because gallinaceous poultry are not natural hosts of IAVs, sustained epizootic and enzootic transmission in poultry leads to viral genetic changes not found in IAVs adapted to other hosts, such as wild birds or mammals.

Although LPAI poultry epizootics may not be recognized because infections are asymptomatic, fatal HPAI outbreaks in domestic chicken and turkey flocks are obvious and have been described worldwide for >225 years. An Asian lineage of HPAI H5N1, designated A/goose/Guangdong/1/1996 H5N1, emerged in 1996 and has since spread throughout much of Asia, Europe, the Middle East, and Africa, causing almost 900 serious human infections and >400 deaths. This alarmingly high number probably does not represent virulence for or adaptation to humans but rather the existence of rare host susceptibilities to AIVs, considering that millions of persons have been exposed (2). In the past 2 decades, this H5N1 lineage has developed into multiple sublineages and has undergone multiple reassortment events leading to major alteration of internal genes but until recently has retained its original N1 subtype neuraminidase (NA).

During 2013–2015, a sublineage of HPAI H5N1, referred to as clade 2.3.4.4, which had first been reported in 2008 in China, suddenly spread explosively to birds in much of the rest of the world (3,4). In doing so, this sublineage underwent genetic reassortment with various naturally occurring LPAI viruses and repeatedly switched out its long-stable N1 subtype for importations of several new NA subtypes, including N2, N3, N5, N6, and N8. This unprecedented series of events resulted in multiple so-called H5Nx viruses (i.e., H5 clade 2.3.4.4 viruses coupled with any NA subtype that reassorted into the preexisting complex of viral genes). During 2013–2015, H5Nx viruses spread panzootically outward from China (5). Exported H5N6 viruses predominated in Asia, and H5N8 viruses were exported in independent sublineages westward to Europe and eastward to North America. In North America, H5Nx virus reassorted into H5N1 and H5N2 viruses, spreading in early 2015 to 21 states in the United States and causing the loss of >50 million poultry, at a cost of $5 billion. After ≈6 months, these viruses all but disappeared from North America and receded dramatically in Europe. In southern China, however, H5N6 has become so widespread in duck populations that it has largely replaced H5N1 as the dominant AIV seen in poultry markets (4).

During summer 2016, H5N8 clade 2.3.4.4 viruses once again began spreading explosively in a second panzootic wave along migratory bird pathways from Russia/Mongolia, separately into Europe and North Africa, the Middle East, and India (6). At the same time, H5N6 clade 2.3.4.4 viruses continue spreading throughout Asia, and there are fears that North America will experience similar resurgent panzootic waves. In the original 2014–2015 panzootic wave, wild bird deaths were uncommon, as is expected for poultry-adapted HPAI viruses. In contrast, the still-ongoing 2016 European/African/Middle Eastern/Indian resurgent panzootic wave has resulted in the deaths of many ducks and a wide variety of wild bird species.

Historically, HPAI viruses are believed to spread by high-production poultry farming and the movements of infected birds, bird material, or surface contamination from infected flocks to susceptible flocks. However, the aggressive

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spread of the new HPAI H5Nx viruses in migratory birds along established flyways after breeding and molting seasons strongly implicates migratory wild birds in the 2 recent panzootic waves. Although genetic bases for panzootic explosivity have not been demonstrated, scientists speculate that H5Nx viruses have become more transmissible than other IAVs or more stable in the environment or in wild birds over long migrations.

In this context, the study of Guo et al. (7) in this issue of Emerging Infectious Diseases is of particular interest. It has long been believed that changes in the HA receptor-binding domain are key to viral adaptation in new hosts, specifically enabling wild bird–origin IAVs to adapt to domestic poultry, to mammals, and to humans. Avian-adapted IAVs usually bind preferentially to glycan receptors that terminate in a sialic acid bound to an underlying galactose with an α2,3 linkage. NA acts to cleave cell-bound and virion-bound sialosides, enabling newly formed virions to be released. Because HA binds sialosides while NA cleaves them, it has long been assumed that a functional balance is required between HA and NA activity (4).

Glycan array studies have demonstrated that modifications to the antepenultimate sugar in these sialosides (e.g., sulfation or fucosylation) often affect HA binding affinity. HA binding to α2,3-fucosylated sialosides, which may be a feature of galliform poultry adaptation, is also acquired by H5Nx viruses from wild birds and in poultry-adapted IAVs of a variety of H5 subtypes.

The study by Guo et al. (7) looked specifically at H5 clade 2.3.4.4 mutations and sialic acid receptor binding properties associated with emergence and spread of a 2014 European chicken H5N8 virus. The authors report mutations in HA residues 222 and 227 (H3 subtype numbering) associated with a change in HA receptor binding specificity. In comparisons of HA glycan array binding properties of an ancestral H5N1 to those of the newer mutated H5N8 clade 2.3.4.4 virus, the newer HA retained its ability to bind to nonfucosylated sialosides while at the same time acquiring the ability to bind to several α2,3 fucosylated sialosides. Examination of additional H5Nx viruses from wild birds and domestic poultry is necessary to understand the prevalence of these binding patterns in H5 clade 2.3.4.4 H5Nx viruses.

The unexpected acquisition of this new property raises several questions. Is the sudden emergence and spread of H5Nx lineage viruses related directly to fucosylated sialoside binding, to the blended HA binding specificity observed, to H5 pairing with new NAs, or to other unappreciated genomic mutations acting alone or in concert? The founder A/goose/Guangdong/1/1996 H5N1 lineage, despite multiple reassortment events over a 20-year period, did not replace its N1 subtype until the recent emergence of H5Nx viruses. Did altered receptor specificity for fucosylated sialosides enable H5 to efficiently partner with a variety of different NA subtypes that ancestral H5 viruses were unable to incorporate into their gene complex via reassortment, because of some yet-unappreciated HA/NA functional mismatch? Are the H5Nx viruses more transmissible or more stable in wild bird species, or more environmentally stable? Is the HPAI phenotype expressed differently than it is with ancestral H5N1 lineages (e.g., in pathogenic effects on a different spectrum of wild bird species)?

What are the implications for humans, who are not commonly productively infected by LPAI or HPAI poultry viruses? Mammalian (and human) adaptation is associated with non-fucosylated sialosides especially with α2,6-linked sialoside receptor binding. If enhanced binding to fucosylated sialosides occurs in poultry-adapted viruses without changing the overall binding preference for α2,3-linked sialoside (characteristic of galliform poultry adaptation), then these viruses are presumably less capable of back-adapting to pose a risk for humans. At the same time, lectin histochemical studies should be performed to look for the presence and distribution of fucosylated sialosides along the respiratory tract of humans, in mammals that either sustain IAV enzootic spread or that can be infected experimentally (e.g., swine, horses, ferrets) and in wild and domestic bird species.

How IAVs evolve, switch hosts, and stably adapt to new hosts remain poorly understood but undoubtedly reflects multiple independent pathways. A better understanding of the molecular bases of these host-adaptation events may help us to recognize genetic signatures of emerging IAVs that can infect humans, domestic animals, and wildlife and to better prevent and control transmission.

In addition to providing insight on the mechanisms by which a novel panzootic virus is emerging, the study of Guo et al. (7) reminds us of the ability of the influenza virus to surprise us with a remarkable repertoire of multidirectional evolution, which presents us with newer and more complicated challenges. In the past several decades, influenza viruses have been moving about globally in new and different ways. If we hope to control them, we need to understand what they are doing, and how they are doing it.

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http://wwwnc.cdc.gov/eid/articles/issue/17/12/table-of-contents
Diffuse Unilateral Subacute Neuroretinitis Caused by *Ancylostoma* Hookworm

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Diffuse unilateral subacute neuroretinitis is an ocular infectious disease caused by several distinct nematodes. Definite identification of the involved nematodes is rarely achieved. We report on the molecular-based genetic identification of an *Ancylostoma ceylanicum* hookworm implicated in a case of diffuse unilateral subacute neuroretinitis in a child.

Diffuse unilateral subacute neuroretinitis (DUSN) is an ocular infectious disease caused by migrating larvae of nematodes. Patients typically have vitritis, papillitis, and gray-white retinal lesions (1,2). DUSN primarily occurs in the United States, the Caribbean, and South America, although several cases also have been reported in Europe, Africa, India, and China (2). Several nematodes can induce DUSN, in particular *Ancylostoma* spp., *Baylisascaris procyonis*, and *Toxocara canis* (3), but the actual cause remains unknown. Because the nematodes are only rarely surgically extracted from the eye, a definite identification is hardly ever achieved (3,4). Noninvasive laser therapy is the treatment of choice for DUSN because it leads to the death of the nematode, thereby stopping the inflammatory process (1). Anthelminthic therapy with albendazole also has been described as successful, albeit primarily in cases where a worm cannot be visualized in the patient’s eye (5). Left untreated, DUSN can progress toward optic nerve atrophy and permanent vision loss.

In their larval form, hookworms infect their hosts by penetrating intact skin. The larvae circulate through the blood to the heart and then reach the lungs, before being coughed up and swallowed, thus entering the gastrointestinal tract. In the intestines, the larvae develop into adult worms and start to reproduce. This leads to the fecal shedding of eggs into the environment. *A. ceylanicum* is a zoonotic hookworm predominantly found in dogs and cats in Southeast Asia, India, and Australia (6). It is the only animal hookworm species known to cause patent intestinal infections in humans (6).

We report on a 10-year-old boy born in Columbia who had been living with his foster parents in Germany for the previous 6 years. He had acute loss of vision in his right eye. Ophthalmoscopy revealed retinal vasculitis, exudative retinal detachment, and proliferative vitreoretinopathy. Because of the retinal detachment, we performed a vitrectomy, during which a white worm of ≈10 mm in length was observed moving in the subretinal space (Figure). During surgical removal, the worm was completely destroyed.

We isolated DNA from the intraoperative rinsing fluid and applied previously described PCR assays to target the mitochondrial 12S rRNA, Cox1, and intergenic transcribed spacer 1 and 2 of various nematodes (7–9). We then sequenced the PCR products obtained. Phylogenetic analysis with the intergenic transcribed spacer 1 and 2 sequences (GenBank accession no. KM066110.1) identified the worm as *A. ceylanicum* (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/1/14-2064-Techapp1.pdf). A blastn search (https://blast.ncbi.nlm.nih.gov) with the Cox1 sequence (GenBank accession no. KM066109.1) showed 99% and 87% identity with *A.
ceylanicum and A. caninum, respectively. The 12S rRNA sequence (GenBank accession no. KM066111.1) for A. ceylanicum revealed 94% and 92% identity to A. caninum and A. duodenale, respectively.

Additional diagnostic results included negative serum antibody tests for the parasites Fasciola hepatica, Strongyloides spp., Trichinella spp., and Taenia solium; negative blood samples for filariasis; and 3 negative stool samples for intestinal helminths. An ELISA result for serum antibody to the helminth Toxocara canis (DRG, Marburg, Germany) was weakly positive, whereas the confirmatory immunoblot test result (Lobio Diagnostics, Lyon, France) was negative.

In addition to performing a vitrectomy, retinotomy, and implantation of silicon oil, we started the patient on anti-inflammatory therapy with oral prednisone. Referring to the treatment recommendation of a case series (5) and ensuring that no additional worm could survive, we administered a 30-day course of anthelminthic therapy with albendazole, even though no signs of additional organ manifestation or blood eosinophilia were observed. Unfortunately, a permanent loss of visual acuity to 0.05 could not be averted.

DUSN is an inflammatory eye disease caused by migrating nematode larvae. Because of the surgical intervention necessary in the case we describe, we were able to amplify and determine DNA sequences of the hookworm A. ceylanicum. The finding of a hookworm in DUSN seems plausible because Ancylostoma spp. nematodes have been repeatedly proposed as an etiologic agent in DUSN (1,3,4).

The source and time of infection in our patient remains unclear. After the patient’s adoption and his move from Columbia to Germany at 4 years of age, the patient and his adoptive family spent vacations in Spain but never traveled outside Europe. We do not know whether the infection was acquired in Columbia or Spain and subsequently survived (e.g., in a hypobiotic state) or whether the infection was acquired in Germany. Although the definitive source and time of infection cannot be confirmed, molecular methods nevertheless unquestionably identified the species A. ceylanicum.

In this case, we obtained a positive ELISA result for Toxocara canis, another helminth implicated in DUSN (3). Because the confirmatory immunoblot test was negative, we assume the ELISA result to most likely have been caused by unspecific cross-reactions. In the past, tests based on serologic testing alone might have falsely attributed nematodes to DUSN. In future cases of exudative retinal detachment caused by DUSN, intraoperative material should be used for molecular studies to identify the responsible nematode.

Dr. Poppert is a medical microbiologist and is currently working as attending physician at the Department of Infectious Diseases at the University Medical Center, Hamburg-Eppendorf, Germany. His main field of research is the development and application of molecular tests for the detection of human pathogens.

References

Azithromycin-Nonsusceptible Shigella flexneri 3a in Men Who Have Sex with Men, Taiwan, 2015–2016

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We report an outbreak of azithromycin-nonsusceptible Shigella flexneri 3a infection in Taiwan associated with men who have sex with men. The bacterial strains belonged to the sublineage A of a recently reported outbreak lineage associated with men who have sex with men, characterized by reduced azithromycin susceptibility and circulation in shigellosis low-risk regions.

Shigellosis among men who have sex with men (MSM) is a major public health concern worldwide (1,2). Shigella flexneri serotype 3a was responsible for a prolonged MSM-associated outbreak in England and Wales initially detected in 2009 and ongoing at the time of study publication (3). More recently, S. flexneri 3a strains with a genetic lineage distinct from Africa- and Asia-associated lineages were identified among MSM in Europe, Australia, and North America (4). All strains in this lineage carried a mobile element with 4 antimicrobial resistance genes (bla\textsubscript{OXA-1}, cat\textsubscript{A1}, adaA\textsubscript{1}, and tetB), and most recently emerging strains harbored a conjugative IncFII plasmid pKSR100, which conferred high-level resistance to azithromycin (4).

During 2005–2014, the incidence of domestically acquired shigellosis in Taiwan was low (0.7/100,000 persons) (5). Shigellosis is reported through the Web-based National Notifiable Disease Surveillance System (NDSS), which has been in operation since 1997 to monitor all notifiable diseases in Taiwan (6,7). The NDSS detected the first shigellosis outbreak in MSM during March–May 2015; the outbreak was determined to be caused by the global spread of a ciprofloxacin-resistant S. sonnei clone (8). During June 2015–May 2016, a total of 200 shigellosis cases were reported to the NDSS, of which 21 were domestically acquired S. flexneri 3a infections in northern and central Taiwan. All 21 of the S. flexneri 3a infections were in men 22–44 years of age, including 17 self-reported MSM. Of the 21 case-patients, most had reported other infections before S. flexneri 3a diagnosis: HIV (n = 16), 0–162 (median 49) months earlier; syphilis (n = 17), 0–85 (median 9.5) months earlier; gonorrhea (n = 6), 14–130 (median 26) months earlier; amebiasis (n = 2), 0–112 (median 56) months earlier; acute hepatitis A (n = 1), 0 months earlier; and S. sonnei infection (n = 1), 3 months earlier.

We tested the 21 S. flexneri 3a isolates for antimicrobial resistance by using a custom-made 96-well Sensititre MIC panel (Trek Diagnostic Systems Ltd., West Grinstead, UK) and the Etest kit (bioMérieux, Marcy l’Etoile, France). All 21 were resistant to ampicillin, chloramphenicol, streptomycin, and tetracycline, and 19 were nonsusceptible to azithromycin (MIC 64–96 μg/mL).

We also used the PulseNet pulsed-field gel electrophoresis (PFGE) protocol to analyze the isolates (9) and compared the PFGE patterns with those of 30 S. flexneri 3a isolates isolated in Taiwan during 2000–2011. The NotI digested PFGE patterns of 20/21 of the isolates were identical to the earlier isolates, and the remaining isolate showed a highly similar pattern. Clustering analysis of the PFGE patterns by using BioNumerics version 6.6 software (Applied Maths, Sint-Martens-Latem, Belgium) revealed that the 21 isolates were genetically distant from the 2000–2011 S. flexneri 3a isolates.

We used the Illumina MiSeq platform (Illumina, San Diego, CA, USA) for whole-genome sequencing of 4 isolates, of which 3 were azithromycin-nonsusceptible (codes R15.1162, R16.001, and R16.0013) and 1 was azithromycin-susceptible (R15.3406). We deposited the raw sequence reads in the National Center for Biotechnology Information Short Read Archive database (accession no. SRP081767; BioProject PRJNA353684). We used CLC Genomics Workbench version 9.0.1 (CLC bio, Aarhus, Denmark) for de novo assembly of the reads. We uploaded contigs for each isolate to the website of Center for Genome Epidemiology (http://www.genomicsepidemiology.org/) and used its tools to identify sequence type (Multi Locus Sequence Typing toolkit), plasmid type (PlasmidFinder), and antimicrobial resistance genes (ResFinder 2.1).

The 4 isolates were sequence type 245 (ST245), harbored plasmids belonging to incompatibility groups IncFII and Col, and had resistance genes bla\textsubscript{OXA-1'}, cat\textsubscript{A1}, adaA\textsubscript{1}, tetB, ermB, mphpA, and bla\textsubscript{TEM}. Although isolate R15.3406 carried an intact mphpA gene, it was phenotypically azithromycin susceptible. We compared genome single-nucleotide polymorphisms and constructed trees by using the CSI Phylogeny pipeline (https://cge.cbs.dtu.dk/services/CSIPhylogeny/) (10) to infer phylogenetic relationships between the 4 isolates and the 331 strains used in the study by Baker et al. (4). These analyses showed that the 4 isolates recovered in Taiwan belonged to the sublineage A of the MSM-associated outbreak lineage reported by Baker et al. (4).

In conclusion, we report an azithromycin-nonsusceptible S. flexneri 3a outbreak associated with MSM in Taiwan. The 21 isolates were genetically distant from the S. flexneri 3a isolates recovered in Taiwan from 2000–2011 and belonged to the sublineage A of the recently reported
MSM-associated outbreak lineage characterized by reduced azithromycin susceptibility and circulation in shigellosis low-risk regions (4). The introduction of this MSM-associated S. flexneri 3a lineage into Taiwan in 2015 illustrates that the pathogen can spread rapidly across continents, possibly through intensified sexual networks among MSM (2,8). We recommend continued surveillance for antimicrobial resistance genes in S. flexneri to inform clinical management of shigellosis among MSM and public health interventions where needed, including appropriate antimicrobial drug stewardship.

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Ms. Liao is a senior researcher in the Centers for Disease Control, Taiwan. Her research interests include molecular subtyping methods in the epidemiology of foodborne bacterial disease.

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Fatal Emmonsia sp. Infection and Fungemia after Orthotopic Liver Transplantation

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We report a fatal case of disseminated Emmonsia sp. infection in a 55-year-old man who received an orthotopic liver transplant. The patient had pneumonia and fungemia, and multysystem organ failure developed. As human habitats and the number of immunocompromised patients increase, physicians must be aware of this emerging fungal infection.

*Emmonsia* species are ubiquitous, soil-dwelling saprophytic fungi. Two species, *E. crescens* and *E. parva*, cause pulmonary disease (adiaspiromycosis) in rodents and other small animals. After inhalation, the conidia (adiaspores) grow without replication or dissemination and can cause pulmonary granulomas. Human cases are rare and usually occur in immunocompromised hosts (1,2). However, disseminated infections caused by *E. pasteuriana*–like species have been reported primarily in HIV-infected patients in South Africa (3,4). A recent review implicated novel *Emmonsia* spp.–like fungi as emerging agents of disseminated infection (1). We report a case of fatal disseminated infection after orthotopic liver transplantation caused by a novel *Emmonsia* sp.
A 55-year-old man received an orthotopic liver transplant because of alcoholic cirrhosis. He was discharged on posttransplant day (PTD) 7 after an unremarkable posttransplant course. Immunosuppression included induction with rabbit antithymocyte globulin and tacrolimus. He did not receive antifungal prophylaxis.

On PTD 19, he was readmitted with right lower quadrant pain and acute kidney injury. Abdominal computed tomography (CT) showed intraabdominal subacute hemorrhage and bilateral pleural effusions with lower lobe compressive atelectasis versus consolidation and a left lower lobe pulmonary nodule. On PTD 24, respiratory distress developed. A chest CT showed new bilateral ground glass opacities and diffuse centrilobular nodules (Figure, panel A). Thoracentesis of the right pleural effusion yielded blood-tinged, turbid, yellow fluid (total protein 1,494 mg/dL, 407 leukocytes/µL [70% polymorphonuclear leukocytes, 29% monocytes, and 1% lymphocytes]), and cultures grew a mold believed to be a contaminant. Antifungal therapy was not initiated.

On PTD 32, after the patient had a fever (temperature 101.5°F), repeat chest CT showed enlargement of the right pleural effusion. A pigtail catheter was inserted, and pleural fluid cultures again grew a mold. Sputum culture yielded normal flora. Three of 4 blood cultures collected on PTD 33 and 1 of 4 blood cultures collected on PTD 36 grew the same mold. The patient was given voriconazole, but treatment was changed to liposomal amphotericin B because of worsening liver function and delirium.

Despite aggressive antifungal therapy, broad-spectrum antimicrobial drugs, and reduction of immunosuppression, multisystem organ failure developed, requiring inotropic support, hemodialysis, and mechanical ventilation. The patient died on PTD 46. No autopsy was performed. The patient owned a snake farm in rural northern California and trapped small mammals to feed his snakes and practice taxidermy. He stopped these activities 1–2 years before receiving the transplant.

**Figure.** *Emmonsia* sp. infection in a 55-year-old man who received an orthotopic liver transplant. A) Chest computed tomography scan showing right pleural effusion and diffuse centrilobular nodules. B) Velvety white colonies of *Emmonsia* sp. (Sabouraud dextrose agar plate) isolated from the patient. C) Colonies stained with lactophenol cotton blue showing hyphae and conidiophores (incubated at 30°C) (original magnification ×400).
The mold isolated from pleural fluid and blood of the patient produced velvety, white colonies on Sabouraud dextrose agar (Figure, panel B). D1D2 rDNA sequencing identified the mold as *E. parva*. Because we found no previous reports of *E. parva* disseminated infections, we sent the isolate to a reference laboratory (University of Alberta Microfungus Collection and Herbarium, Edmonton, Alberta, Canada). Using culture characteristics and internal transcribed spacer and D1D2 sequences, the laboratory identified the fungus as a novel *Emmonsia* species not yet formally described (Figure 1 in Schwartz et al. [1]; L. Sigler, University of Alberta, Edmonton, Alberta, Canada, 2016, pers. comm.). When grown on different culture media incubated at 30°C, the fungus lacked conidia but formed helically coiled, yellow-brown hyphae (Figure, panel C). When incubated on potato dextrose agar at 35°C, the fungus converted into a yeast-like form: clusters of small, irregularly shaped cells extending into short filaments.

Antifungal susceptibility testing of the mold phase was performed at the Fungal Testing Laboratory, University of Texas (San Antonio, TX, USA). The following MICs were obtained: amphotericin B, 0.125 µg/mL at 24 and 48 h; caspofungin, 0.5 µg/mL at 24 h and 2 µg/mL at 48 h; voriconazole 0.125 µg/mL at 24 and 48 h; and posaconazole, <0.03 µg/mL at 24 and 48 h.

A literature review of human *Emmonsia* infections is challenging because these organisms have undergone multiple taxonomic revisions (2). Most reports of adiaspiromycosis base the diagnosis solely on the appearance of adiaspores in histopathologic specimens (5,6), and some published *Emmonsia* cases might have misidentified the causative organism (1).

Disseminated *Emmonsia* infection appears to be a separate clinical entity from adiaspiromycosis (1). Human adiaspiromycosis is primarily a self-limited pulmonary infection caused by *E. crescens*, which is not associated with immunosuppression or fungemia. Disseminated *Emmonsia*

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**Emmonsia** [ĕ-monˈse-a]

*Emmonsia* is a genus of soil fungus that can cause adiaspiromycosis, a pulmonary disease common in wild animals, but rare in humans, as well as disseminated disease. When aerosolized spores are inhaled, they enlarge dramatically, from 2–4 µm to 40–500 µm in diameter. Because these swollen cells do not replicate, Emmons and Jellison termed them “adiaspores” (from the Greek *a* [“not”] + *dia* [“by”] + *spora* [“sowing”]. *Emmonsia* was first described by Chester W. Emmons, senior mycologist with the US Public Health Service, as *Haplosporangium parvum* in 1942. In 1958, it was reclassified into a separate genus and named in honor of Emmons. Recent phylogenetic analyses have concluded that fungi in this genus are polyphyletic, and proposed taxonomic changes may render the genus name obsolete.

**Sources**


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**etymologia**

Ronnie Henry

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infection is caused by a novel cluster of *Emmonsia*-like species (1); involves fungemia; appears to be associated with immunosuppression, including renal transplant (7–9) and orthotopic liver transplantation and HIV (10); and has a high case-fatality rate.

The timing of this infection raised concern for a donor-derived infection. However, we confirmed with the United Network for Organ Sharing (https://www.unos.org/) that no other organ recipients from the same donor had a similar posttransplant infection. Reported soil and rodent exposure for the patient and previous granulomatous disease identified by pretransplant chest imaging raised the possibility that his infection was a reactivation of a latent infection.

The unfamiliar mold isolated from the patient’s pleural fluid was initially identified as a contaminant, and the patient died despite favorable in vitro antifungal susceptibilities. In immunosuppressed patients with a compatible clinical syndrome, fungi isolated from a sterile site should be identified. More cases of *Emmonsia*-like infections will probably be diagnosed as laboratories use sequencing to identify uncommon fungal pathogens.

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Outbreak of Legionnaire’s Disease Caused by *Legionella pneumophila* Serogroups 1 and 13

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In Japan, hot springs and public baths are the major sources of legionellosis. In 2015, an outbreak of Legionnaires’ disease occurred among 7 patients who had visited a spa house. Laboratory investigation indicated that *L. pneumophila* serogroup 1 and 13 strains caused the outbreak and that these strains were genetically related.

Infection with *Legionella* bacteria is one of the major causes of community-acquired pneumonia (1). In Japan, the major sources of *Legionella* infection are hot springs and public

1These authors contributed equally to this article.
baths (2). Among Legionella species, L. pneumophila serogroup 1 accounts for most human infections (3). Legionellosis outbreaks caused by a combination of L. pneumophila serogroup 1 or other serogroups have rarely been reported. We report an outbreak of Legionnaires’ disease caused by L. pneumophila strains of serogroup 1 and serogroup 13.

During June 1–17, 2015, the Health Centers in Kanagawa and Shizuoka Prefectures, Japan, were notified of 7 cases of Legionella pneumonia. All patients with pneumonia were admitted to 1 of 5 hospitals. All patients were male (mean age 66.3 years), 4 had diabetes mellitus, and 1 had hepatic cirrhosis and liver cancer. Diagnosis of pneumonia at the hospitals was based on clinical presentation and immunochromatographic detection of L. pneumophila serogroup 1 antigen in urine. All patients recovered and were discharged.

In epidemiologic interviews, all 7 patients stated that they had visited a spa house in Odawara, Kanagawa, Japan, before illness onset. The latent period was not accurately determined because 5 of the 7 patients frequently visited this spa house and some patients had visited it again after illness onset. The spa house had 7 circulating systems, including filtration and heating components, and 9 baths for men. Sputum samples from 5 urinary antigen–positive patients and environmental samples from the spa house were collected for epidemiologic investigations and cultured for Legionella at the Kanagawa Prefectural Institute of Public Health. L. pneumophila serogroup 1 was detected in sputum from 4 patients (Table), and L. pneumophila serogroups 1 and 13 were detected in sputum from 1 patient (patient 2). Baths 1–5, but not baths 6–9, contained L. pneumophila. Epidemiologic investigation and laboratory results revealed that failure to adequately chlorinate the bath water and the circulating systems resulted in colonization of Legionella at the spa house.

Pulsed-field gel electrophoresis (PFGE) comparison (4) of clinical and environmental isolates revealed that the L. pneumophila serogroup 1 strains produced 3 PFGE profiles: A and B, with a 1-band difference, and C (Table). Patient 7 was infected with 2 L. pneumophila serogroup 1 isolates possessing PFGE profiles A and B. The PFGE profile B of L. pneumophila serogroup 13 was identical to that of serogroup 1. We determined the sequence type (ST) of L. pneumophila strains (5,6) and identified 4 new STs: ST2113, ST2114, ST2115, and ST2121. ST2114 differed from ST2121 by only 1 nt in neuA and differed from ST2113 (serogroup 13) by only 2 alleles (mip and neuA), suggesting that these 3 STs are closely related and that 1 of 3 strains (ST2113, ST2114, or ST2121) may be derived from another by homologous recombination. All examined isolates lacked the lag-I gene, a virulence-associated marker (7).

By using whole-genome sequencing, we applied core-genome multilocus sequence typing (cgMLST) with 50 genes (8) different from the 7 genes in sequence-based typing, thereby confirming the sequence-based typing data (Table). ST2114 and ST2115 isolates were each divided into 2 cgMLST profiles. cgMLST profile B differed from profile A by only 1 nt on lpg1503 and differed from profile d by 5 nt on lpg0812, near the lipopolysaccharide coding region, suggesting that the strain of profile d may be derived from another by homologous recombination. However, the remaining cgMLST profiles (c, e, and f) from strains not isolated from patients differed from profile b by 30, 42, and 43 alleles, respectively.

Multiple L. pneumophila strains with different genetic characteristics exist in the environment and pose infection risks (9). Among the strains studied, dual infections with L. pneumophila serogroup 1 and serogroup 13 strains (patient 2) and L. pneumophila serogroup 1 strains with different

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**Table.** Genetic characteristics of *Legionella pneumophila* isolates from patients with pneumonia and from environmental samples, Japan, 2015*

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>PGFE profile†</th>
<th>ST‡</th>
<th>ST profile, flaA, pilE, asd, mip, mompS, proA, neuA</th>
<th>Sample source</th>
<th>cgMLST profile§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>ST2114</td>
<td>6, 10, 21, 3, 17, 14, 9</td>
<td>Patients 1, 7</td>
<td>a, b</td>
</tr>
<tr>
<td> </td>
<td></td>
<td> </td>
<td> </td>
<td>Bath 1 (bath water)¶</td>
<td>b</td>
</tr>
<tr>
<td> </td>
<td></td>
<td> </td>
<td> </td>
<td>Bath 2 (spout swab)¶</td>
<td>b</td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>ST2121</td>
<td>6, 10, 21, 3, 17, 14, 57</td>
<td>Bath 1 (bath water)¶</td>
<td>b</td>
</tr>
<tr>
<td>1</td>
<td>B</td>
<td>ST2114</td>
<td>6, 10, 21, 3, 17, 14, 9</td>
<td>Patients 2, 5, 7</td>
<td>b, b, b</td>
</tr>
<tr>
<td> </td>
<td></td>
<td> </td>
<td> </td>
<td>Bath 1(bathub swab)¶</td>
<td>b</td>
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<tr>
<td> </td>
<td></td>
<td> </td>
<td> </td>
<td>Bath 2 (bath water)¶</td>
<td>b</td>
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<tr>
<td>1</td>
<td>C</td>
<td>ST1447</td>
<td>6, 10, 20, 13, 9, 4, 11</td>
<td>Bath 3 (hair trap debris)¶</td>
<td>c</td>
</tr>
<tr>
<td>13</td>
<td>B</td>
<td>ST2113</td>
<td>6, 10, 21, 10, 17, 14, 209</td>
<td>Patient 2</td>
<td>d</td>
</tr>
<tr>
<td> </td>
<td></td>
<td> </td>
<td> </td>
<td>Bath 1 (bath water)¶</td>
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</tr>
<tr>
<td> </td>
<td></td>
<td></td>
<td> </td>
<td>Bath 2 (bath water)¶</td>
<td>d</td>
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<tr>
<td> </td>
<td></td>
<td> </td>
<td> </td>
<td>Bath 2 (spout swab)¶</td>
<td>Not done</td>
</tr>
<tr>
<td>10</td>
<td>D</td>
<td>ST2115</td>
<td>7, 10, 17, 3, 13, 14, 207</td>
<td>Bath 4 (spout swab)¶</td>
<td>e</td>
</tr>
<tr>
<td> </td>
<td></td>
<td> </td>
<td> </td>
<td>Bath 5 (bathub swab)¶</td>
<td>f</td>
</tr>
</tbody>
</table>

*cgMLST, core genome multilocus sequence typing; PFGE, pulsed-field gel electrophoresis; ST, sequence type.
†Profiles A and B were obtained from clinical and environmental samples. Profiles C and D were obtained from environmental samples only.
‡New STs from this study were assigned ST2113, ST2114, ST2115, and ST2121.
§Each profile letter indicates a tentative cgMLST profile of 1 strain.
¶Concentrations of *L. pneumophila* in bath water were 800 CFU/L in bath 1 and 1,100 CFU/L in bath 2.
genomic subtypes (patient 7) were detected. Results from 3 genetic methods revealed that *L. pneumophila* serogroup 1 and 13 strains are closely related, although the serogroups differ. Results of this study were consistent with the hypothesis that multiple infections are more likely with less virulent strains and more likely in persons with medical conditions predisposing them to Legionnaires’ disease (10).

Our study of this outbreak suggests that the spa house was colonized by several *L. pneumophila* strains that were genetically related despite belonging to different serogroups and that 2 strains caused infection. Further analysis of the divergence of outbreak strains in genomes related to *Legionella* serogroup and sequence types is ongoing. This analysis clarifies the in-depth genetic relations among *L. pneumophila* strains, such as recombination sites and periods required for divergence. We recommend that the spa house provide high quality management and effective infection control practices according to an infection control manual (e.g., completion of documentation relating to infection control practices and training of employees) and that customers be aware of the sanitary status of spa houses.

**Acknowledgments**

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**Diphyllobothrium nihonkaiense** Tapeworm Larvae in Salmon from North America

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Diphyllobothriasis is reemerging because of global importation and increased popularity of eating raw fish. We detected *Diphyllobothrium nihonkaiense* plerocercoids in the musculature of wild pink salmon (*Oncorhynchus gorbuscha*) from Alaska, USA. Therefore, salmon from the American and Asian Pacific coasts and elsewhere pose potential dangers for persons who eat these fish raw.
The Japanese broad tapeworm, *Diphyllobothrium nihonkaiense* (Yamane, Kamo, Bylund et Wikgren, 1986) (Cestoda: Diphyllobothriidea), is the second most common causative agent of diphyllobothriosis in humans; ≈2,000 cases have been reported, mainly from northeastern Asia (1). However, recent studies that used molecular methods indicate that the number of human cases caused by this tapeworm may have been highly underestimated (1). In addition, increasing popularity of eating raw fish is probably responsible for the increased number of imported cases in regions where this infection is not endemic (1).

In 1986, the Japanese broad tapeworm was recognized as a human parasite separate from the most common broad fish tapeworm, *Diphyllobothrium latum* (L.), in Japan (2). The validity of the Japanese broad tapeworm was later confirmed by molecular data, especially the *cox1* gene sequences (1,3). Evidence indicates that virtually all previous cases of diphyllobothriosis in humans in Japan, South Korea, and the Pacific coast of Russia that were attributed to *D. latum* tapeworms were caused by *D. nihonkaiense* tapeworms (1,3). Moreover, *D. klebanovskii* (Muratov et Posokhov, 1988) described from the Pacific coast of Russia was recently synonymized with the Japanese broad tapeworm (1,4).

Studies on the transmission of the Japanese broad tapeworm in Japan and eastern Russia (Primorsky Region) have identified 4 species of Pacific salmon as the principal sources of human infection: chum salmon (*Oncorhynchus keta*), masu salmon (*O. masou*), pink salmon (*O. gorbuscha*), and sockeye salmon (*O. nerka*). These anadromous fish become infected in brackish water along the coast of the North Pacific Ocean (1,5). Tapeworm larvae infective for humans (plerocercoids) have been described in only a few studies performed in eastern Russia and Japan, (e.g., as plerocercoids type F from the musculature of chum salmon in Kamchatka, Russia) (2,6,7).

For decades, the possible occurrence of the Japanese broad tapeworm on the Pacific coast of North America was ignored; but since 2008, human infection with adult tapeworms and natural infection of carnivores (wolves and bears) with adult tapeworms have been confirmed by use of molecular markers (1,8–10). We report finding Japanese broad tapeworm plerocercoids in North America. Our main intent is to alert parasitologists and medical doctors about the potential danger of human infection with this long tapeworm resulting from consumption of infected salmon imported (on ice) from the Pacific coast of North America and elsewhere.

In July 2013, we examined 64 wild Pacific salmon of 5 species: 1 chinook salmon (*O. tshawytscha*), 1 coho salmon (*O. kisutch*), 23 pink salmon, 8 rainbow trout (*O. mykiss*), and 31 sockeye salmon in south-central Alaska, USA. The salmon were collected by angling (under permit no. SF2013–218) or obtained from local fishermen. The musculature was filleted to narrow slices, and internal organs were observed under a magnifying glass. Several morphotypes of diphyllobothrid plerocercoids were found, including a single larva in the musculature of pink salmon collected in Resurrection Creek (near Hope, Alaska). This plerocercoid, which was later identified as that of the *D. nihonkaiense* tapeworm, was found unencysted, deep in the musculature of the anterior part of the fish, near the spinal cord (Figure). It was highly motile, had a retracting scolex, and measured 8–15 mm long, depending on the state of elongation or contraction (Figure; Video, https://wwwnc.cdc.gov/EID/article/23/2/16-1026-V1.htm). After fixation with hot water, the plerocercoid was 10 mm long, had an elongate scolex 1.05 mm long and 0.60 mm wide, and possessed 2 narrow bothria opened on the apical end (Figure). The sequences of the *cox1* and 28S rRNA genes (lsrDNA)
were almost identical to those of the Japanese broad tapeworm available in the GenBank database (sequence similarities of 99% [GenBank accession no. KY000483] and 100% [KY000484], respectively), thus providing unequivocal support that this plerocercoid was a larva of the *D. nihonkaiense* tapeworm reported from North America.

This report provides additional evidence that salmon from the Pacific coast of North America may represent a source of human infection. Because Pacific salmon are frequently exported unfrozen, on ice, plerocercoids may survive transport and cause human infections in areas where they are not endemic, such as China, Europe, New Zealand, and middle and eastern United States (*I*). It is probable that most diphyllobothriosis cases originally attributed to *D. latum* may have been caused by *D. nihonkaiense* tapeworms. For more effective control of this human foodborne parasite, detection of the sources of human infection (i.e., host associations), and critical revision of the current knowledge of the distribution and transmission patterns of individual human-infecting tapeworms are needed.

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Hepatitis E Virus Infection in Solid Organ Transplant Recipients, France

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The rate of transfusion-transmitted hepatitis E virus (HEV) in transplant recipients is unknown. We identified 60 HEV-positive solid organ transplant patients and retrospectively assessed their blood transfusions for HEV. Seven of 60 patients received transfusions; 3 received HEV-positive blood products. Transfusion is not the major route of infection in this population.
Hepatitis E virus (HEV; family Hepeviridae, genus Orthohepeivirus) is a single-stranded, positive-sense RNA virus of 7.2 kb. At least 4 genotypes are responsible for hepatitis E in humans (HEV-1–4). HEV-1 and HEV-2 infect only humans, while HEV-3 and HEV-4 have animal reservoirs (1). In developed countries, the main source of HEV transmission is the consumption of raw or undercooked, infected meat or direct contact with infected animals. Cases of bloodborne transmission have also been reported (1–3).

Transfusion-transmitted infections in solid organ transplant (SOT) patients remains a major concern; the frequency at which these infections occur is unknown (4). SOT patients receiving transfusions are at risk of contracting HEV because systematic screening for the virus is rare. For SOT patients exposed to HEV, infection can become chronic, with rapidly progressing liver disease (1). Because of the high incidence of HEV infection in the Midi-Pyrénées area (1), SOT patients are regularly screened for HEV RNA, and diagnosis of HEV infection is made at the time of alanine aminotransferase elevation.

We investigated retrospectively the extent to which transfusion-transmitted HEV infections occurred in a cohort of 60 SOT patients infected with HEV from January 1, 2009, through June 30, 2014. We found that 7 (11.7%) of these SOT patients were potentially infected through transfused blood products because they were given transfusions in the 6 months preceding the diagnosis (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/2/16-1094-Techapp1.pdf); the remaining 53 HEV-positive patients were infected by other modes.

The median HEV RNA concentration in recipient blood was 5.4 log copies/mL (range 3.6–6.8 log copies/mL) or 5.2 log IU/mL (range 3.4–6.6 log IU/mL). The median interval between transfusion and diagnosis was 4 months (range 0.2–5.0 months). HEV infections developed in 4 patients (R1, R3, R4, and R5) 6 months after transplantation. Transmission of HEV by the graft was excluded in these patients by examining the samples from the organ donors at the time of donation. None of them tested positive for HEV RNA.

We collected the 231 blood samples corresponding to the 7 patients’ donors (stored by the French Blood Agency) and tested them individually for HEV RNA and HEV IgM/IgG (online Technical Appendix). Of these samples, 7 (3.0%) tested positive for HEV RNA (online Technical Appendix Table 2). This analysis revealed that 3 patients (recipients R1, R2, and R3) received ≥1 blood components derived from the 7 HEV RNA-positive donations; 4 patients were not given viremic donations.

The median HEV RNA dose given to the recipients was 5.1 log copies (range 3.8–8.4 log copies) or 4.9 log IU (range 3.6–8.2 log IU). Recipient R1 received blood components from 1 viremic donor (D1), while R2 and R3 received blood components from 3 HEV RNA-positive donors (D2.1–D2.3 and D3.1–D3.3, respectively). Phylogenetic analyses of the 348-nt partial sequences of the open reading frame (ORF) 2 region (online Technical Appendix) showed that R1/D1 and R3/D3.1 sequences clustered together (Figure); nucleotides were >99.0% identical in both cases, confirming transfusion-transmitted HEV infection. Phylogenetic analysis of R2/D2.3 showed they clustered together but had a lower sequence identity (84.2%), suggesting transmission could have been mediated by another mechanism.

Another study conducted from January 2004 through June 2009 in France found that the risk factors associated with HEV transmission in SOT patients were eating pork meat, game, and mussels (5). Thus, the risk for transfusion-transmitted HEV infection is lower than the risk for acquiring an HEV infection from other sources in the environment in this population. Our study supports these results; we identified viremic donors as the source of infection in 2 (or possibly 3) of 60 HEV-positive SOT patients using phylogenetic analyses.

In France, HEV-positive samples were found in 1/2,218 blood donations, with HEV RNA concentrations of <60 to 29,796 IU/mL (6). In the Netherlands, 1/2,671 donations was viremic, with HEV RNA concentrations from <25 to 470,000 IU/mL (7). Published data indicate that the minimum infectious dose in donations is 7,056 IU (3.85 log IU) (8). A recent study found that donations associated with HEV-transmission had higher HEV RNA concentrations than did those that were not associated with HEV transmission (9). In our study, the 3 blood donations implicated in HEV transmission had HEV RNA doses >5.7 log copies (i.e., 5.5 log IU). Another parameter that must be considered is the presence of HEV antibodies in the donor or in the recipient, although the concentration needed to protect against an HEV infection is still unclear.

We conclude that, although transfusion-transmitted HEV infection can occur in SOT patients, blood transfusion is not the main source of transmission in these patients in France. Optimal policies for screening blood donations for HEV must be defined according to epidemiologic data.

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The authors have no conflicts of interest to declare.

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Figure. Phylogenetic tree of hepatitis E virus (HEV) isolates from 3 HEV-positive blood donors and 3 solid organ transplant recipients (shown in bold), France, compared with reference isolates. The tree was constructed by using partial open reading frame 2 sequences (348 nt). HEV genotypes are indicated at right. A confirmed case of transfusion-transmitted HEV infection requires evidence of infection in the recipient and donor and that the nucleotide sequences of these isolates be identical. The isolates from France were deposited in GenBank under accession nos. KX452928–KX452935; accession numbers, sources, and location of isolation for other isolates are indicated. Scale bar indicates nucleotide substitutions per site.
Emergence of \textit{bla}_{NDM-7}–Producing \textit{Enterobacteriaceae} in Gabon, 2016

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Reports of carbapenemase-producing \textit{Enterobacteriaceae} in Africa remain rare and assess mostly \textit{bla}_{OXA-48}–producing isolates from Mediterranean countries and South Africa. We identified \textit{bla}_{NDM-7}–producing \textit{Enterobacteriaceae} in Gabon in 2016. The isolates contained \textit{bla}_{NDM-7} IncX3 plasmids that were unusual and similar to the one described in a colistin-resistant \textit{Klebsiella pneumoniae} SZ04 isolate from China.

Carbapenems are used as last-line antimicrobial drugs for treating infections caused by multidrug-resistant gram-negative bacilli. Their effectiveness is challenged by the emergence of carbapenemase-producing \textit{Enterobacteriaceae} (CPE). A new type of β-lactamase, \textit{bla}_{NDM} was reported from a patient in Sweden in 2007 (1). Since then, \textit{bla}_{NDM} CPE have been identified worldwide and described as endemic to the Indian subcontinent and the Balkans (2). In countries to which they are nonendemic, CPE are reported mainly from patients with a history of hospitalization in a CPE-endemic area and, more rarely, in patients without history of travel (3). Reports on CPE in Africa are scarce, likely because monitoring of antimicrobial resistance remains uncommon. \textit{bla}_{OXA-48} and \textit{bla}_{NDM-1} CPE have been reported from the Maghreb area, Nigeria, Kenya, and South Africa, and single cases of \textit{bla}_{NDM-4} and \textit{bla}_{NDM-5} \textit{Escherichia coli} have been reported in Cameroon, Algeria, and Uganda (2,4,5).

In January 2016, we conducted a point-prevalence study in all patients at the military general hospital of Libreville, Gabon. We collected demographic and clinical data and screened patients by rectal swabbing with Amies medium transport swabs (Copan Italia SPA, Brescia, Italy). The swabs were placed in 0.5 mL sterile water; 0.1 mL of the resulting suspension was streaked onto a selective agar plate provided for the identification of bacteria resistant to third-generation cephalosporins (CHROMagar, Paris, France). The plates were incubated for 48 h at 35°C. We used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry technology (Bruker, Bremen, Germany) to confirm each isolate thought to be \textit{Enterobacteriaceae}.

We performed antimicrobial drug susceptibility testing by the agar disk diffusion method (http://www.eucast.org/). Isolates resistant to second- and third-generation cephalosporins were investigated for MIC of ertapenem using Etest (bioMérieux, Marcy-L’Étoile, France) and for carbapenemase production by the CarbaNP test (bioMérieux). For molecular characterization, we performed Sanger sequencing of PCR amplicons of the gene. Purified genomic DNA of the \textit{bla}_{NDM-7}–producing isolates was subjected to whole-genome sequencing on a HiSeq system (Illumina, San Diego, CA, USA). Reads were filtered for quality with fastq-mcf (E-utils: http://code.google.com/p/ea-utils).
We used Edena version 2 for genome assembly (3); the genome was annotated by the National Center for Biotechnology Information pipeline. The resistome of the isolates was investigated through the ARG-ANNOT database.

The study was performed in accordance with French and Gabonese recommendations. Ethical approval was obtained at the local level.

We enrolled a total of 138 patients (84 women, 54 men; median age 32 years) in the study. The population had poor health status (a fatal illness likely to occur within the next 5 years for 35.5%; cancer and immunodepression in 5.8% and 22.5%, respectively). Recent hospitalization and next 5 years for 35.5%; cancer and immunodepression in poor health status (a fatal illness likely to occur within the men; median age 32 years) in the study. The population had obtained at the local level.

Ethical approval was ob

NOT database.

of the isolates was investigated through the ARG-ANNOT database. Ethical approval was obtained at the local level.

Three isolates harbored $bla_{OXA-48}$ and 4 a $bla_{NDM-7}$ determinant (LC154935.1) (Table). $bla_{NDM-7}$ is an infrequent allele described in Singapore and in patients returning from India (2,6) and was recently identified in patients not connected to a CPE-endemic area (3) and in nosocomial outbreaks (7,8). $bla_{NDM}$ determinants linked to $ble_{MBL}$ are frequently described on conjugal self-transferable IncX3 plasmids. Genome sequence analysis of the 4 Gabon isolates revealed $bla_{NDM}$, and $ble_{MBL}$ carried within a transposon element on a plasmid differing from the $bla_{NDM}$ IncX3 plasmids carried on India isolates but highly similar (20 single-nucleotide polymorphisms) to an IncX3 plasmid in a $mer-1$ $bla_{NDM}$-producing Klebsiella pneumoniae strain isolated from a patient from China (9). The 4 patients from whom these isolates were obtained (3 neonates and an 83-year-old woman from the orthopedic unit) had no epidemiologic link with any foreign countries; their acquisition of the $bla_{NDM}$ CPE was unexplained.

| Table. Characteristics of $bla_{NDM}$-producing Enterobacteriaceae isolates from patients in a military hospital, Gabon, 2016* |

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Isolate</th>
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<tr>
<td><strong>Species</strong></td>
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<tr>
<td>No. contigs</td>
<td>K. pneumoniae</td>
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<tr>
<td>Genome and plasmid size, bp</td>
<td>83</td>
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<tr>
<td><strong>Resistome genes according to antibiotic class</strong></td>
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<tr>
<td>$β$-lactams</td>
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</tr>
<tr>
<td>$bla_{SHV-28}$</td>
<td>+</td>
</tr>
<tr>
<td>$bla_{CTX-M-15}$</td>
<td>+</td>
</tr>
<tr>
<td>$bla_{NDM}$</td>
<td>+</td>
</tr>
<tr>
<td>act-17</td>
<td>+</td>
</tr>
<tr>
<td>ampR</td>
<td>+</td>
</tr>
<tr>
<td>$bla_{TEM-104}$</td>
<td>+</td>
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<tr>
<td><strong>Aminoglycosides</strong></td>
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<tr>
<td>aac-6′-lb</td>
<td>+</td>
</tr>
<tr>
<td>aac-3-lia</td>
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<td>ant3</td>
<td>+</td>
</tr>
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<td>aac3</td>
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</tr>
<tr>
<td><strong>Sulfamids</strong></td>
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<td>folA</td>
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</tr>
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<td>sul1</td>
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</tr>
<tr>
<td>dfr1</td>
<td>+</td>
</tr>
<tr>
<td><strong>Others</strong></td>
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</tr>
<tr>
<td>mhpA</td>
<td>+</td>
</tr>
<tr>
<td>cat</td>
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</table>

*aac3, aminoglycoside 3-N-acetyltransferase; aac3-lia, aminoglycoside N-acetyltransferase AAC(3)-Iia; aac6′-lb-cr, AAC(6′)-Ib-cr family aminoglycoside N(6′)-acetyltransferase; aac6′-lb, AAC(6′)-Ib family aminoglycoside 6′-N-acetyltransferase; act-7, cephalosporin-hydrolyzing class C β-lactamase ACT-17; ampR, ampicillin chromosomal-mediated β-lactamase; ant3, streptomycin 3′-adenylyltransferase; arr-ms, rifampin ADP-ribosyl transferase; $bla_{CTX-M-15}$, class A extended-spectrum β-lactamase CTX-M-15; $bla_{KPC-2}$, New Delhi metallo-β-lactamase NDM-7; $bla_{OXA-51}$, oxacillin-hydrolyzing class D β-lactamase OXA-51; $bla_{OXA-58}$, class A β-lactamase SHV-12; $bla_{OXA-23}$, class A β-lactamase SHV-28; $bla_{TEM-101}$, β-lactamase TEM-101; cat, chloramphenicol acetyltransferase; dfr1, dihydrofolate reductase type 1; folA, dihydrofolate reductase; fosA, fosfomycin resistance glutathione transferase FosA; $bla_{TEM-104}$, class A β-lactamase LAP-2; qnrS1, quinolone resistance pentapeptide repeat protein QnrS1; mhpA, Mhp(A) family macrolide 2′-phosphotransferase; sul1, sulfonamide-resistant dihydropteroate synthase Sul1; sul2, sulfonamide-resistant dihydropteroate synthase Sul2; tetA-2, tetracycline resistance protein, class C; tetD, tetracycline efflux MFS transporter TetD.

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Because of the social and economic relationships between China and Gabon, the travels of asymptomatic CPE carriers from China to Gabon can be expected to have facilitated the spread of CPE in Gabon. Several multidrug-resistant clones of K. pneumoniae, including sequence type 307 (ST307), have been recognized as having emerging epidemic potential worldwide. The genome analysis of the 3 blaNDM-7-producing K. pneumoniae isolates from Gabon revealed clonal isolates (2 and 5 single-nucleotide polymorphisms between them) of sequence type 307. This result suggests an uncontrolled spread in the hospital intensive care unit.

This description of blaNDM-7 in Africa highlights the international dissemination of carbapenemase determinants and the combination of 2 aggravating factors, resulting in an alarming situation: the identification of blaNDM-7 within a transposon element on a conjugative plasmid with a potentially very high level of transmissibility, and the implication of the presence of K. pneumoniae, a pathogen with a high potential to persist and disperse in the hospital environment. Urgent measures are required, including the rational use of antimicrobial drugs, public education on the importance of hygiene, and diligent surveillance to control the spread of these multidrug-resistant organisms in the hospital setting.

Dr. Moussounda is a doctor of pharmacy in the Hôpital d’Instruction des Armées Omar Bongo Ondimba in Libreville, Gabon. He conducted this study in the course of his master’s research in tropical infections at the École Doctorale Régionale in Franceville, Gabon.

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Novel Reassortant Clade 2.3.4.4 Avian Influenza A(H5N8) Virus in Wild Aquatic Birds, Russia, 2016


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The emergence of novel avian influenza viruses in migratory birds is of concern because of the potential for virus dissemination during fall migration. We report the identification of novel highly pathogenic avian influenza viruses of subtype H5N8, clade 2.3.4.4, and their reassortment with other avian influenza viruses in waterfowl and shorebirds of Siberia.
Highly pathogenic avian influenza virus (HPAIV) subtype H5N1 was first isolated from a goose in 1996 in Guangdong China (Gs/GD). This virus evolved into multiple hemagglutinin (HA) genetic clades and underwent reassortment with different neuraminidase and internal genes to generate subtype H5N8 clade 2.3.4.4 Gs/GD HPAIV, which first appeared in an outbreak in poultry in China in 2013 (1), followed closely by outbreaks in South Korea in January 2014 (2). During these outbreaks, 2 distinct groups of H5N8 viruses were identified; group A (Buan-like) and group B (Gochang-like). There have been no further reports of group B virus since its original detection in China and South Korea during 2014 (3,4). In contrast, in early 2014, group A viruses predominated in South Korea (5) and in September of that year were subsequently isolated from a Eurasian wigeon (Anas penelope) in Sakha Republic in northeast Siberia (6). On the basis of aquatic bird migration patterns, we hypothesized that HPAIV (H5N8) reached Siberia during the 2014 spring bird migration (7). The virus was probably carried by birds from Siberia to various countries of Asia, Europe, and North America during the fall migration, representing an intercontinental group A (iA) (7). We report detection of novel HPAIV (H5N8) from wild aquatic birds sampled in western Siberia during the summer of 2016.

In June 2016, we collected samples from 13 dead and 30 hunter-harvested wild aquatic birds around Uvs-Nuur Lake (Tyva Republic) at the Russia–Mongolia border. We isolated a total of 11 subtype H5 influenza viruses from birds of various species: the black-headed gull (Larus ridibundus), gray heron (Ardea cinerea), common tern (Sterna hirundo), great crested grebe (Podiceps cristatus), and great cormorant (Phalacrocorax carbo) (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/23/2/16-2125-Techapp1.pdf). We characterized 3 of the viruses—A/great crested grebe/Uvs-Nuur Lake/341/2016(H5N8), A/common tern/Uvs-Nuur Lake/26/2016(H5N8), and A/great heron/Uvs-Nuur Lake/20/2016(H5N8)—by sequencing, phylogenetic analysis, and intravenous pathogenicity index (IVPI) testing (online Technical Appendix).

We confirmed that all 3 isolates were HPAIV on the basis of amino acid sequence at the HA proteolytic cleavage site (PLREKRRKR/G) and individual IVPIs of 2.75-2.84 in chickens (online Technical Appendix Table 1). The 3 isolates shared 99.2%-100% nucleotide identity across all 8 genes: HA, neuraminidase (NA), polymerase basic 2 (PB2), polymerase basic 2 (PB1), polymerase acidic (PA), nucleoprotein (NP), matrix (M), and nonstructural (NS). BLAST (https://www.ncbi.nlm.nih.gov/blast/) search results showed that the isolates shared >98% identity with low pathogenicity avian influenza virus (LPAIV) from Mongolia and China over 5 gene segments (PB1, PB2, PA, NP, and M) and >98.5% identity with the 2014 H5N8 clade 2.3.4.4 group B HPAIV for the remaining 3 gene segments (HA, NA, and NS) (Table). Phylogenetic analysis showed that the HA, NA, and NS genes clustered with H5N8 clade 2.3.4.4 group B HPAIV viruses identified in eastern China in 2014 (online Technical Appendix Figure). The PB1, PB2, PA, NP, and M genes clustered with LPAIV identified in Mongolia, China, and Vietnam.

Wild aquatic birds migrate to and congregate in Siberian wetlands for breeding and molting. Major wild aquatic bird migration routes overlap in Siberia, connecting this broad geographic area to the wintering grounds of Eurasia and Africa. This unique ecosystem has been implicated as a pathway for the dissemination of HPAIV during southward autumn migration of waterfowl, as seen in the spread of H5N1 clade 2.2 in 2005–2006 (8) and H5N8 clade 2.3.4.4 in 2014 (6,7). Uvs-Nuur Lake is a key habitat for 46 resident waterfowl species and 215 kinds of birds migrating south from Siberia (9). During widespread dissemination of the HPAIV clade 2.2 in 2006 and clade 2.3.2 in 2009, these viruses were also detected from wild aquatic birds at Uvs-Nuur Lake, suggesting this area is a useful site for surveillance of HPAIV in wild aquatic birds (10). Because numerous species of migratory shorebirds and waterfowl use the summer breeding grounds of Siberia, the identification of HPAIV infection in wild aquatic birds in this area signifies the potential for wide dissemination of these novel reassortant Group B H5N8 viruses during the 2016 fall migration.

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Table. Nucleotide identity of near homologs in GenBank to the influenza A(H5N8) virus from Uvs-Nuur Lake, Russia, as of June 30, 2016*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Virus Details</th>
<th>Classification</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB2</td>
<td>A/duck/Mongolia/30/2015(H3N8)</td>
<td>Eurasian LPAI</td>
<td>98.7</td>
</tr>
<tr>
<td>PB1</td>
<td>A/chicken/Hunan/S1267/2010(H4N6)</td>
<td>Eurasian LPAI</td>
<td>98.1</td>
</tr>
<tr>
<td>PA</td>
<td>A/duck/Mongolia/996/2015(H3N8)</td>
<td>Eurasian LPAI</td>
<td>98.7</td>
</tr>
<tr>
<td>HA</td>
<td>A/duck/eastern China/S1109/2014(H5N8)</td>
<td>H5N8 clade 2.3.4.4</td>
<td>99.1</td>
</tr>
<tr>
<td>NP</td>
<td>A/duck/Mongolia/129/2015(H3N3)</td>
<td>Eurasian LPAI</td>
<td>98.7</td>
</tr>
<tr>
<td>NA</td>
<td>A/duck/eastern China/S1109/2014(H5N8)</td>
<td>H5N8 clade 2.3.4.4</td>
<td>98.9</td>
</tr>
<tr>
<td>M</td>
<td>A/duck/Mongolia/179/2015(H5N8)</td>
<td>Eurasian LPAI</td>
<td>98.5</td>
</tr>
<tr>
<td>NS</td>
<td>A/duck/eastern China/S1109/2014(H5N8)</td>
<td>H5N8 clade 2.3.4.4</td>
<td>99.3</td>
</tr>
</tbody>
</table>

*HA, hemagglutinin; LPAI, low pathogenicity avian influenza; MP, matrix; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; PA, polymerase acidic; PB, polymerase basic.
Detection of Vaccinia Virus in Urban Domestic Cats, Brazil

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References


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We investigated possible vaccinia virus (VACV) in urban house cats in Brazil. Serum samples from 6 cats were positive for VACV by PCR, indicating likely VACV circulation among house cats in urban areas of Brazil. This finding highlights the importance of epidemiologic surveillance to avoid outbreaks among urban human populations.

Vaccinia virus (VACV) outbreaks, first reported in Brazil in 1999, affect dairy cattle and humans in rural areas (1). Although studies have shown evidence of VACV circulation among several mammal species (1–3), no consensus exists regarding the role of these animals in the VACV transmission chain or which animal is the natural reservoir. In fact, domestic or wild mammals could be asymptomatic hosts and also contribute to VACV transmission (3).

In contrast to VACV, cowpox virus (CPXV) circulates in urban environments in Europe but also in surrounding wild and rural areas (4). CPXV is transmitted to humans mainly by cats, which play a link between the natural reservoirs and humans in the urban environment (4,5). In cats, the clinical course of CPXV infection varies from no symptoms to widespread skin necrotic lesions and can ultimately lead to death (6). Some studies have shown serologic evidence of orthopoxvirus infection in cats from Europe and have addressed the role of these animals in orthopoxvirus transmission to humans (7,8).

Because VACV and CPXV share some epidemiologic features and cats have a prominent role in the urban CPXV transmission chain, we decided to investigate whether urban domestic cats have evidence of exposure to VACV in Brazil. This study was approved by the Animal Experimentation Committee of the Universidade Federal de Minas Gerais (registration protocol 315/2014).

We performed a retrospective study of serum samples from 277 house cats, collected during September 2012–December 2014 in 5 states in Brazil (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/23/2/16-1341-Techapp1.pdf). The states in this study were those whose veterinary clinics agreed to submit samples. We screened serum samples for neutralizing antibodies by using a ≥70% plaque-reduction neutralization test (9). To detect VACV DNA in serum samples, we performed real-time PCR targeting the C11R and A56R genes (9). We directly sequenced A56R fragments in both orientations and in triplicate by using the Mega-BACE sequencer (GE Healthcare, Buckinghamshire, UK). We used ClustalW (http://www.genome.jp/tools/clustalw) and MEGA7 soft-
ware (http://www.megasoftware.net) to align nucleotide sequences and construct a phylogenetic tree (neighbor-joining method with 1,000 bootstraps).

The cats’ ages ranged from 3 months to 15 years; 150 (54.2%) of the cats were female. Thirteen cats (4.7%) had outdoor access, and 37 (13.4%) were admitted to the veterinary clinic for ≥1 night. Some cats had clinical illness inconsistent with orthopoxvirus infection, which can overlap with other common dermatologic diseases affecting cats (online Technical Appendix Table). Most (8/53 [15.1%]) seropositive cats were from the Pampulha region of the city of Belo Horizonte (Minas Gerais State) (online Technical Appendix Figure 1), followed by the eastern region of the city. We detected neutralizing antibodies in 16 animals (5.8%), with titers ranging from 100 to 1,600 neutralizing units/mL; of these, 13 (4.7%) were positive for C11R gene and 6 for A56R gene (online Technical Appendix Table). Alignment of the A56R fragments showed high similarity to the homologous gene of VACV isolates from Brazil (online Technical Appendix Figure 1). For the phylogenetic tree, we grouped sequences with VACV group 1 and 2 isolates (Figure).

We describe evidence of VACV circulation in cats in an urban environment in Brazil. Many studies have attempted to elucidate VACV outbreaks and risk factors in rural and wild areas (1–3). Our findings reveal a seropositivity rate of...
5.8%, which is lower than the rate observed in a previous study from Norway (8) and higher than the rate observed in a study of cats in Austria (7). Notably, the Pampulha region, where most seropositive animals were detected, corresponded to areas of relatively low elevation that feature houses with green areas, cottage houses, and ecologic parks, with forested areas making up the remaining portion of the land (online Technical Appendix Figure 1).

Recent data from our research group revealed that caimans (Hydrochoerus hydrochaeris) from the Pampulha region tested positive for VACV (10). These data, corroborated by molecular detection of VACV groups 1 and 2 in house cats from Belo Horizonte, further indicate the presence of VACV in an urban environment (online Technical Appendix Figure 2). In this study, PCR-positive cats showed no clinical signs that would indicate orthopoxvirus infection at the time of sample collection (online Technical Appendix Table), unlike what was observed among cats infected with CPXV in Europe (4,5). Furthermore, cats 4, 10, and 15 (online Technical Appendix Table), in which we detected ongoing VACV DNA, had no clinical signs. Although we detected group 2 VACV (virulent strains) in 4 samples, our findings corroborate the results of Bennett et al. (6), which showed that cats infected with VACV had asymptomatic infection.

Limitations of our study include selection bias of animals; it was not possible to use a convenience sample from the 5 Brazilian states. We were also unable to obtain detailed clinical information of all animals and unable to collect additional clinical samples to better understand the clinical course of VACV infection in cats. In Brazil, no records of VACV-like detection in urban populations are available, despite the fact that VACV was recently found in urban areas (10). In fact, potential sources of infection for cat populations (e.g., small rodents) should be considered. Cats could possibly seroconvert without the onset of classical illness. Hence, VACV could be circulating in cats from urban environments. The potential role of cats in infecting humans should be investigated further to determine whether VACV can emerge in urban human populations and pose a threat to public health.

Acknowledgments
We thank Associação Bichos Gerais, who provided some cat serum samples from Belo Horizonte. We also thank colleagues from the Laboratório de Vírus (ICB-UFMG) for their excellent technical support.

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References

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Cutavirus in Cutaneous Malignant Melanoma

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A novel human protoparvovirus related to human bufavirus and preliminarily named cutavirus has been discovered. We detected cutavirus in a sample of cutaneous malignant melanoma by using viral enrichment and high-throughput sequencing. The role of cutaviruses in cutaneous cancers remains to be investigated.

Parvoviruses are small nonenveloped DNA viruses with a single-stranded linear genome of ≈5 kb. In 2016, a novel species within the Protoparvovirus genus was discovered in fecal samples from children with diarrhea in Brazil and subsequently detected in samples of mycosis fungoides lesions (cutaneous T-cell lymphoma) of patients in France (1). This virus, provisionally named cutavirus, shows highest identity to the human bufaviruses of the Primate protoparvovirus 1 species. Bufaviruses are found in human fecal samples in low percentages (2–7). Using viral enrichment methods, we detected a cutavirus strain in an additional type of cancer, cutaneous malignant melanoma, further expanding the range of tissue types harboring cutaviruses and adding to the knowledge of the human virome.

We subjected a clinical sample of a cutaneous malignant melanoma lesion from a patient in Denmark to enrichment of virion-associated nucleic acids and enrichment of circular DNA molecules, followed by high-throughput sequencing (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/23/2/16-1564-Techapp1.pdf). BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=Download) analysis originally identified contigs related to human bufaviruses in de novo assembled contigs from both datasets. In light of the recently published cutavirus genomes (1), we compared these sequences with the cutaviruses and found high similarity to the cutaviruses. From overlapping contigs, we obtained the 4,452 bp (from start nonstructural protein 1 [NS1] to end viral protein 1 [VP1]) near-complete genome of a novel cutavirus strain, CutaV CGG5–268 (GenBank accession no. KX685945). Similar to the other cutavirus genomes, CutaV CGG5–268 included NS1 and VP1 open reading frames (ORFs) encoding proteins of 659 aa and 707 aa, respectively. The CutaV CGG5–268 sequence also contained the small putative 333-nt middle ORF, starting at position 2021, and a 270-nt ORF located within the VP2 coding region, starting at position 2768. Further testing is required to determine whether these ORFs encode proteins.

We performed phylogenetic analysis based on the NS1 and VP1 amino acid sequences (Figure). Because 4 of the 7 published cutavirus genomes contain partial NS1 sequences, we included only 3 cutavirus strains in the phylogenetic analysis of NS1. NS1-based analysis placed CutaV CGG5–268 closest to CutaV FR-F identified in a mycosis fungoides patient in France, whereas VP1-based analysis placed CutaV CGG5–268 closest to CutaV BR-450 identified in the feces of a child in Brazil.

Cutaviruses were discovered in human fecal samples by use of metagenomics and subsequently detected in 4 of 17 samples of mycosis fungoides lesions; however, 21 skin samples, including samples from skin cancers and parapsoriasis lesions, tested negative for cutavirus (1). Our discovery of cutavirus in a sample of cutaneous malignant melanoma shows that extraneous presence of cutaviruses is not limited to skin infiltrated by neoplastic T cells. The detection of cutaviral DNA after virion enrichment may indicate viral replication taking place in the affected tissue. Human bufaviruses have so far been detected only in fecal samples, predominantly from patients having diarrhea or gastroenteritis, and in only 0.27%–4% of samples (2–8). Another virus of the Parvoviridae family, human parvovirus B19, is shown to persist in multiple tissue types, in most cases without an established correlation to disease (9). Animal protoparvoviruses have also been detected in several sample types, as discussed elsewhere (1). Thus, future studies may reveal an expanded range of tissue types harboring cutaviruses. So far, cutaviruses have only been detected in the tissues investigated, and their direct involvement in disease has not been established. One limitation of this study is the lack of healthy controls for assessing whether cutavirus can also be detected in healthy skin. Furthermore, screening of a larger number of samples is necessary to determine the prevalence of cutavirus in malignant melanoma.

In 9 additional melanoma samples investigated in our laboratory, we did not identify contigs with similarity to
those of cutavirus or bufavirus. All 10 samples were tested for cutaviral DNA by real-time PCR, but only the sample in which the cutaviral contigs were detected had positive results (online Technical Appendix). We can only speculate regarding the cell tropism of cutaviruses; nevertheless, our study opens the possibility that cutaviruses replicate in melanocytes, which are present in the epidermal layers of the skin, where cutavirus DNA was detected by in situ hybridization (1). Melanocytes are also present in low numbers in the enteric epithelium, where melanomas can occur, though rarely (10). However, the cell tropism and potential pathogenicity of human protoparvoviruses remain to be investigated.

This study was supported by the Innovation Fund Denmark (The GenomeDenmark platform, grant no. 019-2011-2), the Danish National Research Foundation (grant no. DNRF94), and the Lundbeck Foundation.

Dr. Mollerup is a postdoctoral researcher at the Centre for GeoGenetics at the University of Copenhagen. Her research topics cover virus discovery, virome characterization, and metagenomics.
We report reoccurrence of highly pathogenic avian influenza A(H5N2) virus clade 2.3.4.4 in a wild mallard in Alaska, USA, in August 2016. Identification of this virus in a migratory species confirms low-frequency persistence in North America and the potential for re-dissemination of the virus during the 2016 fall migration.

Historically, apparently effective geographic barriers (Bering and Chukchi Seas of the North Pacific Ocean) appeared to limit dissemination of Asian-origin, highly pathogenic avian influenza virus (HPAIV), such as influenza A(H5N1) virus A/goose/Guangdong/1/1996 (Gs/GD), between the Old and New Worlds (1). However, such barriers are incomplete; occasional spillovers of virus genes move from 1 gene pool to another (2). Asian-origin HPAIV H5N8 was identified in North America at the end of 2014 (3).

Novel HPAIVs H5N1, H5N2, and H5N8 emerged in late 2014 by reassortment with North American low pathogenicity avian influenza viruses (4). A novel reassortant H5N2 virus originating from Asian-origin H5N8 virus clade 2.3.4.4 and containing Eurasian polymerase basic 2, polymerase acidic, hemagglutinin, matrix, and nonstructural protein genes and North American lineage neuraminidase (NA), polymerase basic 1 (PB1), and nucleoprotein genes was identified on poultry farms in British Columbia, Canada, and in wild waterfowl in the northwestern United States. This virus subsequently predominated during influenza outbreaks in the United States in 2015.

During the boreal summer, birds from 6 continents (North America, South America, Asia, Africa, Australia, and Antarctica) fly to Alaska, USA, to breed. Thus, Alaska is a potentially major location for intercontinental virus transmission (1,2). Recent data provide direct evidence for viral dispersal through Beringia (5,6). Genetic evidence and waterfowl migratory patterns support the hypothesis that H5 virus clade 2.3.4.4 was introduced into North America through the Beringian Crucible by intercontinental associations with waterfowl (3). In addition, low pathogenicity avian influenza viruses were collected in Alaska before initial detection of H5 HPAIV clade 2.3.4.4, which contained genes that had recent common ancestry with reassortant H5N2 virus PB1, nucleoprotein, and NA (N2 subtype) genes and H5N1 virus PB1, polymerase acidic, NA (N1 subtype), and nonstructural protein genes of HPAIVs (7).

We report detection of an HPAIV H5N2 subtype from wild mallard sampled in Alaska during August 2016. Influenza A virus was detected in 48/188 dabbling duck

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Reoccurrence of Avian Influenza A(H5N2) Virus Clade 2.3.4.4 in Wild Birds, Alaska, USA, 2016

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We report reoccurrence of highly pathogenic avian influenza A(H5N2) virus clade 2.3.4.4 in a wild mallard in Alaska, USA, in August 2016. Identification of this virus in a migratory species confirms low-frequency persistence in North America and the potential for re-dissemination of the virus during the 2016 fall migration.

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samples collected during a live bird banding effort near Fairbanks, Alaska, during August 6–15, 2016. One sample of H5 virus from an adult mallard was identified as an HPAIV H5N2 on the basis of complete genome sequencing. We conducted comparative phylogenetic analysis of A/mallard/Alaska/AH0008887/2016(H5N2) virus, hereafter known as 8887/2016(H5N2) virus, to trace its origin and understand its genetic relationship to HPAIV H5N2 isolated in 2014–2015 (online Technical Appendix (https://wwwnc.cdc.gov/EID/article/23/2/16-1616-Techapp1.pdf).

We considered 8887/2016(H5N2) virus an HPAIV on the basis of amino acid sequence at the hemagglutinin proteolytic cleavage site (PLRERRRKR/G), as shown for other Gs/GD HPAIV H5Nx subtypes in subclade 2.3.4 (http://www.offlu.net/fileadmin/home/en/resource-centre/pdf/Influenza_A_Cleavage_Sites.pdf). Homology BLAST searches showed that all genes had >99.2% nucleotide similarity with genes of H5N2 virus outbreak strains collected during late February–March 2015 (online Technical Appendix Table).

Phylogenetic analysis showed that the concatenated genome of 8887/2016(H5N2) virus formed a cluster with viruses from initial detections in the midwestern United States, including a snow goose in Missouri, a backyard poultry farm in Kansas, and a turkey farm in Minnesota (Figure). Our epidemiologic investigation data suggested that point-source introductions by indirect contact with wild waterfowl were the most probable source of infection for these backyard poultry in Kansas and a turkey farm in Minnesota (8). This genetic cluster was supported by a maximum-likelihood bootstrap value of 80 and a Bayesian posterior probability of 1.00.

The mean time to most recent common ancestry of viruses in this genetic cluster was estimated to be the end of January 2015 (mean time to most recent common ancestry January 27, 2015, 95% Bayesian credible interval January 11–February 10, 2015). Consistent clustering of 8887/2016(H5N2) virus with other H5N2 outbreak viruses in phylogenies for each gene suggests that the 8887/2016(H5N2) virus probably evolved through genetic drift from common ancestors of outbreak viruses in the absence of further reassortment (online Technical Appendix Figure 2). The mean rate of the nucleotide substitution obtained by Bayesian analysis was 6.064 × 10⁻³ (95% Bayesian credible interval 4.43–7.82 × 10⁻³) substitutions/site/year. In the root-to-tip regression plot of maximum-likelihood phylogeny, we found that 8887/2016(H5N2) virus fell below the regression line, which indicated sequences that are slightly less divergent than average of 2014–2015 H5N2 outbreak viruses (online Technical Appendix Figure 3).

The last reported detection during the influenza outbreak in the United States in 2015 was from a Canada goose in Michigan on June 17. There were 2 detections by wild waterfowl were the most probable source of infection for these backyard poultry in Kansas and a turkey farm in Minnesota (8). This genetic cluster was supported by a maximum-likelihood bootstrap value of 80 and a Bayesian posterior probability of 1.00.

The mean time to most recent common ancestry of viruses in this genetic cluster was estimated to be the end of January 2015 (mean time to most recent common ancestry January 27, 2015, 95% Bayesian credible interval January 11–February 10, 2015). Consistent clustering of 8887/2016(H5N2) virus with other H5N2 outbreak viruses in phylogenies for each gene suggests that the 8887/2016(H5N2) virus probably evolved through genetic drift from common ancestors of outbreak viruses in the absence of further reassortment (online Technical Appendix Figure 2). The mean rate of the nucleotide substitution obtained by Bayesian analysis was 6.064 × 10⁻³ (95% Bayesian credible interval 4.43–7.82 × 10⁻³) substitutions/site/year. In the root-to-tip regression plot of maximum-likelihood phylogeny, we found that 8887/2016(H5N2) virus fell below the regression line, which indicated sequences that are slightly less divergent than average of 2014–2015 H5N2 outbreak viruses (online Technical Appendix Figure 3).

The last reported detection during the influenza outbreak in the United States in 2015 was from a Canada goose in Michigan on June 17. There were 2 detections by
PCR (3 assays, 2 gene targets, no virus recovered, no sequence obtained) from mallards in July (bird banding effort in Utah) and November (hunter harvest in Oregon) during surveillance in 2015–2016. Sequence of the HPAIV H5N2 from a wild mallard during surveillance in 2016–2017, evidence for continued evolution of this virus lineage, widespread detections of HPAIV H5N2 in healthy wild birds (9), and lack of pathobiological effects in experimentally infected waterfowl (10) collectively provide strong evidence for maintenance of HPAIV H5N2 in wild birds in North America. Detection of HPAIV in a mallard might imply the potential for dissemination of HPAIV H5N2 during the southward fall migration of waterfowl in 2016.

Acknowledgments
We thank Michael J. Petrula and David Sinnett for collecting samples; Kerrie Franzen, Meredith Grady, Andrew Hubble for providing technical assistance; the Washington State Animal Disease Diagnostic Laboratory for their participation in wild bird surveillance activities, and the originating and submitting institution (Kagoshima University, Kagoshima, Japan) for A/crane/Kagoshima/KU1/2014(H5N8) sequences (accession no. EPI169390] from the GISAID EpiFlu Database (http://platform.gisaid.org).

Dr. Lee is postdoctoral researcher at the US Department of Agriculture, Athens, GA. His research interests include molecular epidemiology and host–pathogen interactions for avian influenza viruses.

References

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Increase in Urgent Care Center Visits for Sexually Transmitted Infections, United States, 2010–2014

William S. Pearson, Guoyu Tao, Karen Kroeger, Thomas A. Peterman

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: http://dx.doi.org/10.3201/eid2302.161707

During 2010–2014, urgent care centers saw a >2-fold increase in the number of visits for chlamydia and gonorrhea testing and a >3-fold increase in visits by persons with diagnosed sexually transmitted infections. As urgent care becomes more popular, vigilance is required to ensure proper management of these diseases.

Sexually transmitted infections (STIs) are the most commonly reported nationally notifiable diseases in the United States (1), and annual medical costs for these diseases are estimated to exceed $16 billion (2). Reported rates of gonorrhea, chlamydia, and syphilis all increased from 2014 to 2015, and antimicrobial drug–resistant gonorrhea remains an important concern (3). Therefore, proper diagnosis and
treatment of these diseases is essential to reduce STI-associated morbidity rates and prevent further drug resistance (4).

Urgent care centers have been identified as appropriate sources of care for nonemergency conditions that would otherwise be treated in a more costly emergency department setting (5). These centers are proliferating across the country because of public demand for convenient care and the need to contain healthcare costs (6). The Urgent Care Association of America estimates that >9,000 of these centers are currently operating in the United States and, on average, each center sees ≈14,000 visits per year (7). Additionally, STI clinics are closing across the country because of decreased funding (8); therefore, urgent care centers might increasingly be a typical setting for STI diagnosis and treatment.

We found no literature describing the frequency of diagnosis and treatment of STIs in urgent care settings. Therefore, we set out to estimate the number of visits to urgent care centers for the testing and diagnosis of chlamydia and gonorrhea.

For these analyses, we used data from the MarketScan commercially insured medical claims database for 2010, 2012, and 2014 (9). We only included claims for visits to urgent care centers and aggregated these claims to provide numbers of visits for each patient. We then searched the claims for Current Procedural Terminology (CPT) codes and codes from the International Classification of Diseases, Ninth Revision, that indicated the testing or diagnosis of chlamydia, gonorrhea, or an “unspecified venereal disease” (Table). We counted visits that involved a test or diagnosis for each of the indicated diseases for each year and stratified these results by percentage of female patients and the average age of the patients. We then used weights supplied in the dataset and calculated weighted numbers of visits.

All analyses were conducted by using SAS 9.3 (SAS Institute, Cary, NC, USA).

Overall, we estimated a ≈2.5-fold increase during 2010–2014 for all visits to urgent care centers (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/2/16-1707-Techapp1.pdf). Among these visits, we observed increases in the numbers of visits that involved STI testing or the treatment of patients with diagnosed STIs. During 2010–2014, a ≈1.5-fold increase occurred in visits that involved chlamydia testing and a ≈2-fold increase in visits involving gonorrhea testing. We observed even larger increases in visits that involved diagnosed STIs. During the same period, we observed a ≈6-fold increase in the numbers of visits that involved

Table. Number of urgent care center visits by commercially insured patients during which the patient was tested for gonorrhea or chlamydia or treated for a diagnosed sexually transmitted infection, United States, 2010–2014.*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>2010</th>
<th>2012</th>
<th>2014</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visits during which a chlamydia test was performed (CPT codes 87110, 87270, 87320, 87490, 87491, 87492, 87810)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unweighted</td>
<td>1,197,720</td>
<td>2,603,234</td>
<td>4,075,379</td>
</tr>
<tr>
<td>Weighted†</td>
<td>47,000,000</td>
<td>99,000,000</td>
<td>155,000,000</td>
</tr>
<tr>
<td>% Female</td>
<td>81.5</td>
<td>81.7</td>
<td>81.9</td>
</tr>
<tr>
<td>Average age, y</td>
<td>30.7</td>
<td>35.6</td>
<td>36.3</td>
</tr>
<tr>
<td>Visits during which a gonorrhea test was performed (CPT codes 87590, 87591, 87592, 87850)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unweighted</td>
<td>1,293</td>
<td>2,090</td>
<td>3,711</td>
</tr>
<tr>
<td>Weighted†</td>
<td>51,701</td>
<td>76,746</td>
<td>136,167</td>
</tr>
<tr>
<td>% Female</td>
<td>81.8</td>
<td>79.4</td>
<td>76.6</td>
</tr>
<tr>
<td>Average age, y</td>
<td>31.0</td>
<td>29.6</td>
<td>30.7</td>
</tr>
<tr>
<td>Visits during which diagnosed chlamydia was treated (ICD-9 codes 079.88, 079.98, 099.41, 099.50–099.56, 099.59)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unweighted</td>
<td>1,174</td>
<td>1,885</td>
<td>3,665</td>
</tr>
<tr>
<td>Weighted†</td>
<td>47,747</td>
<td>69,665</td>
<td>134,403</td>
</tr>
<tr>
<td>% Female</td>
<td>82.6</td>
<td>80.1</td>
<td>76.5</td>
</tr>
<tr>
<td>Average age, y</td>
<td>31.2</td>
<td>29.8</td>
<td>29.9</td>
</tr>
<tr>
<td>Visits during which a diagnosed “unspecified venereal disease” was treated (ICD-9 codes 099.8, 099.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unweighted</td>
<td>133</td>
<td>430</td>
<td>988</td>
</tr>
<tr>
<td>Weighted†</td>
<td>4,004</td>
<td>12,152</td>
<td>29,291</td>
</tr>
<tr>
<td>% Female</td>
<td>59.4</td>
<td>56.7</td>
<td>61.3</td>
</tr>
<tr>
<td>Average age, y</td>
<td>29.7</td>
<td>27.0</td>
<td>26.8</td>
</tr>
<tr>
<td>Visits during which diagnosed gonorrhea was treated (ICD-9 code 098)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unweighted</td>
<td>116</td>
<td>224</td>
<td>522</td>
</tr>
<tr>
<td>Weighted†</td>
<td>3,000</td>
<td>6,074</td>
<td>13,783</td>
</tr>
<tr>
<td>% Female</td>
<td>41.8</td>
<td>53.5</td>
<td>47.0</td>
</tr>
<tr>
<td>Average age, y</td>
<td>30.3</td>
<td>29.1</td>
<td>30.2</td>
</tr>
</tbody>
</table>

*Includes all patients seeking care at urgent care centers for any reason.
†Estimates are rounded to the nearest million.
diagnosed chlamydia, a >3-fold increase in the numbers of visits that involved diagnosed gonorrhea, and a ≈6-fold increase in the numbers of visits that involved an unspecified diagnosed STI. Most visits that involved STI testing were made by female patients; the average age for all patients at these visits was 28.1 years. Most visits by a patient for diagnosed chlamydia were made by female patients; the average age for all patients at these visits was 27.8 years. The number of visits by patients for an unspecified diagnosed STI was nearly evenly split between male and female patients; the average age of all patients at these visit was 30.4 years. The visits for diagnosed gonorrhea were predominantly made by male patients; the average age of all patients at these visits was 29.9 years.

Visits to urgent care centers have increased over time, and our findings demonstrate that visits to urgent care centers for STI care in particular have dramatically increased. Previous work has highlighted differences in the use of antibiotics to treat chlamydia in emergency departments compared with physician offices (10) suggesting that differences might also exist in the treatment of STIs in urgent care centers compared with other healthcare settings. Given the increases in STIs, increases in antimicrobial drug resistance, and increases in use of urgent care centers for STI care, further work is needed to determine how STIs are being managed in this venue to ensure quality care.

Dr. Pearson is a health scientist in the Division of STD Prevention, National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention, Centers for Disease Control and Prevention, Atlanta. His primary research interests include the organization, financing, and delivery of healthcare services.

References

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Cerebrospinal Fluid Findings in an Adult with Human Metapneumovirus–Associated Encephalitis

Natalie Jeannet, Bernadette G. van den Hoogen, Joerg C. Schefold, Franziska Suter-Riniker, Rami Sommerstein

Author affiliations: University of Bern, Bern, Switzerland (N. Jeannet, J.C. Schefold, F. Suter-Riniker, R. Sommerstein); Erasmus Medical Center, Rotterdam, the Netherlands (B.G. van den Hoogen); Bern University Hospital, Bern (J.C. Schefold, R. Sommerstein)

DOI: http://dx.doi.org/10.3201/eid2302.161337

To the Editor: Acute encephalitis/encephalopathy associated with human metapneumovirus (HMPV) has been documented in children (1–3). Recently, Fok et al. (4) described an encephalitis case in an adult but were unable to test cerebrospinal fluid (CSF) for HMPV. Following authors’ recommendations, we performed diagnostic testing on the CSF of an adult with HMPV-associated encephalitis.

A previously healthy 61-year-old man came to our institution with headache and seizures 5 days after onset of an influenza-like illness. A lumbar puncture on admission revealed pleocytosis (36 cells/µL) and a mononuclear predominance of 98%. Results of magnetic resonance imaging and computed tomography of the head and chest radiography on admission were inconclusive. The patient was treated in the intensive care unit for possible viral and bacterial meningoencephalitis. Although results of routine CSF-workup for infectious causes were unremarkable, CSF protein level was elevated at 1.39 g/L (reference range 0.2–0.4 g/L). A nasopharyngeal swab specimen was positive for HMPV (cycle threshold 28.6) using duplex reverse transcription PCR (r-gene; Biomérieux, Marcy l’Etoile, France).

To the Editor

The patient did not fulfill diagnostic criteria for meningitis and cerebrospinal fluid (CSF)-workup for infectious causes were unremarkable, to the knowledge of the authors. Intrathecal IgG synthesis was calculated using the formula (IgG<sub>CSF</sub>/HMPV/IgG<sub>Serum</sub> HMPV)/(IgG<sub>CSF</sub> total/IgG<sub>Serum</sub> total) and was lower than the cut-off value of 4, indicating absence of intrathecal IgG against HMPV (Table).

As in the study by Fok et al. (4), our case supports consideration of HMPV as a causative agent of acute encephalitis after respiratory tract infection in adults. We could not demonstrate direct or indirect evidence of HMPV CSF invasion as the cause for HMPV-associated encephalitis in an adult, in contrast to a case in a child in which detection of HMPV in CSF suggested a causative role in acute encephalitis (1). Our data may point toward the role of nonspecific inflammatory response as the main pathogenic factor in HMPV-related encephalitis in adults.

Acknowledgments

We thank the patient for giving his permission to publish his clinical data.

References


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Table. Results of PCR and immunofluorescent assay testing in adult patient with HMPV

<table>
<thead>
<tr>
<th>Test</th>
<th>Nasopharyngeal swab</th>
<th>CSF</th>
<th>HMPV IgG index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcription PCR</td>
<td>Positive (cycle threshold 28.6)</td>
<td>Negative</td>
<td>HMPV IgG index</td>
</tr>
<tr>
<td>Immunofluorescence assays</td>
<td>Vero-118 cells infected with HMPV NL/1/00</td>
<td>1:8,192</td>
<td>1:32</td>
</tr>
<tr>
<td></td>
<td>Vero-118 cells infected with HMPV NL/1/99</td>
<td>1:8,192</td>
<td>1:84</td>
</tr>
</tbody>
</table>

* A duplex reverse transcription PCR (r-gene) for detection of human metapneumovirus (HMPV) was performed from a nasopharyngeal swab specimen and cerebrospinal fluid (CSF). For immunofluorescence assays, 96-well plates coated with Vero 118 cells were infected with HMPV NL/1/00 and NL/1/99, respectively. Twenty-four hours later, infected cells were incubated with serial dilutions of patient serum and CSF for 1 h at 37°C. After washing with phosphate-buffered saline, plates were incubated with anti-human IgG conjugated with fluorescein isothiocyanate for 1 h at 37°C. Lowest dilution giving a positive result was determined by UV microscopy. Intrathecal IgG synthesis was calculated using the formula (IgG<sub>CSF</sub>/HMPV/IgG<sub>Serum</sub> HMPV)/(IgG<sub>CSF</sub> total/IgG<sub>Serum</sub> total). Indices below 4 indicate absence of intrathecal IgG antibody synthesis.
Blue Marble Health: An Innovative Plan to Fight Diseases of the Poor amid Wealth


Neglected tropical diseases (NTDs) comprise 17 helminthic, protozoan, bacterial, and vectorborne viral diseases that disproportionately affect the world’s poor (1). This diverse group of infections and infestations includes hookworms, leishmaniasis, Chagas disease, dengue fever, and trachoma. In Blue Marble Health, Dr. Peter Hotez, dean of the National School of Tropical Medicine and founding editor of PLoS Neglected Tropical Diseases, shifts the focus of global health from a traditional developed versus developing world paradigm toward impoverished populations living amid wealthy countries, who suffer heavily from NTDs. The book’s title invokes an iconic image of the earth as seen from space by the Apollo astronauts.

On the basis of previous work, Dr. Hotez asserts that nearly every person in poverty is infected with at least 1 NTD, “the most important diseases you’ve never heard of.” Blue Marble Health contains helpful summaries of 11 major NTDs in Chapter Two. The main body of the text is organized according to the book’s geographic focus on the G20 countries (the world’s major economies) plus Nigeria. These featured countries account for half of the world’s NTDs. It might surprise many to learn that 12 million US residents live with a neglected parasitic infection. Despite this widespread burden of disease, only 0.003% of world gross domestic product is spent on NTD research.

The book’s readability is enhanced by its reasonable length and its organization according to geography. Although the lack of detailed attention to zoonotic transmission issues is surprising, the book addresses broader forces driving NTD emergence, including co-morbidity with noncommunicable diseases, climate change, and regional conflict. Timely content includes discussion of the West Africa Ebola epidemic and the emergence of Zika virus in the context of NTDs.

The book contains copious figures and tables, which are just as likely to focus on economic indicators as on direct measures of NTDs. Minor formatting issues detract from the book’s readability. For example, magazine-style text inserts crowd the layout at times. Because the field of global health is replete with acronyms and initialisms, a glossary would have been a helpful addition. Although many of the book’s references come from the journal the author edits, these are balanced by citations from other sources.

Much of the book’s focus on interventions to combat NTDs concerns wider use of mass drug administration campaigns. However, as president of the Sabin Vaccine Institute, Hotez briefly describes vaccines for hookworm disease and schistosomiasis that are currently in clinical trials. Hotez’ enthusiasm for and deep knowledge about NTDs comes through clearly. Drawing on his experience as a US Science Envoy, the author clearly wants his book to be read and acted on by the leaders of G20 countries. His ideas for multinational policy solutions include the need for increased surveillance along with local research and development investment.

The intended audience for this book goes well beyond public health and tropical medicine specialists. The policy-heavy content and clear advocacy tone make it appropriate for students and teachers of global health, public policy, and foreign affairs. The book should also be of value to persons interested in social determinants of health, health economists, and historians of public health and international development.

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DOI: http://dx.doi.org/10.3201/eid2302.161801

Reference

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Maynard Dixon was born near Fresno, California, in the center of the San Joaquin Valley, on January 24, 1875. He had asthma as a child and spent much of his time drawing. In 1891, when he was 16, Dixon sent two of his sketchbooks to Frederic Remington, an artist he admired. Remington encouraged him, replying, “You draw better at your age than I did at the same age. If you have the ‘sand’ to overcome difficulties, you could be an artist in time. No one’s opinion of what you can do is of any consequence—time and your character will develop that.”

Dixon lived and worked for several years in San Francisco. However, according to biographer Donald J. Hagerty, Dixon “periodically roamed the West’s plains, mesas, and deserts on foot, horseback, buckboard—even by automobile—drawing, painting, and writing, pursuing a transcendent awareness of the region’s spirit.” When Dixon visited Arizona near the beginning of the 20th century, he proclaimed that “he had found his country” and developed his signature style of depicting western themes.

Although the 1906 San Francisco earthquake and fire destroyed his studio and most of his early creations, over his lifetime Dixon created an abundant body of work including illustrations for novels, paintings in several genres, murals, and poetry. His paintings of western US landscapes

Maynard Dixon (1875–1946), Shiprock Mesa (1942). Oil on canvas, 12 in × 16 in/30.5 cm × 40.6 cm. Mark Sublette Medicine Man Gallery, 6872 E. Sunrise Dr, Ste 130, Tucson, AZ, USA.

Stillness, Light, and Distance

Byron Breedlove

Maynard Dixon was born near Fresno, California, in the center of the San Joaquin Valley, on January 24, 1875. He had asthma as a child and spent much of his time drawing. In 1891, when he was 16, Dixon sent two of his sketchbooks to Frederic Remington, an artist he admired. Remington encouraged him, replying, “You draw better at your age than I did at the same age. If you have the ‘sand’ to overcome difficulties, you could be an artist in time. No one’s opinion of what you can do is of any consequence—time and your character will develop that.”

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Although the 1906 San Francisco earthquake and fire destroyed his studio and most of his early creations, over his lifetime Dixon created an abundant body of work including illustrations for novels, paintings in several genres, murals, and poetry. His paintings of western US landscapes
found throughout are considered remarkable for their realism and detail. Through his art, Dixon also documented economic and social hardships during the Great Depression, lifestyles of Native American cultures, and lingering vestiges of cowboy life. Though Dixon often featured the expansive, remote vistas of the western United States, he typically created these scenes, including “Shiprock Mesa” this month’s cover art, on small canvases.

The mesa dominates this painting, its characteristic flat top and stair-stepped edges etched against a pale, shimmering blue sky. Red and sand-colored strata vibrantly contrast with the immense dark blue shadows playing across the steep cliff faces. The softer shapes and textures of the scrub vegetation that carpets the sandy plains contrast with the bold depiction of the mesa and cloudless sky. Hagerty wrote, “Through long and sympathetic observation, Dixon learned how plains rise and fall as they flow toward the horizon, and how the architecture of mesa and butte marches rhythmically over the landscape into the infinite freedom of a deep blue sky.”

Dixon’s nearly photographic painting, a relaxing study in stillness, light, and distance, appears deceptively simple. Los Angeles art critic Arthur Millier wrote that “Dixon is so steeped in desert forms and colors that these little pictures appear to come from his brush like effortless lyrics.” In describing Dixon’s landscapes, the Pasadena Museum of California Art notes that “His modernist approach to painting Western landscapes featured simple compositions and powerful color fields that shifted the genre away from the more typically sentimental treatment of familiar subject matter.”

In 1894, three years after Dixon wrote to Remington and a year before he landed his first paying job illustrating western scenes for a newspaper, a case of disseminated coccidioidomycosis, or Valley fever as it is commonly known, was first reported in California. This reemerging infectious disease is caused by *Coccidioides immitis*, a soil fungus native to San Joaquin Valley, Dixon’s birthplace, and by *C. posadasii*, which is common to other arid-to-semiarid areas of the southwestern United States, northern portions of Mexico, and areas of Central America and South America. Approximately 60% of reported cases of coccidioidomycosis in the United States occur in Arizona, Dixon’s beloved “country.”

Coccidioidomycosis is usually acquired by breathing microscopic *Coccidioides* fungal spores released after humans, animals, or the weather disturbs contaminated soil or dust. There has been a striking increase in cases of coccidioidomycosis during the past several decades, including a major outbreak in California in 1991–1994. Some of this increase may be related to greater recognition and diagnosis, as well as improved reporting. Some factors linked to this increase throughout the western expanses, where Dixon traveled and worked, are the upsurge in susceptible human populations including older individuals and those with immune deficiencies from non–coccidioidomycosis-endemic areas; rise of cities and towns in areas with *Coccidioides*-contaminated soil; occupational exposures; and changing weather patterns.

**Bibliography**


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- Bartonella ancashensis Identified by Whole-Genome Analysis of Human Pathogens Causing Verruga Peruana, Rural Ancash Region, Peru
- Zika Virus RNA Replication and Persistence in Brain and Placental Tissue
- Comparison of Sputum Culture Conversion for Mycobacterium bovis and M. tuberculosis
- Spatiotemporal Fluctuations and Triggers of Ebola Virus Spillover
- Molecular, Spatial, and Field Epidemiology Suggesting TB Transmission in Community, Not Hospital, Gaborone, Botswana
- Rhodococcus Infection in Solid Organ and Hematopoietic Stem Cell Transplant Recipients
- Zoonotic Transmission of the mcr-1 Colistin Resistance Gene from Nonintensive Poultry Farms, Vietnam
- Genetically Diverse Filoviruses in Rousettus and Eonycteris spp. Bats, China
- Likely Autochthonous Transmission of Trypanosoma cruzi to Humans, Southcentral Texas, USA
- Outbreaks Associated with Food Imported into the United States, 1996–2014
- Pneumonic Plague Transmission, Moramanga, Madagascar, 2015
- Mycobacterium chimaera Closely Related to American and British Isolates in Heater–Cooler Units, Denmark
- pncA Gene Mutations and Pyrazinamide Resistance in Drug-Resistant Tuberculosis, South Africa and Georgia
- Two Cases of Neisseria meningitidis Proctitis in HIV-Positive Men Who Have Sex with Men
- Mycobacterium bovis in a Free-Ranging Black Rhinoceros (Diceros bicornis) in Kruger National Park, South Africa 2016
- Zika Virus Vector Competency of Mosquitoes, Gulf Coast, United States
- Worldwide Endemicity of a Multidrug-Resistant Staphylococcus capitis Clone Involved in Neonatal Sepsis

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

Upcoming Infectious Disease Activities

February 6–8, 2017
American Society for Microbiology 2017 Biothreats
Washington, DC USA

February 13–16, 2017
CROI
Conference on Retroviruses and Opportunistic Infections
Seattle, WA, USA
http://www.croiconference.org/

March 29–31, 2017
SHEA
Society for Healthcare Epidemiology of America
St Louis, MO, USA
http://www.shea-online.org/

April 10–12, 2017
World Vaccine Congress
Washington, DC, USA
http://www.terrapinn.com/conference/world-vaccine-congress-washington

April 22–27, 2017
ECCMID
European Congress of Clinical Microbiology and Infectious Diseases
Vienna, Austria
http://www.eccmid.org/

June 1–5, 2017
ASM
American Society for Microbiology
New Orleans, LA, USA

June 19–21 2017
Transmission of Respiratory Viruses
Harbour Grand Hong Kong
https://transmission2017.med.hku.hk/mass_email.html

March 1–4, 2018
18th International Congress on Infectious Diseases (ICID)
Buenos Aires, Argentina
http://www.isid.org/icid/

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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
Multidrug-Resistant *Candida haemulonii* and *C. auris*, Tel Aviv, Israel

CME Questions

1. Which of the following statements regarding the phylogenetic trees of *Candida auris* and *C. haemulonii* in the current study is most accurate?
   A. Both *Candida* strains were closely linked with singular global international clones
   B. *C. auris* was distinct from other global isolates; *C. haemulonii* was similar to a single global isolate
   C. *C. auris* was similar to a single global isolate; *C. haemulonii* was distinct from other global isolates
   D. Both *Candida* strains were distinct from other global isolates

2. Which of the following statements regarding patient characteristics in the current study of *C. auris* and *C. haemulonii* infection is most accurate?
   A. Most cases of *C. auris* infection were isolated from skin samples
   B. Carriers of *C. haemulonii* tended to be younger and healthier compared with noncarriers
   C. *C. haemulonii* was most frequently recovered from sites of healed lower extremity ulcers
   D. Cultures of medical personnel and equipment implicated chronic facility colonization with *C. haemulonii*

3. *C. auris* and *C. haemulonii* were most likely to be resistant to which of the following antifungals in the current study?
   A. Fluconazole
   B. Anidulafungin
   C. Micafungin
   D. Flucytosine

4. What else did the current study demonstrate regarding the pathologic potential of *C. auris* and *C. haemulonii*?
   A. *C. auris* demonstrated greater potential for azole resistance compared with *C. haemulonii*
   B. The potential for azole resistance in *C. auris* and *C. haemulonii* was not as strong as that of *C. glabrata*
   C. Only *C. auris* grew poorly at high temperatures
   D. Both *C. auris* and *C. haemulonii* grew poorly at high temperatures

Activity Evaluation

<table>
<thead>
<tr>
<th>Question</th>
<th>Strongly Disagree</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Strongly Agree</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The activity supported the learning objectives.</td>
<td>Strongly Disagree</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>Strongly Agree</td>
<td>5</td>
</tr>
<tr>
<td>2. The material was organized clearly for learning to occur.</td>
<td>Strongly Disagree</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>Strongly Agree</td>
<td>5</td>
</tr>
<tr>
<td>3. The content learned from this activity will impact my practice.</td>
<td>Strongly Disagree</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>Strongly Agree</td>
<td>5</td>
</tr>
<tr>
<td>4. The activity was presented objectively and free of commercial bias.</td>
<td>Strongly Disagree</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>Strongly Agree</td>
<td>5</td>
</tr>
</tbody>
</table>
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Article Title

Risk Factors for Disseminated Coccidioidomycosis, United States

CME Questions

1. Your patient is a 56-year-old immunosuppressed man suspected of having disseminated coccidioidomycosis. According to the case series and review by Odio and colleagues, which of the following statements about clinical and epidemiologic factors of disseminated coccidioidomycosis is correct?

A. The US Centers for Disease Control and Prevention (CDC) reported that cases of coccidioidomycosis doubled from 1998 to 2011
B. Approximately 15% of cases of coccidioidomycosis disseminate
C. Dissemination may be single or multisite, is associated with more severe outcomes, and requires prolonged treatment
D. Coccidioides infection is usually severe and chronic

2. According to the case series and review by Odio and colleagues, which of the following statements about risk factors for disseminated coccidioidomycosis is correct?

A. Risk factors for coccidioidomycosis dissemination include exogenous immunosuppression (steroids and biologics), pregnancy, race, and specific genetic defects
B. Nervous system dissemination occurred in 10% of the pregnant women in this series
C. Mortality rate of disseminated coccidioidomycosis is approximately 25% in those immunocompromised by HIV, cancer, organ transplantation, antigraft rejection medications, anti-inflammatory biologics, or chemotherapy
D. Blacks were more likely to have central nervous system (CNS) infection, whereas whites were more likely to have osteomyelitis

3. According to the case series and review by Odio and colleagues, which of the following statements about genetics underlying host defense against disseminated coccidioidomycosis is correct?

A. Interleukin 12/interferon-gamma (IL-12/IFN-γ) and STAT3 axes are critical host defense pathways
B. IFN-γ therapy was ineffective in severe or refractory disseminated coccidioidomycosis
C. Older patients with severe or relapsing disseminated coccidioidomycosis should be considered for genetic screening for primary immune defects
D. The role of STAT3 regarding immunity to Coccidioides is unknown

Activity Evaluation

1. The activity supported the learning objectives.
   Strongly Disagree 1 2 3 4 5 Strongly Agree

2. The material was organized clearly for learning to occur.
   Strongly Disagree 1 2 3 4 5 Strongly Agree

3. The content learned from this activity will impact my practice.
   Strongly Disagree 1 2 3 4 5 Strongly Agree

4. The activity was presented objectively and free of commercial bias.
   Strongly Disagree 1 2 3 4 5 Strongly Agree
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**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 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 50 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. 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 eliminations 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 public health. Topics may include science and the human condition, the unanticipated side of epidemiologic 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.

**Research Letters Reporting Cases, Outbreaks, or Original Research.** EID publishes letters that report cases, outbreaks, or original research as Research Letters. Authors should provide a short abstract (50-word maximum), references (not to exceed 10), and a short biographical sketch. These letters should contain no more than 850 words (including the abstract) and may include either 1 figure or 1 table. Do not divide Research Letters into sections.

**Letters Commenting on Articles.** Letters commenting on articles should contain a maximum of 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article’s publication.

**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.

**Etymology.** Etymology (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

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